Novel Genomic and Evolutionary Insight of WRKY Transcription Factors in Plant Lineage

Tapan Kumar Mohanta¹, Yong-Hwan Park² & Hanhong Bae²

The evolutionarily conserved WRKY transcription factor (TF) regulates different aspects of gene expression in plants, and modulates growth, development, as well as biotic and abiotic stress responses. Therefore, understanding the details regarding WRKY TFs is very important. In this study, large-scale genomic analyses of the WRKY TF gene family from 43 plant species were conducted. The results of our study revealed that WRKY TFs could be grouped and specifically classified as those belonging to the monocot or dicot plant lineage. In this study, we identified several novel WRKY TFs.

To our knowledge, this is the first report on a revised grouping system of the WRKY TF gene family in plants. The different forms of novel chimeric forms of WRKY TFs in the plant genome might play a crucial role in their evolution. Tissue-specific gene expression analyses in Glycine max and Phaseolus vulgaris showed that WRKY11-1, WRKY11-2 and WRKY11-3 were ubiquitously expressed in all tissue types, and WRKY15-2 was highly expressed in the stem, root, nodule and pod tissues in G. max and P. vulgaris.

Plants are continuously subjected to biotic and abiotic stresses throughout their life cycle. Hence, they have developed an evolutionarily complex series of signaling mechanisms to perceive and respond to different signals via different signaling pathways. Transcriptional regulation plays remarkable roles in response of different signaling events. It has progressed from ancient life forms to advanced life forms and is inseparably connected through developmental progression. Such transcriptional progression mechanisms are regulated by different types of transcriptional machinery commonly known as transcription factors (TFs). The TFs possess the ability to activate or repress the expression of target genes responsible for the regulation of different signaling cascades. The WRKY TF is one such TF found in plants. WRKY TFs are characterized by the presence of a unique WRKY domain of approximately 60 amino acid residues. The domain contains a highly conserved WRKYGQK heptapeptide amino acid sequence and conserved C2H2 or C2HC zinc finger motif. The conserved WRKY domain plays a crucial role by binding to the W-box DNA motif TTGACC/T of the target gene. Almost all WRKY TFs identified thus far preferentially binds to a specific core DNA sequence. In addition to binding to the W-box DNA motif, some WRKY TFs also bind to other sites. For example, Oryza sativa OsWRKY13 binds to PRE4 (pathogen-responsive element; TGGCGTT), and Hordeum vulgare HvWRKY46 binds to SURE (sugar-responsive element) (TAAAGATTACTAATAGGAA). The binding of a WRKY TF to the W-box and other elements leads to synergistic transcriptional activation in plants.

In addition to this process, the conserved WRKY amino acid sequences are occasionally replaced by WRRY, WSKY, WKRY or WKKY domains. The model plant Arabidopsis thaliana encodes 74 WRKY TFs in its genome. Based on the similarity in sequence and phylogenetic relationships, WRKY TFs are divided into three groups (I, II, and III); group II is further divided into several sub-groups (e.g. IIa, IIb, IIC, IID, IIE, IIIF, and IIIG). There are two different types of WRKY TFs: (1) contains a single WRKY domain at the C-terminal end, (2) the other contain two WRKY domains, one at the N-terminal and other at the C-terminal end. The WRKY proteins that contain a single WRKY domain fall in group II and III while the WRKY protein that contains double WRKY domain (N- and C-terminals) are fall in group I. The WRKY proteins that contain two WRKY domains are functionally redundant. The N-terminal WRKY domain increases the affinity and specificity to bind the target gene, whereas the C-terminal WRKY domain constitutes the major DNA-binding domain. The single WRKY domain-containing WRKY TFs

¹Free Major of Natural Sciences, College of Basic Studies, Yeungnam University, Gyeongsan, 38541, Republic of Korea. ²School of Biotechnology, Yeungnam University, Gyeongsan, 38541, Republic of Korea. Correspondence and requests for materials should be addressed to T.K.M. (email: nostoc.tapan@gmail.com) or H.B. (email: hanhongbae@ynu.ac.kr)
(groups II and III) are considerably more similar in sequence to the C-terminal WRKY domain rather than to the N-terminal domain of group I WRKY TFs. These findings suggest that the C-terminal WRKY domain of group I WRKY TFs and the single WRKY domain of groups II and III WRKY TFs are functionally consensurate, and share the major DNA-binding domain.

The WRKY TFs have been reported to play important roles in cellular and physiological processes, including seed germination, root development, plant growth, seed development, and senescence. Furthermore, they are involved in diverse responses to biotic stress caused by insect herbivores, bacterial pathogens, fungi, and viruses. They respond to different signaling molecules such as indole-3-acetic acid, jasmonic acid, salicylic acid, abscisic acid, and gibberellic acid. In addition, WRKY TFs respond to different abiotic stresses, such as UV radiation, high and low temperatures, H2O2, and salt and drought stresses. Therefore, understanding the basic biology and genomics of WRKY TFs in plants is very important.

Numerous studies have been conducted with WRKY TFs in different plant species, including Arabidopsis thaliana, Brachypodium distachyon, Gossypium raimondii, Lotus japonicus, Oryza sativa, Ricinus communis, Setaria italica, Solanum lycopersicum, Triticum aestivum, and Vitis vinifera. Different research groups have provided different grouping systems for the WRKY TFs, leading to lack of consistency in the grouping system. Thus, it was highly important to formulate a new and clear grouping system for all WRKY TFs of the plant kingdom identified so far. Xi et al. reported about the presence of a deduced WRKY domain. Therefore, we were also very interested in determining whether WRKY TFs possess any additional novel, modified WRKY domains in its genome. Rinerson et al. reported the presence of chimeric WRKY TFs that contain combinations of novel protein domains and WRKY TF domains as well. Hence, it was also very interesting to elucidate more details about these chimeric proteins. Genome sequencing data from different plant species are currently increasing rapidly that has provided an excellent platform for better understanding the WRKY TF gene family. Therefore, we conducted genome-wide identification of the WRKY TF gene family from 43 plant species and analysed their genomic, phylogenetic, and other basic characteristics to decipher their novel genomic constitution.

Results
Identification of WRKY TFs. Genome-wide identification of WRKY TF gene family members was performed using 43 plant species across the evolutionary lineage of the plant kingdom (Table 1). These plant species included a wide mixture of dicots (27), monocots (7), algae (5), bryophytes (1), gymnosperms (1) and amoebae (1). In total, 3035 WRKY TFs were identified from these species. Of the studied species, the monocot plant Panicum virgatum encoded the maximum number of WRKY TFs (167), whereas, the green algae Chlamydomonas reinhardtii and Coccomyxa subellipsiidea encoded the minimum (only one). Among dicots, Brassica rapa and Glycine max encoded 145 WRKY TFs, whereas the amoeba Dictyostelium purpureum encoded nine. The WRKY TFs of the algae C. reinhardtii, C. subellipsiidea, and M. pusilla contained only a single WRKY domain (C-terminal WRKY domain) whereas O. lucimarinus and V. carteri contain both single and double WRKY domains. The WRKY TF gene family of the amoeba D. purpureum contained both single (C-terminal) and double (N- and C-terminals) WRKY domains.

Genomics of WRKY TFs. The transcript organization of WRKY TFs has been shown to be highly variable in nature. F. vesca FvWRKY70–7 contains the largest transcript, encoding an open reading frame (ORF) of 5949 nucleotides (1982 amino acids). Similarly, the M. domestica MdWRKY61-2 encodes the smallest WRKY TF containing only 135 nucleotides (44 amino acids). The intron organization of WRKY TFs is very dynamic, ranging from zero to twenty introns per gene. The number of plant WRKY TFs that contain various numbers of introns is as follows: zero (46), one (338), two (1440), three (488), four (375), five (223), six (67), seven (20), eight (5), nine (9), ten (12), eleven (4), twelve (3), thirteen (3), fourteen (0), fifteen (2), sixteen (1), seventeen (0), eighteen (2), nineteen (0), and twenty (2).

Novel WRKY TFs. In general, WRKY TFs are characterized by the presence of either one (Fig. 1) or two WRKY domains. In this study, we identified 16 chimeric forms of WRKY TFs in plants (Fig. 2). In addition, we identified different WRKY TFs that contain three (GrWRKY12, GrWRKY21-5, and LuWRKY3-7) (Fig. 2-A); and four (AcWRKY1, SmWRKY4-2) (Fig. 2-B) WRKY domains; three WRKY domains with the ZF_SBP TF domain (LuWRKY3-5, LuWRKY3-6) (Fig. 2-C); a single WRKY domain with three CBS domains (BrWRKY36-2) (Fig. 2-D); a kinase domain followed by a single WRKY domain (FvWRKY59) (Fig. 2-E); a kinase domain followed by two WRKY domains (PhWRKY59) (Fig. 2-F); two WRKY domains followed by a kinase domain (BrWRKY58-1, BrWRKY58-2) (Fig. 2-G); a PAH domain followed by two WRKY domains and one kinase domain (AtWRKY19) (Fig. 2-H); an ULP_protease domain followed by a WRKY domain (OsWRKY57, PvWRKY57-1, and SbWRKY57) (Fig. 2-I); a TIR domain followed by a WRKY domain (FvWRKY52, GmWRKY55-3) (Fig. 2-J); a TIR domain followed by two WRKY domains (Fv WRKY70-7) (Fig. 2-K); a TIR domain followed by seven LRR domains and a WRKY domain (PvWRKY16) (Fig. 2-L); two LRR domains followed by an NAC domain and two WRKY domains (ShWRKY59) (Fig. 2-M); an ATP_GRASP domain followed by a WRKY domain (AIWRKY16) (Fig. 2-N); a B3 domain followed by a WRKY domain (PvWRKY94-1) (Fig. 2-O); and a WRKY domain followed by a ZF_SBP domain (Fig. 2-P).

Conserved domains of WRKY TFs. To understand the conserved domains of WRKY TFs, multiple sequence alignments of single (C-terminal domain) and double WRKY domain (both N- and C-terminal domain) proteins were analyzed separately. The single WRKY domain (C-terminal)-containing proteins included the conserved W-R-K-Y-G-Q-K, P-R-x-Y-Y-x-C-x-C-x-C-x-C-x-K-x-V, and H-x-H domains as well as several conserved amino acid residues (Supplementary Figure 1). The N-terminal region of double WRKY domain proteins contain conserved D-G-Y-N-W-R-K-Y-G-Q-K and R-S-Y-Y-x-C-x-C-x-C-x-C-x-C-x-C-x-H-x-H domains. The C-terminal region...
of the double WRKY domain protein contains conserved D-G-Y-R-W-R-K-Y-G-Q-K, R-S-Y-Y-x-C-x 4-C, V-R-K-H-V-E, and H-x-H domains (Supplementary Figure 2). In some cases, the conserved WRKY amino acids in the WRKY domain were replaced with some other amino acids including W-K-K-Y (BrWRKY10-4, CcWRKY57-2, CsWRKY10-2, EgWRKY49-2, BrWRKY10, GmWRKY4-3, W-R-I-S (GrWRKY12), W-R-Q-V (LuWRKY58-1), G-R-K-Y (LuWRKY41-1), W-L-K-Y (PhWRKY31-2), W-R-E-Y (PhWRKY101), A-R-K-M (PvWRKY57-1, PvWRKY57-2, PvWRKY57-3), W-W-K-N (PvWRKY57-2, PvWRKY57-3), W-R-M-Y (PvWRKY82-2), W-R-K-R (SlWRKY20-3), W-I-K-Y (SlWRKY2-2), W-S-K-Y (SlWRKY72-2), W-Q-K-Y (SlWRKY72-1), W-H-K-C (StWRKY29), W-R-C-I

| Sl. No | Name of Plant Species | Classification | Ploidy level | Abbreviation of WRKY Gene | Single WRKY domain proteins | Double WRKY proteins | Novel WRKY proteins | Total No. of WRKY TFs |
|--------|----------------------|----------------|-------------|---------------------------|----------------------------|---------------------|----------------------|-----------------------|
| 1      | Aquilegia coerulea    | Dicot          | Diploid     | AcWRKY                    | 26                         | 6                   | 1                    | 33                    |
| 2      | Arabidopsis lyrata    | Dicot          | Diploid     | AlWRKY                    | 68                         | 10                  | 1                    | 79                    |
| 3      | Arabidopsis thaliana  | Dicot          | Diploid     | AtWRKY                    | 62                         | 11                  | 1                    | 74                    |
| 4      | Brachypodium distachyon| Monocot      | Diploid     | BdWRKY                    | 64                         | 17                  | 3                    | 81                    |
| 5      | Brassica rapa         | Dicot          | Diploid     | BrWRKY                    | 118                        | 24                  | 3                    | 145                   |
| 6      | Capsella rubella      | Dicot          | Diploid     | CrWRKY                    | 61                         | 11                  | 2                    | 72                    |
| 7      | Carica papaya         | Dicot          | Diploid     | CpWRKY                    | 44                         | 6                   | 1                    | 50                    |
| 8      | Chlamydomonas reinhardtii | Algae      | Haploid     | CreinWRKY                  | 0                          | 1                   | 1                    | 1                     |
| 9      | Citrus clementina     | Dicot          | Diploid     | CcWRKY                    | 45                         | 7                   | 1                    | 53                    |
| 10     | Citrus sinensis       | Dicot          | Diploid     | CsWRKY                    | 44                         | 9                   | 1                    | 53                    |
| 11     | Cuscomyxa subelliptoidea | Algae      | Haploid     | CsubWRKY                   | 0                          | 1                   | 1                    | 1                     |
| 12     | Curtisia sativus      | Dicot          | Diploid     | CsWRKY                    | 52                         | 10                  | 2                    | 62                    |
| 13     | Dicyostelium purpureum| Algae         | Haploid     | DpWRKY                    | 2                          | 7                   | 1                    | 9                     |
| 14     | Eucalyptus grandis    | Dicot          | Diploid     | EgWRKY                    | 63                         | 15                  | 2                    | 78                    |
| 15     | Fragaria vesca        | Dicot          | Diploid     | FvWRKY                    | 42                         | 10                  | 5                    | 56                    |
| 16     | Glycine max           | Dicot          | Tetraploid  | GmWRKY                    | 120                        | 24                  | 1                    | 145                   |
| 17     | Gossypium raimondii   | Dicot          | Diploid     | GrWRKY                    | 100                        | 18                  | 2                    | 120                   |
| 18     | Linum usitatissimum   | Dicot          | Diploid     | LuWRKY                    | 76                         | 26                  | 3                    | 105                   |
| 19     | Malus domestica       | Dicot          | Diploid     | MdWRKY                    | 103                        | 23                  | 1                    | 126                   |
| 20     | Manihot esculenta     | Dicot          | Diploid     | MeWRKY                    | 89                         | 13                  | 1                    | 102                   |
| 21     | Medicago truncatula   | Dicot          | Diploid     | MtWRKY                    | 63                         | 13                  | 2                    | 76                    |
| 22     | Micromonas puilla     | Algae          | Haploid     | MpWRKY                    | 2                          | 0                   | 1                    | 3                     |
| 23     | Mollus guttus         | Dicot          | Diploid     | MgWRKY                    | 53                         | 12                  | 1                    | 65                    |
| 24     | Oryza sativa          | Monocot        | Diploid     | OsWRKY                    | 86                         | 14                  | 1                    | 102                   |
| 25     | Ostracococcus lucimarmus | Algae      | Haploid     | OiWRKY                    | 1                          | 1                   | 1                    | 2                     |
| 26     | Paniucum hallii       | Monocot        | Diploid     | PhWRKY                    | 87                         | 9                   | 1                    | 97                    |
| 27     | Paniucum virgatum     | Monocot        | Tetraploid  | PvWRKY                    | 150                        | 17                  | 2                    | 168                   |
| 28     | Phaseolus vulgaris    | Dicot          | Diploid     | PvuWRKY                   | 73                         | 15                  | 1                    | 88                    |
| 29     | Physcomitrella patens | Bryophytes    | Haploid     | PpWRKY                    | 30                         | 5                   | 1                    | 35                    |
| 30     | Picea abies           | Gymnosperm     | Diploid     | PaWRKY                    | 56                         | 5                   | 1                    | 62                    |
| 31     | Populus trichocarpa   | Dicot          | Diploid     | PtWRKY                    | 80                         | 22                  | 2                    | 102                   |
| 32     | Prunus persica        | Dicot          | Diploid     | PrpWRKY                   | 50                         | 10                  | 2                    | 66                    |
| 33     | Ricinus communis      | Dicot          | Diploid     | RcWRKY                    | 48                         | 9                   | 1                    | 57                    |
| 34     | Selaginella moellendorffii | Pteridophytes| Haploid     | SmWRKY                    | 15                         | 4                   | 1                    | 19                    |
| 35     | Setaria italica       | Monocot        | Diploid     | SiWRKY                    | 93                         | 13                  | 1                    | 106                   |
| 36     | Solanum lycopersicum  | Dicot          | Diploid     | SlWRKY                    | 64                         | 14                  | 1                    | 79                    |
| 37     | Solanum tuberosum     | Dicot          | Diploid     | StWRKY                    | 66                         | 13                  | 1                    | 79                    |
| 38     | Sorghum bicolor       | Monocot        | Diploid     | SbWRKY                    | 77                         | 10                  | 3                    | 90                    |
| 39     | Thellangiella halophila| Dicot         | Diploid     | ThWRKY                    | 54                         | 12                  | 1                    | 66                    |
| 40     | Theobroma cacao       | Dicot          | Diploid     | TcWRKY                    | 49                         | 10                  | 2                    | 59                    |
| 41     | Vitis vinfera         | Dicot          | Diploid     | VvWRKY                    | 46                         | 11                  | 2                    | 56                    |
| 42     | Volves carteri        | Algae          | Haploid     | VcWRKY                    | 1                          | 1                   | 1                    | 2                     |
| 43     | Zea mays              | Monocot        | Diploid     | ZmWRKY                    | 100                        | 16                  | 1                    | 116                   |

Table 1. WRKY TF gene family of 43 species. Different species encode different numbers of WRKY TFs loci. Amoeba species D. purpureum encode for 9 WRKY TFs. Transcript variants were not included in this study.
(TcWRKY52), F-R-K-Y (PtWRKY34), R-S-Q-Y (EgWRKY75-1), W-T-K-Y (EgWRKY44-2), W-K-K-C (PvulWRKY33-4) and W-R-K-C (StWRKY29-1) (Fig. 3).

Phylogeny of WRKY TFs. The phylogenetic trees of plant WRKY TFs were constructed in order to better understand the phylogenetic relationship among them. Five phylogenetic trees were constructed by dividing the WRKY TFs into different groups to better understand the grouping and phylogenetic relationship among them. In the first case, the WRKY TFs of monocots, dicots and basal eukaryotic (amoebae, algae, bryophytes, pteridophytes and gymnosperms) plants were combined and used to construct a phylogenetic tree. The results showed the presence of eight phylogenetically distinct and independent groups that were denoted as groups I (red), II (lime), III (black), IV (blue), V (pink), VII (green) and VIII (black) (Fig. 4, Table 2). The phylogenetic tree generated from monocots and lower eukaryotic plants formed six phylogenetically distinct groups and named as groups I (red), II (lime), III (green), IV (blue), V (pink) and VI (green) (Fig. 5, Table 3). The sub-group of group II was absent in monocot plants. The phylogenetic tree formed from dicot and lower eukaryotic WRKY TFs yielded three groups namely, groups I (pink), IIa (red), IIb (lime), III (green) (Fig. 6, Table 4). When all the WRKY TFs of monocot, dicot, and lower eukaryotic plants that contain only C-terminal WRKY domain were combined, the phylogenetic tree resulted in six groups namely groups I (red), II (lime), III (blue), IV (pink), V (green) and VI (purple) (Fig. 7, Table 5). Similarly, all WRKY TFs of monocot, dicot and lower eukaryotic plants that contained both N- and C-terminal WRKY domains were combined; this resulted in the generation of a phylogenetic tree containing seven groups. The groups are named as group I (red), II (lime), III (blue), IV (purple), V (pink), VI (green) and VII (purple) (Fig. 8, Table 6).

The substitution pattern and evolution rates were estimated by analyzing the shape parameters for the discrete gamma distributions. The rates were estimated using the Jones-Taylor-Thornton (JTT) model (+G). A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G). The mean evolutionary rates for dicot and lower eukaryotic WRKY protein were 0.15, 0.42, 0.75, 1.23 and 2.44 substitutions per site. The amino acid frequencies were 7.69% (A), 5.11% (R), 4.25% (N), 5.13% (D), 2.03% (C), 4.11% (Q), 6.18% (E), 7.47% (G), 2.30% (H), 5.26% (I), 9.11% (L), 5.95% (K), 2.34% (M), 4.05% (F), 5.05% (P), 6.82% (S), 5.85% (T), 1.43% (W), 3.23% (Y), and 6.64% (V). For estimating ML values, a tree topology was automatically computed. The maximum log likelihood for this computation was –19363.118. The analysis involved 774 amino acid sequences. The mean evolutionary rates for monocot and lower eukaryotic WRKY proteins were 0.15, 0.42, 0.75, 1.23 and 2.44 substitutions per site. The amino acid frequencies were 7.69% (A), 5.11% (R), 4.25% (N), 5.13% (D), 2.03% (C), 4.11% (Q), 6.18% (E), 7.47% (G), 2.30% (H), 5.26% (I), 9.11% (L), 5.95% (K), 2.34% (M), 4.05% (F), 5.05% (P), 6.82% (S), 5.85% (T), 1.43% (W), 3.23% (Y), and 6.64% (V).
Figure 2. Novel WRKY TFs of plants. In addition to the presence of classic WRKY TFs in plants, the present study revealed the presence of novel WRKY TFs. These novel WRKY TFs are as follows: (A) WRKY TFs with three WRKY domains (GrWRKY12, GrWRKY21-5, LuWRKY3-7), (B) WRKY TFs with four WRKY domains (AcWRKY1, SiWRKY4-2), (C) WRKY TFs with three WRKY domains followed by a ZF_SBP TF domain (LuWRKY3-5, LuWRKY3-6), (D) WRKY domain followed by three calcium binding CBS domains (BrWRKY36-2), (E) kinase domain followed by one WRKY domain (FvWRKY59), (F) kinase domain followed by two WRKY domains (PhWRKY59), (G) two WRKY domains followed by a kinase domain (BrWRKY58-1, BrWRKY58-2), (H) PAH domain followed by two WRKY domain and kinase domain (AtWRKY19), (I) protease domain followed by a WRKY domain (OsWRKY57, PwWRKY57-1, SbWRKY57), (J) TIR domain followed by WRKY domain (FvWRKY52, GmWRKY55-3), (K) TIR domain followed by a WRKY domain twice (FvWRKY70-7), (L) TIR domain followed by a LRR domain and a WRKY domain (FvWRKY16), (M) LRR and NAC domain followed by two WRKY domains (SbWRKY59), (N) ATP_GRASP domain followed by a WRKY domain (AtWRKY16), (O) B3 domain followed by a WRKY domain (PwWRKY94-1), and (P) WRKY domain followed by a ZF_SBP domain (SiWRKY59-2).

(F), 5.05% (P), 6.82% (S), 5.85% (T), 1.43% (W), 3.23% (Y) and 6.64% (V). The maximum log likelihood for this computation was -13476.656. The analysis involved 445 amino acid sequences. The mean evolutionary rates for WRKY proteins that contained
double WRKY domains were 0.11, 0.36, 0.70, 1.22, and 2.60 substitutions per site. The amino acid frequencies were 7.69% (A), 5.11% (R), 4.25% (N), 5.13% (D), 2.03% (C), 4.11% (Q), 6.18% (E), 7.47% (G), 2.30% (H), 5.26% (I), 9.11% (L), 5.95% (K), 2.34% (M), 4.05% (F), 5.05% (P), 6.82% (S), 5.85% (T), 1.43% (W), 3.23% (Y), and 6.64% (V). The maximum log likelihood for this computation was -30333.349. The analysis involved 480 amino acid sequences. All positions with less than 95% site coverage were eliminated. Thus, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Statistical analysis of WRKY TFs. Tajima's relative rate test was conducted to determine the statistical significance of the investigated WRKY TFs. In all three replicate analyses, the p-values were found to be significant. The X² –test results with one degree of freedom were 5.76 (for monocot, dicot and lower eukaryotic WRKY TFs), 13.76 (for monocot and lower eukaryotic WRKY TFs), 4.45 (for dicot and lower eukaryotic WRKY TFs), 5.00

Figure 3. Substitute WRKY domain of plants. Different novel substitutes of WRKY domains were found in the N- and C-terminal regions of WRKY TFs. The conserved WRKY amino acids were replaced by different types of amino acids. The N- and C-terminal WRKY domains of *A. thaliana* AtWRKY were aligned with these novel substitutes of WRKY domains. This indicates that WRKY amino acids have been replaced by these novel amino acids. Multiple sequence alignment of WRKY TF was performed using multalin software (http://multalin.toulouse.inra.fr/multalin/) by using the protein weight matrix BLOSUM62.
Figure 4. Unrooted phylogenetic tree of WRKY TFs of monocot, dicot, and lower eukaryotic (amoeba, algae, bryophyte, pteridophyte, and gymnosperm) plants. The phylogenetic tree shows eight independent groups. We named them as groups I (red), II (lime), III (black), IV (blue), V (black), VI (pink), VII (green), and VIII (black). To get details about distribution of different WRKY TF in different group, please refer to Supplementary Figure 3. The phylogenetic tree revealed that, the WRKY family members of one group overlapped with another group. The phylogenetic tree was constructed using MEGA6.

| Group I (red) | Group II (lime) | Group III (black) | Group IV (blue) | Group V (black) | Group VI (pink) | Group VII (green) | Group VIII (black) |
|---------------|----------------|-------------------|-----------------|----------------|-----------------|-------------------|--------------------|
| 3, 5, 7, 8, 10, 11, 13, 16, 17, 19, 22, 23, 24, 25, 26, 28, 29, 33, 34, 36, 43, 45, 48, 49, 50, 51, 56, 57, 58, 59, 67, 68, 71, 72, 75, 77, 84, 102, 103, 106 | 1, 2, 3, 4, 5, 10, 19, 20, 24, 25, 26, 30, 32, 33, 34, 35, 44, 45, 53, 57, 58, 59, 70, 78, 80, 81, 82, 84, 85, 90, 96, 105 | 1, 2, 3, 4, 5, 6, 9, 12, 18, 27, 28, 31, 32, 36, 40, 42, 43, 47, 60, 61, 62, 71, 73, 76, 97 | 10, 12, 16, 17, 49, 52, 57, 60, 103 | 2, 4, 6, 7, 11, 12, 13, 14, 15, 16, 17, 21, 22, 25, 27, 29, 31, 35, 37, 39, 43, 51, 52, 57, 64, 65, 66, 68, 69, 74, 83, 87, 88, 94 | 4, 15, 16, 18, 19, 20, 21, 22, 30, 33, 38, 40, 41, 44, 45, 46, 47, 48, 50, 51, 52, 53, 54, 55, 56, 59, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 74, 79, 81, 82, 86, 90, 91, 93, 94, 95, 98, 100, 103, 104 | 21, 22, 50, 69, 89, 94 |

Table 2. Phylogenetic tree of WRKY TFs of monocot, dicot, and lower eukaryotic (amoeba, algae, bryophyte, pteridophyte, and gymnosperm) plants. The phylogenetic tree revealed eight different groups, but the WRKY TF gene families were not restricted to any specific group, and one or more member of WRKY TFs were distributed in two or more groups. The numbers indicate the number of WRKY TFs (for example 1, 2, and 3 and others indicate WRKY1, WRKY2, and WRKY3 and so on). The phylogenetic tree was constructed using the MEGA6 software and the Poisson substitution model by using 1000 bootstrap replicates.
Table 3. Phylogenetic tree of WRKY TFs of monocot and lower eukaryotic plants. Representative WRKY TF members belonging to monocot and lower eukaryotic (amoeba, algae, bryophyte, pteridophyte and gymnosperm) plants. The phylogenetic tree contained six distinct groups. The members of the WRKY TFs were significantly specific to their respective groups. The numbers indicate the WRKY TF members distributed in different groups (1, 2, etc. indicates WRKY1, and WRKY2 and so on). This table confirms that the nomenclature of the entire WRKY TFs gene family is accurate. The phylogenetic tree was constructed using MEGA6 software and the Poisson substitution model using 1000 bootstrap replicates.

(for single WRKY domain containing WRKY TFs), and 7.41 (for double WRKY domain containing WRKY TFs) (Table 7).
Gene expression profile of WRKY TFs. The expression profile of the WRKY TFs was elucidated by investigating the gene expression data for *G. max* and *P. vulgaris* and analyzing their transcription levels. In *G. max*, the transcription profile was determined for different tissue samples, including roots, root hair, leaves, stems, flowers, pods, seeds, nodules and shoot apical meristem. In *G. max*, the expression level of *GmWRKY65-1* was found to be the highest (105.342) among all other WRKY transcription factors (Supplementary Table 2). The expression levels of *GmWRKY6-4* and *GmWRKY6-5* in the root were found to be 74.668 and 43.341, respectively. Some other WRKY TFs, the expression levels of which were relatively higher than others were *GmWRKY6-6*, *GmWRKY11-2*, *GmWRKY11-3*, *GmWRKY11-4*, *GmWRKY11-6*, and *GmWRKY15-2* (Supplementary Table 2). Further, *GmWRKY4-3*, *GmWRKY5-1*, *GmWRKY5-2*, *GmWRKY10*, *GmWRKY13-4*, *GmWRKY18*, *GmWRKY33-2*, *GmWRKY33-3*, *GmWRKY35-1*, *GmWRKY35-2*, *GmWRKY47-1*, *GmWRKY47-2*, *GmWRKY47-3*, *GmWRKY50-1*, *GmWRKY50-2*, *GmWRKY54-1*, *GmWRKY57-1*, *GmWRKY69-1*, *GmWRKY69-2*, *GmWRKY70-3*, *GmWRKY71-2*, *GmWRKY72-1*, and *GmWRKY72-2* were not expressed in the root tissues (Supplementary Table 2). Unlike the higher expression in roots, the expression of *GmWRKY63-1(35.199)* was also found to be the highest in the root hair. Some other WRKY TFs that were expressed relatively at higher levels were *GmWRKY6-4*, *GmWRKY11-1*, *GmWRKY11-2*, *GmWRKY11-3*, *GmWRKY11-4*, *GmWRKY11-6*, *GmWRKY11-7*, *GmWRKY11-8*, *GmWRKY15-1*, and *GmWRKY15-2* (Supplementary Table 2). The WRKY TFs, the expression of which was not detected in root tissues, were *GmWRKY4-3*, *GmWRKY6-3*, *GmWRKY10*, *GmWRKY65-1*.

![Unrooted phylogenetic tree of WRKY TFs of dicot and lower eukaryotic (amoeba, algae, bryophyte, pteridophyte, and gymnosperm) plants.](image-url)}
GmWRKY13-4, GmWRKY29-1, GmWRKY54-1, GmWRKY54-2, and GmWRKY56-1 (Supplementary Table 2). In the leaf tissue, the expression level of GmWRKY6-5 (81.847) was found to be highest among other WRKY TFs. The expression of GmWRKY26-2 in the leaf tissue was found to be 80.957. Some other WRKY TFs, the
expression of which was found to be higher in the leaf tissue, were GmWRKY6-4, GmWRKY15-1, GmWRKY15-2, GmWRKY26-3, GmWRKY41-1, GmWRKY41-2, GmWRKY41-3, and GmWRKY41-7 (Supplementary Table 2). The WRKY TFs, expression of which was not detected in the leaves were GmWRKY6-3, GmWRKY10, GmWRKY13-4, GmWRKY40-1, GmWRKY40-9, GmWRKY41-4, GmWRKY47-1, GmWRKY50-1, GmWRKY50-2, GmWRKY51-1, GmWRKY51-2, GmWRKY51-3, GmWRKY51-4.

Table 5. Phylogenetic tree of WRKY TFs of monocot, dicot and lower eukaryotic plants that contain only a single WRKY domain (C-terminal WRKY TFs). The phylogenetic tree was divided into six distinct phylogenetic groups. The numbers indicate WRKY TF members distributed in different groups (1, 2, etc. indicate WRKY1, WRKY2, etc.). Different WRKY TF members are distributed redundantly. For example, WRKY5 is distributed in group I, III and V. The phylogenetic tree was constructed by using the MEGA6 software and the Poisson substitution model using 1000 bootstrap replicates.

| Group I (red)          | Group II (lime)     | Group III (blue)   | Group IV (pink)      | Group V (green) | Group VI (purple) |
|------------------------|---------------------|--------------------|-----------------------|----------------|------------------|
| 4, 5, 15, 18, 19, 20, 21, 22, 30, 32, 38, 40, 41, 44, 45, 46, 47, 48, 50, 52, 53, 54, 55, 56, 58, 62, 63, 64, 65, 66, 67, 69, 70, 74, 75, 79, 81, 93, 98, 100, 101 | 3, 7, 8, 10, 11, 16, 17, 23, 26, 28, 29, 45, 48, 49, 50, 51, 57, 59, 67, 68, 71, 77, 80 | 5, 13, 23, 24, 34, 36, 43, 56, 102 | 1, 6, 7, 11, 12, 13, 14, 15, 17, 22, 25, 27, 29, 31, 35, 37, 39, 42, 51, 65, 66, 68, 69, 88, 89, 92 | 1, 5, 6, 9, 12, 18, 21, 27, 28, 31, 32, 36, 39, 40, 42, 43, 47, 60, 61, 62, 71, 72, 73, 74, 76, 83, 87, 97 | 16, 18, 20, 25, 33, 49, 52, 59, 60, 95, 103, 106 |

Figure 8. Unrooted phylogenetic tree of N- and C-terminal WRKY domains containing WRKY TFs. The phylogenetic tree shows the presence of seven phylogenetically distinct groups, I (red), II (lime), III (blue), IV (purple), V (pink), VI (green) and VII (purple). The phylogenetic tree was constructed by using MEGA6.
containing gaps and missing data were eliminated. Evolutionary analyses were conducted using MEGA6. The analysis involved three amino acid sequences in each group. All positions for double domain containing group as per default selection in the MEGA program in Tajima’s relative rate test.

The sequences of A (SmWRKY35), and B (SmWRKY55), with sequence C (SmWRKY9) being analysed for monocot, dicot and lower eukaryotic domain. The sequences of A (SmWRKY1, WRKY2, etc.). Analysis showed that different WRKY TFs overlap among groups. The phylogenetic tree contained seven distinct groups. The numbers indicate the WRKY TF members distributed in different groups (1, 2, etc. indicate WRKY1, WRKY2, etc.).

Table 7. Tajima’s relative rate test. Equality of evolutionary rate analysis between sequences A (SmWRKY54) and B (SmWRKY55), with sequence C (SmWRKY9) being analysed for monocot, dicot, and lower eukaryotic WRKY TFs. The sequences of A (SmWRKY35), and B (SmWRKY6), with sequence C (SmWRKY9) being considered for monocot with lower eukaryotic group; sequences A (SmWRKY15), and B (MdWRKY11-6), with sequence C (SmWRKY9) being considered for dicots with lower eukaryotic group; sequences A (SmWRKY54), and B (ThWRKY50), with sequence C (CsWRKY23) being considered for single WRKY domain containing group; and sequences A (SmWRKY35), and B (PaWRKY72), with sequence C (AtWRKY60) were considered for double domain containing group as per default selection in the MEGA program in Tajima’s relative rate test. The statistical results are presented in Table 7. A P-value of less than 0.05 was used to reject the null hypothesis of equal rates between lineages. The analysis involved three amino acid sequences in each group. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted using MEGA6.

| Configuration | Monocot, Dicot and lower eukaryotes | Monocot with lower eukaryotes | Dicot with lower eukaryotes | Single Domain | Double domain |
|---------------|-----------------------------------|-------------------------------|-----------------------------|---------------|--------------|
| Identical sites in all three sequences | 20 | 31 | 20 | 24 | 8 |
| Divergent sites in all three sequences | 14 | 20 | 24 | 63 | |
| Unique differences in Sequence A | 16 | 19 | 9 | 15 | 37 |
| Unique differences in Sequence B | 5 | 2 | 2 | 5 | 17 |
| Unique differences in Sequence C | 5 | 0 | 7 | 12 | 4 |
| P-value | 0.01638 | 0.00021 | 0.03481 | 0.02535 | 0.00650 |
| X² test | 5.76 | 13.76 | 4.45 | 5.00 | 7.41 |
| Degree of freedom | 1 | 1 | 1 | 1 | 1 |

In *P. vulgaris*, the expression of WRKY TFs in different tissue samples, including young trifoliates, leaves, flowers, flower buds, young pods, stems, roots, and nodules was analysed (Supplementary Table 2).
trifoliates, *PvulWRKY17* (37.519) showed the highest expression. Some other genes that showed relatively higher expression in young trifoliates included *PvulWRKY11-2* (21.790), *PvulWRKY15-1* (18.590), *PvulWRKY15-2* (24.308), and *PvulWRKY19-1* (24.328). In contracts, *PvulWRKY9-2*, *PvulWRKY27-1*, *PvulWRKY29-1*, *PvulWRKY35*, *PvulWRKY43-1*, *PvulWRKY47-2*, *PvulWRKY51-1*, *PvulWRKY59-1*, *PvulWRKY59-2*, *PvulWRKY69-1*, *PvulWRKY73-2*, *PvulWRKY73-3*, *PvulWRKY73-4*, and *PvulWRKY79-1* were not expressed in young trifoliates. In the leaf tissue, *PvulWRKY11-2* (25.292) and *PvulWRKY26-1* (25.724) showed higher expression. Some other genes that showed higher expression in leaves were *PvulWRKY7* (19.048), *PvulWRKY19-1* (16.433), *PvulWRKY23-1* (19.076), and *PvulWRKY58* (18.863). In contrast, *PvulWRKY1-2*, *PvulWRKY5-1*, *PvulWRKY9-2*, *PvulWRKY14*, *PvulWRKY29-1*, *PvulWRKY35*, *PvulWRKY43-1*, *PvulWRKY47-2*, *PvulWRKY51-1*, *PvulWRKY59-1*, *PvulWRKY59-2*, *PvulWRKY69-1*, *PvulWRKY73-2*, *PvulWRKY73-3*, *PvulWRKY73-4*, and *PvulWRKY79-1* were not expressed in the leaves. In flowers, *PvulWRKY19-1* (78.755) showed the highest expression followed by *PvulWRKY15-2* (49.015), *PvulWRKY17* (66.844), *PvulWRKY26-1* (76.970), and *PvulWRKY58* (50.788) whereas, *PvulWRKY59-1*, *PvulWRKY59-2*, *PvulWRKY69-1*, *PvulWRKY73-2*, *PvulWRKY73-4*, and *PvulWRKY79-1* and *PvulWRKY79-3* were not expressed. The expression of *PvulWRKY11-2* (50.119) was highest in flower buds followed by *PvulWRKY17* (46.894), *PvulWRKY19-1* (23.965) and *PvulWRKY44* (19.068), whereas, *PvulWRKY5-1*, *PvulWRKY5-3*, *PvulWRKY9-2*, *PvulWRKY29-1*, *PvulWRKY43-1*, *PvulWRKY43-2*, *PvulWRKY51-1*, *PvulWRKY59-1*, *PvulWRKY59-2*, *PvulWRKY69-1*, *PvulWRKY73-3*, *PvulWRKY79-1* and *PvulWRKY79-3* were not expressed (Supplementary Table 2). In young pods, *PvulWRKY17* (58.155), *PvulWRKY15-2* (41.848), and *PvulWRKY19-1* (38.820) showed higher expression whereas, *PvulWRKY9-2*, *PvulWRKY51-1*, *PvulWRKY59-1*, *PvulWRKY69-1*, *PvulWRKY73-2*, *PvulWRKY73-3*, *PvulWRKY73-4*, and *PvulWRKY79-1* were not expressed (Supplementary Table 2). In stems, *PvulWRKY17* (61.321) showed the highest expression whereas *PvulWRKY9-2*, *PvulWRKY29-1*, *PvulWRKY51-1*, *PvulWRKY59-1*, *PvulWRKY59-2*, *PvulWRKY69-1*, *PvulWRKY73-3*, *PvulWRKY79-1*, *PvulWRKY79-2*, and *PvulWRKY79-3* were not expressed. In roots *PvulWRKY11-2* (134.816) showed the highest expression whereas *PvulWRKY29-1*, *PvulWRKY45-1*, *PvulWRKY45-2*, *PvulWRKY59-1*, *PvulWRKY59-2*, and *PvulWRKY69-1* were not detected. In nodules, *PvulWRKY11-2* (79.023) showed the highest expression followed by *PvulWRKY9-2* (48.761), *PvulWRKY11-1* (36.555), and *PvulWRKY69-3* (45.336), whereas *PvulWRKY29-1*, *PvulWRKY45-1*, *PvulWRKY45-2*, *PvulWRKY59-1*, *PvulWRKY59-2*, and *PvulWRKY69-1* were not detected (Supplementary Table 2).

**Discussion**

**Identification and nomenclature of WRKY TFs.** Advancements in genome sequencing technology and available of well annotated genome database led us to identify the WRKY TF gene family of 43 species. Predicting the potential function and activity of newly sequenced genes and their protein products in every organism is very difficult. The major cellular roles of newly identified genes/proteins can be inferred from previously characterized orthologous gene members of the same family. Large-scale comparative genomic studies can reveal important information regarding the function and evolutionary relationship of orthologous species. The same principle can be applied at the gene family level as well (e.g. WRKY TF gene family). Therefore, we identified and analysed the WRKY TF gene family members from 43 different plant species. All identified WRKY TFs were assigned a specific name according to the orthology based nomenclature system. Providing a unique name to every gene is necessary for its future identification. The role of a genome is insignificant unless a comparative genomics study is conducted.

**Genomics of WRKY TFs.** Availability of large-scale genomic data from various plant species allowed the detailed investigation of the WRKY TF gene family in plants. The WRKY TF gene family members vary across species likely because of gene duplication, whole genome duplication, polypidy, gene deletion or mutation. WRKY TFs are considered to be evolutionary conserved and supposed to be present only in plants. However, the WRKY TF gene family was also found in amoeba, fungi and diplomonad species. *Dictyostelium purpureum*, the amoeba that lives in soil belongs to the phylum mycetozoa. The genome of this species encodes nine WRKY TFs. The tetraploid monocot plant *P. virgatum* encodes the highest number (167) of WRKY TFs, whereas, the unicellular *C. reinhardtii* and *C. subellipsoida* encode for the lowest number (only one) of WRKY TFs. In general it is a general assumption that, larger the genome size more will be the number of WRKY TFs in the genome; however, this concept is not true. Genome size is not directly related to the number of genes of a gene family in the genome (Mohanta et al. 2015; Mohanta et al. 2015; Mohanta et al. 2015). Therefore, the presence of a higher or lower number of genes in a gene family of a particular species can be attributed to its functional requirement and diverse cellular processes. Cai et al. reported the presence of 120 WRKY TFs in *Gossypium raimondii*, which is similar to the number of WRKY TFs identified in our study. Li et al. reported the presence of 47 WRKY TFs in *Ricinus communis*, however, in our study, 57 WRKY TFs were identified. Muthamilarasan et al. reported the presence of 105 WRKY TFs in *Setaria italic*, whereas, in our study 106 WRKY TFs were identified. Wen et al. reported the presence of 86 WRKY TFs in Brachypodium distachyon whereas only 81 WRKY TFs were identified in this study. Wen et al. have included locus ID LOC100843345, LOC100834454, LOC100845846, and LOC100837754 as locus ID for the gene name *BdWRKY52*, *BdWRKY69*, *BdWRKY73* and *BdWRKY75*, respectively (Supplementary Table 1 of Wen et al.); however, we did not find any such sequences from the phytozome database. This indicates that these locus IDs do not belong to *B. distachyon* and hence *B. distachyon* do not encode 86 WRKY TFs. We also compared our results with plant transcription factor databases http://planttfdb.bio.uni-potsdam.de/v3.0/ and http://planttfdb.cbi.pku.edu.cn/. In the majority of the cases, our study results were consistent with those of previous studies where splice variants were excluded as a gene. Splice variants are variants of a particular gene/locus; therefore, they cannot be considered as an independent gene locus. The dicot plant *Linum usitatissimum* encodes the highest number (26) of double WRKY domain proteins, whereas the tetraploids...
plant B. distachyon, which has a larger genome, encodes only 17 double WRKY domain proteins. This shows that the genome size plays no role in determining whether single or double WRKY domain proteins are encoded and this might be completely based on the functional requirement of an organism. Further, we found that the lower eukaryotic organisms Chlamydomonas reinhardtii, Coccomyxa subellipsoidea, Ostreococcus lucimarinus, Physcomitrella patens and Volvox carteri encoded at least one WRKY TF that contained a double WRKY domain. Three and four WRKY domain containing WRKY proteins were absent in lower eukaryotes, and are only present in a few higher eukaryotic plants. This shows that these three and four WRKY domain-containing WRKY TFs might have evolved recently. The WRKY TF gene family of Oryza sativa was previously reported to contain 102 WRKY TFs. In this study, we eliminated OsWRKY94 since it was not found to contain any WRKY domain. Ross et al. also reported the absence of any WRKY domain in OsWRKY94.

In the present study, we identified several novel chimeric WRKY TFs from different plant species (Fig. 2) with varying numbers of WRKY domains and other novel domains fused with them (Fig. A to P). These chimeric WRKY TFs might have evolved recently via fusion with other domains. The kinase domain phosphorylates to its target protein. Thus, determining whether, these fused kinase domains play any crucial role in the auto-phosphorylation events in the WRKY TFs to which they are fused, and hence regulate gene expression. In some cases, the kinase domain is followed by a WRKY domain (Fig. 2F and G), whereas, in other cases the WRKY domain is followed by a kinase domain (Fig. 2G). The kinase domains of WRKY TFs most likely get phosphorylated by the cognate up-stream kinase, and regulate the expression of WRKY TFs. The position of the kinase domain might be speculated to be very important in the regulation of WRKY TFs and the phosphorylation events in plants. In some other cases, the WRKY domain is fused with the toll-interleukin receptor (TIR) domain (Fig. 2K and L), which mediates the interactions between the toll-like receptor and signal transduction components. Plant proteins that harbor TIR motifs are involved in plant defense against pathogen attack. Some WRKY TFs have the TIR domain in their structure (Fig. 2J and K), which mediates the interactions between the toll-like receptor and signal transduction components. In some other cases, the WRKY domain is fused with the TIR domain (Fig. 2K), which mediates the interactions between the toll-like receptor and signal transduction components.

Conserved domains of WRKY TFs. Multiple sequence alignment of C-terminal WRKY TFs revealed the presence of conserved W-R-K-Y-G-Q-K and C-x(5)-C-x(23)-H-x-H domains (Supplementary Figure 1). When multiple sequence alignment was conducted using WRKY TFs that contained only double WRKY domains (both N- and C-terminal), the N-terminal region showed the presence of conserved W-R-K-Y-G-Q-K and C-x(5)-C-x(23)-H-x-H whereas the C-terminal region showed the presence of conserved W-R-K-Y-G-Q-K and C-x(4)-C-x(23)-H-x-H domains (Supplementary Figure 2). Although the W-R-K-Y-G-Q-K heptapeptide sequence was highly conserved, sequence similarity beyond the domain was considerably low among most genes. Instead of harboring the W-R-K-Y-G-Q-K domain, several WRKY TFs were found to contain W-R-K-Y, W-T-K-Y, W-S-K-Y, W-K-K-C, W-K-K-C, W-H-Q-Y, R-S-Q-Y, G-R-K-Y, W-R-E-Y, W-L-K-Y, W-R-K-R, W-R-K-N, W-R-K-D, F-R-K-Y, W-I-K-Y, W-I-I-Y, W-W-K-N and W-W-K-S domains (Fig. 3). These domains were not aligned with the W-R-K-Y domains and hence assumed to be newly evolved. Among these new
domains, W-K-K-Y, W-T-K-Y, W-S-K-Y, W-H-K-C, W-Q-K-Y, W-R-K-C, W-K-K-C, W-H-Q-Y, R-S-Q-Y, G-R-K-Y, W-R-E-Y, W-L-K-Y, and W-R-K-R are present in the N-terminal region, whereas W-R-K-N, W-R-K-D, F-R-K-Y, W-L-K-Y, W-R-I-Y, W-W-K-N and W-W-K-S are present in the C-terminal region (Fig. 3). Therefore, the entire WRKY TF gene family which might result from long-time evolutionary history, represents divergent WRKY domains even in very closely related gene pairs. Characterization of these novel motifs might shed new insight into their functional significance.

**Phylogeny and grouping of WRKY TFs.** The WRKY TF gene family from various plant species, including *A. thaliana*, *B. distachyon*, *G. raimondii*, *O. sativa*, *S. lycopersicum*, and *T. aestivum* has been well elucidated. Surprisingly, when we combined the data from several published reports, none of them were found to be correlated with one another (Table 8). The WRKY TF group members of different species vary and are not consistent (Table 8). Different researchers have used different nomenclature and grouping systems for WRKY TFs. Eulgem *et al.* has grouped WRKY TFs as groups I, IIa, IIb, IIc, IId, IIe and IIII whereas, Wang *et al.* grouped them as IN, IC, Ia, Ibb, IIc, IId, Ile and IIIf. Wu *et al.* grouped the WRKY TF gene family of *O. sativa* as Ia (NTWD (N-terminal WRKY domain), CTWD (C-terminal WRKY domain)), Ib, Ila, IIb, IIc, IId and IIII, whereas Okay *et al.* grouped the WRKY TFs of *T. aestivum* as groups I, Ila, Ibb, IId, Ile, and IIIf. Thus, there are hardly any consistencies in the grouping system of WRKY TFs. Moreover, none of the WRKY TF group members of one research group are consistent with those of other research groups. For example, according to Wang *et al.*, WRKY TFs of *A. thaliana* have been grouped as groups I, IIa, IIb, IIc, IId, IIe, and IIII whereas, Wang *et al.* reported that the WRKY TF gene families of *O. sativa* as groups I, IIa, IIb, IIc, IId, IIe and IIII. The WRKY TF group members of *G. raimondii* are present in groups IN and IC respectively whereas, Eulgem *et al.* reported that the WRKY TF gene families of *B. distachyon* are present in groups I, IIa, IIc, and IIe. The WRKY TF gene family members of *S. lycopersicum* are present in groups I, IIa, IIb, IIc, IId, IIe, and IIII. The WRKY TF gene family members of *T. aestivum* are present in groups I, IIa, IIb, IIc, IId, IIe, and IIII. The WRKY TF gene family members of dicot and lower eukaryotic plants, three different groups were generated; they were named as groups I, II, and III. The WRKY TF gene family members of monocot and lower eukaryotic plants, six phylogenetically distinct groups were formed; they were named as groups I, II, III, IV, V, and VI. The WRKY TF gene family members of dicot and lower eukaryotic plants, three different groups were generated where group II contained three sub-groups (Fig. 6, Table 4). We named the groups as I (pink), II (red), III (blue), and IV (green). We found that the WRKY TF members of groups I and III were very specific to their respective group.
and did not overlap with one another (Table 4). These results clearly showed that the WRKY TF grouping system is very specific to the lineages (monocot/dicot). The WRKY TF grouping system of monocot and dicot plants differs remarkably; this might be one of the most important reasons why co-linearity was absent in the grouping system of WRKY TF gene family members (Table 8). Therefore, in this study, we proposed that WRKY TF grouping should be specific to monocot or dicot plant lineages. The WRKY TFs of monocot plants can be grouped into six groups, and dicot-specific WRKY TFs can be grouped into three groups. Earlier reported grouping systems such as groups I, II (IIa, IIb and IIc) and III can be applied to dicot plants, but it is ensuring that WRKY TF group members are confined to their specific groups is important.

We conducted another analysis by dividing WRKY TFs into single WRKY domain-containing (C-terminal) and double WRKY domain-containing (N- and C-terminal) groups. The phylogenetic analysis in the single WRKY domain group resulted in six phylogenetically distinct groups, whereas the double WRKY domain group resulted in seven phylogenetically distinct groups (Tables 5 and 6). The WRKY TF members of domain specific studies were not confined to any specific group and the group members were overlapped with each other. Although single and double WRKY domain-containing TFs resulted into six and seven phylogenetically distinct groups (Tables 5 and 6), the phylogenetic analysis of WRKY proteins with a single or double domain. This explains why WRKY proteins are highly conserved across the plant lineage. The phylogenetic analysis of all plant species showed that all WRKY TFs were present in monocot, dicot and lower eukaryotes, indicating that the appearance of most WRKY TFs in plants predated the divergence of these species. No species-specific group, or sub-group or clades were observed in the phylogenetic tree. This implies that the WRKY TF gene family was more conserved during evolution. In addition, the WRKY domains from the same lineage tended to cluster together in the phylogenetic tree, which was not observed in this study. This suggests that they experienced duplication after divergence. The WRKY TFs that clustered together are orthologous ones that are evolutionarily closer than others. The phylogenetic similarity found in this study

| Group 1N | Group 1C | Group Ia | Group Ib | Group Ic | Group IId | Group Ile | Group III | References |
|----------|----------|----------|----------|----------|----------|-----------|-----------|------------|
| 1, 2, 3, 4, 10, 20, 25, 26, 32, 33, 44, 45, 58 | 8, 12, 13, 23, 24, 28, 43, 45, 48, 56, 68, 71, 75 | 6, 9, 18, 31, 36, 40, 42, 47, 60, 61, 72 | 7, 11, 14, 15, 17, 21, 22, 27, 29, 35, 39, 52, 65, 69, 74 | 8, 12, 13, 23, 24, 28, 43, 48, 49, 50, 51, 56, 57, 59 | 7, 11, 15, 17, 21, 39 | 14, 16, 22, 27, 29, 35 | 30, 38, 41, 46, 53, 54, 55, 62, 63, 64, 66, 67, 70 | 76 |
| Ia NTWD | Ia CTWD | Ib | 25, 66, 81, 82 | 2, 4, 6, 8, 30, 46, 59, 63 | 27, 35, 38, 40, 44, 73, 102 | 9, 14, 16, 26, 29, 39, 43, 45, 61, 65, 85 | IIIa | 48 |
| 22, 33, 36, 41, 51, 52, 70, 71, 74, 76, 86, 95, 101 | 22, 33, 36, 41, 51, 52, 70, 71, 74, 76, 83, 101 | 1, 3, 7, 10, 12, 13, 15, 23, 24, 28, 34, 37, 42, 47, 56, 57, 58, 62, 67, 92 | 7, 18, 27, 32, 37, 38, 45, 46, 47, 59, 60, 61, 64, 65, 66, 67, 75 | 14, 39, 68 | 1, 4, 5, 12, 48, 70 | 1,3, 16, 23, 24, 26, 30, 33, 41, 49, 50, 52, 53, 54, 56, 57, 58, 71, 72, 76 | 13, 21, 36, 42, 69, 73, 77, 80, 81, 82 | 8, 9, 10, 15, 17, 19, 22, 25, 29, 40, 43, 44, 51, 55, 78, 84, 85 | 14 |
| 1, 2, 3, 4, 5, 14, 15, 18, 20, 31, 32, 33, 34, 36, 44 | 1, 2, 3, 4, 5, 14, 15, 18, 20, 31, 32, 33, 34, 36, 44 | 39, 40, 43, 45, 46 | 7, 18, 27, 32, 37, 38, 45, 46, 47, 59, 60, 61, 64, 65, 66, 67, 75 | 14, 39, 68 | 1, 3, 7, 10, 12, 13, 15, 23, 24, 28, 34, 37, 42, 47, 56, 57, 58, 71, 72, 76 | 12, 13, 23, 28, 30, 38, 47, 48, 50, 51, 55, 56, 57, 61, 71, 75 | 7, 8, 10, 11, 21, 24, 22, 25, 26, 29, 35, 37, 62, 63, 64, 65, 66, 67, 68, 69, 77, 78, 79 | 19, 41, 42, 52, 53, 54, 58, 59, 60, 80, 81 | 51 |
| 1, 2, 14, 17, 18, 19, 26, 27, 37, 53, 61, 65, 78, 82 | 4, 8, 34, 39, 43, 52, 54, 60, 71, 79, 80, 81 | 29, 75, 77 | 2, 3, 6, 10, 14, 18, 20, 23, 24, 27, 36, 38, 44, 48, 49, 50, 57, 58, 66, 67, 72, 86, 90 | 8, 9, 16, 20, 21, 51, 56, 59, 68, 74 | 7, 13, 28, 30, 41, 47, 69 | 1, 5, 10, 11, 12, 13, 15, 19, 25, 31, 32, 33, 35, 40, 41, 42, 45, 46, 55, 62, 63, 64, 70, 73, 74, 76, 83, 84, 85, 87, 88, 89, 91 | 30, 38, 41, 46, 53, 54, 55, 62, 63, 64, 66, 67, 70 | 76 |

Table 8. Classification and grouping of plant WRKY TFs published by different research groups at different times. The results clearly showed that none of the WRKY TF group members of one plant species matched with those of other species. These findings indicate that no previous research groups followed specific and proper principles to name and group the WRKY TFs in plants. The number indicates the name of the WRKY TF for example 1, 2, and others indicate WRKY1, WRKY2, and so on.)
showed that WRKY TFs evolved conservatively. Only few WRKY TFs were found in lower eukaryotes, including *C. reinhardtii*, *C. subellipsoidea*, *M. pusilla*, and *V. carteri* whereas higher plants possessed a larger number of WRKY TF genes. This indicated that the earliest evolutionary origin of the gene containing the WRKY TF was from unicellular green algae. This suggested that WRKY proteins evolved before plants transitioned from an aquatic to a terrestrial habitat. With the continuous evolution of species, land plants have evolved a series of highly sophisticated signaling mechanisms that helped them to adapt to the ever changing environmental conditions, and hence, the number of WRKY TFs increased in different species. Presence of the WRKY TF gene in diplomonads, amoebozoa, and fungi sheds new light on the early evolution of WRKY genes.

Understanding the evolution of the WRKY TFs in plant lineage is very challenging. If the concept of early evolution is considered, in green algae, a BED finger-like C2H2 zinc finger domain incorporated a WRKY domain N-terminal to the zinc finger. This single-domain WRKY TFs served as the progenitor for all other WRKY genes. Subsequently, this single-domain WRKY TFs fused via addition or recombination to yield a double WRKY domain by maintaining the original copy intact. Thereafter, independent lateral gene transfer to non-plant lineage and plant lineage occurred during the early evolution of WRKY TFs. This led to the transfer of WRKY TFs to fungi, amoeba and other species. The amoeba species, *D. purpureum* and the green algae *O. lucimarinus* and *V. carteri* contain both double and single WRKY domain proteins. However, *C. reinhardtii* contains only the double WRKY domain protein. This shows that the single double WRKY domains have coevolved from the green plant lineage. All these events seemed to have occurred before the transition of green plants to a terrestrial habitat. During these evolutionary processes, the chimeric WRKY protein evolved to contain either kinase, NAC, B3, LRR, PAH, CBS, ZF_SBP, ULP_protease, TIR, or ATP_GRASP domain. These chimeric WRKY TFs are not found in all plant species, and are restricted to only the flowering plant lineage. WRKY TFs with other novel domains can be expected from other plant species the genomes of which are yet to be sequenced.

**Gene duplication and evolution.** Evolution by gene duplication is one of the most important processes responsible for the supply of raw genetic material to an organism for its biological evolution. Duplication can occur via recombination, aneuploidy, retro-transposition or whole genome duplication. *A. thaliana* encodes about 16,574 (65%) duplicated genes among its total of 25498 genes. In the present study, we found several duplicated WRKY TFs (Supplementary Table 1). Most duplicated WRKY TF genes are present as paralogous genes. More specifically, gene duplication analysis of some novel WRKY TFs (Fig. 2, Table 9), performed using Pinda (pipeline for intraspecies duplication analysis) server revealed that most of the WRKY TFs are duplicated. Some of the novel WRKY TFs, such as SbWRKY59, PvWRKY94-1 and SiWRKY59-2, were found to be non-duplicated. The Z-score values of these non-duplicated WRKY TFs ranged from 1.11 to 1.78. A z-score value of less than four indicates a non-duplicated gene.

**Statistical analysis.** Tajima’s relative rate test, the simplest test that can be applied to test the molecular evolutionary clock, can be applied to both nucleotide and amino acid sequences. This method yields results as the Chi-square test, and can even be applied when the pattern of substitution is unknown or the substitution rate varies across sites. In Tajima’s relative rate test of WRKY TFs, the p-value and Chi-square test were found to be significant (Table 7).

**Gene expression profile of WRKY TFs.** Understanding the tissue-specific expression of genes can lead to elucidation of the molecular mechanisms and the role of the genes in tissue development and function. Understanding the genes, how they were expressed and were regulated in different tissues is a challenging and fundamental question. Therefore, we investigated the tissue-specific expression of WRKY TFs of *G. max* and *P. vulgaris* (Supplementary Table 2). In *G. max*, expression analysis was conducted in the roots, root hairs, leaves, stems, flowers, pods, seeds, nodules and shoot apical meristem tissue. Of the total of 145 *G. max* WRKY TFs, 143 were found to be expressed in either of the mentioned tissues. Expressions of *GmWRKY65-1* (105.342), *GmWRKY6-4* (74.668), and *GmWRKY6-5* (43.341) were found to be significantly higher than those of others in the roots, suggesting their important role in root development. Expression of 24 *GmWRKY* was not detected in root tissue (Supplementary Table 2), indicating that these genes might not play any active role in root development. The expression level of *GmWRKY65-1* (35.199) was found to be the highest in root hair, suggesting its active role in the development of root hair. Expression levels of at least eight genes were not detected in root hairs. The expression levels of *GmWRKY6-4* (51.394), *GmWRKY6-5* (81.847), *GmWRKY6-2* (80.957), *GmWRKY6-3* (72.911), and *GmWRKY641-3* (72.788) were significantly higher in the leaf tissues than in any other tissues, suggests that these genes might play crucial roles in leaf development. Expression levels of at least 24 genes were not detected in leaf tissues. In stems, the expression levels of *GmWRKY61-2* (47.276), *GmWRKY671-6* (24.872), and *GmWRKY65-2* (24.866) were found to be significantly higher than that of other genes, suggesting their role in stem development. Expression levels of at least 15 genes were not detected in the stem tissue. In flowers, the expression levels of *GmWRKY26-2* (67.456), *GmWRKY26-3* (51.836), *GmWRKY70-6* (61.053) and *GmWRKY70-7* (63.153) were found to be significantly higher than those of other genes, suggesting that these genes might play an important role in flower development. Expression levels of at least eight genes were not detected in flower tissue (Supplementary Table 2). In pods, the expression level of *GmWRKY44-2* (17.882) was found to be significantly higher than that of other genes, suggesting its important role in pod development. The expression levels of at least 19 genes were not detected in pod. In seeds, the expression level of *GmWRKY21-3* (31.762) was found to be significantly higher than that of other genes, suggesting its important role in seed development. In nodules, the expression level of *GmWRKY65-1* (39.186) was significantly higher than that of other genes, suggesting its important role in nodule development. The expression level of *GmWRKY65-1* was higher in root and root hairs as well. Thus, *GmWRKY65-1* might play a crucial role in root, root hair, and nodule development. In the shoot
apical meristem, the expression level of \( \text{PvulWRKY17} \) was found to be significantly higher than that of other genes, suggesting its role in apical meristem development. Expression levels of at least 21 genes were not detected in the apical meristem tissue. Considering the ubiquitous expression of WRKY TFs in \( G. \max \), we found that \( \text{GmWRKY6-4}, \text{GmWRKY6-5}, \text{GmWRKY70-4}, \text{GmWRKY11-1}, \text{GmWRKY11-2}, \text{GmWRKY11-3}, \text{GmWRKY11-4}, \text{GmWRKY11-5}, \text{GmWRKY11-6}, \text{GmWRKY11-7}, \text{GmWRKY11-8}, \text{GmWRKY15-1}, \text{GmWRKY15-2}, \text{GmWRKY20-2}, \text{GmWRKY20-4}, \text{GmWRKY22-3}, \text{GmWRKY22-4}, \text{GmWRKY26-3}, \text{GmWRKY35-3}, \text{and GmWRKY41-7} \) were highly expressed in all the studied tissues (Supplementary Table 2). Similarly, the expression levels of \( \text{GmWRKY10} \) and \( \text{GmWRKY13-4} \) were almost negligible or absent in the major tissue types (Supplementary Table 2).

In \( P. \text{vulgaris} \), expression analysis was conducted in eight tissue types that included young trifoliates, leaves, flowers, flower buds, young pods, stems, roots, and nodules. In young trifoliates, the expression level of \( \text{PvulWRKY17} \) was also found to be highly expressed in the stems, roots, nodules, and pods of \( P. \text{vulgaris} \). Similarly, \( \text{WRKY15-2} \) was also found to be highly expressed in the stems, roots, nodules, and pods of \( G. \max \) and \( P. \text{vulgaris} \), suggesting that this gene might be extensively involved in root and nodule development in both the plants.

Table 9. Gene duplication analysis of novel WRKY TFs identified during this study. The result showed that \( \text{SbWRKY29}, \text{PvWRKY94-1} \) and \( \text{SiWRKY59-2} \) are non duplicated WRKY TFs. A z-score value above four is considered duplicated, whereas a value below four was considered nonduplicated. The duplication analysis was performed as described in Pinda (pipeline for intraspecies duplication analysis)\(^8\)1.

| Figure 1 | Genes | Z-score | Percentage of confidence Level |
|----------|-------|---------|-------------------------------|
| A | GmWRKY12 | 8.12 | 100 |
| B | AtWRKY1 | 6.7 | 100 |
| C | LuWRKY3-5 | 4.42 | 100 |
| D | BrWRKY36-2 | 8.22 | 100 |
| E | FvWRKY59 | 20.58 | 100 |
| F | PwWRKY59 | 19.82 | 100 |
| G | BrWRKY58-1 | 25.80 | 100 |
| H | AtWRKY19 | 25.98 | 100 |
| I | OsWRKY57 | 8.43 | 100 |
| J | FvWRKY52 | 15.34 | 100 |
| K | FvWRKY70-7 | 18.80 | 100 |
| L | FvWRKY16 | 6.67 | 100 |
| M | SiWRKY59 | 1.78 | 92.5 |
| N | AlfWRKY16 | 12.53 | 100 |
| O | PwWRKY94-1 | 1.11 | 73.2 |
| P | SiWRKY59-2 | 1.11 | 73.4 |
Conclusion
Analysis of the WRKY TF gene family across the plant lineage revealed the presence of novel WRKY TFs. The monocot or dicot lineage specific grouping and orthologous-based nomenclature system of WRKY TFs might be crucial in future studies. Expression analysis showed that WRKY11-1, WRKY11-2, and WRKY11-3 were highly expressed in all tissue types in G. max and P. vulgaris. Similarly, WRKY15-2 was found to be highly expressed in the stems, roots, nodules and pods in G. max and P. vulgaris, suggesting its important role in the development of these tissues. Understanding the functional role of novel WRKY TFs will help to understand their functional and evolutionary roles.

Material and Methods
Identification of WRKY TFs. WRKY TFs from the model organisms A. thaliana and O. sativa were downloaded from The Arabidopsis Information Resource (TAIR) database and the Rice Genome Annotation project respectively83,84. The protein sequences of WRKY TFs from A. thaliana and O. sativa were used as query sequences to search the WRKY TFs in other plant species in the phytozome database85. The WRKY TFs from O. sativa were used to search the WRKY TFs from monocot plants, and A. thaliana WRKY TFs were used to search the TFs from dicot and other plant species. Overall, WRKY TFs gene families of 43 plant species were investigated. The Hidden Markov Model (HMM) and BLASTP program was used as well to search the WRKY TFs of the investigated plant species by using the default parameters of the phytozome database. The sequences generated by BLASTP searches were collected for further analysis to confirm whether they were WRKY TFs. All the collected sequences were then analysed using the scanprosite and MEME software to confirm the presence of WRKY domains86,87. Default parameters were used in the scanprosite software to identify the WRKY domains. The identified sequences that contained the WRKY domain were retained for further validation which was accomplished by subjecting the sequences to BLASTP analysis in the TAIR and rice genome annotation project database using the default parameters. Further, all the sequences were analysed using HMMER web server to identify the interactive sequence similarities88. Sequences that resulted in BLASTP hits with WRKY TFs in the TAIR or rice genome annotation database were confirmed as WRKY TFs.

Nomenclature of WRKY TFs. All identified WRKY TFs were assigned a specific name. Nomenclature of the WRKY TFs was assigned according to an orthology-based nomenclature system proposed by different researchers83,85. In the nomenclature system, names were assigned by considering the first letter of the genus in upper case and the first letter of the species in lower case followed by the WRKY and orthology-based number of A. thaliana or O. sativa. When redundancies were found in the nomenclature system, 2 to 4 letters of the species name were considered for the nomenclature. When more than one orthologous gene was found, they were considered as paralogous genes which were numbered by including a hyphen. For example, if there are two OsWRKY46 in O. sativa, they would be named OsWRKY46-1 and OsWRKY46-2.

Multiple sequence alignment. The multiple sequence alignment of WRKY TFs was conducted using the Multalin software (http://multalin.toulouse.inra.fr/multalin/) with default parameters which were as follows: protein weight matrix, Blossum62-12-12; gap penalties at opening, default; gap penalty at extension, default; gap penalty at extremities, none; one iteration only, any; high consensus value, 90% (default); and low consensus value, 50% (default). The multiple sequence alignment of proteins containing single and double WRKY domain was conducted separately by using the same parameters.

Construction of phylogenetic tree. Unrooted phylogenetic trees were constructed to understand the closeeness and evolutionary relatedness of WRKY TFs in plants. We constructed different phylogenetic trees by grouping the WRKY TFs into different groups. Groupings included (1) monocot, dicot and lower eukaryotic plants, (2) monocot and lower eukaryotes, (3) dicot and lower eukaryotes (4) single WRKY domain (C-terminal WRKY domain)-containing WRKY TFs and (5) double WRKY domain (N- and C-terminal WRKY domain)-containing WRKY TFs. To construct the phylogenetic trees, we created clustal files for each group using the clustalW or clustal omega program89,90. The generated clustal files were converted to the MEGA file format, after which the MEGA files were run in MEGA6 software to construct the phylogenetic tree92. Different statistical parameters used to construct the phylogenetic trees included the following: analysis, phylogeny reconstruction; statistical method, maximum likelihood; test of phylogeny, bootstrap method; number of bootstrap replicates, 1000; substitution type, amino acids; model/method, Poisson model; rates among sites, uniform rates; gap/missing data treatment, partial deletion/use all sites; site coverage, 95%; ML heuristic method, nearest-neighbor-interchange (NNI); and branch swap filter, very strong.

Statistical analysis. Different statistical analyses were performed to understand the evolutionary aspects of WRKY TFs using the MEGA6 program92. The MEGA files of all five groups that were used in the construction of the phylogenetic tree were subjected to the MEGA6 program for statistical analysis. Tajima’s relative rate test was conducted to evaluate the statistical significance of WRKY TFs to understand whether there were significant variations in molecular evolution. In this test, sequences 1, 2, and 3 were considered simultaneously where sequence 3 was considered as an out group. If \( n_{ij} \) was the observed number of sites in which sequences 1, 2 and 3 have protein/nucleotides 1, j, and k. under the molecular clock hypothesis, \( E(n_{ijk}) = E(n_{ijk}) \) irrespective of the substitution model used and whether the substitution rate varied across the sites. If the hypothesis is rejected, then the molecular clock hypothesis of evolution can be rejected for the given set of sequences 1, 2 and 3. The statistical parameters used to perform Tajima’s relative rate test were as follows: analysis, Tajima’s relative rate test; scope, for 3 chosen sequences; substitution type, amino acids; and gaps/missing data treatment, complete deletion.
Gene duplication analysis. Gene duplication analysis of some selective WRKY TFs performed using the online server Pinda (http://orion.mbg.duth.gr/Pinda). All the data used in this study were obtained from publicly available database (https://phytozome.jgi.doe.gov/pz/portal.html, http://congenie.org/start) available in the public domain.

Gene expression data. The expression data of *G. max* and *P. vulgaris* were downloaded from the phytomine database (https://phytozome.jgi.doe.gov/phytomine/template.do?name=One_Gene_Expression&scope=global) of phytomine. Locus ID of *G. max* and *P. vulgaris* were used for to searching the expression data in different tissue samples.

References

1. Chen, K. & Rajewsky, N. The evolution of gene regulation by transcription factors and microRNAs. Nat Rev Genet 8, 93–103 (2007).
2. van Verk, M. C., Bol, J. F. & Linthorst, H. J. M. WRKY transcription factors involved in activation of SA biosynthesis genes. BMC Plant Biol. 11, 89 (2011).
3. Bakshi, M. & Oelmüller, R. WRKY transcription factors: Jack of many trades in plants. Plant Signal. Behav. 9, 1–18 (2014).
4. Eulgem, T., Rushton, P. J., Robatzez, S. & Somssich, I. E. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5, 199–206 (2000).
5. Uller, R. & Somssich, I. E. WRKY transcription factors: From DNA binding towards biological function. Curr. Opin. Plant Biol. 7, 491–498 (2004).
6. Yamasaki, K. et al. Structural basis for sequence-specific DNA recognition by an Arabidopsis WRKY transcription factor. J. Biol. Chem. 287, 7683–7691 (2012).
7. Rushton, P. J., Somssich, I. E., Ringler, P. & Shen, Q. J. WRKY transcription factors. Trends Plant Sci. 15, 247–258 (2010).
8. Cai, M. et al. Identification of novel pathogen-responsive cis-elements and their binding proteins in the promoter of OsWRKY13, a gene regulating rice disease resistance. Plant Cell Environ. 31, 86–96 (2008).
9. Sun, C. et al. A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. Plant Cell 15, 2076–2092 (2003).
10. Eulgem, T., Rushton, P. J. & Schmelzer, E. Hahlbrock, K. & Somssich, I. E. Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMB J. 18, 4689–4699 (1999).
11. Xie, Z. et al. Annotation and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of asbscic acid signaling in aleurome cells. Plant Physiol. 137, 176–189 (2005).
12. Dong, J., Chen, C. & Chen, Z. Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol. Biol. 51, 21–37 (2003).
13. Ciolkowski, I., Wanke, D., Birkenbihl, R. P. & Somssich, I. E. Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. Plant Mol. Biol. 68, 81–92 (2008).
14. Wen, F. et al. Genome-wide evolutionary characterization and expression analyses of WRKY family genes in Brachypodium distachyon. DNA Res. 21, 327–339 (2014).
15. de Pater, S., Greco, V., Pham, K., Memelink, J. & Kijne, J. Characterization of a zinc-dependent transcriptional activator from Arabidopsis. Nucleic Acids Res. 24, 4631–4631 (1996).
16. Maro, K., Hayashi, S., Kojima-Suzuki, H., Morikami, A. & Nakamura, K. Role of Conserved Residues of the WRKY Domain in the DNA-binding of Tobacco WRKY Family Proteins. Biosci. Biotechnol. Biochem. 65, 2428–2436 (2001).
17. Rushton, D. L. et al. WRKY transcription factors: key components in abscisic acid signalling. Plant Biotechnol. J. 10, 2–11 (2012).
18. Jiang, W. & Yu, D. Arabidopsis WRKY2 transcription factor mediates seed germination and postgermination arrest of development by abscisic acid. BMC Plant Biol. 9, 96 (2009).
19. Grunewald, W. et al. Transcription factor WRKY23 assists auxin distribution patterns during Arabidopsis root development through local control on flavonoid biosynthesis. Proc. Natl. Acad. Sci. USA 109, 1554–1559 (2012).
20. Yu, F. et al. GhWRKY15, a member of the WRKY transcription factor family identified from cotton (Gossypium hirsutum L.), is involved in disease resistance and plant development. BMC Plant Biol. 12, 144 (2012).
21. Zhang, C.-Q., et al. The WRKY transcription factor OsWRKY78 regulates stem elongation and seed development in rice. Planta 234, 541–554 (2011).
22. He, S. et al. Converting restriction fragment length polymorphism to single-strand conformation polymorphism markers and its application in the fine mapping of a trichome gene in cotton. Plant Breed. 132, 337–343 (2013).
23. Kang, X., Li, W., Zhou, Y. & Ni, M. A WRKY Transcription Factor Recruits the SYG1-Like Protein SHB1 to Activate Gene Expression and Seed Cavity Enlargement. PLoS Genet. 9, e1003347 (2013).
24. Zhou, X., Jiang, Y. & Yu, D. WRKY22 transcription factor mediates dark-induced leaf senescence in Arabidopsis. Molecules and cells 31, (2011).
25. Ricachenkovsky, F. K., Sperrotto, R. A., Menguer, P. K. & Fett, J. P. Identification of Fe-excess-induced genes in rice shoots reveals a WRKY transcription factor responsive to Fe, drought and senescence. Mol. Biol. Rep. 37, 3735–3745 (2010).
26. Beato, S., Li, J. & Palva, E. T. WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in Arabidopsis thaliana. J. Exp. Bot. 63, 2667–2679 (2012).
27. Skibbe, M., Qu, N., Galis, I. & Baldwin, I. T. Induced plant defenses in the natural environment: Nicotiana attenuata WRKY3 and WRKY6 coordinate responses to herbivory. Plant Cell 20, 1984–2000 (2008).
28. Mohanta, T. K. et al. Ginkgo biloba responds to herbivory by activating early signaling and direct defenses. PLoS One 7, e32822 (2012).
29. Zheng, Z., Mosher, S. L., Fan, B., Klessig, D. F. & Chen, Z. Functional analysis of Arabidopsis WRKY23 transcription factor in plant defense against Pseudomonas syringae. BMC Plant Biol. 7, 2 (2007).
30. Xu, X., Chen, C., Fan, B. & Chena, Z. Physical and Functional Interactions between Pathogen-Induced Arabidopsis WRKY18, WRKY40, and WRKY60 Transcription Factors. Plant Cell 18, 1310–1326 (2006).
31. Zheng, Z., Qamar, S. A., Chen, Z. & Mengiste, T. Arabidopsis WRKY53 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 48, 592–605 (2006).
32. Park, C.-J. et al. A hot pepper gene encoding WRKY transcription factor is induced during hypersensitive response to Tobacco mosaic virus and Xanthomonas campestris. Planta 223, 168–179 (2006).
33. Mao, P., Duan, M., Wei, C. & Li, Y. WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. Plant Cell Physiol. 48, 833–842 (2007).
34. Qin, D. et al. OsWRKY13 mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. Mol. plant-microbe Interact. 20, 492–499 (2007).
35. Xie, Z. et al. Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurome cells. Plant J. 46, 231–242 (2006).
36. Wang, Z. et al. A WRKY transcription factor participates in dehyration tolerance in Brachypodium distachyon by binding to the W-box of the galectin synthase (BhGoS1) promoter. *Planta* **230**, 1155–1166 (2009).
37. Zhang, Z.-L. et al. A Rice WRKY Gene Encodes a Transcriptional Repressor of the Gibberellin Signaling Pathway in Aleurone Cells. *Plant Physiol.* **134**, 1500–1513 (2004).
38. Chen, L. et al. The role of WRKY transcription factors in plant abiotic stresses. *Biochim. Biophys. Acta* **1819**, 120–8 (2012).
39. Izaguirre, M. M., Scopec, A. L., Baldwin, J. T. & Ballare, C. L. Convergent responses to stress, Solar ultraviolet-B radiation and Manduca sexta herbivory elicit overlapping transcriptional responses in field-grown plants of Nicotiana longiflora. *Plant Physiol.* **132**, 1755–1767 (2003).
40. Li, S., Fu, Q., Huang, W. & Yu, D. Functional analysis of an Arabidopsis transcription factor WRKY25 in heat stress. *Plant Cell Rep.* **28**, 683–693 (2009).
41. Huang, T. & Dumau, J. G. Cloning and characterization of a thermal hysteresys (antifreeze) protein with DNA-binding activity from winter bittersew nightshade, Solanum dulcamara. *Plant Mol. Biol.* **48**, 339–350 (2002).
42. Vandenaenelee, S. et al. A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. *Proc. Natl. Acad. Sci. USA* **100**, 16113–16118 (2003).
43. Wang, C. et al. A wheat WRKY transcription factor TaWRKY10 confers tolerance to multiple abiotic stresses in transgenic tobacco. *PLoS One* **8**, e65120 (2013).
44. Zhou, Q.-Y. et al. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. *Plant Biotechnol. J.* **6**, 486–503 (2008).
45. Jiang, Y. & Deyholos, M. K. Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. *BMC Plant Biol.* **25**, (2006).
46. Cai, C. et al. Genome-wide analysis of the WRKY transcription factor gene family in Gossypium raimondii and the expression of orthologs in cultivated tetraploid cotton. *Crop J.* **2**, 87–101 (2014).
47. Song, H., Wang, P., Nan, Z. & Wang, X. The WRKY Transcription Factor Genes in Lotus japonicus. *Int. J. Genomics* **2014**, 1–24 (2014).
48. Wu, K., Guo, Z., Wang, H. & Li, J. The WRKY Family of Transcription Factors in Rice and Arabidopsis and Their Origins. *BMC Genomics* **26**, 9–26 (2005).
49. Li, H.-L., Zhang, L.-B., Guo, D., Li, C.-Z. & Peng, S.-Q. Identification and expression profiles of the WRKY transcription factor family in Ricinus communis. *Gene* **503**, 248–253 (2012).
50. Muthamilarasan, M., Bonthala, V. S. & Khanddelwal, R. Global analysis of WRKY transcription factor superfamily in Setaria identifies potential candidates involved in abiotic stress signaling. *Front. Microbiol.* **6**, 1–15 (2015).
51. Huang, S. et al. Genome-wide analysis of WRKY transcription factors in Solanum lycopersicum. *Mol. Genet. genomics* **287**, 495–513 (2012).
52. Okay, S., Derelli, E. & Unver, T. Transcriptome-wide identification of bread wheat WRKY transcription factors in response to drought stress. *Mol. Genet. genomics* **289**, 765–781 (2014).
53. Wang, M. et al. Genome and transcriptome analysis of the grapevine (Vitis vinifera L.) WRKY gene family. *Hortic. Res.* **1**, 16 (2014).
54. Rinnerth, C. I., Rabara, R. C., Tripathi, P., Shen, Q. J. & Rushton, P. J. The evolution of WRKY transcription factors. *BMC Plant Biol.* **15**, 1–18 (2015).
55. Hamel, L.-P. et al. Ancient signals: comparative genomics of plant MAPK and MAPKKK gene families. *Trends Plant Sci.* **11**, 192–198 (2006).
56. Mohanta, T. K., Arora, P. K., Mohanta, N., Purida, P. & Bae, H. Identification of new members of the MAPK gene family in plants shows diverse conserved domains and novel activation loop variants. *BMC Genomics* **16**, 58 (2015).
57. Mohanta, T., Mickael, M., Nibedita, M. & Chidananda, N. K. In-silico identification and phylogenetic analysis of auxin efflux carrier gene family in Setaria italica L. *African J. Biotechnol.* **13**, 211–225 (2014).
58. Mohanta, T. K. & Mohanta, N. Genome wide identification of auxin efflux carrier gene family in physcomitrella patens. *J. Biotechnol. Sci.* **1**, 54–64 (2013).
59. Yamazaki, K., Kigawa, T., Seki, M., Shinozaki, K. & Yokoyama, S. DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. *Trends Plant Sci.* **18**, 267–276 (2013).
60. Zhang, Y. & Wang, L. The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. *BMC Evol. Biol.* **5**, 1 (2005).
61. Pérez-Rodriguez, P. et al. PhITFDB: Updated content and new features of the plant transcription factor database. *Nucleic Acids Res.* **38**, 822–827 (2009).
62. Jin, J., Zhang, H., Kong, L., Gao, G. & Luo, J. PlantITFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. *Nucleic Acids Res.* **42**, D1182–D1187 (2014).
63. Ross, C. A., Liu, Y. & Shen, Q. J. The WRKY Gene Family in Rice (Oryza sativa). *J. Integr. Plant Biol.* **49**, 827–842 (2007).
64. Hamel, L.-P., Sheen, J. & Séguin, A. Ancient signals: comparative genomics of green plant CDPKs. *Trends Plant Sci.* **19**, 79–89 (2014).
65. Shen, H., Liu, C., Zhang, Y. & Wang, X. OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice. *Plant Mol. Biol.* **80**, 241–253 (2012).
66. tiezen, E. & Jones, J. Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem Sci.* **3004**, 454–456 (1998).
67. Anderson, K. V. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* **12**, 13–19 (2000).
68. Xu, Y. et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature* **408**, 111–115 (2000).
69. Wan, H. et al. Analysis of TIR- and non-TIR-NBS-LLR disease resistance gene analogs in pepper: characterization, genetic variation, functional divergence and expression patterns. *BMC Genomics* **13**, 502 (2012).
70. Nandety, R. S. et al. The role of TIR-NBS and TIR-X proteins in plant basal defense responses. *Plant Physiol.* **162**, 1459–1472 (2013).
71. Day, P. et al. Structure of a CBS-domain pair from the regulatory gamma1 subunit of human AMPK in complex with AMP and ZMP. *Acta Crystallogr. D. Biol. Crystallogr.* **63**, 587–596 (2007).
72. Klein, J., Saedler, H. & Huijser, P. A new family of DNA binding proteins includes putative transcriptional regulators of the Antirrhinum major floral meristem identity gene SQUAMOSA. *Mol. Gen. Genet.* **250**, 7–16 (1996).
73. Cardon, G. et al. Molecular characterisation of the Arabidopsis SBF-box gene At3G27280. *Nat Struct Mol Biol* **11**, 738–746 (2004).
74. Fawaz, M. V., Topper, M. E. & Firestone, S. M. The ATP-grasp enzymes. *Bioorg. Chem.* **39**, 185–191 (2011).
75. Wang, Q. et al. WRKY gene family evolution in Arabidopsis thaliana. *Genetica* **139**, 973–983 (2011).
76. Tang, H., Bowers, J. E., Wang, X. & Paterson, A. H. Angiosperm genome comparisons reveal early polyploidy in the monocot lineage. *Proc. Natl. Acad. Sci.* **107**, 472–477 (2010).
77. Tang, H. et al. Synteny and Collinearity in Plant Genomes. *Science* **(80-)**, 320, 486–488 (2008).
78. Zhang, J. Evolution by gene duplication: an update. *Trends Ecol. Evol.* **18**, 292–298 (2003).
80. Arabidopsis, T. & Initiative, G. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* **408**, 796–815 (2000).
81. Kontopoulou, D.-G. & Glykos, N. M. Pinda: a web service for detection and analysis of intraspecies gene duplication events. *Comput. Methods Programs Biomed.* **111**, 711–714 (2013).
82. Tajima, F. Simple Methods for Testing the Molecular Evolutionary Clock Hypothesis. *Genet. Soc. Am.* **135**, 599–607 (1998).
83. Lamesch, P. et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. *Nucleic Acids Res.* **40**, D1202–D1210 (2012).
84. Ouyang, S. et al. The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucleic Acids Res.* **35**, D883–D887 (2007).
85. Goodstein, D. M. et al. Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* **40**, D1178–D1186 (2012).
86. de Castro, E. et al. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res.* **34**, W362–W365 (2006).
87. Bailey, T. L. et al. MEME Suite: Tools for motif discovery and searching. *Nucleic Acids Res.* **37**, 202–208 (2009).
88. Finn, R. D., Clements, J. & Eddy, S. R. HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res.* **39**, W29–W37 (2011).
89. Mohanta, T. K. & Mohanta, N. Genome Wide Identification of Auxin Efflux Carrier Gene Family in Solanum tuberosum L. *J. Nat. Sci.* **1**, 44–51 (2013).
90. Larkin, M. A. et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–8 (2007).
91. Sievers, F. et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
92. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729 (2013).

**Acknowledgements**

This study was carried out with the support of the “Forest Science and Technology” project (Project No. S121414L030110) provided by Korea forest service.

**Author Contributions**

T.K.M.: Conceived the idea, performed the experiments and statistical analysis, drafted and revised the manuscript; Y.P.: revised the manuscript; H.B.: revised the manuscript.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

**How to cite this article:** Mohanta, T. K. et al. Novel Genomic and Evolutionary Insight of WRKY Transcription Factors in Plant Lineage. *Sci. Rep.* **6**, 37309; doi: 10.1038/srep37309 (2016).

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016