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

Ting-Ying Wu[1], Ping Kao[1], Chia-Lun Chang[2], Pang-Hung Hsu[3], Chang-Hung Chou[4], Ing-Feng Chang[1, 2, 5 *]

[1]**Department of Life Science, National Taiwan University, Taipei, Taiwan**
[2]**Institute of Plant Biology, National Taiwan University, Taipei, Taiwan**
[3]**Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan**
[4]**Graduate Institute of Ecology and Evolutionary Biology, China Medical University, Taichung, Taiwan**
[5]**Genome and Systems Biology Degree Program, National Taiwan University and Academia Sinica, Taipei Taiwan**

***Corresponding author: ifchang@ntu.edu.tw**

Abstract

Cogon grass [*Imperata cylindrica* (L.) Beauv. var. *major*] is one of the top-ten 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 phosphopeptides 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 column; SEM_scanning electron microscopy; RAPD_ rapid amplified polymorphic DNA; RFLP_restriction fragment length polymorphism; TMT_tonoplast monosaccharide transporter; PEPC_phosphoenolpyruvate carboxylase; PPDK_pyruvate phosphate dikinase.

Introduction

Phosphorylation is a reaction catalyzed by kinases in the cells. Cells undergo phosphorylation for signal transduction in response to internal or external cues. Therefore, identification of phosphorylation site turns out to be essential in the functional study of signal transduction. Mass spectrometry (MS)-based phosphoproteomic analysis has been introduced to map phosphorylation sites in the plant cells. By use of enrichment method *i.e.* immobilized metal affinity chromatographyn (IMAC) or TiO_2 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-ten 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^{4+}-IMAC or TiO_2 enrichment and LTQ-Velos analysis. By using *Imperata* local protein database, we significantly ($p \leq 0.05$) 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 these 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. S1F-S1G), suggesting ecotype-specific phosphorylation site. The identified phosphopeptides were also validated manually followed by using Sacffold 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 (GDGELELpSVGVR) was not identified before and appears to be novel. In addition, the phosphorylation site (Ser-357) of a phosphorylated peptide of TMT2 (EGDDYApSDHGGDDIEDNLQSPLISR) 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) (QVQDGVApTAEAPATR) 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 (QVQDGVApTAEAPATR) 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 (Truemit, 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 (Nühse et al., 2004). Mass spectrometry analyses showed that AtSUC1 can be phosphorylated at Ser-20 and The-393 (Niittylä et al., 2007; Kühn and Grof, 2010). Tonoplast monosaccharide transporter (TMT) is a tonoplast membrane protein (Neuhaus, 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

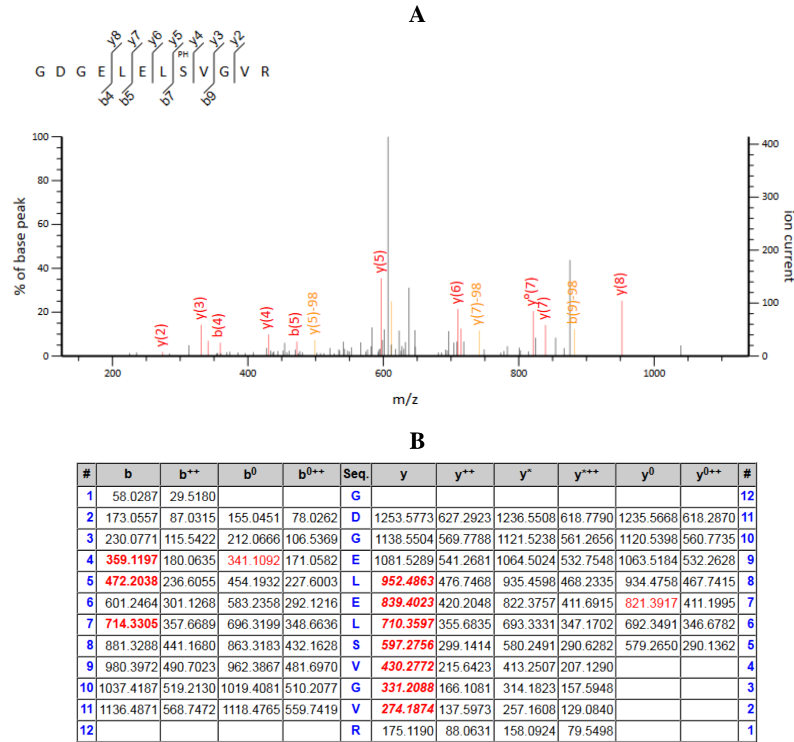
Table 1. Phosphorylated peptides identified in microsomal fractions isolated from *Imperata*.

Protein name	Mascot score	Accession number	Scaffold Probability(%)	Best Ascore	Phosphopeptide sequence	Enrichment method	Mass spectrometry	Organism	*Imperata* population
Membrane protein									
Plasma-membrane H^+-ATPase 2	69	gi| 162458860	100	123.86	GLDIDTIQQNYpTV	Zr^{4+}	LTQ-Velos	*Imperata cylindrica*	Chuwei, Sarlun
Sucrose transporter 1	53	gi| 162463612	100	1000	GDGELELpSVGVR	Zr^{4+}	LTQ-Velos	*Zea mays*	Chuwei
TMT2	70	gi| 226529950	100	74.34, 12.9	GQpSALGpSALGLISR	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Chuwei
TMT2	30	gi| 226529950	53	23.52	EGDDYApSDHGGDDIEDNLQSPLISR	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Sarlun
PIP2;7	39	gi| 514732737	96	46.68, 77.7	ALGpSFRpSTSATV	Zr^{4+}	LTQ-Velos	*Setaria italica*	Sarlun
Photosystem II protein H	60	gi| 11467220	96, 100	28.13	A(pT)Q(pT)VEDSSRPKPK	Zr^{4+}, TiO_2	LTQ-Velos	*Zea mays*	Chuwei, Sarlun
Calcium sensing receptor	52	gi| 514717642	100	18.42	IGpTTSSASR	Zr^{4+}	LTQ-Velos	*Imperata cylindrica*	Chuwei, Sarlun

Protein name	Mascot score	Accession number	Scaffold Probability(%)	Best Ascore	Phosphopeptide sequence	Enrichment method	Mass spectrometry	Organism	*Imperata* population
Non-membrane protein									
TUDOR-SN protein 1	106	gi| 514712805	100	1000	IWQYGDVEpSDEDEQAPAAR	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Chuwei, Sarlun
Pyruvate orthophosphate dikinase	62	gi| 108796050	100	10.28	GGMpTSHAAVVAR	TiO2	LTQ-FT	*Imperata cylindrica*	Chuwei
Chlorophyll a/b binding protein 2	46	gi| 226510115	99	17.74	pTAAKPKPAASGSPWYGADR	TiO2	LTQ-Velos	*Zea mays*	Chuwei
Similar to translational activator	83	gi| 242038321	99	31.81	AILEGGpSDDEGASTEAQGR	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Chuwei, Sarlun
NADP-malate dehydrogenase	48	gi| 242081803	98	14.47	QVQDGVApTAEAPATR	Zr^{4+}	LTQ-Velos	*Sorghum bicolor*	Chuwei
Sucrose-phosphate synthase 1	66	gi| 346685058	100		EA(pT)EDLAEDL(pS)EGEKGDTLGELAPVETAK	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Chuwei, Sarlun
Sucrose-phosphate synthase 1	50	gi| 346685058	100	64.62	NFpSDLTVWSDDNKEK	Zr^{4+}	Q-TOF	*Imperata cylindrica*	Chuwei, Sarlun
PEPcarboxylase 3	79	gi| 10185175	100		HGDFSDEGSATTEpSDIEETLKR	Zr^{4+}	Q-TOF	*Saccharum officinarum*	Sarlun
PEPcarboxylase 3	37	gi| 10185175	96	1000	HHpSIDAQLR	Zr^{4+}	Q-TOF	*Saccharum officinarum*	Sarlun

The amion acid residue in parenthesis indicates alternative phosphorylation site.

A

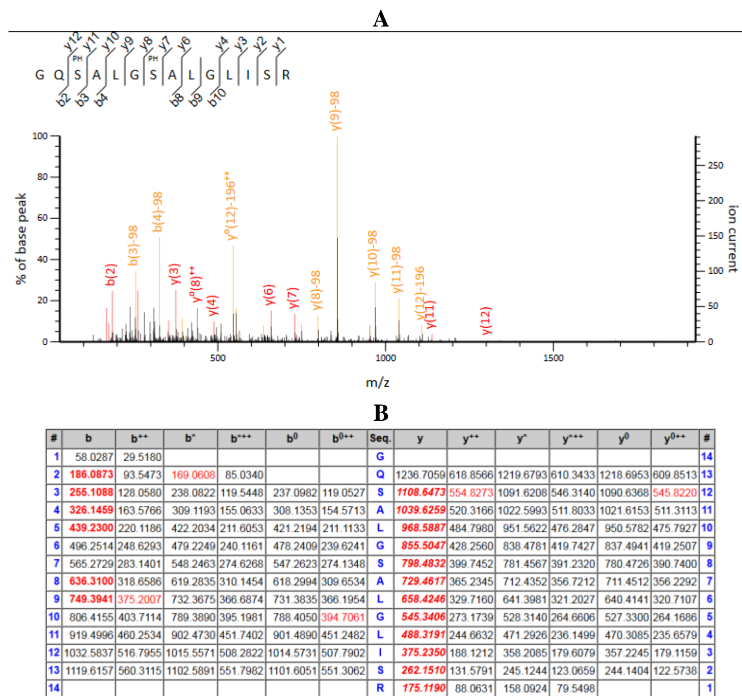


B

#	b	b++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	58.0287	29.5180			G							12
2	173.0557	87.0315	155.0451	78.0262	D	1253.5773	627.2923	1236.5508	618.7790	1235.5668	618.2870	11
3	230.0771	115.5422	212.0666	106.5369	G	1138.5504	569.7788	1121.5238	561.2656	1120.5398	560.7735	10
4	359.1197	180.0635	341.1092	171.0582	E	1081.5289	541.2681	1064.5024	532.7548	1063.5184	532.2628	9
5	472.2038	236.6055	454.1932	227.6003	L	952.4863	476.7468	935.4598	468.2335	934.4758	467.7415	8
6	601.2464	301.1268	583.2358	292.1216	E	839.4023	420.2048	822.3757	411.6915	821.3917	411.1995	7
7	714.3305	357.6689	696.3199	348.6636	L	710.3597	355.6835	693.3331	347.1702	692.3491	346.6782	6
8	881.3288	441.1680	863.3183	432.1628	S	597.2756	299.1414	580.2491	290.6282	579.2650	290.1362	5
9	980.3972	490.7023	962.3867	481.6970	V	430.2772	215.6423	413.2507	207.1290			4
10	1037.4187	519.2130	1019.4081	510.2077	G	331.2088	166.1081	314.1823	157.5948			3
11	1136.4871	568.7472	1118.4765	559.7419	V	274.1874	137.5973	257.1608	129.0840			2
12					R	175.1190	88.0631	158.0924	79.5498			1

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.

A

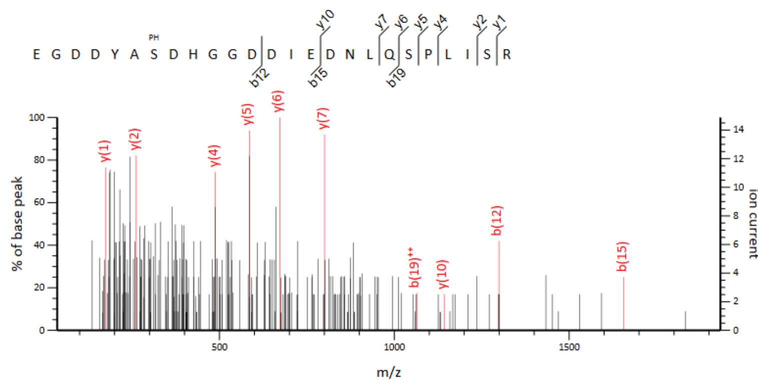


B

#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	58.0287	29.5180					G							14
2	186.0873	93.5473	169.0608	85.0340			Q	1236.7059	618.8566	1219.6793	610.3433	1218.6953	609.8513	13
3	255.1088	128.0580	238.0822	119.5448	237.0982	119.0527	S	1108.6473	554.8273	1091.6208	546.3140	1090.6368	545.8220	12
4	326.1459	163.5766	309.1193	155.0633	308.1353	154.5713	A	1039.6259	520.3166	1022.5993	511.8033	1021.6153	511.3113	11
5	439.2300	220.1186	422.2034	211.6053	421.2194	211.1133	L	968.5887	484.7980	951.5622	476.2847	950.5782	475.7927	10
6	496.2514	248.6293	479.2249	240.1161	478.2409	239.6241	G	855.5047	428.2560	838.4781	419.7427	837.4941	419.2507	9
7	565.2729	283.1401	548.2463	274.6268	547.2623	274.1348	S	798.4832	399.7452	781.4567	391.2320	780.4726	390.7400	8
8	636.3100	318.6586	619.2835	310.1454	618.2994	309.6534	A	729.4617	365.2345	712.4352	356.7212	711.4512	356.2292	7
9	749.3941	375.2007	732.3675	366.6874	731.3835	366.1954	L	658.4246	329.7160	641.3981	321.2027	640.4141	320.7107	6
10	806.4155	403.7114	789.3890	395.1981	788.4050	394.7061	G	545.3406	273.1739	528.3140	264.6606	527.3300	264.1686	5
11	919.4996	460.2534	902.4730	451.7402	901.4890	451.2482	L	488.3191	244.6632	471.2926	236.1499	470.3085	235.6579	4
12	1032.5837	516.7955	1015.5571	508.2822	1014.5731	507.7902	I	375.2350	188.1212	358.2085	179.6079	357.2245	179.1159	3
13	1119.6157	560.3115	1102.5891	551.7982	1101.6051	551.3062	S	262.1510	131.5791	245.1244	123.0659	244.1404	122.5738	2
14							R	175.1190	88.0631	158.0924	79.5498			1

Fig 2. MS/MS fragmentation pattern of a phosphopeptide of IcTMT2 from Chuwei ecotype. A, the phosphopeptide GQpSALGpSALGLISR 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.

A



B

#	b	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	130.0499	65.5286			112.0393	56.5233	E							25
2	187.0713	94.0393			169.0608	85.0340	G	2669.0999	1335.0536	2652.0733	1326.5403	2651.0893	1326.0483	24
3	302.0983	151.5528			284.0877	142.5475	D	2612.0784	1306.5429	2595.0519	1298.0296	2594.0679	1297.5376	23
4	417.1252	209.0662			399.1147	200.0610	D	2497.0515	1249.0294	2480.0249	1240.5161	2479.0409	1240.0241	22
5	580.1885	290.5979			562.1780	281.5926	Y	2382.0245	1191.5159	2364.9980	1183.0026	2364.0140	1182.5106	21
6	651.2257	326.1165			633.2151	317.1112	A	2218.9612	1109.9842	2201.9347	1101.4710	2200.9506	1100.9790	20
7	818.2240	409.6156			800.2135	400.6104	S	2147.9241	1074.4657	2130.8975	1065.9524	2129.9135	1065.4604	19
8	933.2510	467.1291			915.2404	458.1238	D	1980.9257	990.9665	1963.8992	982.4532	1962.9152	981.9612	18
9	1070.3099	535.6586			1052.2993	526.6533	H	1865.8988	933.4530	1848.8722	924.9398	1847.8882	924.4478	17
10	1127.3313	564.1693			1109.3208	555.1640	G	1728.8399	864.9236	1711.8133	856.4103	1710.8293	855.9183	16
11	1184.3528	592.6800			1166.3422	583.6748	G	1671.8184	836.4128	1654.7919	827.8996	1653.8079	827.4076	15
12	**1299.3797**	650.1935			1281.3692	641.1882	D	1614.7970	807.9021	1597.7704	799.3888	1596.7864	798.8968	14
13	1414.4067	707.7070			1396.3961	698.7017	D	1499.7700	750.3886	1482.7435	741.8754	1481.7594	741.3834	13
14	1527.4908	764.2490			1509.4802	755.2437	I	1384.7431	692.8752	1367.7165	684.3619	1366.7325	683.8699	12
15	**1656.5333**	828.7703			1638.5228	819.7650	E	1271.6590	636.3331	1254.6325	627.8199	1253.6484	627.3279	11
16	1771.5603	886.2838			1753.5497	877.2785	D	***1142.6164***	571.8118	1125.5899	563.2986	1124.6058	562.8066	10
17	1885.6032	943.3052	1868.5767	934.7920	1867.5927	934.3000	N	1027.5895	514.2984	1010.5629	505.7851	1009.5789	505.2931	9
18	1998.6873	999.8473	1981.6607	991.3340	1980.6767	990.8420	L	913.5465	457.2769	896.5200	448.7636	895.5360	448.2716	8
19	2126.7459	1063.8766	2109.7193	1055.3633	2108.7353	1054.8713	Q	***800.4625***	400.7349	783.4359	392.2216	782.4519	391.7296	7
20	2213.7779	1107.3926	2196.7513	1098.8793	2195.7673	1098.3873	S	***672.4039***	336.7056	655.3774	328.1923	654.3933	327.7003	6
21	2310.8307	1155.9190	2293.8041	1147.4057	2292.8201	1146.9137	P	***585.3719***	293.1896	568.3453	284.6763	567.3613	284.1843	5
22	2423.9147	1212.4610	2406.8882	1203.9477	2405.9041	1203.4557	L	***488.3191***	244.6632	471.2926	236.1499	470.3085	235.6579	4
23	2536.9988	1269.0030	2519.9722	1260.4898	2518.9882	1259.9977	I	375.2350	188.1212	358.2085	179.6079	357.2245	179.1159	3
24	2624.0308	1312.5190	2607.0043	1304.0058	2606.0202	1303.5138	S	***262.1510***	131.5791	245.1244	123.0659	244.1404	122.5738	2
25							R	***175.1190***	88.0631	158.0924	79.5498			1

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.

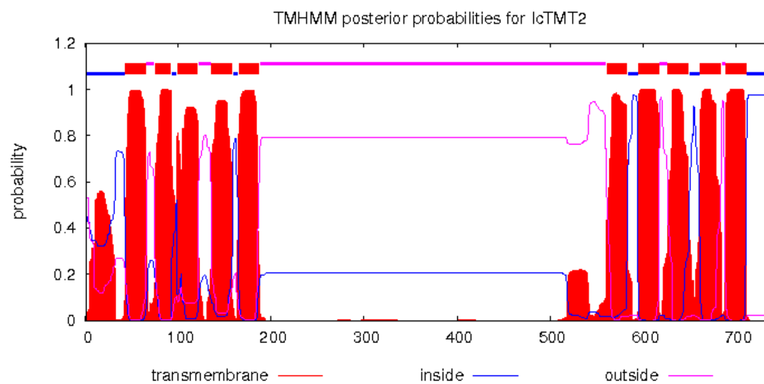


Fig 4. Predicted transmembrane topology of IcTMT2. The full-length protein sequence was subjected to prediction by TMHMM server v 2.0. The identified phosphorylation site is localized in the non-transmembrane domain, which explained the shaving region of IcTMT2 catalyzed by trypsin digestion.

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

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IcTMT2          EAKRVLQRLRGREDVSGEMALLVEGLGVGKDTRIEEYIIGPDDELAYEGL
gi|242040371    EAKRVLQRLRGREDVSGEMALLVEGLGVGKDTRIEEYIIGPDDELADEGL
gi|226529950    EAKRVLQRLRGREDVSGEMALLVEGLGVGKDTRIEEYIIGPDDELADEGL
gi|115483178    EAKRVLQGLRGREDVSGEMALLVEGLGVGKDTKIEEYIIGPDDELADEGL
gi|26986186     EAKRVLQRLRGREDVSGEMALLVEGLGVGKDTHFEEYIIGPDDELADDGL
gi|115445159    EAKKVLQKLRGREDVSGEMALLVEGLEVGADTSIEEYIIGPAIEPADEHV
                ***:*** ****************** ** ** :*******  * * : :

IcTMT2          AP-DPEKIKLYGPEEGLSWVARPVR--GQSALGSALGLISRHGSMAS-QG
gi|242040371    AP-DPEKIKLYGPEEGLSWVARPVR--GQSALGSALGLISRHGSMAS-QG
gi|226529950    AP-DPEKIKLYGPEEGLSWVARPVR--GQSALGSALGLISRHGSMAASQG
gi|115483178    AP-DPEKIKLYGPEEGLSWVARPVH--GQSALGSALGLISRHGSMVS-QG
gi|26986186     AP-DQEKLKLYGAEEGVSWIARPVRXGGQSALGSALGLMSRHGSMVS-QG
gi|115445159    VDGDKDQITLYGPEEGQSWIARPSK--GPSILGSVLSLTSRHGSMVN-QS
                .  * ::::.***.*** **:*** :  * * ***.*.* ******.  *.

IcTMT2          KPLVDPVVTLFGSVHAKMPEIMGSMRSTLFPNFGSMFSVAEQQQVKADWD
gi|242040371    KPLVDPVVTLFGSVHEKMPEIMGSMRSTLFPNFGSMFSVAEQQQVKADWD
gi|226529950    KPLVDPMVTLFGSVHEKMPEIMGSMRSTLFPNFGSMFSVADQQQVKADWD
gi|115483178    KPLVDPVVTLFGSVHEKMPEIMGSMRSTLFPNFGSMFSVAEQQQAKGDWD
gi|26986186     KSLVDPLVTLFGSVHEKMPEVMGSMRSTLFPNFGSMFSVAEQQQAKADWD
gi|115445159    VPLMDPIVTLFGSVHENMPHAGGSMRSTLFPNFGSMFSVTDQHPKVDQWD
                 .*:**:******** :**.  *****************::*:    :**

IcTMT2          TE-SQREGDDYASDHGGDDIEDNLQSPLISRQATSVEGKEIAAP-HGSIM
gi|242040371    AE-SQREGDDYASDHGGDDIEDNLQSPLISRQATSVEGKEIAAP-HGSIM
gi|226529950    AE-SQREGEDYASDHGGDDIEDNLQSPLISRQATSVEGKEIAAP-HGSIL
gi|115483178    AE-SQREGEDYGSDHGGDDIEDSLQSPLISRQATSVEGKEIAAP-HGSIM
gi|26986186     AE-SHRDDEDYASDHGADDIEDNLNSPLISRQATSVEGKEIAAP-HGSIM
gi|115445159    EENLHRDDEEYASDGAGGDYEDNVHSPLLSRQTTSAEGKDIAHHAHRGSA
                 *  :*:.::*.** ...* **.::***:***:**.***:**   * .

IcTMT2          GAVGRSSSLQ-GGEAVSSMGIGGGWQLAWKWTEREGEDGQMEGGFQRIYL
gi|242040371    GAVGRSSSLQ-GGEAVSSMGIGGGWQLAWKWTEREGEDGEKEGGFQRIYL
gi|226529950    GAVGRSSSLQ-GGEAVSSMGIGGGWQLAWKWTEREGEDGQKEGGFQRIYL
gi|115483178    GAVGRSSSLMQGGEAVSSMGIGGGWQLAWKWTEREGADGEKEGGFQRIYL
gi|26986186     GGVE-SSSMQ-GGDAVSSMGIGGGWQLAWKWTEREGADGQKEGGFQRIYL
gi|115445159    LSMRRRSLLEEGGEAVSSTGIGGGWQLAWKWSEREGEDGKKEGGFKRIYL
                 .:    * :  **:**** ***********:**** **: ****:****

IcTMT2          HEEGVQGN-RGSILSLPGG-DVPPGGEFIQAAALVSQPALYSKELLEQRA
gi|242040371    HEEGVQG--RGSILSLPGG-DVPPGGEFVQAAALVSQPALYSKELLEQRA
gi|226529950    HEEGVQGN-RGSILSLPGG-DVPPGGEFIQAAALVSQPALYSKELLEQRA
gi|115483178    HEEGVTGDRRGSILSLPGG-DVPPGGEFVQAAALVSQPALYSKELMEQRL
gi|26986186     HEEGVSGDRRGSILSMPGG-DIPPGGEYIQAAALVSQPALYSKDLIEQQL
gi|115445159    HQEEVPGSRRGSVISLPGGGDAPEGSEFIHAAALVSQPALYSKDIIEQRM
                *:* * *  ***::*:*** * * *.*:::**************:::**:
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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|26986186 (*Hordeum vulgare*), gi|115483178| (*Oryza sativa*), gi|115445159| (*Oryza sativa*), gi|242040371| (*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. Chuwei 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. Plants rhizomes collected from the field were washed by sterilized water and cultured in pots ($60\times 20 \times 20$ cm^3) 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

pump, and the solution was replaced every one week.

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 1× 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 Zr^{4+}–IMAC or TiO_2 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, NCBInr 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. Ascore for each phosphopeptide (Beausoleil *et al.,* 2006) was calculated and phosphopeptides were validated manually and using ScaffoldPTM (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 Zr^{4+}-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.

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