Phosphoproteomic profiling of microsomal fractions in leaves of Cogon grass (Imperata cylindrica)

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Abstract

Cogon grass [Imperata cylindrica (L.) Beauv. var. major] is one of the top-weeds. It is a distributed C₄ plant. In particular, one ecotype (Chuwei) is salt and flooding tolerant. Imperata leaves harvested from Chuwei and Sarlun control site were subjected to microsomal fractionation followed by membrane shaving using trypsin. The resulting peptides were further enriched by columns and mass spectrometry analyses for phosphopeptide identification. The phosphoproteomic analysis identified 20 phosphorylation sites from 16 unique phosphorylated peptides in the microsomal fractions isolated from leaves of Imperata, of which 5 are specific to Chuwei ecotype. Out of these 5 phosphopeptides, 2 belong to sugar transporters, including a sucrose transporter SUT1 which appears to be a novel and C₄-specific phosphorylation site. Whereas the other 3 belong to photosynthesis proteins of which 2 are C₄ enzymes. Interestingly, two independent phosphoproteins of tonoplast monosaccharide transporter 2 (TMT2) were identified in Chuwei ecotype and the other salt-intolerant Sarlun population individually. Our phosphoproteomic study identified differentially phosphorylated peptides of membrane proteins which may play an important role in the salt stress response of Imperata.

Keywords: Imperata cylindrica, salt, C₄ photosynthesis, phosphoproteomics.

Abbreviation: MS_mass spectrometry; IMAC_immobilized metal affinity chromatographyn (IMAC) or TiO₂ column, phosphorylated peptides isolated from cells can be enriched followed by MS analysis for site mapping (Kersten et al., 2009; Silva-Sanchez et al., 2015). For example, many phosphorylation sites have been mapped in Arabidopsis (Arabidopsis thaliana) (Hsu et al., 2009; Nühse et al., 2004; Kline et al., 2010; Chang et al., 2012; Meyer et al., 2012; Yang et al., 2013), rice (Oryza sativa) (Shen et al., 2012), barley (Hordeum vulgare) (Endler et al., 2009), and maize (Zea mays) (Hu et al., 2013).

Imperata cylindrica, a top-weed and a C₄ plant (Holm et al., 1977), is widely used as a medicinal plant in Asia (Matsunaga et al., 1994; Kumar et al., 2011). The medical uses of Imperata cylindrica rely on its rhizomes (Sripanidkulchai et al., 2001; Yoon et al., 2006). Imperata cylindrica (L.) Beauv. var. major (Nees Hubb. was found to be widely distributed in Taiwan (Hsu, 1975). Interestingly, I. cylindrica is growing in Chuwei mangrove salt marsh wetland. In 1997, Cheng examined the leaf structure of I. cylindrica from Chuwei by scanning electron microscope (SEM). The result indicated that Chuwei ecotype was structurally different from others. The stele was empty and the surface of lower stem was coated with wax instead of trichomes (Cheng and Chou, 1997a). The polymorphism among populations was also analyzed by rapid amplified polymorphic DNA (RAPD) (Cheng and Chou, 1997b) and restriction fragment length polymorphism (RFLP) (Chou and Tsai, 1999) on ribosomal DNA (rDNA) (Chiang et al., 1998; Tsai and Chou, 1999). Based on molecular classification, Chuwei population was identified as a unique ecotype of I. cylindrica in Taiwan. Moreover, in 2006, Chang and Chou utilized hydroponic system to test the salt and flood tolerance of Imperata ecotypes, and discovered that Chuwei ecotype was salt and flood-tolerant (Chang and Chou, 2006). For unknown reason, the population size of Chuwei ecotype was found to be decreasing (Kao et al., 2011).

In 2008, Chang utilized proteomic approach and identified three proteins, namely enolase, chloroplast ferredoxin- NADP(H)
oxidoreductase, and mitochondrial malate dehydrogenase that were differentially expressed in Chuwei and Sarlun ecotypes (Chang, 2008). However, the salt tolerance mechanism of Chuwei ecotype is still unknown. In addition, Imperata is not a model organism and does not have genome sequenc information. Many studies have utilized proteomic approach and identified proteins in response to salt stress in C4 plants (Zhao et al., 2013a; Zhao et al., 2013b). In maize (Zea mays), salt-responsive proteins were identified using proteomic tools (Zörb et al., 2009; Zörb et al., 2010). In Sorghum seedlings, a proteomic study found salt-responsive proteins (Ngara et al., 2012). In a C4 dicot Amaranthus cruentus, differential accumulated proteins were identified by a proteomic study (Joaquín-Ramos et al., 2014). However, phosphoproteome of C4 plants is still little studied.

To date, most of the studies were performed in model organisms due to the complete genome sequence information. Little is known in non-model organisms. In the present study, we test if we can identify phosphorylation sites from the non-model organism Imperata. The phosphoproteomic analyses on microsomal fractions of Imperata cylindrica were carried out. Chuwei ecotype (salt-tolerant) and Sarlun population (salt-intolerant) were used as our plant materials. In the phosphoproteomic analyses, we identified 20 phosphorylation sites including Chuwei-specific phosphorylation sites some of which appear to be novel.

Results and Discussion

In 2008, the Santoni group utilized MS and identified novel phosphorylation sites in Arabidopsis aquaporin PIP2;1 following salt stress (Prak et al., 2008). In addition, phosphorylation of water channel aquaporins was found changed in Arabidopsis and rice in response to high salt stress (Hsu et al., 2009; Chang et al., 2012). We tested if we can utilize membrane shaving strategy to identify phosphopeptides from membranes of salt-stressed Imperata cylindrica. We harvested Imperata cylindrica from two different populations (Chuwei salt-tolerant ecotype and Sarlun salt-intolerant population) (Chang and Chou, 2006). Microsomal fractions from both populations were isolated and shaved using trypsin digestion (membrane shaving) followed by Zr⁺⁺-IMAC or TiO₂ enrichment and LTQ-Velos analysis. By using Imperata local protein database, we significantly (p<0.05) identified a total of 9 phosphopeptides containing 10 phosphorylation sites from 8 proteins of which 4 are membrane proteins (Table 1). However, the number of the sites is still limited. By use of NCBI database, by contrast, we identified a total of 16 phosphopeptides containing 20 phosphorylation sites from 13 proteins of which 6 are membrane proteins (Table 1: Supplemental Fig. S1A-S1P). Seven phosphopeptides were identified in leaves of both Chuwei ecotype and Sarlun population, indicating that 9 phosphorylation sites are evolutionarily conserved between Chuwei ecotype and Sarlun population.

Out of the 9 differentially accumulated phosphopeptides, 5 are specific to Chuwei ecotype, including that of a tonoplast monosaccharide transporter 2 (TMT2) and a sugar transporter; 4 are specific to Sarlun population, including TMT2 and PEPCarboxylase 3. Interestingly, two independent phosphopeptides of TMT2 were identified in Chuwei ecotype and the other salt-intolerant Sarlun population individually (Table 1: Fig. 2-3; Supplemental Fig. S1P-S1G), suggesting ecotype-specific phosphorylation site. The identified phosphopeptides were also validated manually followed by using Scaffold software v4.3 and ScaffoldPTM (Proteome Software, USA) (Supplemental Fig. S1A-S1P). To determine whether the identified phosphorylation sites were previously documented, literature and phosphorylation site database (i.e. PhosPhat, P3DB) searching was carried out. We found that the phosphorylation site of a phosphorylated peptide of a sucrose transporter (GDEGELEpSVGVQR) was not identified before and appears to be novel. In addition, the phosphorylation site (Ser-357) of a phosphorylated peptide of TMT2 (EGDDYApSDHHGDDIEDNLQSPPLSR) appears to be novel as well. The MS/MS fragmentation pattern of the phosphopeptide of SUT1 and TMT2 are shown in Fig. 1, Fig. 2 and Fig. 3. Moreover, we identified the phosphorylation site of a phosphorylated peptide of NADP-malate dehydrogenase (MDH) (QVQDVGApTAEAPATR) which was not identified before and appears to be novel. The MS/MS fragmentation pattern of the phosphopeptide of NADP-malate dehydrogenase is shown in Supplemental Fig. S1P.

In order to determine whether the identified phosphorylation site of TMT2 is not localized in the transmembrane domain, a transmembrane domain prediction was carried out by search TMHMM server. As shown in the prediction result in Fig. 4, the identified phosphorylation site of TMT2 is not localized in the transmembrane domain. This result supports that trypsin digestion pattern using membrane shaving by cleaving peptides from non-transmembrane domain of IcTMT2. In addition, to further determine whether the identified phosphorylation site of Imperata TMT2 is evolutionarily conserved, protein sequence alignment was carried out using ClustalW. As shown in Fig. 5, The Ser residue of the phosphorylation site of Imperata TMT2 is evolutionarily conserved in Imperata and barley (Hordeum vulgare).

In the microsomal fractions of Chuwei ecotype, phosphopeptides corresponding to four photosynthesis proteins were identified. These photosynthesis proteins include pyruvate phosphate dikinase (PPDK), malate dehydrogenase, chlorophyll a/b binding protein 2, and phosphoenolpyruvate carboxylase (PEPC) (Table 1). The phosphopeptides were not observed in Sarlun population. The phosphorylation sites of PPDK, chlorophyll a/b binding protein 2, and PEPC were reported in previous studies (Ueno et al., 2000; Reiland et al., 2009; Shen et al., 2012). PEPC was found to be a 14-3-3 interactor (Chang et al., 2009) and phosphorylated by CDPK in vitro (Curran et al., 2011). In particular, Hu identified the same site of PPDK, a salt-responsive protein, in maize leaves (Hu et al., 2013). However, the site of NADP-malate dehydrogenase (QVQDVGApTAEAPATR) and a site of PEPC (HGDFSDEGSATTEpSDIEETLKR) were not reported before and appeared to be novel.

In plant cells, sugars can be transported by different transporters. Sucrose transporters are membrane proteins known to be involved in sucrose loading in the phloem in plants (Truehit, 2001; Kühn and Grof, 2010). Sucrose transporters are plasma membrane (Kühn and Grof, 2010) and vacuolar tonoplast localized. A sucrose transporter, AtSUT4, is a vacuolar sucrose transporter (Endler et al., 2006). In Arabidopsis, phosphorylation of the N-terminus of AtSUC5 was first observed in 2004 (Nilbüs et al., 2004). Mass spectrometry analyses showed that AtSUC1 can be phosphorylated at Ser-20 and Thr-199 (Nittiyālī et al., 2007; Kühn and Grof, 2010). Tonoplast monosaccharide transporter (TMT) is a tonoplast membrane protein (Neuhau, 2007). It is involved in vacuole sugar transport. It is encoded by a small gene family in Arabidopsis. In Arabidopsis AtTMT1 is involved in vacuole monosaccharide transport and abiotic stress response (Wormit et
### Table 1. Phosphorylated peptides identified in microsomal fractions isolated from *Imperata*.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Mascot score</th>
<th>Accession number</th>
<th>Scaffold Probability(%)</th>
<th>Best Ascore</th>
<th>Phosphopeptide sequence</th>
<th>Enrichment method</th>
<th>Mass spectrometry</th>
<th>Organism</th>
<th>Imperata population</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Membrane protein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma-membrane H+-ATPase 2</td>
<td>69</td>
<td>gi</td>
<td>162458860</td>
<td>100</td>
<td>123.86</td>
<td>GLDIDTIQQNYpTV</td>
<td>Zr^{4+}</td>
<td>LTQ-Velos</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>Sucrose transporter 1</td>
<td>53</td>
<td>gi</td>
<td>162463612</td>
<td>100</td>
<td>1000</td>
<td>GDGELELPVGYR</td>
<td>Zr^{4+}</td>
<td>LTQ-Velos</td>
<td><em>Zea mays</em></td>
<td>Chuwei</td>
</tr>
<tr>
<td>TMT2</td>
<td>70</td>
<td>gi</td>
<td>226529950</td>
<td>100</td>
<td>74.34, 12.9</td>
<td>GQpSLGpSLGLISR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei</td>
</tr>
<tr>
<td>TMT2</td>
<td>30</td>
<td>gi</td>
<td>226529950</td>
<td>53</td>
<td>23.52</td>
<td>EGDDYApSDHGDDIEDNLQSPLISR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td></td>
<td>Sarlun</td>
</tr>
<tr>
<td>PIP2;7</td>
<td>39</td>
<td>gi</td>
<td>514732737</td>
<td>96</td>
<td>46.8, 77.7</td>
<td>ALGpSFpPSTSATV</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td></td>
<td>Sarlun</td>
</tr>
<tr>
<td>Photosystem II protein H</td>
<td>60</td>
<td>gi</td>
<td>11467220</td>
<td>96, 100</td>
<td>28.13</td>
<td>A(pT)Q(pT)VEDSSRPKPK</td>
<td>Zr^{4+}, TiO_{2}</td>
<td>LTQ-Velos</td>
<td><em>Zea mays</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>Calcium sensing receptor</td>
<td>52</td>
<td>gi</td>
<td>514717642</td>
<td>100</td>
<td>18.42</td>
<td>IGpTTSSASR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td></td>
<td>Sarlun</td>
</tr>
<tr>
<td><strong>Non-membrane protein</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUDOR-SN protein 1</td>
<td>106</td>
<td>gi</td>
<td>514712805</td>
<td>100</td>
<td>1000</td>
<td>IWQYG&gt;DVpSEDEEQAPAAR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>Pyruvate orthophosphate dikinase</td>
<td>62</td>
<td>gi</td>
<td>108796050</td>
<td>100</td>
<td>10.28</td>
<td>GGMPtSHAAVVAR</td>
<td>TiO_{2}</td>
<td>LTQ-FT</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei</td>
</tr>
<tr>
<td>Chlorophyll a/b binding protein 2</td>
<td>46</td>
<td>gi</td>
<td>226510115</td>
<td>99</td>
<td>17.74</td>
<td>pTAApKPAAGSPWYGADR</td>
<td>TiO_{2}</td>
<td>LTQ-Velos</td>
<td><em>Zea mays</em></td>
<td>Chuwei</td>
</tr>
<tr>
<td>Similar to translational activator</td>
<td>83</td>
<td>gi</td>
<td>242038321</td>
<td>99</td>
<td>31.81</td>
<td>AILEGgpSDDEGASTEAQGR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>NADP-malate dehydrogenase</td>
<td>48</td>
<td>gi</td>
<td>242081803</td>
<td>98</td>
<td>14.47</td>
<td>QVQDGVApTAEAPATR</td>
<td>Zr^{4+}</td>
<td>LTQ-Velos</td>
<td><em>Sorghum bicolor</em></td>
<td>Chuwei</td>
</tr>
<tr>
<td>Sucrose-phosphate synthase 1</td>
<td>66</td>
<td>gi</td>
<td>346685058</td>
<td>100</td>
<td>1000</td>
<td>EA(pT)ELDAEL(pS)EGEKGDTLGLAPVETA</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>Sucrose-phosphate synthase 1</td>
<td>50</td>
<td>gi</td>
<td>346685058</td>
<td>100</td>
<td>64.62</td>
<td>NFpSDLTVWSDDNKEK</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Imperata cylindrica</em></td>
<td>Chuwei, Sarlun</td>
</tr>
<tr>
<td>PEPcarboxylase 3</td>
<td>79</td>
<td>gi</td>
<td>10185175</td>
<td>100</td>
<td>1000</td>
<td>HGDFSDQGSATEpSDIEETLKR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Sacccharum officinarum</em></td>
<td>Sarlun</td>
</tr>
<tr>
<td>PEPcarboxylase 3</td>
<td>37</td>
<td>gi</td>
<td>10185175</td>
<td>96</td>
<td>1000</td>
<td>HHpSIDAQLR</td>
<td>Zr^{4+}</td>
<td>Q-TOF</td>
<td><em>Sacccharum officinarum</em></td>
<td>Sarlun</td>
</tr>
</tbody>
</table>

The amino acid residue in parenthesis indicates alternative phosphorylation site.
Fig 1. MS/MS fragmentation pattern of a phosphopeptide of SUT1 in *Imperata*. The phosphopeptide GDGELELPSVGVR was identified in Chuwei ecotype. The matched daughter ion masses were labeled. B, the matched daughter ion masses marked in red.

Fig 2. MS/MS fragmentation pattern of a phosphopeptide of IcTMT2 from Chuwei ecotype. A, the phosphopeptide GQpSALpSALGLISR was identified in Chuwei ecotype. The matched daughter ion masses were labeled. Ser-276 and Ser-280 are both phosphorylated. B, the matched daughter ion masses marked in red.
Fig 3. MS/MS fragmentation pattern of a phosphopeptide of IcTMT2 from Sarlun population. The phosphopeptide EGDDYApSDHGGDDIEDNLQSPLISR was identified in Sarlun population. Ser-357 is phosphorylated. B, the matched daughter ion masses marked in red.

Fig 4. Predicted transmembrane topology of IcTMT2. The full-length protein sequence was subjected to prediction by TMHMM server v 2.0. The identified phosphorylation site is localized in the non-transmembrane domain, which explained the shaving region of IcTMT2 catalyzed by trypsin digestion.

al., 2006). Moreover, the phosphorylation sites of AtTMT1 were identified in a phosphoproteomic study (Whiteman et al., 2008). Sugar transporters can be post-translationally modified in response to abiotic stress. In 2009, an increase of phosphorylation of a sugar transporter STP1 in response to salt stress was found in a proteomic study in Arabidopsis (Hsu et al., 2009). In the present study, phosphorylation sites of SUT1 and TMT2 were identified from Chuwei ecotype (Table 1). Although the sites of TMT2 were identified in HvSTP of barley (Endler et al., 2009), the site of SUT1 was not reported before and appeared to be novel. We did not detect the same phosphopeptide in Sarlun population. We suspected that the phosphorylation of SUT1 and TMT2 may play important roles in the salt stress response in Chuwei ecotype by regulating sugar homeostasis in plant cells. Since the actual function of phosphorylation of TMT is still unknown, it is required to perform further studies. Interestingly, another phosphorylated peptide of TMT2 (EGDDYApSDHGGDDIEDNLQSPLISR) was identified in Sarlun population. The phosphorylation site was not previously documented and appeared to be novel. Therefore, we identified two individual phosphorylation sites of the same protein TMT2 in Chuwei ecotype and Sarlun.
Fig 5. Sequence alignment of TMT2 from plants. Protein region containing amino acid # around 200-500 was compared. Alignment using ClustalW2 was carried out for protein sequences of TMT2 from Imperata, gi|226529950 (Hordeum vulgare), gi|115445159 (Oryza sativa), gi|242040371 (Oryza sativa), gi|226529950 (Oryza sativa), gi|115445159 (Sorghum bicolor), and gi|226529950 (Zea mays). The phosphorylated peptide and site identified in the Chuwei ecotype and Sarlun population in the present study is underlined individually. The phosphorylation sites identified in the present study and in barley are marked in bold face.

population, which supports the ecotypic variation among different Imperata populations on the molecular level (Cheng and Chou, 1997b; Chou and Tsai, 1999). Whether the identified differential phosphorylation sites are involved in salt-tolerance of Imperata requires further studies. Since TMT2 is a vacuolar sugar transporter, it is possible that Imperata utilizes TMT2 to maintain sugar homeostasis in response to salt stress. In C4 plants, photosynthesis is a lot different from C3 plants in that C4 pathway is involved. The C4 pathway includes key enzymes PEPC, malic enzyme, malate dehydrogenase, and PPDK. These enzymes increase the efficiency of photosynthesis in C4 plants (Sage et al., 2012). In a previous study in maize, activities of PEPC and NADP-MDH in C4 photosynthesis pathway increased under salinity condition (Omoto et al., 2012). In Sorghum leaves, PEPC activity was increased by salt stress (Echevarria et al., 2001; Monreal et al., 2013). A possible correlation between salt tolerance and C4 pathway was proposed (Bromham and Bennett, 2014). Studies have utilized transcriptomic approach to identify salt responsive genes and proteins in C4 plants. For example, expression of genes in response to salt stress changed in maize kernel (Andjelkovic and Thompson, 2006). A transcriptomic study on Sorghum bicolor in response to dehydration, high salinity, and abscisic acid (ABA) was carried out (Buchanan et al., 2005). Glycine-rich RNA binding protein gene expression was found to be regulated by salinity in Sorghum (Aneeta et al., 2002). In the present study, we identified differentially accumulated phosphorylated peptides of C4 enzymes. Whether the phosphorylation sites of these C4 enzymes are involved in salt stress response requires further studies.

Materials and Methods

Sampling site and plant materials

Imperata cylindrica (L.) Beauv. var. major (Nees) Hubb, Cogon grass, was used as plant material in this study. Chuwai mangrove salt-marsh wetland Taipei, Taiwan was selected as a sampling site. Imperata cylindrica collected from Sarlun sandy beach in Taipei, Taiwan was used as a control sample. Plant leaves were harvested from sampling site. During harvesting, each leaf sample was excised by sterilized scissors and stored in zip-block in ice bucket with dry ice to keep it fresh and brought back to lab immediately for study the same day. Plant rhizomes collected from the field were washed by sterilized water and cultured in pots (60x 20 x 20 cm³) in greenhouse for two weeks, then transplanted to Kimura’s culture solution (Ma et al., 2001) to grow plantlets. The culture solution was aerated with air.
Membrane shaving and phosphopeptide enrichment

Plants harvested from Chuwei and Sarlun were subjected to membrane shaving as previously described (Hsu et al., 2009; Chang et al., 2012). Two ml extraction buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 1x protease inhibitor cocktail (EDTA-free, Roche) was added to the mortar containing 1 mg of plant tissue. The crude extract was filtered using Miracloth at 4°C. The crude extract was then centrifuged (Beckman J2-MC) for 30 min at 4°C to collect the supernatant. The supernatant was centrifuged again in an ultracentrifuge (Hitachi CP80WX) at 136400 x g for 75 min at 4°C to collect the microsomal fractions. Microsomal proteins were shaved using trypsin in the presence of 60% methanol at 37°C overnight. Phosphopeptides were enriched using Zr⁴⁺-IMAC or TiO₂ magnetic beads as previously described (Hsu et al., 2009; Chang et al., 2012).

MS analysis and protein identification

The ESI-MS/MS mass spectrometers utilized for peptide analysis were a Q-TOF (Micromass, UK), LTQ-Velos, or LTQ-FT (Thermo Scientific, USA). Collision induced dissociation (CID) was used as the fragmentation method. For Q-TOF analyses, the peptide mixture was subjected to the CapLC system (Waters, Milford, MA). Capillary column (75 µm i.d., 10 cm in length, MST, Taiwan) was used with a linear gradient from 5% to 50% acetonitrile containing 0.1% formic acid for a 46-min LC run. The scan range for MS was from m/z 400 to 1600, and for MS/MS was from m/z 50 to 2000. PKL files were generated using MassLynx 4.0 by processing raw data. For LTQ-Velos analyses, the flow rate for peptide mixture analyses was 70 µl/min by using Thermo Biobasic column (5 µm, 1 mm x 150 mm) with a linear gradient for a 40-min LC run. For LTQ-FT, an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies, Palo Alto, CA), and a Famos autosampler (LC Packings, San Francisco, CA) were used. Chromatographic separation was performed on a self-packed reversed phase C18 nano-column (75 µm I.D. x 200 mm, 3 µm, 100 Å) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 80% acetonitrile as mobile phase B operated at the flow rate of 300 nL/min. The mass scanning range was set from m/z 350 to 2,000 for the survey full-scan MS modeand data dependent MS/MS acquisition. The MS/MS fragmentation pattern was analyzed by using the MASCOT v2.3 searching engine (www.matrixscience.com) (Perkins et al., 1999). The search parameters were defined as follows: Database, NCBI nr 20130909, a local Imperata protein database; Taxonomy, Viridiplantae (Green Plants); Enzyme, Trypsin; Variable modifications, phosphorylation; Peptide MS tolerance, ± 0.8 Da for LTQ-Velos data and ± 0.6 Da for Q-TOF data; Fragment MS tolerance, ± 0.8 Da for LTQ-Velos data and ± 0.6 Da for Q-TOF data, with allowance of one missed cleavage site. For LTQ-FT, 0.1 Da was set for peptide MS tolerance and fragment MS tolerance. Ascor for each phosphopeptide (Beausoleil et al., 2006) was calculated and phosphopeptides were validated manually and using Scaffold/PTM (Proteome Software, USA). In addition, manual inspection of mass spectrum for each phosphopeptide was performed by checking the neutral loss fragments (Schroeder et al., 2004). To determine whether the identified phosphorylation sites are novel, phosphorylation site databases PhosPhat (Heazlewood et al., 2008) and P3DB (Yao et al., 2014) were searched against. The transmembrane domain of membrane protein was predicted by using TMHMM server v 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Conclusion

In conclusion, by use of membrane shaving and Zr⁴⁺-charged magnetic bead-based phosphopeptide isolation, we identified phosphorylation sites in membrane proteins from Imperata cylindrica which appears to be novel. Our phosphoproteomic study identified differentially expressed phosphorylation sites which suggest ecotypic variation of Imperata on the proteome level. The differential phosphorylation may play an important role in Chuwei ecotype in response to salt stress.

Acknowledgments

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