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CSS-Palm 2.0: an updated software for palmitoylation sites prediction

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Protein palmitoylation is an essential post-translational lipid modification of proteins, and reversibly orchestrates a variety of cellular processes. Identification of palmitoylated proteins with their sites is the foundation for understanding molecular mechanisms and regulatory roles of palmitoylation. Contrasting to the labor-intensive and time-consuming experimental approaches, *in silico* prediction of palmitoylation sites has attracted much attention as a popular strategy. In this work, we updated our previous CSS-Palm into version 2.0. An updated clustering and scoring strategy (CSS) algorithm was employed with great improvement. The leave-one-out validation and 4-, 6-, 8- and 10-fold cross-validations were adopted to evaluate the prediction performance of CSS-Palm 2.0. Also, an additional new data set not included in training was used to test the robustness of CSS-Palm 2.0. By comparison, the performance of CSS-Palm was much better than previous tools. As an application, we performed a small-scale annotation of palmitoylated proteins in budding yeast. The online service and local packages of CSS-Palm 2.0 were freely available at: http://bioinformatics.lcd-ustc.org/css_palm.

Keywords: clustering and scoring strategy/CSS-Palm/palmitoylated proteins/palmitoylation

Introduction

As a special class of post-translational modifications, numerous proteins could be covalently modified by a variety of lipids, including myristate (C14), palmitate (C16), farnesyl (C15), geranylgeranyl (C20), glycosylphosphatidylinositol (GPI) and so on (Casey, 1995; Nadolski and Linder, 2007; Resh, 2006a, 2006b). Although most of lipid modifications are irreversible, protein *S*-palmitoylation, also called as thioacylation or *S*-acylation, could reversibly attach 16-carbon saturated fatty acids to specific cysteine residues in protein substrates through thioester linkages (el-Husseini Ael and Bredt, 2002; Bijlmakers and Marsh, 2003; Dietrich and Ungermann, 2004; Smotrys and Linder, 2004; Resh, 2006a, 2006b; Roth *et al*., 2006; Greaves and Chamberlain, 2007; Linder and Deschenes, 2007; Nadolski and Linder, 2007; Wan *et al*., 2007). Palmitoylation will enhance surface hydrophobicity and membrane affinity of protein substrates, and plays important roles in modulating proteins' trafficking (Draper *et al*., 2007; Linder and Deschenes, 2007), stability (Linder and Deschenes, 2007), sorting (Greaves and Chamberlain, 2007) and so on. Also, protein palmitoylation has been involved in numerous cellular processes, including signaling (Casey, 1995; Resh, 2006a, 2006b), apoptosis (Chakrabandhu *et al*., 2007), neuronal transmission (el-Husseini Ael and Bredt, 2002) and so on. Although many efforts have been made in this field, the molecular mechanisms underlying protein palmitoylation still remain to be inexplicit.

Identification of palmitoylation proteins with their sites is fundamental for elucidating the molecular mechanisms and dynamics of palmitoylation processes. However, experimental identification of palmitoylation substrates with their sites is quite difficult, because there is not a common motif for palmitoylation recognition (el-Husseini Ael and Bredt, 2002; Bijlmakers and Marsh, 2003; Dietrich and Ungermann, 2004; Smotrys and Linder, 2004; Roth *et al*. 2006; Linder and Deschenes, 2007; Nadolski and Linder, 2007). Conventionally, palmitoylation sites were usually mapped by mutagenesis of candidate cysteine residues. Without any guidance or pre-prediction, such a procedure is time-consuming and labor-intensive. Recently, with a high-throughput, tandem mass spectrometry (MS/MS)-based proteomic methodology of MudPIT (multi-dimensional protein identification technology), a large-scale experiment was performed to identify ∼50 palmitoylated proteins in *Saccharomyces cerevisae* (Roth *et al*., 2006; Wan *et al*., 2007). However, the *bona fide* palmitoylation sites in most of these substrates still remained to be dissected. In this regard, computational prediction of palmitoylation sites *in silico* is urgent and greatly useful for further experimental verification.

In the field of computational lipid modifications, we and other researchers have taken great efforts to develop a variety of predictors (Eisenhaber *et al*., 1999; Eisenhaber *et al*., 2003; Bologna *et al*., 2004; Eisenhaber *et al*., 2004; Podell and Gribskov, 2004; Fankhauser and Maser, 2005; Maurer-Stroh and Eisenhaber, 2005; Xue *et al*., 2006; Zhou *et al*., 2006). In 1999, Eisenhaber *et al*. constructed the first web server of 'big-Pi predictor' to predict potential GPI-anchor sites from protein sequences (Eisenhaber *et al*., 1999). The model combined several distinct features of GPI-anchor sites with 11 upstream and 10 downstream amino acid residues (Eisenhaber *et al*., 1999, 2003, 2004). And Fankhauser *et al*. employed an artificial neural network algorithm to develop the GPI-SOM, with a window length of 32 amino acid residues (Fankhauser and Maser, 2005). For prediction of N-myristoylation proteins, there were at least three web tools constructed, including NMT (Maurer-Stroh *et al*., 2002a, 2002b; Eisenhaber *et al*., 2003), Myristoylator (Bologna *et al*., 2004) and PlantsP (Podell and Gribskov, 2004). And for prediction of prenylated proteins, Eisenhaber *et al*. developed the Prenylation Prediction Suite (PrePS) (Maurer-Stroh and Eisenhaber, 2005). Previously, we constructed two online severs of CSS-Palm 1.0 and NBA-Palm 1.0 to predict palmitoylation sites (Xue *et al*., 2006; Zhou *et al*., 2006). The CSS-Palm 1.0 was implemented in Clustering and Scoring Strategy (CSS) algorithm (Zhou *et al*., 2006), whereas the

NBA-Palm 1.0 was constructed with the Naïve Bayesian Algorithm (NBA) (Xue *et al.*, 2006).

In this work, we updated our previous CSS-Palm 1.0 into version 2.0. We manually collected the experimentally verified palmitoylation sites from scientific literature. The non-redundant training data contained 263 palmitoylation sites from 109 distinct proteins. Then an improved version of CSS algorithm was deployed. The leave-one-out (Loo) validation and 4-, 6-, 8- and 10-fold cross-validations were calculated to evaluate the prediction performance and system robustness of CSS-Palm 2.0. Again, the prediction performance was also tested on an additional data set not included in the training data set, with 53 palmitoylation sites in 26 proteins. By comparison with our previous CSS-Palm1.0 and NBA-Palm 1.0, the performance of CSS-Palm 2.0 was greatly improved. Finally, the online service and local packages of CSS-Palm 2.0 were implemented in JAVA 1.4.2 with high speed. The CSS-Palm 2.0 could predict potential palmitoylation sites for ~1000 proteins (with an average length of ~1000 amino acids) within 3 min. Taken together, we proposed that the CSS-Palm 2.0 will be a useful tool for experimentalists. The online service and local packages of CSS-Palm 2.0 were freely available at: http://bioinformatics.lcd-ustc.org/css_palm.

Materials and methods

Data preparation

We searched the scientific literature from PubMed with keywords of ‘palmitoylation’ or ‘palmitoylated’, and manually collected 340 experimentally verified palmitoylation sites in 145 proteins which were published before 8 October 2007. In this work, we arbitrarily took the 284 palmitoylation sites from 116 proteins published before November 2006 as the training data set. And the remaining 56 sites in 29 proteins were not included in training as an additional data set for performance evaluation. The protein sequences were retrieved from UniProt database (http://cn.expasy.org/uniprot).

As previously described (Xue *et al.*, 2006), we regarded the cysteine (C) residues that undergo palmitoylation modification as positive data (+), while all other non- palmitoylated cysteine residues were taken as negative data (−). The positive data set (+) for training might contain several homologous sites from homologous proteins. If the training data were highly redundant with too many homologous sites, the prediction accuracy would be overestimated. To avoid the overestimation, we clustered the protein sequences with a threshold of 40% identity by CD-HIT (Li and Godzik, 2006). If two proteins were similar with ≥40% identity, we re-aligned the proteins with BL2SEQ, a program in the BLAST package (Altschul *et al.*, 1997), and checked the results manually. If two palmitoylation sites from two homologous proteins were at the same position after sequence alignment, only one item was reserved while the other was discarded. Finally, the non-redundant data set for training contained 263 positive sites and 1150 negative sites from 109 substrates. And the non-redundant new data set contained 53 positive sites from 26 proteins. The training and new data sets are freely available upon request.

An upgraded algorithm of CSS

In CSS-Palm 1.0, the algorithm of CSS was employed (Zhou *et al.*, 2006). And the experimentally verified palmitoylation sites were automatically clustered into three clusters by different thresholds of peptides similarity (Zhou *et al.*, 2006). The clustering procedure was terminated, when the prediction performance was not significantly increased any more. Given a putative palmitoylation site for prediction, the CSS-Palm 1.0 will calculate a score between the sites with each cluster dependent on BLOSUM62 matrix, respectively. If the largest score was greater than the cut-off value, the putative site would be predicted as a positive hit.

In CSS-Palm 2.0, an updated version of CSS algorithm was used. First, we manually classified the known palmitoylation sites into three clusters, including Type I (sites follow a −CC− pattern, C is a cysteine residue), Type II (sites follow a −CXXC− pattern, C is a cysteine residue and X is a random residue) and Type III (other sites) group. Thus, the clustering procedure was based on experimental evidence rather than randomness. Then, we defined a *potential palmitoylation peptide PPP*(m, n) as a C residue flanked by m residues upstream and n residues downstream. By exhaustively testing, we chose *PPP*(25, 7), *PPP*(25, 16) and *PPP*(23, 15) for Type I, Type II and Type III palmitoylation sites, respectively. The training and prediction processes were separately performed on Type I, Type II and Type III palmitoylation sites, while the prediction results were integrated to calculate the final performance. Also, to improve the prediction performance, we developed a simple approach of matrix mutation (MaM). First, the BLOSUM62 was chosen as the initial matrix, and the Loo performance was calculated. Then, we fixed the specificity (Sp) as 85% to improve the sensitivity (Sn) by randomly picking out an element of the matrix for mutation. The procedure was terminated when the Sn value was not increased any more.

Performance evaluation

As previously described (Zhou *et al.*, 2006), we used four measurements such as Sn, Sp, accuracy (*Ac*) and *Mathew correlation coefficient* (MCC) to evaluate the prediction performance of the CSS-Palm 2.0. The four measurements were defined as below:

$$\mathrm{Sn} = \frac{\mathrm{TP}}{\mathrm{TP}+\mathrm{FN}}, \quad \mathrm{Sp} = \frac{\mathrm{TN}}{\mathrm{TN}+\mathrm{FP}},$$

$$\mathrm{Ac} = \frac{\mathrm{TP}+\mathrm{TN}}{\mathrm{TP}+\mathrm{FP}+\mathrm{TN}+\mathrm{FN}},$$

and

$$\mathrm{MCC} = \frac{(\mathrm{TP}\times\mathrm{TN})-(\mathrm{FN}\times\mathrm{FP})}{\sqrt{(\mathrm{TP}+\mathrm{FN})\times(\mathrm{TN}+\mathrm{FP})\times(\mathrm{TP}+\mathrm{FP})\times(\mathrm{TN}+\mathrm{FN})}}.$$

In this work, the Loo validation and 4-, 6-, 8- and 10-fold cross-validations were performed on the training data set (263 positive sites and 1150 negative sites). And the receiver operating characteristic (ROC) curves were drawn (Fig. 1). Also, the area under ROC (AROC) values were calculated as 0.8993 (Loo validation), 0.8732 (4-fold cross-validation, 4-fold), 0.8730 (6-fold cross-validation, 6-fold), 0.8864 (8-fold cross-validation, 8-fold) and 0.8982 (10-fold cross-validation, 10-fold). Thus, the results of 4-, 6-, 8- and 10-fold cross-validations were very similar with the Loo validation. In this regard, we took the Loo validation as an indicator of prediction performance of CSS-Palm 2.0.

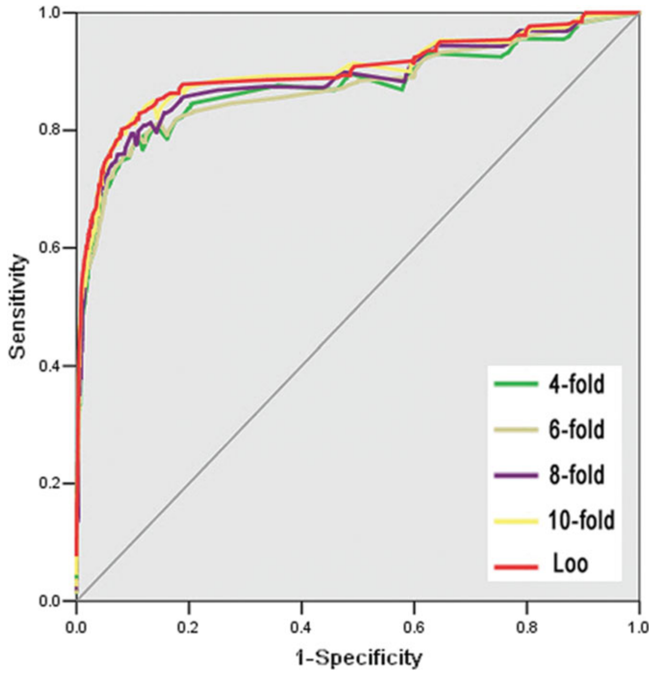


Fig. 1. The receiver operating characteristic (ROC) curves of leave-one-out (Loo) validation and 4-, 6-, 8- and 10-fold cross-validations (4-, 6-, 8- and 10-fold).

Also, we evaluated the robustness of CSS-Palm 2.0 with a new data set, including 53 verified palmitoylation sites in 26 substrates (published after Nov., 2006).

Implementation of the online service and local packages

The online service and local packages of CSS-Palm 2.0 were implemented in JAVA and freely available at http://bioinformatics.lcd-ustc.org/css_palm/prediction.php. For the online service, we tested the CSS-Palm 2.0 on a variety of internet browsers, including Internet Explorer 6.0, Netscape Browser 8.1.3 and Firefox 2 under Windows XP Operating System (OS), Mozilla Firefox 1.5 of Fedora Core 6 OS (Linux) and Safari 3.0 of Apple Mac OS X 10.4 (Tiger) and 10.5 (Leopard). For Windows and Linux systems, a latest version of Java Runtime Environment (JRE) package (JAVA 1.4.2 or later versions) of Sun Microsystems should be pre-installed for using the CSS-Palm 2.0 program. However, for Mac OS, the CSS-Palm 2.0 could be used directly without any additional packages. The online service of CSS-Palm 2.0 uses the local CPU for computation. Thus, the computing time is dependent on the users' computers. In our laptop (IBM ThinkPad R51, 1.60 GHz, 768 MB), it only cost <3 min to predict palmitoylation sites for 1000 protein sequences (average length ~1000 amino acids). For convenience, we also developed the local packages of CSS-Palm 2.0. The stand-alone software of CSS-Palm 2.0 supported three major OSs, including Windows, Linux and Mac.

Results

Development of the CSS-Palm 2.0 software

In this work, we used an updated version of CSS algorithm. The experimental results proposed that there is not a general consensus sequence for protein palmitoylation (el-Husseini Ael and Bredt, 2002; Bijlmakers and Marsh, 2003; Dietrich and Ungermann, 2004; Smotrys and Linder, 2004; Roth *et al.*, 2006; Linder and Deschenes, 2007; Nadolski and Linder, 2007). However, there are still some sequence patterns for a large proportion of palmitoylation sites. For example, in budding yeast, a DHHC cysteine-rich domain protein of Akr1p was identified as a palmitoyl transferase, to dual modify the casein kinase Yck2p at its C-terminal –CC– sequences (Roth *et al.*, 2002; Dietrich and Ungermann, 2004). Also, H-Ras was verified to be dual palmitoylated at its –CXXC– motif (Hancock *et al.*, 1989). Based on the experimental observations, we classified the known palmitoylation sites into three clusters, including Type I (sites follow a –CC– pattern, C is a cysteine residue), Type II (sites follow a –CXXC– pattern, C is a cysteine residue and X is a random residue) and Type III (other sites) cluster. Although several other motifs were also proposed, we adopted only the two major motifs for protein palmitoylation by performance comparisons. To improve the prediction performance, we also developed a simple method of MaM. By exhaustively testing, we fixed the Sp as 85% to improve the Sn by MaM. Successfully, both of the Loo validation and the performance on the new data set were greatly improved (Table I). Also, the *Ac* of Loo validation is very similar with the performance on the new data set. In this regard, the CSS-Palm 2.0 system is accurate and robust.

Finally, the online service and local packages of CSS-Palm 2.0 was implemented in JAVA 1.4.2 (J2SE). As an instance, the prediction results of human CD82 was shown (Fig. 2). The human CD82 (UniProt accession number: P27 701), also called as KAI1, is a member of

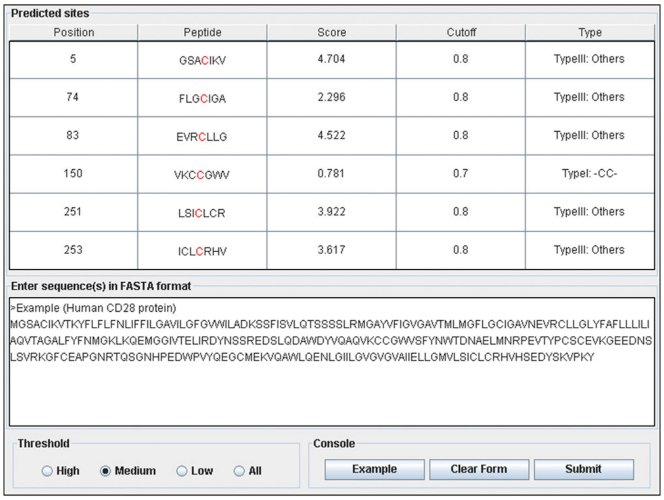
Table I. The performance of CSS-Palm 2.0 was greatly improved by matrix mutation (MaM)

CSS-Palm 2.0	Threshold	Leave-one-out				New data set			
		Ac (%)	Sn (%)	Sp (%)	MCC (%)	Ac (%)	Sn (%)	Sp (%)	MCC
Before MaM[a]	High	88.68	77.19	91.30	0.6495	89.00	56.60	93.82	0.5084
	Medium	82.38	82.89	82.26	0.5541	81.91	69.81	83.71	0.4256
	Low	69.43	87.83	65.22	0.4153	71.88	75.47	71.35	0.3303
After MaM[b]	High	89.60	77.19	92.43	0.6709	89.49	56.60	94.38	0.5227
	Medium	85.92	82.89	86.61	0.6142	86.31	73.58	88.20	0.5207
	Low	77.00	87.83	74.52	0.5024	76.28	81.13	75.56	0.4089

Both of the leave-one-out validation and the performance on the new data set were calculated and shown.

[a] Performance before MaM.

[b] The performance after MaM.



Predicted sites

Position	Peptide	Score	Cutoff	Type
5	GSACIKV	4.704	0.8	TypeIII: Others
74	FLGCIGA	2.296	0.8	TypeIII: Others
83	EVRCLLG	4.522	0.8	TypeIII: Others
150	VKCCGWV	0.781	0.7	TypeI: -CC-
251	LSICLCR	3.922	0.8	TypeIII: Others
253	ICLCRHV	3.617	0.8	TypeIII: Others

Enter sequence(s) in FASTA format

```
>Example (Human CD28 protein)
MGSACIKVTKYFLFLFNLIFFILGAVILGFGVWILADKSSFISVLQTSSSSLRMGAYVFIGVGAVTMLMGFLGCIGAVNEVRCLLGLYFAFLLLILI
AQVTAGALFYFNMGKLKQEMGGIVTELIRDYNSSREDSLQDAWDYVQAQVKCCGWVSFYNWTDNAELMNRPEVTYPCSCEVKGEEDNS
LSVRKGFCEAPGNRTQSGNHPEDWPVYQEGCMEKVQAWLQENLGIILGVGVGVAIIELLGMVLSICLCRHVHSEDYSKVPKY
```

Threshold ○ High ● Medium ○ Low ○ All

Fig. 2. The snapshot of CSS-Palm 2.0 JAVA applet. The prediction results of human CD28 protein were shown as an instance.

tetraspanin superfamily. Palmitoylation of CD82/KAI1 plays an essential role in inhibiting the migration and invasion of cancer cells (Zhou *et al*., 2004). The experimentally verified palmitoylation sites on CD82/KAI1 were mapped at position 5, 74, 83, 251 and 253 (Zhou *et al.*, 2004). With the default threshold (medium threshold), the CSS-Palm 2.0 could correctly predict the five sites as positive hits (Fig. 2). In addition, the C150 was also predicted as a positive hit to follow a –CC– (Type I) pattern. Thus, this site might also be a highly potential palmitoylation site and need further experimental verifications.

Comparison of CSS-Palm 2.0 with previous tools

Here, we compared the prediction performance of CSS-Palm 2.0 to CSS-Palm 1.0 and NBA-Palm 1.0. Previously, the NBA-Palm 1.0 was compared with CSS-Palm 1.0 on an old data set (210 experimental sites in 83 proteins) (Xue *et al.*, 2006; Zhou *et al.*, 2006). Since the training data set of CSS-Palm 2.0 is much larger than previous tools, it is not strange that the performance of CSS-Palm 2.0 is much higher on the training data set. To dissect whether the updated algorithm of CSS-Palm 2.0 is superior, we re-trained the CSS-Palm 2.0 with the old data set. The default thresholds were chosen for CSS-Palm 1.0 and NBA-Palm 1.0, respectively. Then, we fixed the Sn values of CSS-Palm 2.0 to be identical with previous tools and compared the Sp values (Table II). The prediction performance of CSS-Palm 2.0 was greatly improved against previous tools on the old data set. In this regard, the updated CSS algorithm was more useful and accurate. Also, we compared the prediction performance of CSS-Palm 2.0 with previous tools on the new data set (Table II). Again, the prediction results of CSS-Palm 2.0 were much better than the previous tools. Taken together, we proposed that CSS-Palm 2.0 would be more useful for experimentalists.

Annotation of palmitoylated proteins in budding yeast

Recently, Roth *et al*. (2006) carried out a large-scale experiment to identify palmitoylated proteins in *S. cerevisae*. Totally, there were 16 known palmitoylated proteins and 35 novel palmitoylated proteins reported. Then, we used the CSS-Palm 2.0 with high threshold to predict potential palmitoylation sites for these known and novel palmitoylated proteins (Table III). Under the high threshold, the *Ac*, Sn, Sp and MCC of CSS-Palm 2.0 were 89.60, 77.19, 92.43 and 0.6709, respectively. Successfully, CSS-Palm 2.0 could predict 12 of 16 (75%) known palmitoylated proteins with at least one site. And 26 of 35 (∼74%) novel palmitoylated proteins were predicted with at least one site.

Also, for the known palmitoylated proteins, we searched the UniProt database and scientific literature for their palmitoylation sites information. The ambiguous information with ‘By similarity’, ‘Potential’ and ‘Probable’ in UniProt database was not adopted. In our results, most of real palmitoylation sites were correctly predicted by CSS-Palm 2.0 (Table III). Only one site of Snc1 C95 was missed. And our predictions provided additional information and were useful for further experimental design. For example, although Yck1, Yck2 and Yck3 were verified as palmitoylated proteins, only the palmitoylation sites in Yck2 were clearly mapped as C545 and C546 (Roth *et al.*, 2006). Our prediction results proposed that Yck1 and Yck3 might be palmitoylated at C537, C538 and C517, C518, C519, C520, C522, C523 and C524, respectively. Again, although Gpa2 was proposed as a real palmitoylated protein, its palmitoylation sites information is still ambiguous (Roth *et al.*, 2006). Our results suggested that Gpa2 might be palmitoylated on a single cysteine residue at position 4 (Table III).

In the novel palmitoylated proteins, the palmitoylation sites on Rho2 and Rho3 were mapped at C188 and C5, respectively (Roth *et al.*, 2006). Our results could correctly predict these sites as positive hits (Table III). Again, eight amino acid permeases (AAPs) including Agp1, Bap2, Gap1, Gnp1, Hip1, Sam3, Tat1 and Tat2 were suggested to be palmitoylated at C-teminal cysteines (Table III) (Roth *et al.*, 2006). And our results predicted most of these C-terminal cysteine residues as positive hits.

Table II. Comparisons of CSS-Palm 2.0 with CSS-Palm 1.0 and NBA-Palm 1.0

Predictor	Old data set				New data set			
	Ac (%)	Sn (%)	Sp (%)	MCC (%)	Ac (%)	Sn (%)	Sp (%)	MCC
CSS-Palm 2.0	88.81	82.38	90.68	0.6982	89.49	64.15	93.26	0.5527
	90.31	67.62	96.94	0.7082	92.42	43.40	99.72	0.6161
CSS-Palm 1.0	82.94	82.16	83.17	0.5877	81.42	64.15	83.99	0.3887
NBA-Palm 1.0	86.67	67.46	92.25	0.6102	88.26	43.40	94.94	0.4287

The old data set included 210 palmitoylation sites from 83 proteins (Zhou *et al.*, 2006), while the new data set contained 53 palmitoylation sites in 26 proteins. The default thresholds were chosen for CSS-Palm 1.0 and NBA-Palm 1.0. Then we fixed the Sn values of CSS-Palm 2.0 to be identical with previous tools and compared the Sp values.

Table III. The prediction results for 16 known palmitoylated proteins and novel palmitoylated proteins in budding yeast

Protein	UniProt	Exp. sites	Predicted sites	Predicted palmitoylated peptides
Known palmitoylated proteins				
Ras1	P01119	305	305, 306, 309	303-GG**CC**II**C**-309
Ras2	P01120	318	318, 319	316-GG**CC**IIS – 322
Ste18	P18852	106	56, 106, 107	55-A**C**L-57, 104-SV**CC**TLM-110
Gpa1	P08539	3	3	1-MG**C**TV-5
Vac8	P39968	4, 5, 7	4, 5, 7, 106, 149	4-**CC**S**C**LK-9, 105-A**C**A-107, 148-G**C**I-150
Gpa2	P10823		4	2-GL**C**AS-6
Yck1	P23291		537, 538	534-KLG**CC-**538
Yck2	P23292	545, 546	545, 546	542-KLG**CC**-546
Yck3	P39962		517, 518, 519, 520, 522, 523, 524	515-KY**CCCC**F**CCC**-524
Bet3	P36149	80	80	78-PR**C**EN-82
Lcb4	Q12246	43, 46	43, 46, 358, 359	41-LS**C**LS**C**LD-48, 356-LM**CC**S-360
Akr1	P39010		443, 598	441-PG**C**LP-445, 596-QI**C**KG-600
Snc1	P31109	95		
Snc2	P33328			
Tlg1	Q03322			
Syn8	P31377			
Novel palmitoylated proteins				
Rho2	P06781	188	188, 189	186-AN**CC**IIL – 192
Rho3	Q00245	5	5, 228	3-FL**C**GS-7, 226-SS**C**TI-230
Ycp4	P25349		243, 244	241-LS**CC**TVM-247
Psr1	Q07800		9, 10	7-IL**CC**SS-12
Psr2	Q07949		9, 10	7-IL**CC**SS-12
Meh1	Q02205		7, 8	5-LS**CC**RN-10
Ygl108c	P53139		4	2-GL**C**GS-6
Ypl236c	Q12003		13, 14, 15	11-NL**CCC**RG-17
Lsb6	P42951		607	605-TW**C**-607
Ypl199c	Q08954		235	231-IFCN**C**IQ-237
Ykl047w	P36090		511, 516	509-PE**C**LGNL**C**-516
Ybr016w	P38216		119, 122	117-AL**C**IC**C**TM-124
Pin2	Q12057		4, 66, 79, 81, 82, 84	3-V**C**K-5, 65-T**C**F-67, 77-FI**C**W**CC**R**C**-84
Sna4	Q07549		2, 3, 5, 7, 8	1-M**CC**Y**C**V**CC**TV-10
Mnn1	P39106		17	15-RS**C**TIP-20
Ylr001c	Q07895		780	778-LF**C**II-782
Mlf3	P32047		2, 450	1-M**C**VYKS-5, 447-FNS**C**DT-452
Mse1	P48525		12, 169	10-SY**C**SP-14, 167-RC**C**AHL-172
Nuc1	P08466		2	1-M**C**SRI-5
Sso1	P32867			
Sso2	P39926			
Vam3	Q12241			
Tlg2	Q08144			
Mnn10	P50108			
Mnn11	P46985			
Tvp18	A6ZMD0			
Ylr326w	Q06170			
Amino acid permeases (AAPs)				
Agp1	P25376			
Bap2	P38084	609	435, 609	433-IV**C**CVF-438, 607- FW**C**-609
Gap1	P19145		397, 602	395-YA**C**SR-399, 600-FW**C**-602
Gnp1	P48813	663	193, 663	191-GS**C**VY-195, 601-FW**C**-603
Hip1	P06775	603	339, 397, 400, 603	338-G**C**L-340, 397-**C**SR**C**-400, 601-FW**C**-603
Sam3	Q08986		123, 377, 587	122-F**C**V-124, 376-S**C**V-378, 585-FW**C**-587
Tat1	P38085	619	619	617-FW**C**-619
Tat2	P38967		289, 592	288-T**C**L-290, 590-FW**C**-592

The predicted palmitoylation sites were marked in bold underline. The experimentally verified sites were taken from UniProt annotation or scientific literature. Eight amino acid permeases (AAPs) were proposed to be palmitoylated at C-teminal cysteines (Roth *et al.*, 2006).

Furthermore, Roth *et al.* (2006) suggested a novel sequence pattern for palmitoylation recognition. Thirteen palmitoylated proteins, including Snc1, Snc2, Tlg1, Syn8, Sso1, Sso2, Vam3, Tlg2, Mnn10, Mnn11, Pin2, Mnn1 and Ylr001c (Table III), were proposed to be potentially palmitoylated at cysteines cytoplasmically adjacent to their single transmembrane domains. However, these potential palmitoylation sites were still not experimentally verified during the past one and a half year. Thus, the new sequence pattern for palmitoylation was not adopted in current CSS-Palm 2.0. And the CSS-Palm 2.0 with high threshold generated only poor prediction on these proteins. We believed that the prediction performance of CSS-Palm 2.0 will be improved if these potential sites were experimentally verified and included into training data set.

Discussion

In this work, we updated our previous CSS algorithm with great improvement (Zhou *et al.,* 2006). First, the experimentally verified palmitoylation sites were classified into three clusters, including Type I (sites follow a –CC– pattern, C is a cysteine residue), Type II (sites follow a –CXXC– pattern, C is a cysteine residue and X is a random residue) and Type III (other sites) cluster. Both of training and prediction processes were separately performed on three types of palmitoylation sites. Also, the threshold values for three types of sites were different, dependent on final prediction performance. In addition, we developed a simple method as MaM to improve the prediction performance of CSS-palm 2.0.

Although it is very fast to predict potential palmitoylation sites for a single protein sequence, the speed of previous tools will be greatly slowed down if several users input multiple sequences simultaneously for prediction. Thus, both CSS-Palm 1.0 and NBA-Palm 1.0 only permitted a few proteins (<100) in FASTA format as input. The CSS-palm 2.0 was implemented in JAVA and used local CPU for computation. Thus, the calculating time is dependent on the users' computers. Also, the code of CSS-Palm 2.0 was greatly optimized. We tested the speed of CSS-palm 2.0 on a variety of computers. Even on a laptop (e.g. IBM ThinkPad R51, 1.60 GHz, 768 MB), CSS-palm 2.0 will predict out potential palmitoylation sites for ~1000 proteins (average length ~1000 amino acids) within 3 min. Thus, the CSS-palm 2.0 is more convenient for a large-scale scan. Moreover, the local packages of CSS-Palm 2.0 were developed and could support three major OSs, including Windows, Linxu/Unix and Mac.

As an application of CSS-Palm 2.0, we annotated the palmitoylation sites information for palmitoylated proteins in budding yeast. These substrates were generated from a large-scale experiment (Roth *et al.*, 2006). And the palmitoylation sites in most of these proteins are not experimentally verified. Our results could accurately predict out the known palmitoylation sites. Furthermore, our predictions provided more information and were useful for further experimental consideration. Taken together, we proposed that CSS-Palm 2.0 will be more useful for its fast-speed and superior performance.

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