Genome-wide identification and characterization of ABA receptor PYL gene family in rice

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Abstract

Background: Abscisic acid (ABA), a key phytohormone that controls plant growth and stress responses, is sensed by the pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of the ABA receptor (RCAR) family of proteins. Comprehensive information on evolution and function of PYL gene family in rice (Oryza sativa) needs further investigation. This study made detailed analysis on evolutionary relationship between PYL family members, collinearity, synteny, gene structure, protein motifs, cis-regulatory elements (CREs), SNP variations, miRNAs targeting PYLs and expression profiles in different tissues and stress responses.

Results: Based on sequence homology with Arabidopsis PYL proteins, we identified a total of 13 PYLs in rice (BOP clade) and maize (PACCMAD clade), while other members of BOP (wheat – each diploid genome, barley and Brachypodium) and PACCMAD (sorghum and foxtail millet) have 8-9 PYLs. The phylogenetic analysis divided PYLs into three subfamilies that are structurally and functionally conserved across species. Gene structure and motif analysis of OsPYLs revealed that members of each subfamily have similar gene and motif structure. Segmental duplication appears to be the driving force for the expansion of PYLs, and the majority of the PYLs underwent evolution under purifying selection in rice. 32 unique potential miRNAs that might target PYLs were identified in rice. Thus, the predicted regulation of PYLs through miRNAs in rice is more elaborate as compared with B. napus. Further, the miRNAs identified to in this study were also regulated by stresses, which adds additional layer of regulation of PYLs. The frequency of SAPs identified was higher in indica cultivars and were predominantly located in START domain that participate in ABA binding. The promoters of most of the OsPYLs have cis-regulatory elements involved in imparting abiotic stress responsive expression. In silico and q-RT-PCR expression analyses of PYL genes revealed multifaceted role of ABARs in shaping plant development as well as abiotic stress responses.

Conclusion: The predicted miRNA mediated regulation of OsPYLs and stress regulated expression of all OsPYLs, at least, under one stress, lays foundation for further validation and fine tuning ABA receptors for stress tolerance without yield penalty in rice.

Keywords: ABA receptors (ABARs), Abiotic stresses, Collinearity, miRNAs, Single amino acid polymorphism (SAP), Single nucleotide polymorphism (SNP), Stress responsive cis-elements, Synteny

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Background

Abscisic acid (ABA) plays a pivotal role in plant growth and development including cell elongation and division, embryo maturation, desiccation tolerance of seeds, seed dormancy, germination, leaf senescence, induction of root growth and fruit ripening. In addition ABA regulates stomatal aperture [1–6] and is the primary hormone imparting cellular tolerance to various biotic and abiotic stresses in plants [7–10].

Over the past one decade mammal advancements have been made in unravelling the mechanism of ABA signalling including the discovery of ABA receptors [11]. The PYLs are currently the largest plant hormone receptor family known [12]. ABA is perceived by soluble cytosolic pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/Regulatory component of ABA receptor (RCAR) protein family in Arabidopsis [13–15]. Binding of ABA to PYL leads to conformational change in PYL enabling it to bind to clade A type 2C protein phosphatases (PP2Cs). Binding of PYL to PP2C releases class III sucrose non-fermenting 1-related protein kinase 2 s (SnRK2s) from inhibition by PP2Cs [4, 11–13, 15–22]. Activated SnRK2s phosphorylate downstream targets like Abscisic acid responsive element (ABREs)/Abscisic acid binding factor (ABF)/ABI5 clade of bZIP transcription factors and other regulatory proteins promoting ABA induced physiological responses [16–19]. Thus, trinity of PYLs, PP2Cs and SnRKs constitute the core ABA signalling modules which are highly conserved in land plants which need abiotic stress tolerance for survival [15–26].

Voluminous efforts have been made in characterization of PYL receptors from model plant Arabidopsis which encodes for 14 PYL members that are highly conserved in amino acid sequence as well as in functional domain structure [13, 14, 27]. AtPYR1, AtPYL1 and AtPYL2 are dimeric, while AtPYL4 to AtPYL10 are monomeric in apo-receptor state. AtPYL3 exists in both monomeric and dimeric state. PYL receptors negatively regulate PP2Cs in an ABA independent manner [20]. Based on sequence similarity, ABA receptors of Arabidopsis have been broadly classified into 3 subfamilies [13]. ABA receptors belonging to subfamily I and II are monomeric, while subfamily III are dimeric in nature. The overall PYL structure exhibits the helix-grip fold, a hallmark of START (star-related lipid transfer) domain/Bet v 1-fold proteins, which is characterised by the presence of a central β-sheet surrounded by N- and C-termini α-helices, with a long C terminal α-helix packing tightly against the β-sheet. The helix-grip fold creates a large cavity constituting the ABA binding pocket [20, 23].

Extensive studies with PYLs in Arabidopsis have shown that PYL family ABA receptors play a diverse role in plant development and combating abiotic stresses. AtPYR1, AtPYL1, AtPYL2, AtPYL4, AtPYL5, AtPYL8 and AtPYL9 have been shown to promote ABA induced seed germination, stomatal closure and root growth; AtPYL6 and AtPYL13 have been shown to inhibit seed germination [13, 28–31]. Apart from playing key role in growth and development, AtPYL5 and AtPYL9 were found to provide drought tolerance [15, 32].

Since the discovery of PYL family of ABA receptors, a lot of efforts have been made to unravel the function of PYL members in diverse plant species and agriculturally important crops, including Arabidopsis [15, 22, 28, 32–37], Artemisia annua [38], Vitis vinifera [39, 40], Oryza sativa [41–47], Triticum aestivum [48, 49], Zea mays [50, 51], Solanum lycopersicum [52], Glycine max [53], Populus [54], Hevea brasiliensis [55], strawberry [56], Gossypium hirsutum [57–59], Brassica rapa [60] and Brachypodium distachyon [61, 62]. As compared with dicotyledonous model plant Arabidopsis, signalling modules in the monocot rice are similar in type and number which proves the conserved nature of functional ABA signalling pathway across plant species [49, 63–65].

Abiotic stresses such as cold, drought and salinity have detrimental effect on the agricultural crops leading to yield losses worldwide [66]. Despite the advancements achieved towards the comprehension of the role of PYL gene family in Arabidopsis, functional diversity and redundancy of PYLs in development and stress responsive processes in agronomically important crop like rice is relatively less investigated. In rice OsPYL2, OsPYL8, OsPYL9, OsPYL10 and OsPYL11 receptors have been functionally characterized by overexpressing the PYL genes while CISPR/Cas9 knockout mutants of PYL genes in rice has been found to moderate abiotic stress tolerance and yield [41–47]. Most of these works were focused in japonica rice while very less information is available in indica rice. Most of the mentioned approaches involved characterization using either overexpressing or knocking down individual OsPYL gene. Moreover previous expression studies of PYL genes were confined to a particular stage and stress. A detailed genome wide analysis of ABA receptors in terms of evolution, promoter analysis, miRNA targets and functional characterization has not been reported in rice. Functional validation of ABA receptor is pivotal for plant genetic engineering towards improving important agricultural traits such as plant biomass, yield and tolerance to abiotic stresses.

In the present study, genome wide identification and characterization of PYL gene family in Oryza sativa spp. indica was carried out using comparative genomic tools and experimental verification. A total of 13 OsPYL genes were identified from rice genome. Further we discerned PYL gene family in other agriculturally imperative crops and phylogenetic relationship amongst them. Genomic organization, gene structure, motif composition,
subcellular localization and miRNA targets and expression analysis were characterized using in silico approaches. Collinearity and syntenic relationship of PYL gene across different taxa was studied. Non synonymous SNPs were identified across popular rice accessions to study polymorphism amongst them. We also carried out a detailed investigation of cis-regulatory elements in promoter region of ABA receptors in relation to their role in stress responsiveness and development. A comprehensive differential gene expression profiling of OsPYL gene family in spatiotemporal manner was carried out under different stresses (drought, ABA, low temperature, salinity, high temperature) and tissues using in silico data and quantitative PCR analysis. Our results provide a foothold in understating functions to further illuminate OsPYL genes under different stresses and development and identification of targets for improving abiotic stress tolerance in rice.

Results
Genome-wide identification and phylogenetic analyses of the OsPYL gene family in rice
To identify all the OsPYL gene members in rice, Hidden Markov model and BLASTp (e-value <=1e-10) searches were carried out to search rice genome annotation project (RGAP) (http://rice.plantbiology.msu.edu/) using 14 Arabidopsis PYL amino acid sequence as queries [67, 68]. A total of 13 OsPYL genes were identified in the genome of rice. Nomenclature of the identified OsPYL genes was done in accordance with previous study of OsPYLs [45]. Among these, two (OsPYL7 and OsPYL12) are thought to be non-functional ABA receptors as they have large deletion in the N and C terminal of the gene, respectively [26]. The identified rice OsPYL genes encode protein with size ranging from 125 (OsPYL12) to 229 (OsPYL6) amino acid residues. The other characteristics of the OsPYL genes, including gene length, open reading frame (ORF) length, the isoelectric point (pl), molecular weight (MW), and exons, are presented in (Table 1, Additional files 1, 2).

Table 1 Basic information of OsPYL family genes and their proteins in Oryza sativa spp. indica

| Gene  | Locus ID     | Accession No. | Gene length (bp) | ORF length (bp) | No. of Exon | Predicted Protein Size (aa) | MW (kDa) | pl  |
|-------|--------------|----------------|------------------|-----------------|-------------|-----------------------------|----------|-----|
| OsPYL1 | LOC_Os10g42280 | KJ634481       | 639              | 639             | 1           | 212                         | 23,083.91| 5.45|
| OsPYL2 | LOC_Os06g36670 | KJ634482       | 931              | 624             | 1           | 207                         | 22,344.15| 6.31|
| OsPYL3 | LOC_Os01g13330 | KM371729       | 1069             | 633             | 1           | 210                         | 22,760.58| 6.45|
| OsPYL4 | LOC_Os01g61210 | KJ634480       | 627              | 627             | 1           | 208                         | 22,295.33| 8.26|
| OsPYL5 | LOC_Os05g39580 | KJ634479       | 1476             | 654             | 1           | 217                         | 22,721.74| 8.29|
| OsPYL6 | LOC_Os03g18600 | KJ634478       | 1286             | 690             | 1           | 229                         | 23,815.95| 6.89|
| OsPYL7 | LOC_Os06g33480 | KJ634477       | 3010             | 441             | 2           | 146                         | 16,691.17| 9.26|
| OsPYL8 | LOC_Os06g33640 | KJ634477       | 3411             | 621             | 1           | 206                         | 23,321.71| 5.99|
| OsPYL9 | LOC_Os06g33690 | KM371729       | 2062             | 621             | 3           | 206                         | 23,397.74| 6.45|
| OsPYL10| LOC_Os02g15640 | KF925265       | 4129             | 615             | 3           | 204                         | 23,068.25| 6.46|
| OsPYL11| LOC_Os05g12260 | KJ634476       | 2468             | 630             | 3           | 209                         | 22,229.04| 5.69|
| OsPYL12| LOC_Os02g15620 | KJ634476       | 2049             | 378             | 2           | 125                         | 13,682.57| 5.16|
| OsPYL13| LOC_Os06g33490 | KJ634470       | 2517             | 477             | 3           | 158                         | 17,721.26| 5.37|

*Sequences with Accession numbers are cloned and sequenced from drought tolerant rice cv. Nagina 22 in our lab and are available in the NCBI https://www.ncbi.nlm.nih.gov/nucleotide/; Sequences where 3K genome is given in place of accession number, are the sequences of respective PYLs of Nagina 22 downloaded from rice 3000 genome database https://snp-seek.irri.org/
**Fig. 1** Phylogenetic relationship, gene architecture and conserved motifs of PYL genes. 

**a** Phylogenetic relationship of PYLs from Arabidopsis and rice. Tree was constructed by the Maximum likelihood method. The blue, green, and red boxes depict the subfamily I, II and III, respectively.

**b** Exon/intron architectures of PYLs. Grey colour boxes indicate exons and lines represent introns. The lengths of the exons and introns for each PYL gene can be calculated following the scale at the bottom.

**c** Distributions of conserved motifs in PYL proteins. Motifs are indicated by 10 different colour boxes.

**d** Legend depicting the protein sequence of the corresponding motifs.
Conserved motifs of OsPYLs
Among 13 OsPYLs, motif 1 harbouring the trademark Gate–Latch domain was conserved across all PYLs (Fig. 1c and Fig. 1d), while motif 2 and 3 were found to be conserved among all receptors except OsPYL7 and OsPYL12 (Table 1). OsPYLs have two (OsPYL12) to six (OsPYL2, 3, 8 and 9) motifs (Fig. 1c). Apart from sharing conserved motifs, each subfamily members have unique motifs. Putative functions of these motifs are given in Additional file 3 Table S1. These results indicate that OsPYL members clustered in the same subfamily show similar motif characteristics, suggesting functional similarities among members, while presence of unique motifs might carry out unique/specialized biological functions.

Chromosomal distribution of OsPYLs across rice genome
OsPYL genes were found to be unevenly distributed across 12 rice chromosomes (Fig. 2). Rice chromosome 6 harbours 5 PYLs (OsPYL2, OsPYL7, OsPYL8, OsPYL9 and OsPYL13), chromosome 2 harbours 3 PYLs, (OsPYL3, OsPYL10 and OsPYL12), and chromosome 5 harbours 2 PYLs (OsPYLS and OsPYL12). Six of the 12 chromosomes do not harbour PYL genes.

Identification of PYL members in other species
To study the evolutionary relationships between rice OsPYLs and PYLs of other grass family members, amino acid sequence OsPYLs were used as queries to search genome of wheat, maize, brachypodium, sorghum, foxtail millet and barley (Additional file 4). We identified 26 TaPYLs as previously reported [49]. Nine TaPYLs were identified in each diploid genome except TaPYL2 which was absent in B genome. In maize, Brachypodium, foxtail millet, barley and sorghum 13, 10, 9, 9 and 8 PYLs, respectively, were identified. In OsPYL7, 8, 9 and 13 typical latch residues (HRL) are replaced by HML (Additional file 4).

Based on the phylogenetic analysis, PYLs could be broadly classified to 3 subfamilies. There are 29, 41 and
31 members in Subfamily I, II and III, respectively in the eight species analysed. Therefore, PYL subfamily I and II have the lowest and highest PYL members, respectively, in grass family (Fig. 3).

**Genome wide synteny analysis of PYL gene family**

Gene duplication events including tandem and segmental duplications play an important role in broadening gene family during the evolutionary process [72]. Therefore, to gain insight into the genetic origins and evolution of the PYL gene family across six species, genome wide collinearity analysis was performed. Collinearity network grouped PYL genes across different taxa into five clusters where nodes represent individual PYL gene, while edges (lines between points) represent syntenic relationship amongst them (Fig. 4). Each cluster depicts high sequence similarity which might be a result of tandem gene duplication in the course of evolution. Interestingly, all PYL genes in cluster 1 belonged to subfamily II based on phylogenetic classification (Fig. 3). Notably cluster 3 comprising of PYL2 and PYL3 of different taxas was found to be least conserved as all the PYL genes form a closed interconnected network. On the other hand, PYL genes in cluster 1 were found to be most conserved. Further, we analysed whole genome duplication (WGD) events for PYL gene family between Arabidopsis and rice genomes by drawing whole genome Synteny blocks using CIRCOS (Fig. 5). Out of total 27 genes queried (14 Arabidopsis PYLs and 13 rice PYLs), only 20 PYL gene pairs formed collinearity blocks (11 Arabidopsis PYLs and 9 rice PYLs). Arabidopsis PYL genes were highly duplicated on chromosome two and four, while collinear gene pair between Arabidopsis and rice PYL genes was highest between Arabidopsis chromosome 2, 4, 5 and rice chromosome 1, 2 and 5. Some of the PYL genes exhibited multiple collinearity. We also found that collinearity of PYL4, PYL5 and PYL6 genes belonging to

![Fig. 3 Phylogenetic analysis of PYL proteins from Arabidopsis (14 AtPYL), Brachpodium (9 BdPYL), Rice (13 OsPYL), Wheat (26 TaPYL), Maize (13 ZmPYL), Sorghum (8 SbPYL), Barley (9 HvPYL) and Foxtail millet (9 SiPYL) were used using the maximum likelihood method. The PYL proteins are classified into 3 subfamilies: I, II and III depicted by blue, green and red colour respectively]
Fig. 4 Synteny network of PYL genes across six different plant species. Nodes represent syntenic genes and edges (lines) represent a syntenic connection between two nodes. 181 homologous gene pairs exist among PYL from *A. thaliana*, *B. distachyon*, *O. sativa*, *Z. mays*, *S. bicolor* and *H. vulgare* at genome wide scale. Each cluster represents genes with high sequence similarity.
subfamily II was highest between the genome of two species. Moreover, *AtPYL2* and *AtPYL3* of subfamily III, *AtPYL12* of subfamily II, and *OsPYL8*, 9, 11 and 13 of subfamily I do not form any collinearity blocks (Additional file 5 Table S2). These results further fortify our findings that *PYL* genes belonging to subfamily II and III have been evolutionarily conserved, while genes of subfamily I are least conserved. Collinearity blocks at genome scale of Arabidopsis and rice was also constructed which showed gene duplication was high within the species (Additional file 6 Fig. S1). Further to infer extent of selection pressure in the divergence of *PYL* genes, the non-synonymous (Ka) and synonymous (Ks) values were evaluated for the orthologous gene pairs (Table 2). A total of 65 ortholog pairs were formed for which the average Ka/Ks value was 0.070, suggesting that *PYL* family across the species were under purifying or stabilizing selection during evolution (Additional file 7).

**Identification of miRNAs targeting *OsPYL* genes in rice**

In order to predict miRNAs that may target *OsPYLs*, the cDNA sequences of *OsPYL* genes of rice were used as
Table 2 The Ka/Ks values of the homologous PYL gene family in *Oryza sativa* among genome of *A. thaliana, B. distachyon, S. bicolor, Z. mays* and *H. vulgare*

| PYL (Oryza sativa) | PYL Ortholog | S   | N   | Ka    | Ks    | Ka/Ks |
|--------------------|--------------|-----|-----|-------|-------|-------|
| OsPYL1             | AtPYL1       | 108.60 | 453.40 | 66.4125 | 0.2662 | 0.0040 |
| OsPYL1             | BdPYL1       | 64.60  | 547.40 | 1.1968  | 0.0989 | 0.0826 |
| OsPYL1             | HvPYL1       | 63.30  | 539.70 | 0.8162  | 0.0679 | 0.0832 |
| OsPYL1             | SbPYL1       | 66.60  | 543.40 | 0.7075  | 0.0779 | 0.1101 |
| OsPYL1             | ZmPYL1       | 57.10  | 536.90 | 0.6550  | 0.0766 | 0.1169 |
| OsPYL2             | BdPYL2       | 90.70  | 521.30 | 84.1710 | 0.1534 | 0.0018 |
| OsPYL2             | BdPYL3       | 83.20  | 501.80 | 1.7773  | 0.0621 | 0.0349 |
| OsPYL2             | HvPYL2       | 79.30  | 502.70 | 0.4656  | 0.0539 | 0.1157 |
| OsPYL2             | OsPYL3       | 86.20  | 534.80 | 1.1002  | 0.1543 | 0.1402 |
| OsPYL2             | SbPYL3       | 69.40  | 524.60 | 1.1691  | 0.0812 | 0.0694 |
| OsPYL2             | ZmPYL3       | 64.80  | 499.20 | 0.7683  | 0.1144 | 0.1489 |
| OsPYL3             | BdPYL2       | 85.30  | 535.70 | 0.9195  | 0.0863 | 0.0938 |
| OsPYL3             | BdPYL3       | 81.70  | 506.30 | 1.8213  | 0.1321 | 0.0725 |
| OsPYL3             | HvPYL2       | 70.90  | 505.10 | 1.0597  | 0.0933 | 0.0880 |
| OsPYL3             | SbPYL3       | 68.80  | 525.20 | 1.3384  | 0.1073 | 0.0802 |
| OsPYL3             | SbPYL2       | 67.50  | 535.50 | 0.6936  | 0.0961 | 0.1385 |
| OsPYL3             | ZmPYL3       | 57.10  | 503.90 | 0.5076  | 0.0532 | 0.1048 |
| OsPYL4             | AtPYL4       | 137.50 | 462.50 | 55.6376 | 0.1013 | 0.0428 |
| OsPYL4             | AtPYL5       | 109.20 | 442.80 | 64.5965 | 0.2737 | 0.0059 |
| OsPYL4             | AtPYL6       | 115.00 | 449.00 | 62.4790 | 0.3296 | 0.0053 |
| OsPYL4             | BdPYL6       | 85.30  | 502.70 | 1.7956  | 0.1362 | 0.0759 |
| OsPYL4             | BdPYL7       | 86.00  | 514.00 | 1.5294  | 0.1325 | 0.0866 |
| OsPYL4             | HvPYL2       | 79.60  | 502.40 | 1.1115  | 0.0729 | 0.0656 |
| OsPYL4             | HvPYL6       | 82.60  | 511.40 | 2.5193  | 0.1279 | 0.0508 |
| OsPYL4             | OsPYL5       | 80.20  | 522.80 | 2.3641  | 0.1013 | 0.0428 |
| OsPYL4             | SbPYL6       | 90.90  | 530.10 | 1.6537  | 0.2132 | 0.1289 |
| OsPYL4             | SbPYL5       | 82.40  | 511.60 | 3.1779  | 0.1080 | 0.0340 |
| OsPYL4             | ZmPYL7       | 82.30  | 523.70 | 2.3808  | 0.1514 | 0.0636 |
| OsPYL4             | ZmPYL13      | 90.40  | 506.60 | 1.3615  | 0.1732 | 0.1272 |
| OsPYL5             | AtPYL4       | 121.70 | 436.30 | 58.5149 | 0.2987 | 0.0051 |
| OsPYL5             | AtPYL5       | 110.70 | 453.30 | 65.0593 | 0.2808 | 0.0043 |
| OsPYL5             | AtPYL6       | 120.10 | 473.90 | 62.9904 | 0.3365 | 0.0053 |
| OsPYL5             | BdPYL6       | 76.10  | 505.90 | 0.9116  | 0.0703 | 0.0772 |
| OsPYL5             | BdPYL7       | 88.50  | 520.50 | 1.2382  | 0.1170 | 0.0945 |
| OsPYL5             | HvPYL4       | 90.70  | 515.30 | 1.2614  | 0.0797 | 0.0632 |
| OsPYL5             | HvPYL6       | 77.80  | 525.20 | 0.6748  | 0.0590 | 0.0874 |
| OsPYL5             | SbPYL6       | 82.50  | 529.50 | 2.5775  | 0.1714 | 0.0665 |
| OsPYL5             | SbPYL5       | 79.80  | 547.20 | 0.6968  | 0.0664 | 0.0953 |
| OsPYL5             | ZmPYL7       | 86.80  | 525.20 | 9.7639  | 0.1419 | 0.0145 |
| OsPYL5             | ZmPYL13      | 90.50  | 524.50 | 2.5277  | 0.1514 | 0.0599 |
| OsPYL6             | AtPYL5       | 109.10 | 463.90 | 66.9917 | 0.2994 | 0.0045 |
| OsPYL6             | AtPYL6       | 116.70 | 480.30 | 65.0515 | 0.3476 | 0.0053 |
input in psRNATarget [73] against all the rice mature miRNAs available in miRbase [74] (Additional file 8). Total of 32 unique potential miRNAs targeting the OsPYL family members of rice were identified with mature miRNAs of 21–23 nucleotide long, Watson-Crick or G/U base pairing and stable minimal folding free energy (MFE). Some of the miRNAs were found to target specific subfamily. For example osa-miR5832 targets OsPYL1–OsPYL3 of subfamily III, while osa-miR5075 targets OsPYL4 and OsPYL5 of subfamily-II and OsPYL3 of subfamily I. Two members of osa-miR5157-3p family (osa-miR5157a-3p and osa-miR5157b-3p) were predicted to target OsPYL8, OsPYL9 and OsPYL10 of subfamily I. The miRNAs that potentially target OsPYLs ranged from minimum one (OsPYL4) to maximum of eight (OsPYL2) (Fig. 6a). The prominent inhibitory action by most of the miRNAs predicted to target OsPYL members across the three subfamilies was through cleavage (Additional file 8). Interestingly, most of the miRNAs were found to play a key role in stress responsiveness and development (Additional file 8). Microarray expression analysis of the identified miRNAs showed that majority of them are downregulated in different tissues and under stress conditions, while osa-miR820a and osa-miR408-3p targeting OsPYL1 and OsPYL6, respectively, showed the highest expression level in all tissues and under different abiotic stresses (Fig. 6b). Thus, regulation of OsPYL genes through miRNA mediated sequence specific interaction might play a decisive role for plants to respond to growth and environmental stimuli.

Identification of SNPs in OsPYL members
To get a deeper insight into allelic variation of OsPYL members across 12 rice varieties selected based on stress responses [75]. Rice SNP-Seek database [76] (https://snp-seek.irri.org/) was queried SNPs in OsPYL genes using Nipponbare reference genome. Among 13 PYL genes in rice, non-synonymous SNPs were identified in 10 OsPYLs, but not in OsPYL1, OsPYL5 and OsPYL10 (Table 3). This shows that PYL1, PYL5 and PYL10 genes are highly conserved across different rice genotypes. Interestingly, on protein structural basis, we found that majority of the Single Amino acid Polymorphisms (SAPs) were located across the helix grip fold domains, while some were located on CL loops surrounding ABA binding pocket [20, 23] (Additional file 9).

**Table 2** The Ka/Ks values of the homologous PYL gene family in *Oryza sativa* among genome of *A.thaliana*, *B. distachyon*, *S. bicolor*, *Z. mays* and *H. vulgare* (Continued)

| OsPYL (Oryza sativa) | PYL Ortholog | S     | N     | Ka   | Ks   | Ka/Ks |
|----------------------|--------------|-------|-------|------|------|-------|
| OsPYL6               | BdPYL4       | 100.60| 520.40| 2.6849| 0.1572| 0.0585|
| OsPYL6               | BdPYL5       | 87.50 | 578.50| 1.0429| 0.0763| 0.0732|
| OsPYL6               | HvPYL7       | 88.60 | 577.40| 0.7845| 0.0630| 0.0804|
| OsPYL6               | SbPYL4       | 87.20 | 572.80| 1.4762| 0.0709| 0.0480|
| OsPYL6               | ZmPYL6       | 76.70 | 577.30| 1.3192| 0.0616| 0.0467|
| OsPYL6               | ZmPYL5       | 76.70 | 577.30| 1.3192| 0.0616| 0.0467|
| OsPYL7               | BdPYL8       | 104.80| 297.20| 2.4005| 0.2484| 0.1035|
| OsPYL7               | HvPYL8       | 105.70| 296.30| 1.1841| 0.2365| 0.1997|
| OsPYL7               | OsPYL10      | 99.10 | 311.90| 1.9137| 0.2234| 0.1167|
| OsPYL7               | SbPYL8       | 106.80| 313.20| 1.4210| 0.2666| 0.1876|
| OsPYL7               | ZmPYL11      | 106.30| 319.70| 2.0012| 0.2953| 0.1475|
| OsPYL7               | ZmPYL10      | 109.80| 310.20| 1.3024| 0.2482| 0.1906|
| OsPYL11              | BdPYL9       | 130.50| 475.50| 0.8664| 0.0327| 0.0377|
| OsPYL11              | SbPYL7       | 111.10| 479.90| 1.1054| 0.0939| 0.0850|
| OsPYL11              | ZmPYL8       | 102.00| 489.00| 1.7432| 0.0996| 0.0572|
| OsPYL12              | AtPYL7       | 104.30| 258.70| 44.4530| 0.3180| 0.0072|
| OsPYL12              | AtPYL8       | 107.10| 252.90| 18.1991| 0.3106| 0.0171|
| OsPYL12              | BdPYL8       | 90.60 | 272.40| 7.1185| 0.2313| 0.0325|
| OsPYL12              | HvPYL8       | 89.20 | 246.80| 5.3474| 0.3427| 0.0641|
| OsPYL12              | SbPYL8       | 75.80 | 287.20| 9.5204| 0.2396| 0.0252|
| OsPYL12              | ZmPYL11      | 66.30 | 206.70| 16.3826| 0.4896| 0.0299|
| OsPYL12              | ZmPYL10      | 80.20 | 282.80| 8.0420| 0.2375| 0.0295|
Fig. 6 (See legend on next page.)
Identification of cis-regulatory elements (CREs) in promoters of OsPYLs

Rice, being a sensitive crop to various biotic and abiotic stresses, needs to adapt swiftly to frequently changing stresses [66]. The control of gene transcription via CREs in the promoter remains a pivotal mode of regulation of gene expression. To investigate the potential CREs in OsPYL gene family in rice, the promoter sequences of approximately 2.0-kb upstream from the translation start sites of individual PYL receptors, were taken from RGAP [68] and searched against New PLACE [77] (https://www.dna.affrc.go.jp/PLACE/?action=newplace). A total of 193 putative CREs were predicted across thirteen OsPYL genes with a range from 62 to 105 CREs (Additional file 10). The prevalence distribution of 20 key CREs are schematically depicted (Fig. 7). Some of these elements are conserved across OsPYL family, and might be critical in imparting stress and developmental regulation. We have identified certain CREs that can be functionally attributed to individual OsPYL members or subfamily.

Table 3 Single Amino acid Polymorphisms (SAPs) in PYL proteins of selected rice genotypes

| Genotype   | OsPYL2 | OsPYL3 | OsPYL4 | OsPYL6 | OsPYL7 | OsPYL8 | OsPYL9 | OsPYL11 | OsPYL12 | OsPYL13 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|            | G125   | A125   | F49    | A86    | V132   | R21    | G3     | V144   | P22    | N62    |
|            | G125   | K101   | C49    | A86    | V132   | R21    | A11    | V144   | S22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | P22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | S22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | P22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | S22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | P22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | S22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | P22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | S22    | N62    |
|            | G125   | M101   | F49    | A86    | V132   | R21    | A11    | V144   | P22    | N62    |
example OsPYL8 and OsPYL9 has endosperm and seed specific CREs like -300CORE [78], and 2SSEEDPROTNAPA [79]. Previously these elements have been reported to be associated with endosperm specific activity of OsPYL8 and OsPYL9 [46]. Likewise ABREZMRAB28 is induced by ABA and is a binding site for CBFs [80]. It has been recently reported that overexpression of OsPYL10 imparts dehydration and freezing tolerance in transgenic rice [47]. We have identified cis-element TAAAGSTKST1 [81] which is a target site for transcription factor governing guard cell specific gene regulation in stomata. All the identified CREs have been grouped into four major categories based upon function and their response to stimuli (Fig. 8). In this study, hormone responsive elements like abscisic acid responsive elements (ABRE) form the major proportion of CREs. Interestingly gibberellic acid responsive elements (GARE) and auxin responsive elements (ARE) were also found in abundance in the promoters, suggesting potential hormonal cross talk at the expression level of ABA receptors (Fig. 8a). Amongst stress responsive elements, dehydration responsive elements (DRE) forms the major group followed by low temperature responsive elements (LTRE) (Fig. 8b). On the basis of metabolic functions, the proportion of amylose/starch responsive elements (34%) followed by carbohydrate responsive elements (27%) was notably highest, while elements involved in amino acid metabolism comprised least (6%) of the CREs identified (Fig. 8c). The proportion of elements that might be involved in photosynthetic machinery and light responsive elements (LRE) was found to be highest (34%) followed by seed and endosperm specific elements (19%) (Fig. 8d). All OsPYL promoters had more than one stress-response-related CREs. CREs associated with hormonal regulation, including ABRE, AuxRR-core, CGTCA motif, P-box, TCA-element, and TGA-element were identified in most of the OsPYL genes promoter. TC-rich repeats, which are involved in low temperature, drought inducibility and defence responsiveness, were also

![Fig. 7 cis-regulatory Elements (CREs) in the promoter of PYL genes. Positional distribution of predicted CREs on OsPYL promoters are shown as vertical bars. Promoter sequences (~ 2000 bp) of thirteen OsPYL genes were analyzed by using NewPLACE. Legend depicting the colour of individual cis elements](image-url)
found in many OsPYLs (Additional file 11). This suggests that OsPYL genes are regulated by diverse development and stress responses.

**In silico expression analysis of OsPYL genes**

Spatiotemporal and stress responsive expression analysis of OsPYL genes was carried out using GENEVESTIGATOR database [82] (https://genevestigator.com/gv/). OsPYL1, OsPYL2, OsPYL6, OsPYL10 and OsPYL11 showed expression in most tissues across developmental stages (Fig. 9a and b). OsPYL7, OsPYL8 and OsPYL9 were found to be specifically expressed in endosperm and embryo suggesting their potential role in seed development in rice. In response to rice blast fungus (*Magnaporthe oryzae*) infection, OsPYL5 and OsPYL6 showed upregulation in leaf of indica cv. Pusa Basmati 1. Bacterial leaf streak pathogen (*Xanthomonas oryzae pv. oryzicola*) inoculation also induced these two PYLs and OsPYL12 (Fig. 10a). Ethylene moderately upregulated only OsPYL5 (Fig. 10b). Alkali treatment induced the expression of OsPYL6 to > 1.5 folds in leaves. Drought stress induced the expression of OsPYL1, OsPYL8 and OsPYL10 by > 1.5 folds in leaves. Drought stress induced the expression of OsPYL10 by > 1.5 fold leaf but it was downregulated in the roots (Fig. 10c). Heat stress upregulated OsPYL1 and downregulated OsPYL6 in leaf. Both drought and salt stresses downregulated OsPYL5 and OsPYL6 (Fig. 10c).

**Real time qRT-PCR expression profiling of OsPYL genes in different tissues**

Tissue and stress responsive expression of PYL genes were analysed to understand their role in development
OsPYL8 and OsPYL9 showed with highest expression in seeds, while OsPYL1, OsPYL11 and OsPYL13 showed highest expression in panicle among the tissues (Fig. 11). Among the PYLs, OsPYL2 showed highest expression in roots of the plants at reproductive stage as compared with that in other tissues. Interestingly, many PYLs (OsPYL1, OsPYL4, OsPYL5, OsPYL8, OsPYL9, OsPYL11, OsPYL12 and OsPYL13) also showed high levels of expression in stem tissue at reproductive stage as compared with that in seedling roots (Fig. 11).
Real time qRT-PCR expression profiling of OsPYL genes in responses to abiotic stresses

All the subfamily III OsPYLs were significantly upregulated by all the abiotic stresses at least in one tissue at seedling stage in drought tolerant rice cv. Nagina 22 (Fig. 12). OsPYL1 was significantly upregulated in both root and shoot at seedling stage by abiotic stresses except NaCl which downregulated its expression in root (Fig. 12). Similarly contrasting tissue specific regulation was observed for OsPYL2 under osmotic (PEG) stress and ABA, where it was upregulated in shoot and downregulated in root at seedling stage. OsPYL3 was upregulated only in shoot by PEG stress, while it was upregulated both in root and shoot by NaCl and heat stress, and downregulated in the both the tissue by cold stress (Fig. 12). The subfamily II OsPYLs (PYL4, PYL5 and PYL6) were mostly downregulated both in root and shoot by all stresses and none were upregulated by stress at seedling stage (Fig. 12). Among subfamily I OsPYLs (PYL7-PYL13), OsPYL8, OsPYL9 and OsPYL13 were significantly upregulated by osmotic stress (PEG) in shoot. Salt stress significantly upregulated OsPYL7, OsPYL8 and OsPYL11 in shoot, and OsPYL8 and OsPYL9 in root. Cold stress significantly upregulated OsPYL7, OsPYL9 and OsPYL13 in both root and shoot at seedling stage. Heat stress significantly upregulated OsPYL8, OsPYL9 and OsPYL10 in both root and shoot, and OsPYL11 and OsPYL12 only in shoot at seedling stage (Fig. 12). ABA regulated the expression of all OsPYLs except OsPYL9, OsPYL11 and OsPYL13 at least
in one tissue at seedling stage in rice ABA significantly upregulated OsPYL2, OsPYL7 and OsPYL12 in shoot, and OsPYL1, OsPYL8 and OsPYL10 in root at seedling stage (Fig. 12).

At reproductive stage, tissue specific regulation of PYLs was observed under drought stress. In root tissue, drought stress significantly upregulated the expression of OsPYL4, while it downregulated rest of the OsPYLs except OsPYL12. In contrast, in the stem tissue, drought stress significantly upregulated most OsPYLs, except OsPYL7 and OsPYL13 which were downregulated, and OsPYL10 which was unaltered. Drought stress significantly upregulated OsPYL2 and OsPYL4 in flag leaf and OsPYL8, OsPYL9 and OsPYL13 in panicles (Fig. 13). Thus, at reproductive stage, drought stress either up- or down-regulated the expression all the OsPYLs in most of the four different tissues examined (Fig. 13).

Heat stress at reproductive stage differentially regulated the expression of all OsPYLs in vegetative tissues (root, stem, leaf) except that of OsPYL1 in stem, while in panicle it regulated most PYLs except OsPYL7, OsPYL10 and OsPYL12. Among the subfamily III members, OsPYL3 was significantly upregulated under heat stress in all four tissues (Fig. 13). Among the subfamily II members, OsPYL6 was downregulated in all four tissues, OsPYL4 was upregulated in stem and leaf but downregulated in root and panicle, and OsPYL5 was downregulated in roots and upregulated in rest of the three tissues under heat stress (Fig. 13). Among the subfamily I members, OsPYL8, OsPYL9, OsPYL10 and OsPYL12 were significantly upregulated in both root and stem, OsPYL7, OsPYL8 and OsPYL9 in flag leaf and OsPYL13 in panicle under heat stress (Fig. 13).

Discussion
Gene structure and evolution
ABA is a key phytohormone that governs various plant development and stress response processes. ABA is perceived by PYL family of receptors, which are the largest plant hormone receptor family known [12]. Despite arduous efforts investigation on PYL gene family has largely been confined to characterization of PYL genes mainly in Arabidopsis, while only limited efforts have been made on genome wide characterization of PYL gene family for elucidating their role in multiple stresses and their evolutionary relationship in major crops. In the present study, a genome wide comprehensive analysis of PYL genes in rice and its potential role in development and abiotic stress responses was investigated using bioinformatic and experimental approaches. A total of 13 OsPYLs were identified from rice genome using Arabidopsis PYL protein sequence as queries as previously reported [44]. The sequences of all 13 OsPYLs from upland indica rice variety Nagina22 were used in the present study. Based on phylogenetic relationship with Arabidopsis, OsPYL family can be broadly classified into three subfamilies: I, II and III (Fig. 1a). Subfamily II and III PYLs of rice and Arabidopsis are intronless, while all seven members of OsPYLs (Fig. 1b). The organizations of intron/exon and the number of exons in the surveyed rice ABA receptors were similar to those orthologs in
Fig. 12 (See legend on next page.)
maize, tomato, rubber, cotton, brachypodium and sorghum [54, 56, 59, 61, 63, 83]. These results imply that the exon/intron organizations of OsPYLs are closely related to the phylogenetic relationship of the genes (Fig. 1a). Introns play key role in post-transcriptional regulation gene expression by splicing-dependent and splicing-independent intron-mediated enhancement of mRNA accumulation [84]. All the PYL members of subfamily I has evolved introns to fine tune their regulation.

To further understand the diversification of PYL gene family at protein level, ten conserved motifs were acquired for each protein using MEME Suite (Fig. 1c). All the PYL proteins had motif 1 that has the conserved Gate and Latch domain of PYL receptor [23, 28]. Motif 2 and motif 3 were present in all PYL members except the non-functional OsPYL7 and OsPYL12. HHpred analysis to affirm if the motifs obtained from the MEME analysis are similar to any of the known protein motifs revealed that Motif1, 2 and 3 belongs to Polyketide cyclase/dehydrase and lipid transport superfamily protein. It was observed that these novel motifs did not show any significant similarity with the known motifs (Additional file 3). This indicates that all the identified OsPYLs have typical subfamily features and the proteins classified into the same subgroup share similar protein motifs.

ExPaSy analysis of physical properties of the OsPYLs from indica rice cultivar Nagina 22 showed that except OsPYL7, OsPYL12 and OsPYL13, all other receptors had similar protein length and physical properties (Table S1). Amino acid sequence of most ABA receptor were quite similar between japonica and indica barring few SNPs in OsPYL2, OsPYL3 and OsPYL9 (NCBI Accession no. KJ634482, KM371729, KM371729). Compared to japonica rice, OsPYL3 in indica rice has an insertion of nine nucleotides, increasing the amino acid sequence by three residues.

Chromosomal distribution of PYL genes across 12 rice chromosomes showed uneven distribution of PYLs with as many as five PYLs located on six chromosomes. OsPYL8 and OsPYL9 having 95.5% sequence similarity at nucleotide levels are located in nearby positions on chromosome 6 suggesting a tandem gene duplication event that might have caused evolution of one these receptors. Unequal number of PYLs across seven different plant species ranging from 8 to 14 (sorghum and Arabidopsis) identified in the current study.

We analysed ABA receptors in seven members of grass family. Phylogenetic analysis of PYL protein sequences from rice, maize, sorghum, barley, brachypodium, wheat and foxtail millet showed that PYL gene family across seven species can be broadly grouped into three subfamilies similar to that in Arabidopsis (Fig. 3). Subfamily II has the maximum number of PYL members (41), followed by Subfamily III (31 PYL genes) and Subfamily I has the least members (29 PYL genes). These members of grass family analysed here have evolved from a common ancestor about 96 Ma, and BOP (Bambusoideae-Oryzoideae-Pooidaeae) and PACCMAD (Panicoideae, Arundinoideae, Chloridoideae, Centothecoideae, Aristidoideae, Danthonioideae) clades diversified about 70 Ma. Arabidopsis and rice which diverged from a common ancestor about 120–200 Ma have similar number of PYLs. It is interesting to note that rice (BOP clade) and maize (PACCMAD clade) have 13 PYLs each, while other members of BOP (wheat – each diploid genome, barley and Brachypodium) and PACCMAD (sorghum and foxtail millet) have only 8–9 PYLs, respectively. The sub-family III of all seven members of grass family analysed here have 3 PYLs (PYL1-PYL3), while it varied among grasses in sub-family II (4–7 PYLs) and family I (2–7 PYLs). The conservation of three subfamilies albeit with different number of members suggests non-redundant roles for each subfamily.

Collinearity results showed that approximately 181 (Additional file 12 Table S3) homologous gene pairs existed among PYLs from A. thaliana, B. distachyon, O. sativa, Z. mays, S. bicolor and H. vulgare at genome wide scale (Fig. 4), grouping PYL genes into 5 clusters with each cluster depicting high sequence similarity and might therefore share same functional domains. Surprisingly PYLs in cluster 5 belonged to subfamily III to phylogenetic classification. Synteny analysis of PYL family between Arabidopsis and rice showed that collinearity blocks between PYL members of subfamily II were highest, while members of subfamily III formed least collinearity pairs (Fig. 5). These results suggest that PYL family expanded through segmental duplication events during evolution. The evolutionary history subfamily II members might provide more clues to the origin and evolution of the PYL gene family.

Non synonymous SAPs in OsPYLs identified from 3 K SNP seek database showed that frequency of SAPs was high across indica rice cultivars as compared to landraces (Table 3). Moreover most of the SAPs were present across START domain that might modulate the
ABA binding ability of ABA receptors in response to different types and magnitude of stresses.

**Regulation of gene expression**

We carried out a systematic analysis of CREs in promoter regions of PYLs and identified various types of CREs such as stress responsive elements, hormone responsive elements, metabolic responsive elements and elements involved in growth and development (Additional file 10). The number of ABA and stress responsive elements was found to be highest in the promoters across *OsPYL* genes. Most *OsPYL* promoters contain CREs stress signalling and pathogen response. Each promoter of *OsPYL* genes possessed more than one cis elements involved in photosynthesis and light responsiveness, while elements involved in carbohydrate and starch metabolism was equally high across all promoters. This suggests that *OsPYL* genes might be regulated by photosynthesis and carbohydrate metabolism signalling. The knowledge of presence of regulatory elements in the promoters of PYL genes can further help in functional characterization and tailoring PYL receptors in rice and other commercially important crops.

We identified 32 candidate miRNAs that may potentially target *OsPYLs* mRNA to regulate their expression in rice (Fig. 6a). Most of these miRNAs identified have been implicated in stress responsiveness and development (Additional file 8). In an earlier study with *Brassica napus*, 26 miRNAs targeting 11 BnPYL genes were identified, and predicted that 10 members of miR169 target BnPYL1–4 [85]. For better understanding, each *OsPYL* gene targeted by different miRNAs is depicted by different colour and shape (Fig. 6c). In our study, many *PYLs* were targeted by multiple miRNAs with maximum of 8 different miRNAs targeting *OsPYL2*. Although *OsPYL8* and *OsPYL9* have very high sequence similarity, *OsPYL9* is targeted by 7 miRNAs while *OsPYL8* is targeted by only 4 miRNAs. The miR5075 targets five *OsPYL* genes and thus may play a critical role in fine tuning the expression of PYLs. In silico expression analysis of the identified miRNAs under different tissues and abiotic stresses revealed that apart from osa-miR820a and osa-miR408-3p, other miRNAs were downregulated (Fig. 6b). It is important to note that ABA-activated SnRK2 kinases interact with and phosphorylate SERRATE (SE) and HYPOSTATIC LEAVES (HYL1) proteins involved in miRNA biogenesis [86]. The predicted miRNA mediated regulation of PYLs suggests that miRNA mediated regulation of PYLs may be important not only for stress response of rice but may also play a key role in feedback regulation of overall miRNA biogenesis through PYL-mediated regulation of SnRK2.2/3/6. Further functional characterization of the predicted miRNAs would enable us to better understand the regulatory mechanism underlying ABA receptors and miRNA biogenesis.

Analyzing the spatiotemporal pattern of gene expression across the broad spectrum of different tissues, developmental stages and stress conditions would provide insight into the physiological and developmental functions of *OsPYLs*. In silico as well as q-RT-PCR expression analysis of *OsPYLs* different stress treatments and developmental stages showed that *PYL* genes are regulated in a tissue and developmental dependent manner and by multiple stresses (Fig. 10, 11, 12). Two members of subfamily II (*OsPYL5* and *OsPYL6*) and all 3 members of subfamily III showed higher expression potential in different tissues across developmental stages, while only *OsPYL10* and *OsPYL11* among the 7 members of subfamily I showed higher expression potential in different tissues across developmental stages (Fig. 9). This suggests that these PYLs have multiple roles throughout the growth and development of rice. The *PYL8* and *PYL9* orthologs in Arabidopsis have been shown to play important role in lateral root formation during seedling growth [5]. Interestingly, *OsPYL7, OsPYL8* and *OsPYL9* showed expression during germination stage and seed development (embryo, endosperm and caryopsis) (Fig. 9).

Real-time qRT-PCR analysis also showed that *OsPYL8* and *OsPYL9* were highly expressed in panicles and seeds (Fig. 11). In a previous study also it was found that these two PYLs were highly expressed in seeds [42]. These results suggest that *OsPYL7, OsPYL8* and *OsPYL9* may have specific role in germination as well seed development. Although *OsPYL7* was predicted as non-functional due to lack of C-terminal sequences (motif2), it was found to be co-expressed with *OsPYL8* and *OsPYL9* in seeds (embryo, endosperm and caryopsis) (Fig. 9). Further studies may illuminate whether *OsPYL7* interact *OsPYL8* and *OsPYL9* to regulate rice seed development. In our qRT-PCR expression analysis also *OsPYL8* and *PYL9* showed very high levels of expression in panicle and seeds as compared with other tissues (Fig. 11). Notably we also identified CREs 25SEEDPROTBANAPA and –300 CORE, which are involved in seed and endosperm specific expression, specifically present in promoter region of *OsPYL8* and *OsPYL9*. This further
strengthens the proposed roles of OsPYL8 and OsPYL9 in seed and endosperm specific activity. OsPYL5, OsPYL9, OsPYL11 and OsPYL13 may play an important role in regulation of source activity as their expression was higher in flag leaf which contributes to > 70% of current assimilation for grain development. A previous study showed that only OsPYL6 (their nomenclature, OsPYL4) was upregulated, while OsPYL2, OsPYL3, OsPYL4 and OsPYL10 were downregulated by 200 μM in 14 days old seedlings of japonica cv. Nipponbare [42]. In contrast, our analysis showed that 100 μM ABA significantly upregulated OsPYL1, OsPYL8 and OsPYL10 root, and OsPYL1, OsPYL2, OsPYL5, OsPYL7 and OsPYL12 in shoots of drought tolerant indica rice cv. Nagina22 (Fig. 12). In consistent with Tian et al. [42], in our study also ABA downregulated OsPYL2, OsPYL3 and OsPYL4 roots, OsPYL4 and OsPYL10 in shoots. Thus, ABA-mediated regulation of OsPYLs appears to be genotype and tissue dependent. Previous functional validation studies have shown that constitutive/stress-inducible overexpression of OsPYL2 [42], OsPYL10 [42, 47, 87], and OsPYL1 (=RCAR5) [44] conferred tolerance to abiotic stresses. In a previous study, expression analysis of OsPYL1 (=RCAR5) [44] under salt, PEG and ABA treatments showed that it is significantly downregulated by ABA and salt stress. In our analysis ABA downregulated the expression of OsPYL11 in root, while salt stress upregulated in shoots of drought and heat tolerant cv. Nagina 22. In a previous study, OsPYL10 [47] expression was found to be downregulated by PEG, NaCl and cold stresses in the roots, but was found to be upregulated by ABA in roots. In this study also similar expression of OsPYL10 was found under these treatments. However, in none of the previous studies expression of all OsPYLs under different stresses were examined. In this study, qRT-PCR expression analysis showed that all the OsPYLs are regulated by one or more abiotic stresses (osmotic/PEG, drought, salt and cold) in at least one tissue/development stage of rice plant (Fig. 12, 13). This suggests that all PYLs play important roles in abiotic stress responses of rice. In general subfamily I and III PYLs were upregulated by different abiotic stresses and ABA at seedling stage of drought and heat tolerant rice cv. Nagina 22, while subfamily II PYLs were downregulated (Fig. 12, 13). This suggests that subfamily-wise stress response role at seedling stage. In consistent with the presence of CCAATBOXI [88] CRE that act as heat responsive elements in the Promoters of PYLs, heat stress upregulated OsPYLs except OsPYL6 and OsPYL11, at least in one tissue (Fig. 13). Over all, drought stress downregulated all PYLs except OsPYL4 which was upregulated and OsPYL12 which was unaltered, while heat stress upregulated OsPYL1, OsPYL3 OsPYL8, OsPYL9, OsPYL12 and OsPYL12 in the roots at reproductive stage (Fig. 13). This suggests contrasting regulation and function of OsPYLs in roots under drought and heat stress. Both drought and heat stress upregulated OsPYL2, OsPYL3 OsPYL4, OsPYL5, OsPYL8, OsPYL9 and OsPYL12 in the stem at reproductive stage, while OsPYL1, OsPYL6 and OsPYL11 were upregulated only by drought and OsPYL10 was upregulated only by heat in the stems (Fig. 13). Interestingly all the three OsPYLs (OsPYL2, OsPYL4 and OsPYL13) upregulated in flag leaf in response to drought stress were also upregulated under heat stress. In the panicle only OsPYL13 was commonly upregulated by both drought and heat at reproductive stage. OsPYL8 and OsPYL9 were specifically upregulated by drought but were downregulated by heat in panicles, while OsPYL3 which was upregulated under heat but was downregulated under drought stress in panicle (Fig. 13). The diverse expression patterns of OsPYLs were indicative of their functional distinctiveness.

Conclusion

The present study is a comprehensive functional identification and characterization of PYL gene family in indica rice at genomic level. Evolutionary relationship of PYL genes in rice and other cereal crops was established which grouped PYL genes into three subfamilies that are structurally and functionally evolutionarily conserved. Identified cis elements could help in understanding the diversified role of PYL receptors in response to different stresses and developmental stages. These data will provide the basis for understanding evolutionary history and the developmental roles of OsPYL genes in rice, and may be helpful for future exploration of the biological functions of OsPYL genes. These findings will also serve to extend our knowledge for identifying candidate genes that improve plant architecture under stress conditions and enable potential breeding and genetic improvements for other agriculture crops.

Methods

Identification of PYL genes from plant species

To identify ABA receptors of different species, Arabidopsis PYLs were used as query in EnsemblPlants database (https://plantsensembl.org/index.html) against their respective species genome with a threshold of 10−4 and Match/Mismatch score of 2 and –3.

Chromosomal distribution of PYL genes in rice

Chromosomal location of OsPYL genes was determined with respect to their position and information retrieved from rice genome sequences. The physical map information on chromosome number, length and gene loci were obtained from rice genome annotation project (RGAP)
Sequence retrieval and phylogenetic analysis
Gene sequences of rice PYLs were identified from the Rice Genome Annotation Project [68] using Arabidopsis PYL protein sequences from Arabidopsis Information Resource [89]. In the present study, Nagina22 sequence for OsPYL7 and OsPYL13 were retrieved from Rice SNP-Seek Database (https://snp-seek.irri.org/), while rest of the PYLs were coned and sequenced from Nagina 22. Sequences of PYLs from Brachypodium, Sorghum, barley, maize, foxtail millet and wheat genomes were retrieved from EnsemblPlants database (https://plants.ensembl.org/index.html). For phylogenetic analysis, amino acid sequences of putative PYL proteins of H. vulgare, S. bicolor, O. sativa and A. thaliana, T. aestivum, Z. mays, S. italica and B. distachyon were analyzed. The genes of PYLs were named based on numbering and sequence homology with A. thaliana orthologs genes. Multiple sequence alignment was executed by ClustalW 2.0 program [90]. Phylogenetic trees were constructed using MEGA X [70] by the maximum likelihood method [69].

Analysis of gene structure, conserved motif and protein properties
Conserved motifs were also predicted for all 13 OsPYL proteins using MEME Suite v5.1.0 [91, 92]. Functional annotations of these motifs were performed using HHpred (http:// toolkit.tuebingen.mpg.de/hhpred) [93]. Maximum number of motifs was specified as 10. Parameters on minimum/maximum width were specified as 6 and 50, while the range for minimum and maximum sites per motif were kept as 2 and 13, respectively. The motifs were serially numbered according to their frequency of occurrence in MEME. Motifs were placed adjacent to their respective OsPYLs in accordance with their subfamily signatures based on phylogenetic relationship. Using the ExPASy database, the isoelectric points and molecular weights of the OsPYLs were predicted.

miRNA identification and in silico expression analysis
miRNA targeting PYL genes in rice were predicted using psRNATarget [73] against all the rice mature miRNAs that were reported in miRBase [74] and the network was created using Cytoscape [94]. In silico expression analysis of identified miRNAs was carried out using miRid as query against rice datasets of plant miRNA expression atlas database PmiRExAt (http://pmirexat.nabi.res.in/index.html) [95].

Genome wide collinearity and Ka/Ks analysis of PYLs
Analysis of homologous gene pairs of PYLs among Arabidopsis thaliana, Oryza sativa, Zea mays, Brachypodium distachyon, Sorghum bicolor and Hordeum vulgare at genome level was carried out. Whole genome primary transcript file of each species was obtained from Phytozone database (https://phytozone.jgi.doe.gov/pz/portal.html) and whole genome reciprocal protein to protein BLAST in all pair wise combinations (36 permutation combinations) was carried out. BLAST result of all the possible combinations was merged. Annotation file of all selected species were imported into Cytoscape and collinearity network was constructed. Similarly for Synteny block calculation of PYL gene family between Arabidopsis and rice at genome scale were used. PYL genes were filtered form whole genome collinearity data along with gene positional information of gene was detected using ‘collinearity with gene families’ were queried in MCScanX and visualized in CIRCOS [96, 97] using default parameters. The values of nucleotide substitution parameter Ka (non-synonymous) and Ks were calculated for PYL gene family in Oryza sativa. Orthologs of rice PYL gene family were noted from collinearity blocks table and calculated using MCScanX software [96]. Pairwise global alignment for all ortholog pairs (protein sequences) was done online by EMBOSS Needle Pairwise Sequence Alignment tool [98]. Online program PAL2NAL [99] was used to convert protein sequence alignment and the corresponding mRNA sequences into a codon alignment and calculating Ka/Ks value from the aligned codon.

Identification of SNPs in PYL genes
Amongst available 3024 accessions, sequence of 13 PYL genes from 12 mega rice varieties comprising of contrasting genotypes in terms of abiotic stress sensitivity were fetched from Rice SNP-Seek database [76] against the reference Nipponbare sequence. Non-synonymous SNPs that could particularly translate to change in the protein sequence were identified across the selected genotypes. Domain based localization of SAPs was done on the basis of secondary structure of OsPYL proteins.

Identification of putative cis-regulatory elements (CREs) in the promoters
The 2000 bp upstream sequences from the translation start site of all of the OsPYL genes were obtained from Rice genome annotation project database. The putative cis-acting regulatory elements in these sequences were predicted using the NewPlace web server [77] and then to identify the putative CREs. Functional annotation of individual CRE was manually curated from place.seq (https://www.dna.affrc.go.jp/PLACE/place_seq.shtml); (Additional file 10).
Expression analysis of OsPYLs in rice
In silico expression analysis of ABA receptors at different developmental stages and stresses were analyzed using GENEVESTIGATOR database (https://genevestigator.com/gv/) [82]. The rice genotype Nagina 22 (Oryza sativa ssp. indica cv. Nagina 22) seeds, from our lab in Division of Plant Physiology, ICAR-IARI, New Delhi, was used for analysis of tissue specific and stress responsive expression of 13 OsPYL genes at seedling stage (14 day old) and reproductive stage using real time qRT-PCR. OsPYL expression was analyzed in different tissues from plants at anthesis stage exposed to control, drought and heat stress treatments. At seedling stage, plants grown in Yosida’s medium (YM) were treated with YM supplemented with 20% PEG 6000 (−0.49 MPa), 200 mM NaCl (−1.01 MPa; 20 dS/m) and cold (4 °C) stresses, Heat (42 °C) and 100 μM ABA for 6 h. Samples were collected from control, and treated plants and frozen in liquid nitrogen. Drought and heat stress was imposed at anthesis stage in Nagina22 grown in pot at green house, IARI, New Delhi conditions of 30 ± 2 °C for control and drought and while temperature of 42 ± 2 °C for heat at a relative humidity (RH) of ~ 60–70%. Drought was imposed at reproductive stage by withholding water till the soil matric potential (SMP) reached up to -80 kPa.

Quantitative real-time RT-PCR
Total RNA was extracted using RNeasyMini Kit (Qiagen, Germany) following manufacturers protocol and treated with DNaseI. Quantification of RNA was carried out using NanoDrop (Thermo Fisher, US). cDNA was synthesized from 2 μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed with Hotstart SYBR Green master mix (KAPA SYBR FAST; Universal). PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 40 s in StepOne Real-Time PCR system (Applied Biosystems). The relative expression levels of OsPYL genes were calculated based on the comparative Ct method using the 2^ΔΔCt method [100] and all expressions were normalized against the Ubiquitin5 gene [101]. Root tissue ΔCt of seedling stage was used as calibrator for tissue specific expression analysis, while expression level control tissue (ΔCt) was used as calibrator for stress responsive expression analysis. The primers used are listed in (Additional file 13 Table S4).

Statistical analysis
The presented values are the means ± SE of three different experiments with three replicated measurements. Unpaired t-Test was used to compare significant differences based on Fisher’s LSD test at significance levels of P < 0.05 and P < 0.01 using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-07083-y.

Additional file 1. Complete CDS sequences of 13Nagina22 OsPYLs.
Additional file 2. Sequence of 13 Nagina22 OsPYL proteins.
Additional file 3: Table S1. Putative function of Motifs identified.
Additional file 4: Sequence of identified PYLs of eight species.
Additional file 5: Table S2. Pair wise collinearity among PYLs of Arabidopsis and rice.
Additional file 6: Figure S1. Synteny blocks between Arabidopsis and rice at genomic level.
Additional file 7: Ka/Ks values of PYL orthologs.
Additional file 8. Sequence and detail of identified miRNAs targeting OsPYLs.
Additional file 9: Figure S2. Sequence alignment of 13 OsPYL proteins depicting four conserved loops CL1–CL4.
Additional file 10. List of identified CRE and their putative function in 13 OsPYL promoter.
Additional file 11: Figure S3. Frequency and distribution of identified CRE in individual OsPYL promoter.
Additional file 12: Table S3. List of homologous gene pair between Arabidopsis and rice.
Additional file 13: Table S4. List of primers used for q-RT expression analysis of 13 OsPYL genes.

Abbreviations
ABA: Abscisic acid; MW: Molecular weight; NJ: Neighbor-joining; ORF: Open reading frame; pl: isoelectric point; PYL: PYR1-Like; PRY: Pyrabactin Resistance; RCAR: Regulatory Component of ABA Receptor; SNP: Single nucleotide polymorphism; SAP: Single amino-acid polymorphism; CRE: cis regulatory elements

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Authors’ contributions
Conceptualization of research (SKY, BC and VC); Methodology (SKY, VVSK, RKV and PY); q-RT-PCR expression analysis (SKY, VSK and RKV); In silico promoter analysis and miRNA identification (PY); Collinearity and synteny analysis (SKY, AS and DPW); Data Analysis (All authors); Manuscript writing (SKY, BC and VC); Supervision (VC). All authors read and approved the final manuscript.

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Availability of data and materials
The sequences of ABA receptors cloned from rice cv. Nagina 22 were deposited in the NCBI and are available in the NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/; Table 1; Additional file 1). The rice genome sequences, the physical map information on chromosome number and length and gene loci are available in RGAP (http://rice.plantbiology.msu.edu/), the Nagina22 sequence for OsPYL7, OsPYL12 and OsPYL13, the sequence of thirteen PYL genes from twelve mega rice varieties, the reference Nipponbare sequence are available SNP-seek (https://snp-seek.irri.org/; Table 1; Additional file 1), and the sequences of PYLs from Arabidopsis (https://www.arabidopsis.org) Brachypodium, Sorghum, barley, maize, foxtail millet and wheat genomes (https://plantsensembl.org/index.html) are available in the respective database. The accession number and web links for the PYLs of these species are given in Additional file 4.
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

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