

Understanding the role of *Oryza sativa* OsPILS (PIN Like) genes in auxin signaling

The phytohormone auxin is one of the most important signaling molecule that undergo accumulation or depletion in temporal or spatial manner due to wide arrays of changes in developmental or stress programme. Proper distribution, maintenance and homeostasis of auxin molecules across the plant system is one of the most important phenomena. The distribution and homeostasis of auxin is maintained by auxin transport system across the plant. Auxin transportation is carried out by auxin transporter family proteins, popularly known as auxin efflux carriers (PINs). Besides, auxin efflux carrier family proteins, a sub-family of auxin efflux carriers (OsPILS) being identified from *Oryza sativa* and reported here. *Oryza sativa* encodes for seven putative sub-cellularly localized transmembrane PILS proteins. Differential expression of OsPILS genes are found to be regulated by auxin and cytokinin dependent manner. In auxin treated plants, all OsPILS genes are up-regulated in leaf tissues and down regulated in root tissues during third week time point. In cytokinin treated plants, maximum of OsPILS genes were up-regulated both in leafs and roots tissues during third week time period. Regulation of gene expression of OsPILS genes by auxin and cytokinin during third week time period reflects its important roles during plant growth and development.

1 **Understanding The Role of *Oryza sativa* OsPILS (PIN Like) Genes in Auxin Signaling**

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1. Introduction

Phytohormone auxin plays important roles in plant growth and development by modulating process like cell division, cell elongation, vascular differentiation, apical dominance, tropic growth, embryogenesis, cell polarity, root architecture and root organogenesis (Sabatini et al. 1999; Friml and Palme 2002; Benková et al. 2003; Feraru et al. 2011). Majority of auxins are synthesized in the aerial parts of the plant and transported up to root the tips via vascular system and polarized auxin distribution system and maintains auxin homeostasis and distribution (Peer et al. 2010; Forestan and Varotto 2012). Besides vascular transport system present in plants, cell to cell transport system also present in the tissues which covers long and short distance auxin distributions across the plant (Zazimalová et al. 2010). Polar auxin efflux carrier (PIN) genes plays important roles in auxin distribution and homeostasis in plants in polarized manner (Friml and Palme 2002; Abas et al. 2006; Barbez et al. 2012). AtPIN1 gene was the first PIN gene to be cloned from plant *Arabidopsis thaliana* (Gälweiler et al. 1998). When it has reported that *Arabidopsis* pin-formed1 (pin1) mutant defective in auxin transport develops pin-like inflorescence, it became clear that PIN protein plays significant role in auxin efflux from the cells (Gälweiler et al. 1998). There are twelve PIN genes present in rice and eight PIN genes in found *Arabidopsis thaliana* (Blilou et al. 2005; Wang et al. 2009a). PIN genes shows distinct pattern of cellular and sub-cellular localization in root and shoot and shows tissue specific expression (Friml et al. 2002; Blilou et al. 2005; Paponov et al. 2005). AtPIN1 localizes polarly in plasma membrane, and upon pharmacological disruption, it immediately relocalizes, suggesting conceptual basis of auxin flux affects tropic response and patterning (Estelle 1998; Friml and Palme 2002; Ikeda et al. 2009; Forestan and Varotto 2012). It has reported that rice OsPIN1 gene expressed in root cap, OsPIN1b, OsPIN1c and OsPIN9 predominantly expressed in stele and OsPIN1b, OsPIN1c, OsPIN5a and OsPIN5b found to be expressed in meristem tissues (Wang et al. 2009b).

The plant specific PIN gene family of auxin efflux carriers are integral membrane proteins that contain inner and outer transmembrane domain and central hydrophilic domain (Petrásek et al. 2006; Křeček et al.

2009; Mravec et al. 2009). The N-terminal and C-terminal region of PIN proteins are conserved and the central hydrophilic loop region is very dynamic in nature among different PIN proteins (Kramer and Bennett 2006; Mohanta and Mohanta 2013a; Mohanta and Mohanta 2013b). Based on the divergence of central hydrophilic loop, PIN proteins are divided into different groups (Křeček et al. 2009). Although several PIN genes from different species and their function has been reported to date, there are only few reports been available regarding the roles of PIN like (PILS) genes in plants (Barbez et al. 2012). As PILS genes are described as PIN like genes, therefore I tried to analyze their roles of OsPILS by treating them with auxin and cytokinin.

2. Materials and Methods

2.1. Bioinformatics analysis

The PIN like (PILS) gene family of *Oryza sativa* was identified from publicly available rice genome annotation database (www.rice.plantbiology.msu.edu) (Ouyang et al. 2007). The OsPILS genes found from *Oryza sativa* genome was named according to orthology based nomenclature of *Arabidopsis thaliana* AtPILS genes (Hamel et al. 2006). Online available TMMOD (Hidden Markov Model for Transmembrane Domain) prediction software was used to analyze the transmembrane domains of OsPILS proteins (Kahsay et al. 2005). Swiss model work space (<http://swissmodel.expasy.org/workspace/>) was used to predict the auxin efflux carrier domains of OsPILS proteins. Multiple sequence alignment of OsPILS proteins with orthologous AtPILS proteins of *Arabidopsis thaliana* was carried out using clustalw software using BLOSUM programme. Phylogenetic tree of OsPILS, AtPILS, OsPIN and AtPIN genes of *Arabidopsis thaliana* and *Oryza sativa* was constructed using MEGA5 software (Tamura et al. 2011). Sub-cellular localization of OsPILS protein was predicted using online available software CELLO v.2.5: sub-cellular localization predictor (Yu et al. 2006).

2.2. Plant Materials and growth conditions

Oryza sativa plants were grown in half MS solid media with 10 μ M of auxin (Reinhardt et al. 2000; Guo et al. 2005; Petrásek et al. 2006) and cytokinin (Lorteau et al. 2001) treatment. Plants were grown up to

four weeks at 12 hour per day light period at 28° C. Light intensity for growth of rice plants was kept at 700 micro mol/m² s⁻¹. Phytohormone treated plants were harvested at each week interval (1st week, 2nd week, 3rd week and 4th week). Harvested plants were immediately transferred to liquid nitrogen and preserved them for further analysis. Three biological replicate samples were prepared for this study. Total RNA was extracted from pulled leaf and root samples separately from treated and non treated plants. Isolated RNA was subjected to cDNA synthesis. cDNA synthesis was carried out using Fermentas RevertAid first strand cDNA synthesis kit following manufacturer instructions. In short, reactions were prepared by adding 1.5 µg total RNA, 2 µl of 10x RT buffer, 2 µl of 10 mM dNTPs mix, 2 µl of random primers, 1 µl of reverse transcriptase, 1 µl ribolock RNase inhibitor and nuclease free sterile water up to 20 µl. Then the reaction mixtures were subjected to thermal incubation at 42°C for 60 minutes followed by reaction termination at 70°C for 5 minutes.

2.3. Expression Analysis of *OsPILS* Genes by qRT-PCR

Primer designing of *OsPILS* genes was carried out using primer3 software and detail list of primers are provided in table 1. Proper care was taken to design the primers for each gene so that there should not be any overlapping amplification between different *OsPILS* genes. As the central hydrophilic region of *OsPILS* genes is very much dynamic in nature, it provided very useful platform for designing specific primer sets. Further specificity of each *OsPILS* amplicon were confirmed by sequencing. Quantitative real time PCR of *OsPILS* genes were carried out by Applied Biosystems® viiA™ 7 real time PCR system using SYBR green PCR master mix (2x) (Fermentas) and ROX as passive reference standard to normalize SYBR green fluorescent signal. The PCR amplification was carried out in a 25 µl reaction mixture containing 1 µl of cDNA as template (1:10 dilution of cDNA from 20 µl RT-PCR product), 12.5 µl SYBR green qPCR master mix (2x), 1 µl of each forward and reverse primer and nuclease free water up to 25 µl. The thermal profile of qRT-PCR reaction was as follows; initial polymerase activation of 10 minutes at 95° C, and 40 cycles of 30 seconds at 95° C, 30 second at 60° C, and 30 seconds at 72° C. Triplicate pulled biological samples were used to analyze qRT-PCR datas. Three reference housekeeping genes (actin, tubulin and glyceraldehyde-3 phosphate dehydrogenase) were used to calibrate and normalize the result of

87 real time PCR. Relative expression of OsPILS genes were calculated using $2^{-\Delta\Delta CT}$ method (Schmittgen and
88 Livak 2008).

89 3. Results

90 3.1. Identification and Bioinformatics analysis of OsPILS genes

91 Rice OsPILS (PIN likes) genes were identified from rice genome annotation database. In total 7 OsPILS
92 genes were identified and detailed information of them are reported in table (Table 2). OsPILS1 is the
93 smallest OsPILS gene that contain open reading frame (ORF) of 1242 nucleotides length and OsPILS5 is
94 the largest OsPILS gene that contain ORF length of 3840 nucleotides length (Table 2). The genomic
95 organization of OsPILS genes show that OsPILS1, OsPILS6a, OsPILS6b, OsPILS7a contain 10 introns
96 each, OsPILS5 have 7, OsPILS7b have 9 and OsPILS2 have only one intron each (Figure 1).
97 Chromosomal distribution of OsPILS genes shows that OsPILS1, OsPILS7a and OsPILS7b are distributed
98 in distal end of chromosome 9 whereas OsPILS2 present in chromosome 8, OsPILS5 in chromosome 7,
99 OsPILS6a in chromosome 1 and OsPILS6b in chromosome 5 (Figure 2).

100 Rice OsPILS proteins were subjected to swiss workspace model to predict their auxin efflux carrier
101 domains. It was found that all OsPILS proteins contain auxin carrier domains (Figure 3). Multiple
102 sequence alignment of OsPILS proteins were carried out to find conserved domains and motifs of OsPILS
103 proteins. Result shows presence of several conserved domains, and motifs within the OsPILS gene
104 sequences (Figure 4). These conserved sequences are present both at N- and C-terminal region of OsPILS
105 proteins and central hydrophilic region is variable in nature. The major N-terminal conserved domains
106 present in OsPILS proteins was N-x-G-N (except OsPILS5). Instead of containing N-x-G-N consensus
107 sequence, OsPILS5 contain G-x-S-S consensus sequence. The C-terminal region of OsPILS proteins also
108 conserved and contains A-P-L and G-G-N-L (Figure 4) consensus sequences. The central hydrophilic
109 domains don't have any conserved domains but contain conserved threonine amino acid residue. Earlier it
110 has reported that central hydrophilic region of PIN proteins don't have any conserved structure and are
111 variable in nature (Křeček et al. 2009; Mohanta et al. 2014). To know more details about the sequence

similarities of PIN proteins with OsPILS proteins, multiple sequence alignment of OsPILS, OsPIN, AtPILS and AtPIN proteins were carried out together. Sequence alignment shows no sequence similarity between PIN and OsPILS proteins (Supplementary Figure).

Auxin efflux carriers are membrane bound (except OsPILS5) proteins (Geldner and Palme 2001; Laxmi et al. 2008). So protein sequences of OsPILS were analyzed to confirm whether they are membrane bound or not. All OsPILS proteins are found to be transmembrane bound and present inside as well as outside of membrane except OsPILS5 (Figure 5). OsPILS5 have only outside domain, whereas the inside as well as the transmembrane domain are absent from it.

3.2. Phylogenetic analysis of *OsPILS*

Phylogenetic analysis of OsPILS proteins with OsPIN, AtPILS and AtPINs proteins were carried out using MEGA5 software. Phylogenetic analysis shows that PIN and OsPILS genes are falls in to two different clades distinctly (Figure 6). The OsPILS genes clades are also sub-grouped into three different sub-groups. In phylogenetic tree OsPILS5, OsPILS7 grouped with AtPILS5; AtPILS7 and OsPILS1 grouped with AtPILS1; OsPILS2 grouped with AtPILS2; OsPILS6a and OsPILS6b grouped with AtPILS6 (Figure 6). From the phylogenetic study, it's clear that there is absence of OsPILS3, and OsPILS4 genes in rice, an orthologous counterpart of AtPILS3 and AtPILS4 gene respectively.

3.3. Expression of *OsPILS* genes in leaf tissue treated with IAA

In order to determine the expression pattern of *OsPILS* genes in rice, leaf tissues treated with 10 μ M IAA were taken for quantitative real time PCR analysis. Part of the open reading frame of *OsPILS* genes were taken for qRT-PCR analysis. Expression level of *OsPILS* genes of untreated rice plants were used as control sample. All the experiments were carried out from 7, 14, 21 and 28 days (1 week, 2 week, 3 week and 4 week) old rice plants.

The expression level of *OsPILS1*, *OsPILS6b*, *OsPILS7a* genes were significantly up-regulated and expression level of *OsPILS2*, *OsPILS5*, *OsPILS6a* were significantly down-regulated in 7 days old rice plants (Figure 7). Expression level of *OsPILS7b* was not detected in 7 days old rice plants. In 14 days old plants; *OsPILS2*, *OsPILS6a* and *OsPILS7b* genes were up-regulated and *OsPILS1*, *OsPILS5*, *OsPILS6b* and *OsPILS7a* genes were down regulated. The expression level of all the *OsPILS* genes were more than two fold up-regulated in 21 days old rice plants. In 28 days old plants; *OsPILS1*, *OsPILS2*, *OsPILS5* and *OsPILS6b* genes were up-regulated and *OsPILS6a*, *OsPILS7a* and *OsPILS7b* genes were down regulated (Figure 7).

3.4. Expression of *OsPILS* genes in root tissue treated with IAA

The expression level of *OsPILS1*, *OsPILS2*, *OsPILS5* and *OsPILS6b* genes were significantly up-regulated and expression level of *OsPILS6a*, *OsPILS7a* and *OsPILS7b* genes were significantly down regulated in 7 days old rice seedlings (Figure 7). In 14 days time periods; only *OsPILS6a* and *OsPILS7a* genes were up-regulated whereas all other *OsPILS* genes were found to be down regulated. In 21 days time point, expression levels of all *OsPILS* genes were down regulated. In 28 days time point, except *OsPILS5*, all other *OsPILS* genes were found to be down regulated (Figure 7).

3.5. Expression of *OsPILS* genes in leaf tissue treated with cytokinin

The expression level of *OsPILS1*, *OsPILS6a*, *OsPILS6b*, *OsPILS7a* and *OsPILS7b* genes were up-regulated and *OsPILS2* and *OsPILS5* genes were down regulated in cytokinin treated 7 days old rice seedlings (Figure 8). In 14 days time period; *OsPILS1*, *OsPILS5*, *OsPILS6a*, *OsPILS6b*, *OsPILS7a* and *OsPILS7b* genes were up-regulated except *OsPILS2* gene. In 21 days time period, all the *OsPILS* genes were up-regulated except *OsPILS5* gene (Figure 8). In 28 days time period, expression level of *OsPILS1*, *OsPILS2* and *OsPILS6b* genes were up-regulated and expression level of *OsPILS5*, *OsPILS6a*, *OsPILS7a* and *OsPILS7b* genes were found to be down regulated.

3.6. Expression of *OsPILS* genes in root tissue treated with cytokinin

Gene expression level of *OsPILS5* and *OsPILS6b* genes in cytokinin treated root tissue were up-regulated and expression level of *OsPILS1*, *OsPILS2*, *OsPILS6a*, *OsPILS7a* and *OsPILS7b* genes were down regulated in 7 days rice seedlings (Figure 8). In 14 days time period; all the *OsPILS* genes were up-regulated except *OsPILS6a*. In 21 days time point, *OsPILS1*, *OsPILS2*, *OsPILS6a*, *OsPILS6b* and *OsPILS7b* genes were up-regulated whereas *OsPILS5* and *OsPILS7a* genes were found to be down regulated (Figure 8).

4. Results and Discussion

Auxin (indole-3-acetic acid) is one of the most important phytohormone found in plants and has unique position among plants as growth regulatory substances (Dubrovsky et al. 2008; Ikeda et al. 2009; Möller and Weijers 2009; Halliday et al. 2009; Scarpella et al. 2010; Krupinski and Jönsson 2010; McSteen 2010; Overvoorde et al. 2010). Auxin acts as a prominent signaling molecule, provided by its local accumulation or depletion in selected cells, a spatial and temporal reference for changes in developmental programmes. The temporal and spatial distribution of auxin relies on its metabolism (biosynthesis, conjugation and degradation) and cellular transports (Friml 2003; Woodward and Bartel 2005). Interaction and coordinated auxin transport systems in plants underlie on a flexible networks that metabolizes auxin in response to several environmental as well as developmental changes encountered by plants (Laxmi et al. 2008; Halliday et al. 2009). Auxin distribution and its differential accumulation levels for different plant tissues are created in response to internal developmental programmes or endogenous signaling cascades (Muday and Murphy 2002; Leyser 2006; Krupinski and Jönsson 2010). This process is carried out by auxin metabolism and transports. Auxin transport system shown to be involved in modulation of plant developments, and multiple transporter proteins are required to maintain directional auxin flows within and between different organs and tissues. Thereby, plants accommodated maximum number of auxin transporter proteins within itself including PINs and PILS genes to broaden its optimum functionalities. These auxin transports are being realized over both short and long distance distributions (Petrásek and Friml 2009; Zazimalová et al. 2010). Auxin molecules can move to long distances through vascular system by mass flow or cell-to-cell movements by polar auxin transport systems (Zazimalová et al. 2010).

Members of auxin efflux carrier proteins associated with auxin transports were characterized by their presence as integral membrane proteins (Muday and Murphy 2002). In the present study all the OsPILS proteins are predicted to be membrane localized and contain putative auxin efflux carrier domains (Figure 3). Each PILS gene posse's central hydrophilic loop flanked each side by presence of five transmembrane domains. Auxin efflux carriers (PINs) are divided as long (PIN1, PIN2, PIN4 and PIN7) and short (PIN3, PIN5) efflux carrier proteins depending upon their length of hydrophilic loop (Zazimalová et al. 2010). The long efflux carriers are mostly shows polar plasmamembrane localization and provided directional auxin transport and play major roles in auxin dependent process in plant developments, embryo development, organogenesis and tropism (Blilou et al. 2005). In contrast to long efflux carriers, the short efflux carriers proteins has reduced central hydrophilic loop and don't localizes to plasmamembrane (Křeček et al. 2009). They usually localized to endoplasmic reticulum (AtPIN5). Its localization in endoplasmic reticulum suggest its role in intracellular auxin distribution and regulation of cellular auxin homeostasis (Mravec et al. 2009). In the present study, it has found that, all the OsPILS (OsPILS1, OsPILS2, OsPILS6a, OsPILS6b, OsPILS7a and OsPILS7b) proteins contains short hydrophilic loop and hence supposed to be endomembrane localized (Table 3). Finding presented in this manuscript also closely matches with finding of Barbez et al., (2012). In the phylogenetic tree, the short transmembrane domain containing proteins OsPIN5 and AtPIN5 are present very closely with OsPILS proteins (Figure 6). Both sub-cellular localization prediction and results from phylogenetic tree gives enough information regarding the presence of short hydrophilic loop of OsPILS genes. Earlier it was reported that, maximum of PIN proteins are plasmamembrane localized and present in polarized fashion but are usually non static in nature (Friml and Palme 2002; Zazimalová et al. 2010; Viane et al. 2013). They undergoes constitutive recycling between plasmamembrane and endomembrane compartments and can easily re-localize rapidly to various parts of cell by transcytosis in response to different environmental signals (Geldner et al. 2001; Dhonukshe et al. 2007; Kleine-Vehn et al. 2008). Maximum of OsPILS genes were found to be sub-cellularly localized either in endoplasmic reticulum and vacuoles (Table 3). Presence of OsPILS proteins in endoplasmic reticulum/vacuoles that facilitates auxin accumulation inside the cells seems to contribute the possibility of compartmentalized regulation of auxin metabolism (Barbez et al. 2012). It could be

211 speculate that auxin conjugation may occurs in the endomembranes including endoplasmic reticulum and
 212 vacuoles (Barbez et al. 2012). But, the specific molecular compartment for the action of auxin metabolism
 213 is yet to be elucidated. Multiple sequence alignment results shows that, OsPILS proteins contain N- and
 214 C-terminal conserved consensus sequences. N-terminal conserved consensus sequence is N-x-G-N and C-
 215 terminal conserved consensus sequences are A-P-L and G-G-N-L. Independent functionalities of these
 216 domains are yet to be reported and hence more in depth investigations are required to infer the
 217 functionalities of N and C-terminal conserved consensus sequences. Although, central hydrophilic loop of
 218 PILS proteins are very variable in nature, still they contain conserved threonine amino acid residue within
 219 it. Threonine amino acid is probable target phosphorylation site of upstream kinases (Sinha et al. 2011).
 220 Presence of conserved threonine amino acid in all PILS genes indicates that their functions are regulated
 221 by kinase modulated phosphorylation process. The sequence alignment show that alignment of OsPILS
 222 protein starts at the end of alignment of PIN proteins (Supplementary Figure). It was reported that PILS
 223 genes are evolutionarily conserved among unicellular algae to higher eukaryotic plants like gymnosperm
 224 and angiosperm (Barbez et al. 2012). Starting of OsPILS proteins alignment at the end of PIN protein
 225 alignment explains, both PINs and OsPILS genes were probably coexisted together in the early time
 226 period of evolution and then diversified and diverged during evolution when plants evolved from simpler
 227 aquatic habitat to complex terrestrial habitat.__

228 In 2012 first report was published regarding presence of PILS genes (sub-family of PIN genes) from
 229 *Arabidopsis thaliana* (Barbez et al. 2012). They reported the presence of seven AtPILS genes in *A.*
 230 *thaliana*. Genome wide identification of PILS genes in *O. sativa* led to identification of seven OsPILS
 231 genes. OsPILS genes are considered as PIN like genes as the topology of OsPILS proteins are found to be
 232 similar to the topology of PIN proteins. Although PIN and PILS proteins share similar predicted protein
 233 topology, they don't share pronounced protein sequence identity (Barbez et al. 2012). That's why it was
 234 very difficult to identify the PILS proteins by conventional BLAST approach. Interpro scan analysis of all
 235 OsPILS genes shows presence of auxin career domain. PILS proteins are conserved throughout the plant

lineage from unicellular algae from *Ostreococcus* and *Chlamydomonas* (where PIN genes are absent) to angiosperm (Barbez et al. 2012). This indicates that PILS proteins are evolutionarily older than PIN proteins. To understand the role of PILS as auxin carrier, Barbez et al., (2012) performed PILS2 oestradiol-inducible tobacco BY-2 cell cultures and performed ³H-IAA accumulation assay. They found that AtPILS2 induction increased the accumulation of radioactivity in BY-2 cells. This indicated that AtPILS2 carry out auxin transport process. In accordance with auxin accumulation assay in BY-2 cells, *pils2 pils5* double-mutant protoplast showed significantly higher level of auxin export indicating reduced auxin retention capacity in loss of function mutant (Barbez et al. 2012). Expression of *AtPILS2*, *AtPILS3* and *AtPILS7* in *Saccharomyces cerevisiae* yeasts also led to increased retention of exogenously applied auxin.

Barbez et al., (2012) also reported that *AtPILS* genes are expressed broadly in different tissue and they found that *AtPILS2-AtPILS7* were transcriptionally up-regulated by application of auxin. In the present study, *O. sativa* plants were treated with auxin and cytokinin (10 µM) respectively and analyzed their expression level in leaf and root tissues. The major finding of this transcriptome analysis was that, all the *OsPILS* genes were up-regulated at third week time points in auxin treated leaf tissues (Figure 7). In contrast to this, all the *OsPILS* genes in auxin treated root tissues were down regulated at third week time point (Figure 7). In cytokinin treated leaf tissues, except *OsPILS5* all other *OsPILS* genes were up-regulated at third week time point (Figure 8). Similarly, except *OsPILS5* and *OsPILS7a* other *OsPILS* genes were up-regulated in cytokinin treated root tissue at third week time point (Figure 8). The exceptional differential gene expression of *OsPILS5* gene may be attributed to its different topology in transmembrane domain with other *OsPILS* genes. Up-regulation of maximum of *OsPILS* genes at third week time point reflects their significant roles in growth and development during this time period. This study also reflects some important aspects of *O. sativa* plant development at third week time period and major hormonal as well as developmental changes might occurred during this time period to shape the plant for its future development. *OsPILS1* gene was up-regulated in first, third and fourth week time points in auxin treated leaf tissues (Figure 7) and down regulated at second, third, and fourth week time

262 period in auxin treated root tissues (Figure 7) respectively. *OsPILS2* gene was consistently up-regulated in
 263 second, third, and fourth week time period in auxin treated leaf tissues (Figure 7) and conversely, down
 264 regulated in second, third and fourth week time period in auxin treated root tissues (Figure 7). This
 265 suggests that application of exogenous auxin reversed the expression of respective *OsPILS* genes in root
 266 tissues. In the case of *OsPILS6a*, it was up-regulated in second week time period both in auxin treated leaf
 267 and root tissues (Figure 7). But in majority of cases, in auxin treated root tissues, *OsPILS6a* was down
 268 regulated in first, third and fourth week time point. Up-regulation of *OsPILS6a* in auxin treated leaf and
 269 root tissues suggest its important role during second week time period. In the case of *OsPILS6b*, its
 270 expression level was up-regulated in auxin treated leaf tissues (except second week time period) (Figure 7)
 271 and was down regulated at second, third and fourth week time period in root tissues (Figure 7).
 272 Application of exogenous auxin reversed the expression of *OsPILS* genes in root tissues. In the case of
 273 *OsPILS7b*, gene expression was down regulated in auxin treated leaf tissues in all time points (Figure 7).
 274 This suggests that exogenous application of auxin has significant effects in *OsPILS7b* gene expression in
 275 the root tissues. In fourth week time period, except *OsPILS5* all other *OsPILS* genes were down regulated
 276 in auxin treated root tissues (Figure 7). So, differential expression of *OsPILS* genes with response to
 277 exogenous auxin treatment indicates their roles in auxin-regulated process in *O. sativa*.

278 In the case of cytokinin treated leaf tissues, *OsPILS1* and *OsPILS6b* genes were up-regulated in all four
 279 time course experiments (Figure 8). Similarly, *OsPILS6a*, *OsPILS7a* and *OsPILS7b* genes were also up-
 280 regulated in first, second and third week time period suggesting their important roles in cytokinin treated
 281 leaf tissues (Figure 8). In the third week time period, except *OsPILS5*, all other genes were up-regulated in
 282 cytokinin treated leaf tissues. Significant different behavior of gene expression of *OsPILS5* may be due to
 283 absence of proper transmembrane domain and presence of little bit dissimilar topology as compared to
 284 other *OsPILS* proteins. Almost similar trends of gene expression of *OsPILS5* gene was observed in auxin
 285 treated leaf tissues in the same time period compared to other *OsPILS* genes. *OsPILS* genes undergoing
 286 up-regulation in leaf tissues at third week time period in both auxin and cytokinin treated plants shows its
 287 significant roles in plant developmental process during these time period. Five *OsPILS* genes (except

288 *OsPILS5* and *OsPILS7a*) among seven *OsPILS* genes were up-regulated in cytokinin treated root at third
289 week time period (Figure 8). This finding is exactly opposite to the gene expression patterns found in
290 auxin treated root tissues where all genes were down regulated. In the fourth week time period, maximum
291 (except *OsPILS5*) of *OsPILS* genes were down regulated in root tissues irrespective of auxin and
292 cytokinin treatment.

293 Potential function of *AtPILS* genes of auxin efflux carrier facilitator in plant development was analyzed
294 and reported earlier (Barbez et al. 2012). They carried out ectopic over expression of *AtPILS1* and
295 *AtPILS3* genes using 35S viral promoter and found that dwarfed/or bushy plants showing sever defects in
296 flower development that led to sterility in T1 generation. Flowers of these *AtPILS* over expressing plants
297 were found to have severe patterning defects like homeotic transformation of flower organs into new
298 flower buds, triplication of gynoecium or fused carpels. They found that *AtPILS2* and *AtPILS5* were the
299 most abundantly expressed genes in seedling stage. Similar result was found in the case of *OsPILS2* gene.
300 They reported that *AtPILS2* and *AtPILS5* showed overlapping expression in root meristematic zone
301 suggesting their redundant role in regulating root growth. This was found to be true in the case of
302 *OsPILS2* and *OsPILS5* genes. Both of these genes were up-regulated in root tissues in early phase (first
303 week) of plant development thus suggesting their crucial roles in root development. *AtPILS5* gain of
304 function mutant had reduced lateral rooting. Expression of *AtPILS1*, *AtPILS3* and *AtPILS5* under root hair
305 specific promoter in auxin treated condition led to repress the root hair growth. This may be due to PILS-
306 dependent regulation of auxin signaling and homeostasis. When *AtPILS5* gain- and *pils2 pils5* loss-of
307 function mutant treated with exogenous auxin, *pils2 pils5* loss- and *AtPILS5* gain of function mutant
308 showed hyper- and hyposensitive root growth respectively. These finding suggests the requirement of
309 PILS protein activity in auxin-dependent growth and regulation.

310 In this manuscript, genome wide identification of *OsPILS* genes has uncovered, a distinct protein family
311 that regulates intracellular auxin transport and homeostasis. Manuscript highlighted the evolutionary and
312 developmental importance *OsPILS* in auxin mediated regulation in rice plants and correlated these
313 findings with *AtPILS* genes. More, in depth investigations are necessary to understand the potential

314 interplay or possible diversified function of endoplasmic reticulum localized OsPILS genes as well as
315 AtPILS genes in plants.

316 **Contribution**

317 Tapan Kumar Mohanta: Conceived the project, carried out the experiment, analyzed the datas and
318 prepared the manuscript draft for publication.

319 Nibedita Mohanta: Analyzed data, drafted the manuscript.

320 **Conflict of interest:** Author declares there is no any conflict of interest in this manuscript.

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425 **Figure 1**

426 Figure showing genomic organization of different OsPILS genes. Black colored boxes represent the exons
427 and lines represent the introns of different OsPILS genes. In majority of cases, maximum of OsPILS gene
428 contains ten exons in their gene.

429 **Figure 2**

430 Figure showing distribution of OsPILS genes in rice chromosomes. All the seven OsPILS genes
431 distributed towards the distal end of chromosome. Chromosome 9 harbors maximum of three OsPILS
432 genes whereas chromosome 1, chromosome 5, chromosome 7 and chromosome 8 harbor one OsPILS
433 gene each.

434 **Figure 3**

435 Figure represents auxin efflux carrier domain of OsPILS gene. Swiss workspace model
436 (<http://swissmodel.expasy.org/workspace/>) was used to predict the auxin efflux carrier domain of OsPILS
437 genes. All the OsPILS genes contain auxin efflux carrier domain.

438 **Figure 4**

439 Multiple sequence alignment of OsPILS proteins show presence of several conserved amino acid residues
440 as well as motifs in N- and C-terminal region. Major conserved motifs present in N-terminal region is N-
441 x-G-N (in box) except OsPILS5. The C-terminal region contain conserved A-P-L and G-G-N-L (in box)
442 consensus sequences. Although central hydrophilic region of OsPIL gene is very dynamic in nature, still it
443 contain conserved T amino acid (Threonine) residue (boxed) in central hydrophilic region.

444 **Figure 5**

445 Prediction of transmembrane domains of OsPILS genes using TMHMM server 2.0
446 (<http://www.cbs.dtu.dk/services/TMHMM/>). Prediction shows that maximum of OsPILS genes contain
447 short transmembrane domain.

448 **Figure 6**

449 Phylogenetic tree of OsPIN, AtPIN, AtPILS and OsPILS genes. Phylogenetic analysis shows, PINs and
450 PILS genes are grouped into two distinct clades. In the phylogenetic tree, short transmembrane domain
451 containing protein OsPIN5 and AtPIN5 lies proximately towards short transmembrane domain containing
452 AtPILS and OsPILS genes. This indicates that PILS genes are short transmembrane domain containing
453 proteins. Phylogenetic tree was constructed using MEGA5 software. Programme used to construct the
454 phylogenetic tree was; statistical method: maximum likelihood, test of phylogeny: bootstrap method, no.
455 of bootstrap replication: 1000, substitution type: amino acids, models/methods: Jones-Taylor-Thornton
456 (JTT) and branch swap filter was very strong.

457 **Figure 7**

458 Relative expression of OsPILS Genes in Leaf and root tissues treated with 10 μ M IAA. Relative gene
459 expression of OsPILS genes shows all the OsPILS genes were up-regulated in auxin treated leaf tissues
460 and down-regulated in auxin treated root tissues at 21 days time period.

461 **Figure 8**

462 Relative expression of OsPILS genes in leaf and root tissues treated with 10 μ M BAP. From the result we
463 can see that maximum of OsPILS genes are up-regulated in leaf tissues. More specifically OsPILS1 and
464 OsPILS6b genes are up-regulated at all 7 days, 14 days, 21 days time points in cytokinin treated leaf. In
465 cytokinin treated root tissues, maximum of OsPILS were up-regulated at 14 and 21 days time period.

466 **Table Legends**

467 **Table 1**

468 List of primers used for analysis of relative expression of OsPILS genes.

469 **Table 2**

470 Table showing OsPILS genes and their genomic information. Table shows that OsPILS5 gene has longest
471 ORF of 3840 nucleotides length. Among seven OsPILS genes, four of them contain 10 introns in their
472 gene. OsPILS2 and OsPILS5 resides in acidic isoelectric point (pI) ranges and all other OsPILS are
473 resides in basic pI ranges.

474 **Table 3**

475 Prediction of sub-cellular localization of OsPILS genes shows that all OsPILS gens are integral
476 transmembrane proteins and localized in sub-cellular compartments like, endoplasmic reticulum and
477 vacuoles. OsPILS7b localized to endoplasmic reticulum as well as plasmamembrane. Prediction of sub-
478 cellular localization was carried out using online available WOLF PSORT protein sub-cellular localization
479 prediction software (http://www.cbrc.jp/cbrc/news/wolf_eng.html)

Figure 1

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Figure showing genomic organization of different OsPILS genes. Black colored boxes represent the exons and lines represent the introns of different OsPILS genes. In majority of cases, maximum of OsPILS gene contains ten exons in their gene.

Figure 1

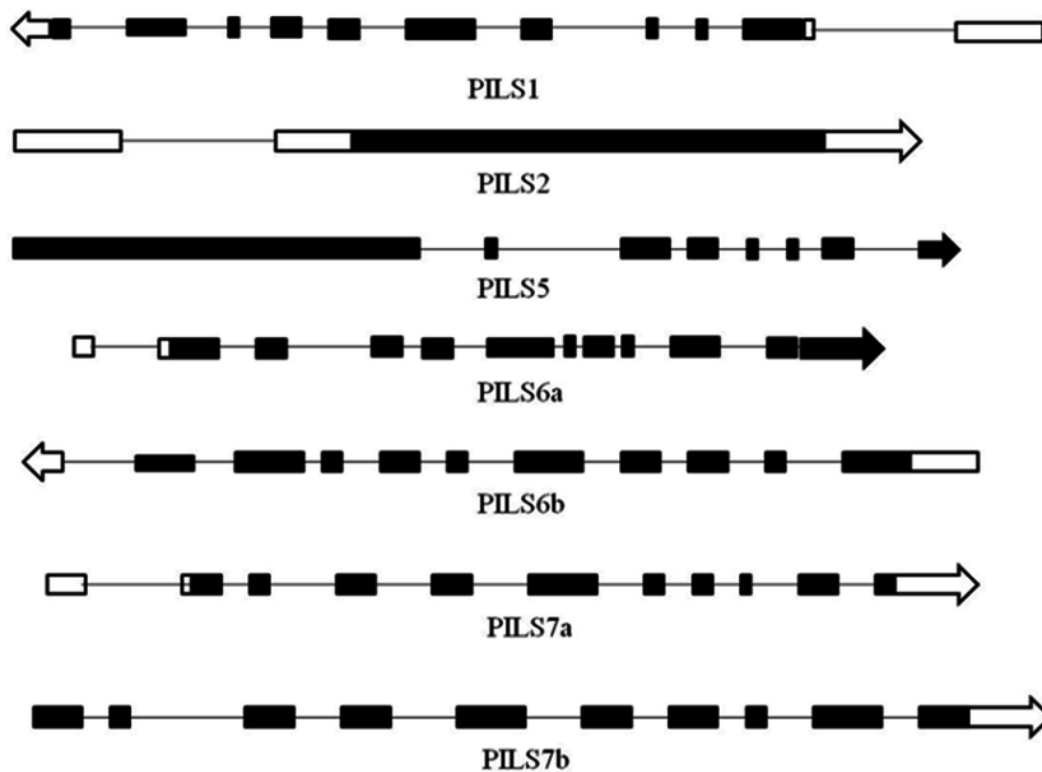


Figure 2

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Figure showing distribution of OsPILS genes in rice chromosomes. All the seven OsPILS genes distributed towards the distal end of chromosome. Chromosome 9 harbors maximum of three OsPILS genes whereas chromosome 1, chromosome 5, chromosome 7 and chromosome 8 harbor one OsPILS gene each.

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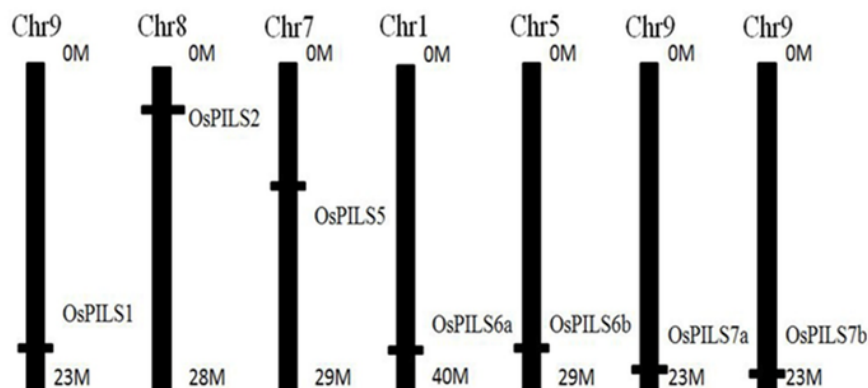


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Figure represents auxin efflux carrier domain of OsPILS gene. Swiss workspace model (<http://swissmodel.expasy.org/workspace/>) was used to predict the auxin efflux carrier domain of OsPILS genes. All the OsPILS genes contain auxin efflux carrier domain.

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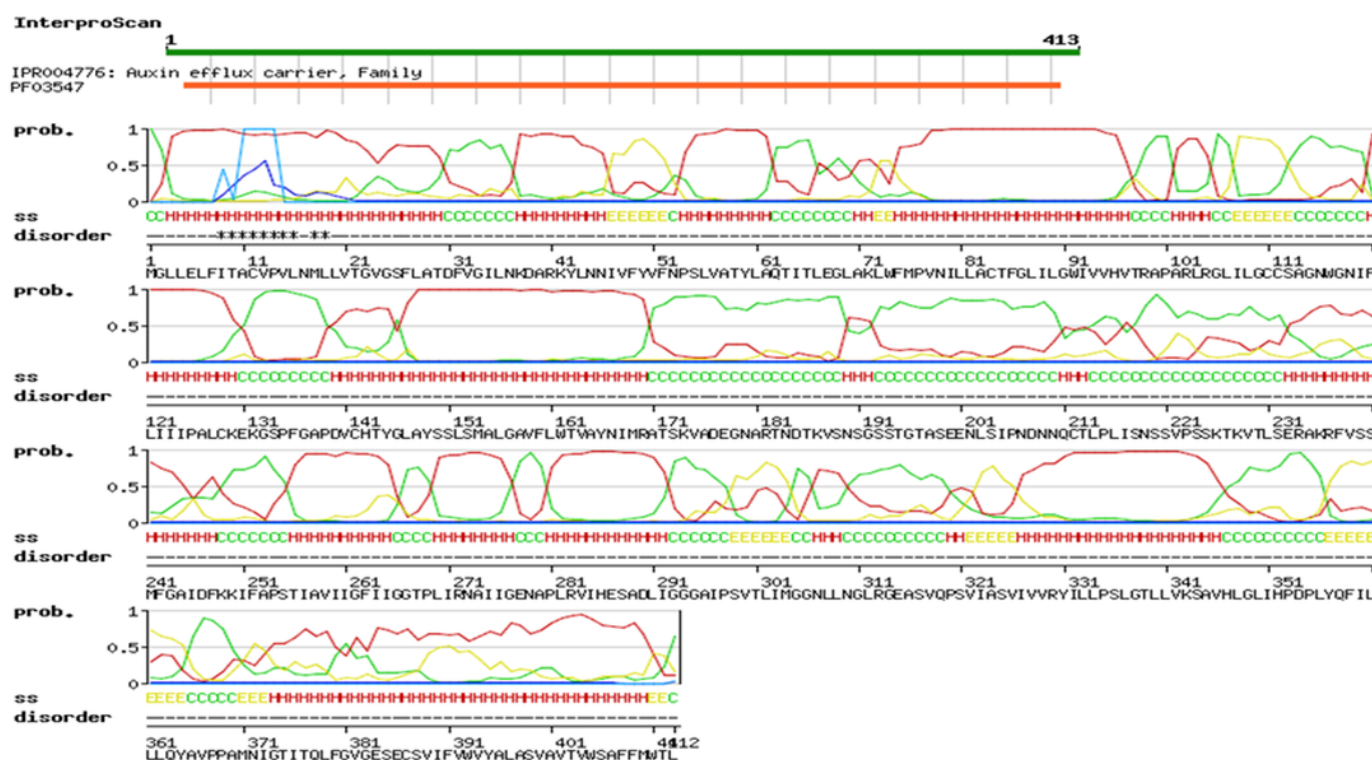


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Multiple sequence alignment of OsPILS proteins show presence of several conserved amino acid residues as well as motifs in N- and C-terminal region. Major conserved motifs present in N-terminal region is N-x-G-N (in box) except OsPILS5. The C-terminal region contain conserved A-P-L and G-G-N-L (in box) consensus sequences. Although central hydrophilic region of OsPIL gene is very dynamic in nature, still it contain conserved T amino acid (Threonine) residue (boxed) in central hydrophilic region.

Figure 4

```

OsPILS7a  -----MGFLSLLLVASMPVQVLLIGVIGAFASGYSKILTSSALRDMNKVVFVTFPSLMFASLAKTVTSDVISWWMFVNIGITFIVGGTIG
OsPILS7b  -----MGFLALLLVASMPVQVLLIGVIGAFASGYSNLTSSALSDMNKVVFVTFPSLMFASLARTVSDVISWWMFVNIGITFMAGGTIGD
OsPILS5    SAGDLEQAMFLIEEMFLRGHASISSFNNLVDGFCRGRKMQETLKLHVIMGRGLVFNALTENIISGLSEAGKLESEVHTIFVELQKTSESAARTFSSLFMDMINQGKIPLDVDDMIRD
OsPILS1    -----MGLLELFITACVPLNMLLVTVGVSFLATDFVGILNKDARKYLNNIVFYVFNPSLVATYLAQITITLEGLAKLWFMFVNILLACTFGLILG
OsPILS6a   -----MMERSLMEALATAAQGG--TVGTSVDFMLKYAVLPKAVFTVCFMGFLMASKYVNILQPNGRKLLNGLVFSLLLPCLIFSQLGRAITIERMLQWYIIPVNIIVGAVSGSLIG
OsPILS6b   -----MMGRSVLEMVVAAGGGGAAGESVLGMFRYAVLPKAVFVVCFMGFLMASKYVGLKPSGRKLLNALVFSLLLPCLIFSQLGRAITIDKIMEWFIIPANIALGAVSASLVG
OsPILS2    -----MDNLSANLQVN---PQVTVHGDWVSVMPLMKLLCLTVIGLVLSNPRQTQIPKATFKLLSKLVFALFLPCLIFVHLGQSVTIQNILDWFIIPVNVLISTAIGCILG

OsPILS7a  WIACKILKPPQHFRGMIIAFCSAENLGNLLIIIVPAVCDDEGNPFQKDRSLCRSRG-----LSYSSLSMALGGLFIITITYSIMQKAGKMYHMQSKSIQCPADSDDEHH
OsPILS7b  WIACKILKPPQHFRGMIIAFCSAENLGNLLIIIVPAVCDDEGNPFQKDRSLCRSLG-----LSYSSLSMALGGLYITITYSIMKKGK-RDQMYHQPNSIQCLDSDDEHH
OsPILS5    HCKEGLNLDKALMLRDVIVAKSAPAGCSSLAIVDNLCKRKGKLEALNLLKEMDKRGNLQPTLVALLGIFWFRHHHILNKLGGLYITITYSIMKKGK-RGQMYHQPNSIQCLDSDNEHH
OsPILS1    WIVVHVITRAPARLRGLILGCCSANNWGNFLIIIPALCKEKGSPFG--APDVCHTYG-----LAYSSLSMALGAVFLITAYINIMRATSKVADEGNARTNDTKVNS---
OsPILS6a   FVVASIIRPPYPYFKFTIITHIGIENIGNPLVLIAALCRDPTNPFQDSKDCNEDGN-----AYISFGQWVGAIIVTVTFKMLAPPP-----
OsPILS6b   LIVALLIVRPPYPYFKFTIITHIGIENIGNPLVLISALCRDQNLNPFQDSNKTQDGN-----AYLSFGQWVGAIIVTVTFKMLAPPP-----
OsPILS2    YIVALICRPPYPYFKFTIITHIGIENIGNPLVIAIGSVCHTIDHPFGG--CHRGKV-----AYVSFAQWVAVILVTVVYHMEPPMQFYIIVGEGNEIVEEPAQISNY

OsPILS7a  PAQGHQVQLDGETAYADEEAALLVSAKLAPEHNEENQMEAPLLTCERE-----IANKGGFTN-LKETVHQWVEELMAPPTVSAILGFVVGVLFWLKSILVIGNSAPLVI
OsPILS7b  SKK----FKANGEAAAYADEEATLFPVSAKLAQHN--EENQMEAPLLSCESEK-----VAKKCSWTTITNLKDTIHHWVEELMAPPTLSAILGFVVGVLFWLKSILVIGDSAPLVI
OsPILS5    AKK----FKANGEAACADEEATLLVSAKLAQHN--EENQMEAPLLSGESE-----IAKKGSWTTITNLKDTIHHWVEELMAPPTLSAILGFVVGVLFWLKSILVIGDSAPLVI
OsPILS1    -----GSSTGTASEENLSIPNDNNQCTPLISNSSVPSSKTKVT-----LSERAKRFVSSMFGAID--FKKIFAPSTIAVIGFIIGGTPILRNAIIGENAPLVI
OsPILS6a   -GESFDSAEEDILPIKASGDNVPEKGYPTSTRISTVPENEPLLSSEG-----DKNVS---TSLGSKIMGIVRSVMVKFLKQKQLLPPIIASVFAIAIGVVPVLKNFVLTDAPLFF
OsPILS6b   -GQTFDSCDEERDKLPKAPNTMSSVAKYPSSAHGNTHREEPLLSIEE-----EEEEGQDVHSLGSKIMPIKGMVRFQKQQLLPPIIASVLAITLGVVPFLKNLILTDAPLFF
OsPILS2    SRSLLEAEWPGMDKETEHSKTPFIARVMSISGSSQNTFPDIDFAEEGTSAGAPSSPKSLRCLAEKPVVRRIRVVAEKTPIQHVLPPTIASLLAIVIGMVPFLKDFVFPDAPLFF

OsPILS7a  QESLQLMNGNTIPCVTLVGGNLIKGLR--KSVLKRTVITIVCIRYVIQPLIGMAVVAAYGVGFLP-HDPLYRYVLMQFALPAMNIGTMAQLFDVQGECSVIFLWTVLIAAIALT
OsPILS7b  QDSIQLMNGNTIPCVTLVGGNLIKGLR--KSELKRTVITIVCIRYVILPLVGIIVVHGAYVVGFLP-HDPLYRYVLMQFALPAMNIGTMAQLFDVQGECSVIFLWTVLVASISLT
OsPILS5    QDSIQLMNGNTIPCVSLVGGNLIKGLR--KLEFKHTVITIVCIRYMIPLVGIIVVHGAYVVGFLP-HDPLYRYVLMQFALPAMNIGTMAQLFDVQGECSVIFLWTVLVSSISLT
OsPILS1    HESADLIGGAIPSVTLMGGNLIKGLR--EASVQPSVIASVIVRYILLPSLGTLLVKSAVHLGLIH-PDPLYQFLMMQYAVPAMNIGTITQLFGVGESECSVIFVWYVALASVAVT
OsPILS6a   TDSCLILGEAMIPCIILLVGGNLDVGPGEGRNRLGVRTTVAIIFARLILVPLAGVGIIVLVKLGFIKPKDKMFKFVLLVQHSMPSTSVLSGAVANLRG-CGKESAAILFVWHIFAVFSMA
OsPILS6b   TDSCLILGEAMIPCIILLVGGNLDVGPGEGRNRLGVRTTVAIIFARLILVPLAGVGIIVLVKLGFIKPKDKMFKFVLLVQHSMPSTSVLSGAVANLRG-CGKESAAILFVWHIFAVFSMA
OsPILS2    TDSLEILAQAVVPVSMVLLVGGMLAEQPKD--NALGIRITIGIIVARLLILPCIGIVVLLADRLHLLVEEDHMYRFVLSLQYSTPSAILLGAIASLRGYAVKESALLFWQHICAVLSLS

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Figure 5

Figure 5

Prediction of transmembrane domains of OsPILS genes using TMHMM server 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Prediction shows that maximum of OsPILS genes contain short transmembrane domain.

Figure 5

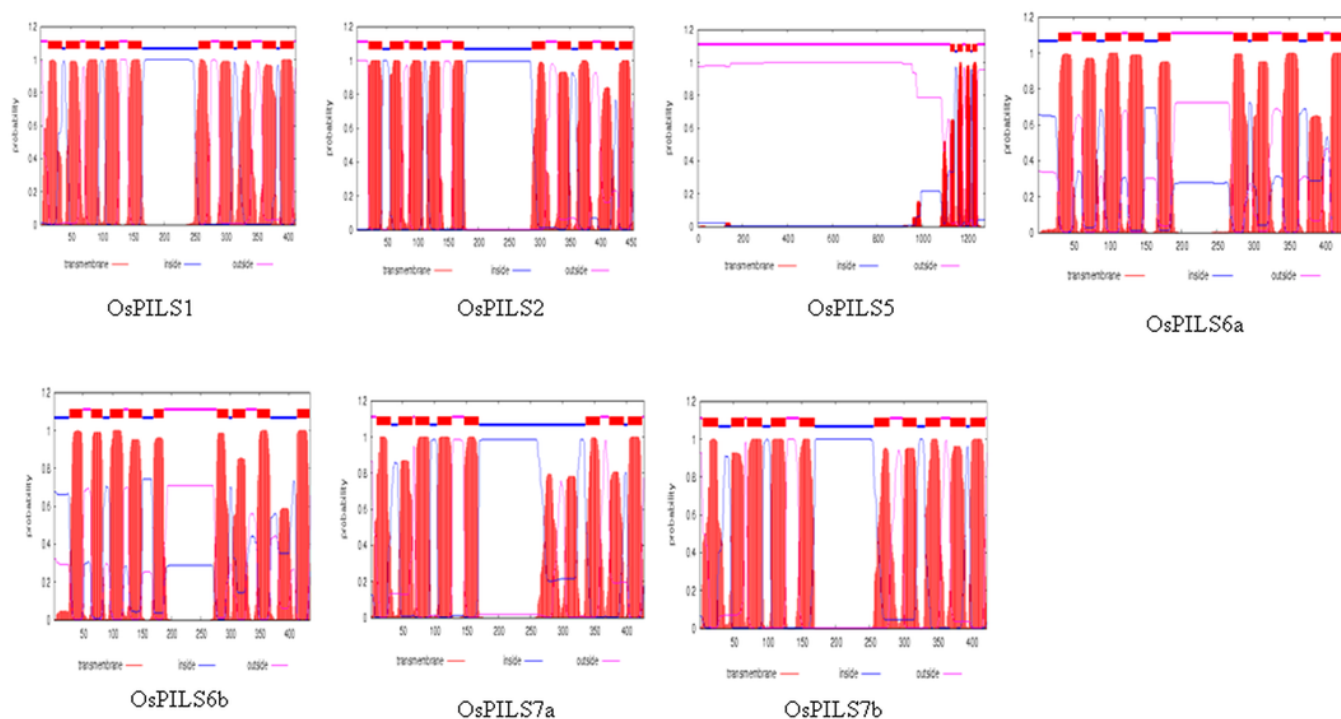


Figure 6

Figure 6

Phylogenetic tree of OsPIN, AtPIN, AtPILS and OsPILS genes. Phylogenetic analysis shows, PINs and PILS genes are grouped into two distinct clades. In the phylogenetic tree, short transmembrane domain containing protein OsPIN5 and AtPIN5 lies proximately towards short transmembrane domain containing AtPILS and OsPILS genes. This indicates that PILS genes are short transmembrane domain containing proteins. Phylogenetic tree was constructed using MEGA5 software. Programme used to construct the phylogenetic tree was; statistical method: maximum likelihood, test of phylogeny: bootstrap method, no. of bootstrap replication: 1000, substitution type: amino acids, models/methods: Jones-Taylor-Thornton (JTT) and branch swap filter was very strong.

Figure 6

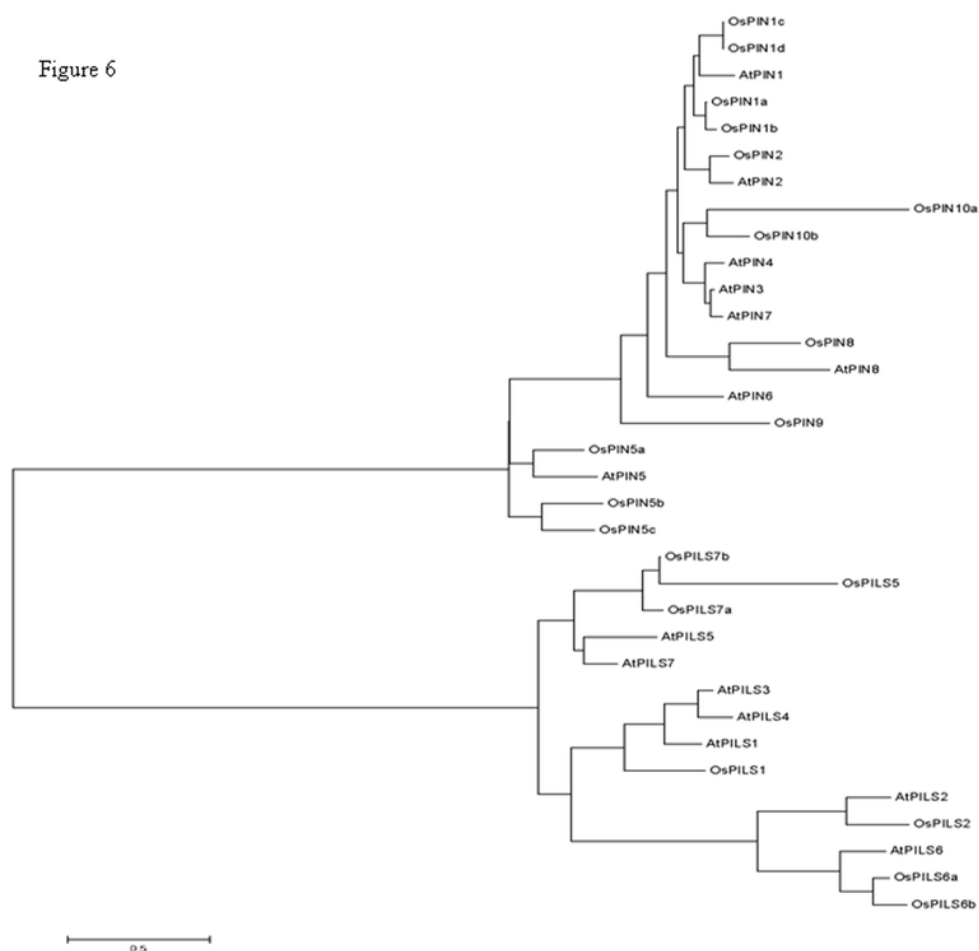


Figure 7

Figure 7 Part 1

Relative expression of OsPILS Genes in Leaf and root tissues treated with 10 μ M IAA. Relative gene 545 expression of OsPILS genes shows all the OsPILS genes were up-regulated in auxin treated leaf tissues and 546 down-regulated in auxin treated root tissues at 21 days time period.

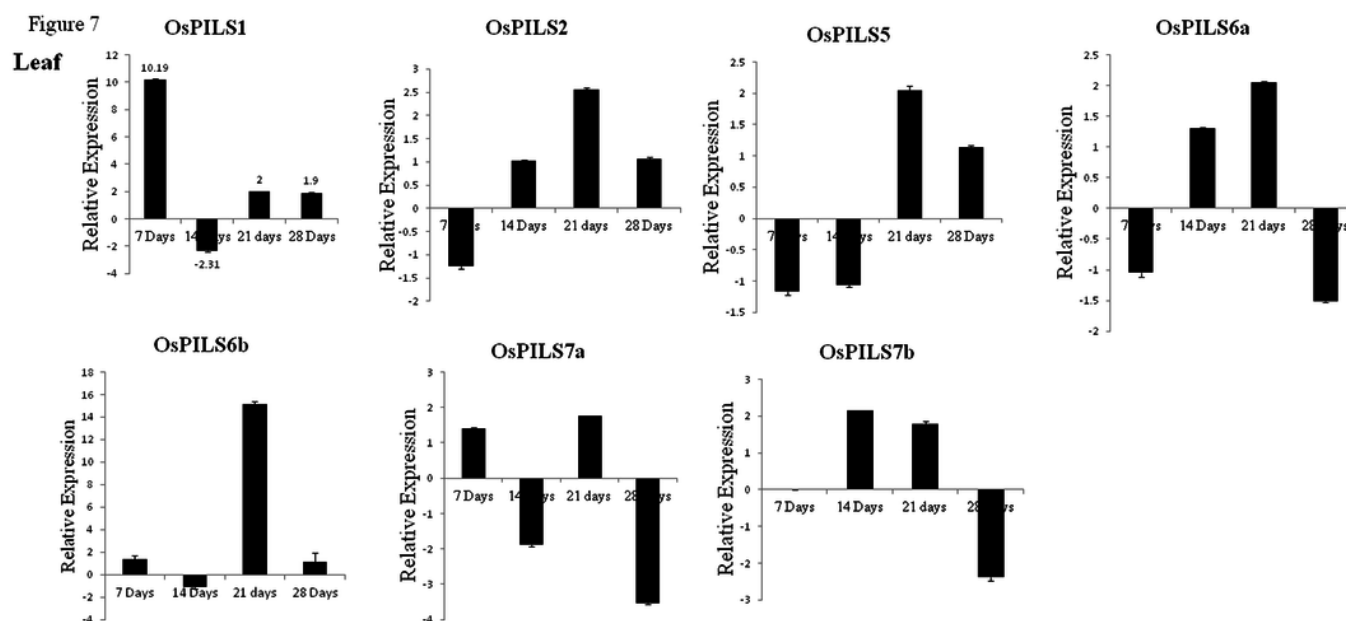


Figure 8

Figure 7 Part 2

Relative expression of OsPILS Genes in Leaf and root tissues treated with 10 μ M IAA. Relative gene 545 expression of OsPILS genes shows all the OsPILS genes were up-regulated in auxin treated leaf tissues and 546 down-regulated in auxin treated root tissues at 21 days time period.

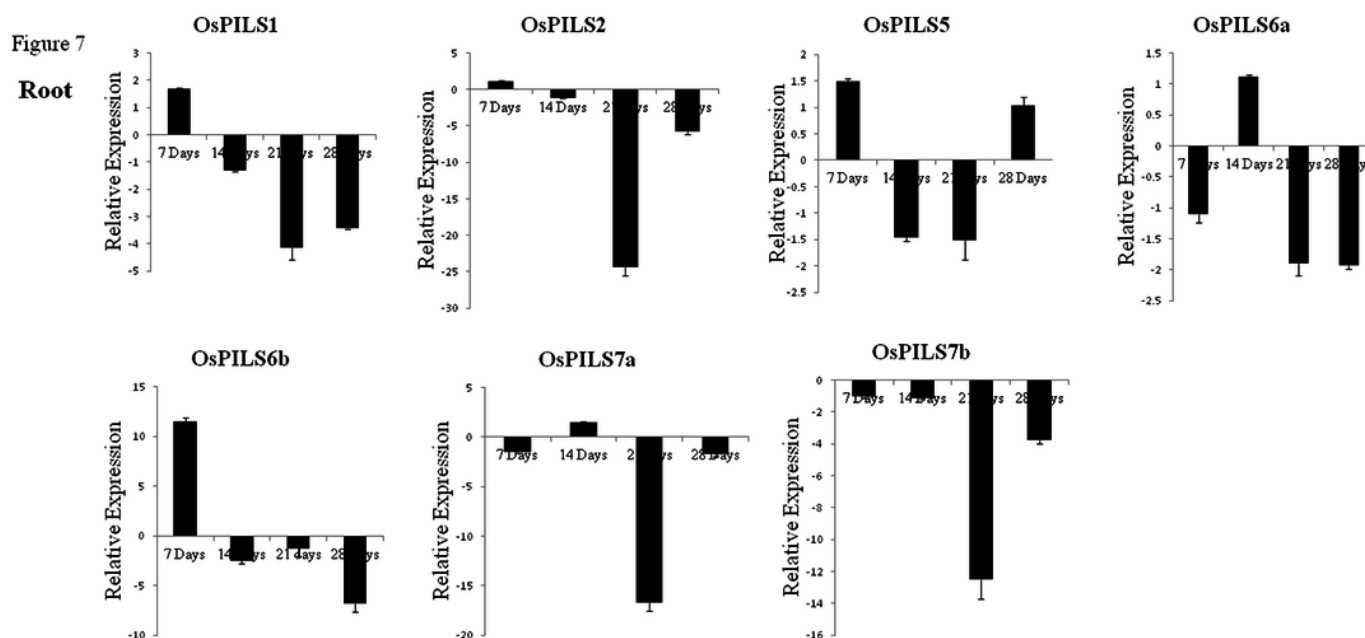


Figure 9

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Relative expression of OsPILS genes in leaf and root tissues treated with 10 μ M BAP. From the result we can see that maximum of OsPILS genes are up-regulated in leaf tissues. More specifically OsPILS1 and OsPILS6b genes are up-regulated at all 7 days, 14 days, 21 days time points in cytokinin treated leaves. In cytokinin treated root tissues, maximum of OsPILS were up-regulated at 14 and 21 days time period.

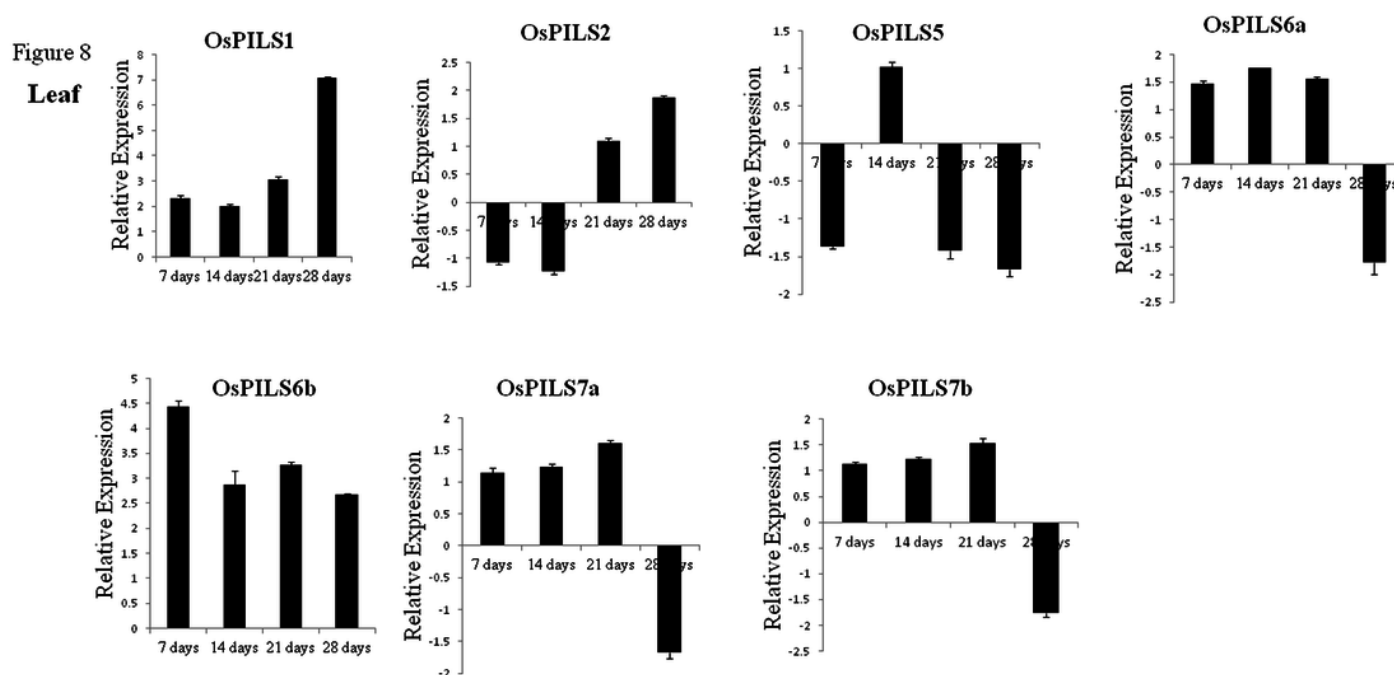


Figure 10

Figure 8 part 2

Relative expression of OsPILS genes in leaf and root tissues treated with 10 μ M BAP. From the result we 556 can see that maximum of OsPILS genes are up-regulated in leaf tissues. More specifically OsPILS1 and 557 OsPILS6b genes are up-regulated at all 7 days, 14 days, 21 days time points in cytokinin treated leafs. In 558 cytokinin treated root tissues, maximum of OsPILS were up-regulated at 14 and 21 days time period. 559 560 561

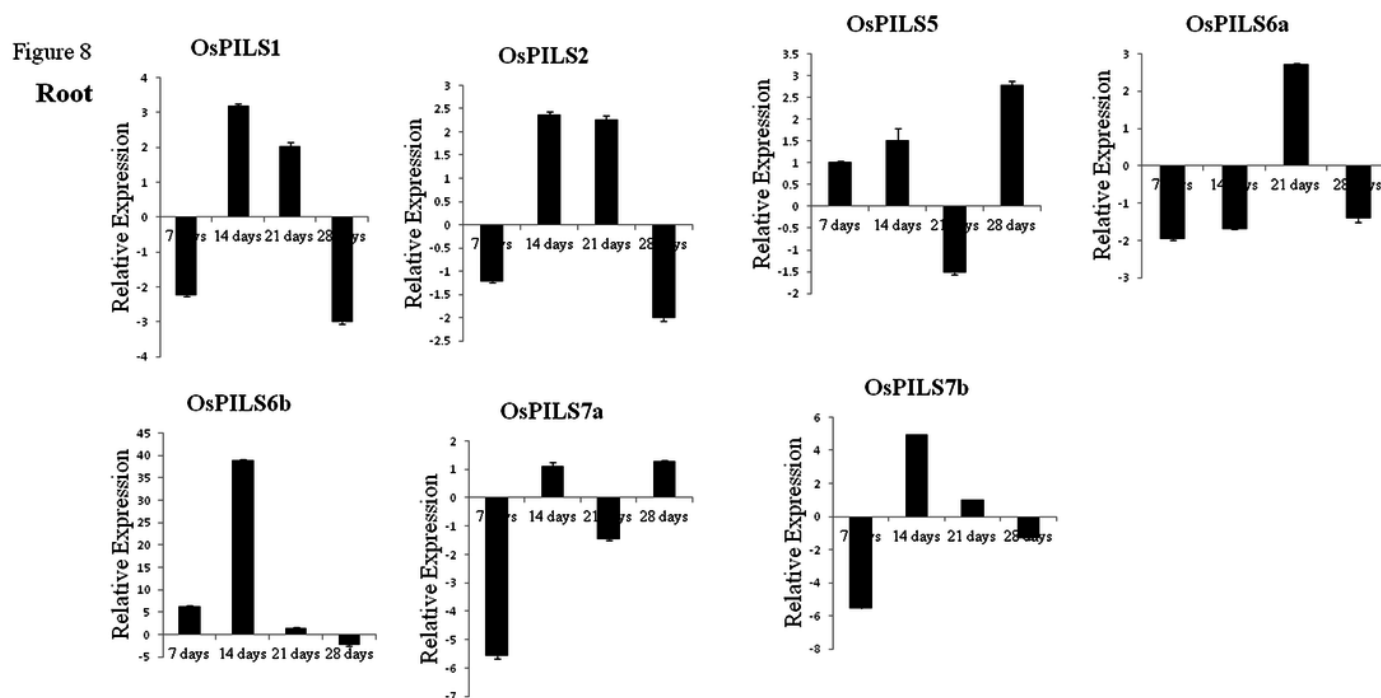


Table 1 (on next page)

Table 1

List of primers used for analysis of relative expression of OsPILS genes

Table 1.

List of primers used for analysis of relative expression of OsPILS genes.

Gene Name	Locus ID	Forward Primer	Reverse Primer
OsPILS1	LOC_Os09g31478.1	AGCAAGCGTCCAGCCTTCAG	CTCTCCCCCACGCCGAACAG
OsPILS2	LOC_Os08g09190.1	CTCCAGCCACCAACCATCG	CGGGAGAATCAGGAGCCTTGC
OsPILS5	LOC_Os07g20510.1	GTCGTCCTCGCCTCCATCCA	CGCACCTTCCAAAGCAGAGC
OsPILS6a	LOC_Os01g60230.1	GATGGAGAGGTCGCTGATGGAG	GCGAGAACACAAGCCCATTG
OsPILS6b	LOC_Os05g40330.1	GAGGTCGGTGCTGGAGATGGTG	GGCAAGGAAGGAGCAGAGAGAACAC
OsPILS7a	LOC_Os09g38130.1	CCGCTCTCGTGGGCTCTCCT	GCCGCCTCCTCATCAGCGTA
OsPILS7b	LOC_Os09g38210.1	GAAGATGGCAACCCGTTCGG	CCTCCTCATCGGCGTAAGCA

Table 2 (on next page)

Table 2

Table representing OsPILS genes and their detailed genomic information. Table show that OsPILS5 gene posses longest ORF of 3840 nucleotides length. Among seven OsPILS genes, four of them contain 10 introns in their gene. OsPILS2 and OsPILS5 resides in acidic isoelectric point (pI) ranges and all other OsPILS are resides in basic pI ranges.

Table 2.

Gene Name	Locus ID	ORF	No. of Amino Acids	No. of Introns	5'-3' Coordinates	pI
OsPILS1	LOC_Os09g31478.1	1242	414	10	Chr9: 18983481-18978136	8.38
OsPILS2	LOC_Os08g09190.1	1368	456	1	Chr8: 5322709-5325262	6.93
OsPILS5	LOC_Os07g20510.1	3840	1280	7	Chr7: 11845862-11851637	6.91
OsPILS6a	LOC_Os01g60230.1	1299	433	10	Chr1: 34834581-34838747	8.02
OsPILS6b	LOC_Os05g40330.1	1314	438	10	Chr5: 23700670-23697738	8.20
OsPILS7a	LOC_Os09g38130.1	1287	429	10	Chr9: 21963498-21968222	7.02
OsPILS7b	LOC_Os09g38210.1	1272	424	9	Chr9: 22007511-22010336	7.23

Table representing OsPILS genes and their detailed genomic information. Table show that OsPILS5 gene posses longest ORF of 3840 nucleotides length. Among seven OsPILS genes, four of them contain 10 introns in their gene. OsPILS2 and OsPILS5 resides in acidic isoelectric point (pI) ranges and all other OsPILS are resides in basic pI ranges.

Table 3 (on next page)

Table 3

Prediction of sub-cellular localization of OsPILS genes show that all OsPILS gens are integral transmembrane proteins and localized to sub-cellular compartment like endoplasmic reticulum and vacuoles. OsPILS7b found to be localized to endoplasmic reticulum as well as plasmamembrane. Prediction of sub-cellular localization was carried out using online available WOLF PSORT protein sub-cellular localization prediction software (http://www.cbrc.jp/cbrc/news/wolf_eng.html).

Table 3.

Prediction of sub-cellular localization of OsPILS genes show that all OsPILS gens are integral

Gene	Protein domains	Putative Subcellular Localaization
OsPILS1	Integral membrane protein	Vacuolar
OsPILS2	Integral membrane protein	Endoplasmic reticulum
OsPILS5	Integral membrane protein	Endoplasmic reticulum
OsPILS6a	Integral membrane protein	Vacuolar
OsPILS6b	Integral membrane protein	Endoplasmic reticulum
OsPILS7a	Integral membrane protein	Endoplasmic reticulum
OsPILS7b	Integral membrane protein	Plasmamembrane & ER

transmembrane proteins and localized to sub-cellular compartment like endoplasmic reticulum and vacuoles. OsPILS7b found to be localized to endoplasmic reticulum as well as plasmamembrane. Prediction of sub-cellular localization was carried out using online available WOLF PSORT protein sub-cellular localization prediction software (http://www.cbrc.jp/cbrc/news/wolf_eng.html).