

Article

Genome-Wide Analysis of the Wall-Associated Kinase (WAK) Genes in *Medicago truncatula* and Functional Characterization of MtWAK24 in Response to Pathogen Infection

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Abstract: The wall-associated kinases (WAKs) can perceive and transmit extracellular signals as one kind of unique receptor-like kinases (RLKs) involved in the regulation of cell expansion, pathogen resistance and abiotic stress tolerance. To understand their potential roles and screen some key candidates in *Medicago truncatula* (*M. truncatula*), genome-wide identification and characterization of MtWAKs were conducted in this study. A total of 54 MtWAK genes were identified and classified into four groups based on their protein domains. They were distributed on all chromosomes, while most of them were clustered on chromosome 1 and 3. The synteny analysis showed that 11 orthologous pairs were identified between *M. truncatula* and *Arabidopsis thaliana* (*A. thaliana*) and 31 pairs between *M. truncatula* and *Glycine max* (*G. max*). The phylogenetic analysis showed that WAK-RLKs were classified into five clades, and they exhibited a species-specific expansion. Most MtWAK-RLKs had similar exon–intron organization and motif distribution. Multiple *cis*-acting elements responsive to phytohormones, stresses, growth and development were observed in the promoter regions of MtWAK-RLKs. In addition, the expression patterns of MtWAK-RLKs varied with different plant tissues, developmental stages and biotic and abiotic stresses. Interestingly, plasm membrane localized MtWAK24 significantly inhibited *Phytophthora* infection in tobacco. The study provides valuable information for characterizing the molecular functions of MtWAKs in regulation of plant growth, development and stress tolerance in legume plants.

Keywords: abiotic stress; biotic stress; expression pattern; *Medicago truncatula*; WAK



Citation: Kong, W.; Shi, J.; Yang, B.; Yu, S.; Zhao, P.; Guo, Z.; Zhu, H. Genome-Wide Analysis of the Wall-Associated Kinase (WAK) Genes in *Medicago truncatula* and Functional Characterization of MtWAK24 in Response to Pathogen Infection. *Plants* **2023**, *12*, 1849. <https://doi.org/10.3390/plants12091849>

Academic Editor: Vagner A. Benedito

Received: 13 March 2023

Revised: 27 April 2023

Accepted: 27 April 2023

Published: 30 April 2023



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1. Introduction

Plants perceive and process various signals on cell surface to modulate biological processes through members of the receptor-like kinase family [1]. WAK is a unique class of RLKs that link cell wall to cytoplasm physically, consisting of extracellular epidermal growth factor (EGF) domain and/or galacturonan-binding (GUB) domain, transmembrane domain and intercellular Ser/Thr kinase domain [2]. Based on the organization of the conserved domains, WAKs are divided into four types: the RLK type, which contains both extracellular domain and kinase domain; the receptor-like cytoplasmic kinase (RLCK), which contains kinase domain alone; the receptor-like protein (RLP), which contains only extracellular domain; and the short protein type, which harbors no domain but shows high similarity in the emerging sequence.

WAKs participate in regulation on plant growth and development as well as biotic and abiotic stress tolerance. Down-regulation of *AtWAK4* or *AtWAK2* expression leads to impaired cell expansion in *Arabidopsis* [3,4]. *OsWAK11* regulates both stem and seed

elongation in rice through monitoring cell wall pectin changes that fine-tune brassinosteroid signaling [5]. OsiWAK1 positively regulates male sterility by binding its extracellular domain to pectic polysaccharides and plays a pivotal role in regulating programmed cell death process [6]. Several WAK genes, such as *AtWAK1* [7], *Htn1* (*ZmWAK*) [8,9], *Stb6* [10] and *Xa4* [11], are involved in plant immunity against fungal and bacterial pathogens. *OsWAK14*, *OsWAK91* and *OsWAK92* expression is up-regulated by inoculation with *Magnaporthe oryzae* and chitin treatment for regulating blast resistance in rice positively. On the contrary, *OsWAK112d* expression is down-regulated by *M. oryzae* and negatively regulates blast resistance [12]. The *AtWAK1* transcript level is induced by oligogalacturonides (OGs) that act as damage-associated molecular patterns (DAMPs) for activating plant immune responses [13], and the cytoplasmic kinase domain is activated by OGs [3]. *AtWAK1* [14], *AtWAKL4* [15] and *OsWAK11* [16] are involved in metal tolerance. *AtWAKL4* expression is induced by Na^+ , K^+ , Cu^{2+} , Ni^{2+} and Zn^{2+} , and the T-DNA insertion of *AtWAKL4* resulted in hypersensitivity to excessive K^+ , Na^+ , Cu^{2+} , and Zn^{2+} [15]. *OsWAK112* negatively regulates plant salt responses by binding with *OsSAMS1/2/3* for inhibiting ethylene production [17].

M. truncatula is a diploid plant with a small genome size. It is a model legume species for functional genomics research. However, there are no investigations on WAKs in *M. truncatula*. Given the important role of WAKs in plants, the genome-wide analysis of WAK family genes was conducted to provide valuable information for understanding the roles of *MtWAKs* in the model legume in the present study. The chromosomal location, gene duplication and coding protein properties of *MtWAK* genes were analyzed. To highlight the putative functions of the classical RLK type (*MtWAK*-RLKs), their phylogenetic relationship, gene structure and conserved domains were characterized. Furthermore, their *cis*-acting elements in the promoter and expression profiles in response to diverse stresses were examined.

2. Results

2.1. Identification and Characterization of WAKs in *M. truncatula*

To comprehensively identify WAK genes in the genome of *M. truncatula*, the data harvested from BLASTP and HMMER searches analysis were integrated. The putative candidates were further verified for the presence of the EGF_CA (PF07645), WAK_assoc (PF14380), GUB_WAK_bind (PF13947) and Pkinase_Tyr (PF07714) domain. A total of 54 non-redundant and full-length *MtWAKs* were obtained (Table S1). According to the classification of *OsWAKs* [18], *MtWAKs* were divided into 4 groups that contained 26 *MtWAK*-RLKs, 9 *MtWAK*-RLCKs, 10 *MtWAK*-RLPs and 9 *MtWAK* short proteins (Table S1). The numbers of amino acid (aa) ranged from 52 (*MtWAK32*) to 771 (*MtWAK29*), while the molecular weight (MW) varied from 5.55 kDa (*MtWAK32*) to 85.6 kDa (*MtWAK29*). The isoelectric point (pI) of *MtWAKs* ranged from 4.47 (*MtWAK54*) to 10.17 (*MtWAK32*), with an average of 6.94 (Table S1).

2.2. Chromosomal Location and Expansion Analysis of *MtWAKs*

Fifty-four *MtWAKs* were unevenly distributed on eight chromosomes (Figure 1). Chromosome 1 had 17 *MtWAKs*, while the other chromosomes had 2 to 9 *MtWAKs*. Interestingly, 20 *MtWAK*-RLKs and 7 *MtWAK*-RLCKs were found in clusters with 10 pairs of tandem duplicated gene pairs, while most *MtWAK*-RLPs and *MtWAK* short proteins were separately distributed on 8 chromosomes (Figure 1, Table S2).

Two pairs of paralogous genes, *MtWAK19*-*MtWAK35* and *MtWAK36*-*MtWAK53*, belong to segmental duplications (Table S2). The synteny analysis showed that there were 11 pairs of orthologous genes between *M. truncatula* and *A. thaliana* and 31 pairs between *M. truncatula* and *G. max* (Figure 2, Table S2).

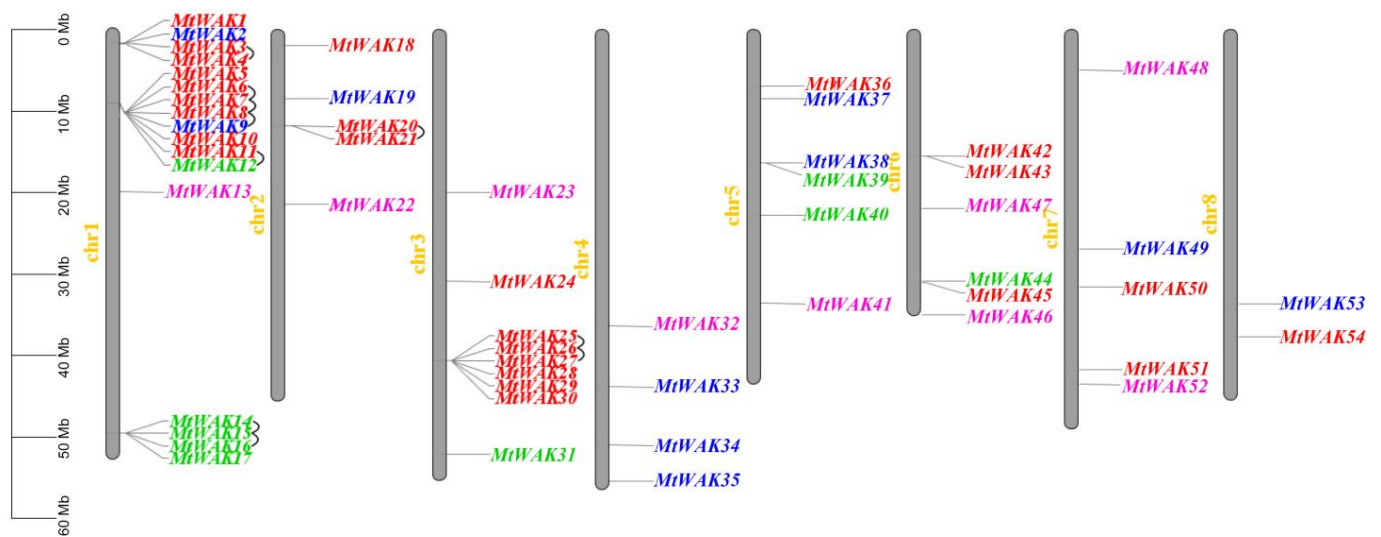


Figure 1. Chromosomal location of *MtWAKs*. Grey bars represent the chromosomes. The chromosome numbers are shown in yellow. The scale bar is shown on the left. WAK gene names are highlighted with different colors. *MtWAK-RLKs*, *MtWAK-RLCKs*, *MtWAK-RLPs* and *MtWAK* short proteins are indicated using red, green, blue, and pink, respectively. Tandem duplicated gene pairs are linked using black lines.

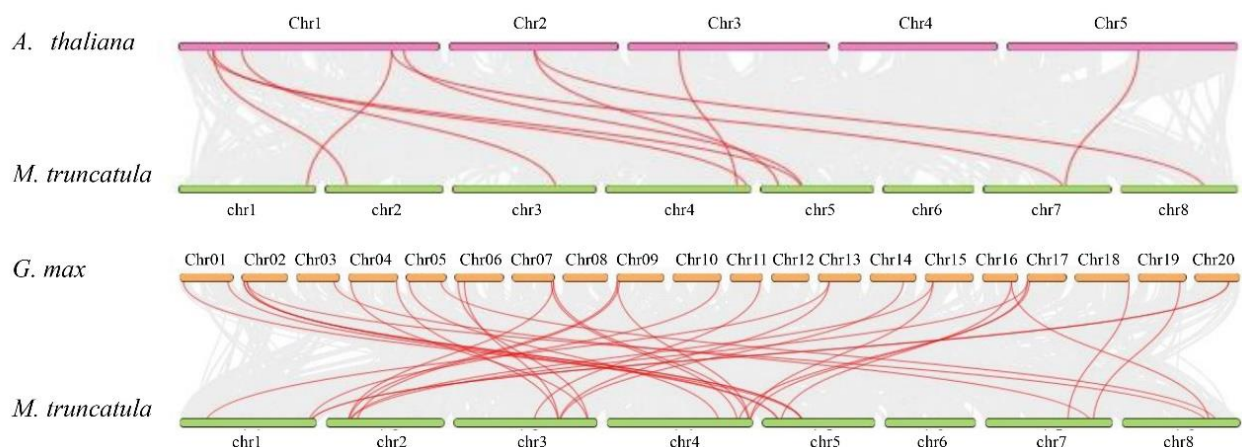


Figure 2. The synteny analysis of *MtWAKs* between *M. truncatula* and *A. thaliana* or *G. max*. Gray lines indicate all collinear blocks within *M. truncatula* and *A. thaliana* or *G. max*, while red lines indicate the synteny of WAK genes between *M. truncatula* and *A. thaliana* or *G. max*.

2.3. Phylogeny, Gene Structure, Protein Domain and Motif Analysis of *MtWAK-RLK* Members

Phylogenetic analysis of WAK-RLKs from *M. truncatula*, *A. thaliana*, *G. max* and *O. sativa* showed that the WAK-RLKs could be divided into five clades. Clade I and V contained only *OsWAK-RLKs*. Clade II contained 5 *AtWAKs*, 14 *GmWAK-RLKs*, 16 *MtWAK-RLKs* and 2 *OsWAK-RLKs*. Clade III contained 14 *AtWAKL-RLKs*, 6 *GmWAK-RLKs* and 2 *MtWAK-RLKs*. Clade IV was comprised of 2 *AtWAKL-RLKs*, 35 *GmWAK-RLKs*, 4 *OsWAK-RLKs* and 8 *MtWAK-RLKs* (Figure 3).

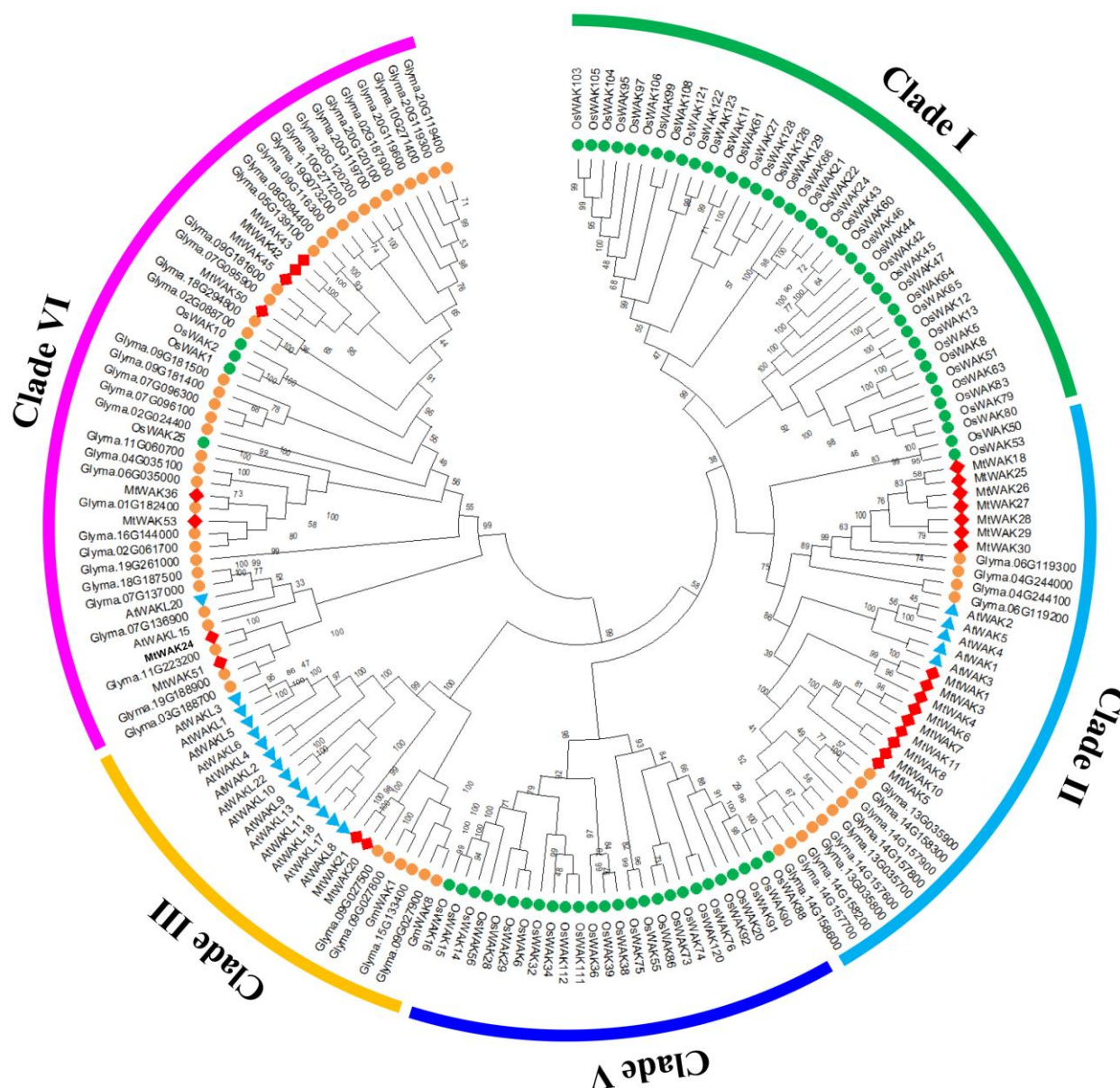


Figure 3. Phylogenetic analysis of WAK-RLKs from *M. truncatula*, *A. thaliana*, *G. max* and *O. sativa*: 26 MtWAK-RLKs (red square), 21 AtWAK-RLKs (blue triangle), 56 GmWAK-RLKs (orange circle) and 71 OsWAK-RLKs (green circle) were aligned using the CLUSTALX software (Version 1.81). The phylogenetic tree was constructed with MEGAX software (version 10.0.5) by the neighbor-joining method with 1000 bootstrap replicates.

The gene structure, protein domain and motif of MtWAK-RLKs were further analyzed (Figures 4 and S1). Most members showed similar gene structure, harboring two long exons at both ends and a short one in the middle, except *MtWAK3* and *MtWAK51* with two exons only and *MtWAK4*, 24, 42, 43 and 50 with four exons (Figure 4B). One or two transmembrane domains were shown in the majority of MtWAK-RLKs except *MtWAK3* and *MtWAK45*. Both Gub_WAK_bind and EGF domains existed in all members of clade II and *MtWAK36*, and there were 51 in clade IV (Figure 4C). Moreover, 10 conserved motifs were predicted using MEME program. Motifs 1 to 7 corresponded to the conserved kinase domain in all MtWAK-RLKs (Figures 4D and S1). A conserved arginine (R) residue was present in front of the motif DxxxxN; thus, all MtWAK-RLKs were classified as RD kinase. In addition, motif 8 was absent in the members of clade IV.

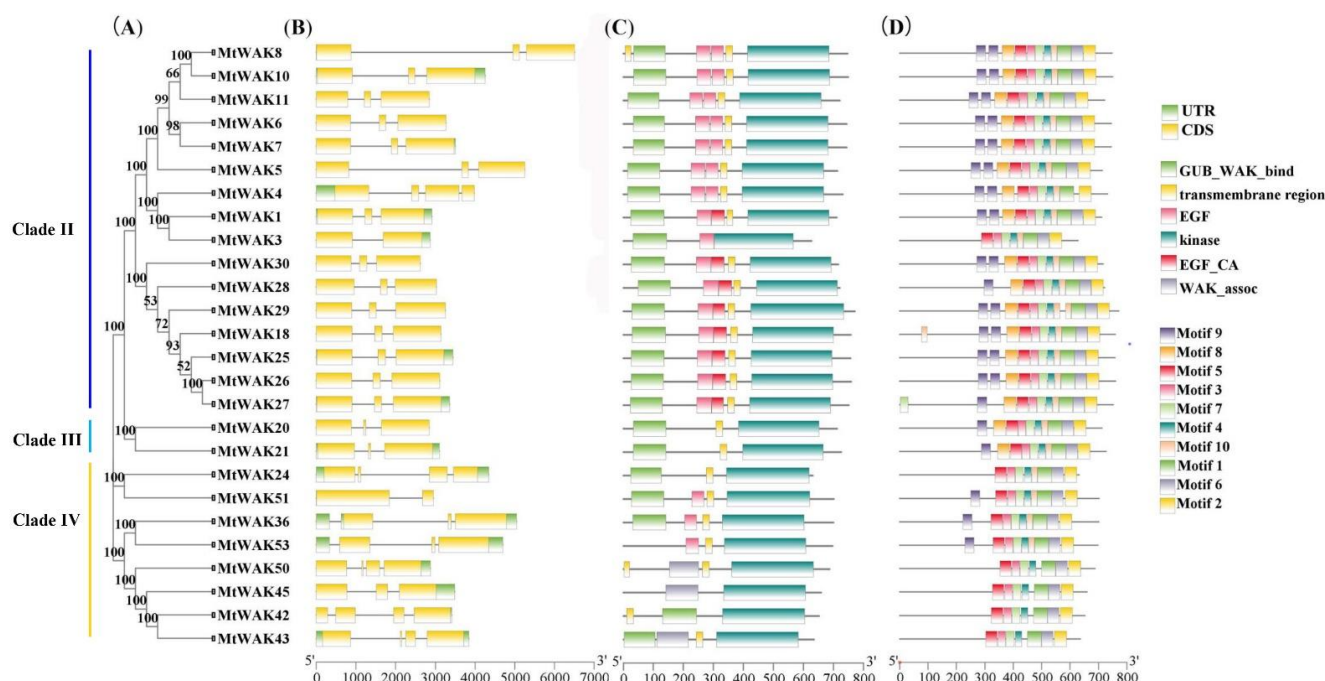


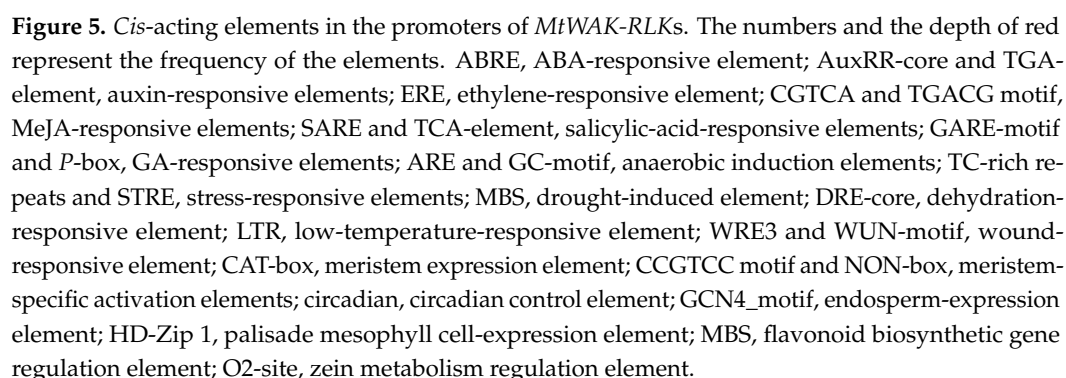
Figure 4. The gene structure, conserved domain and motif analysis of MtWAK-RLKs. (A) The phylogenetic tree of MtWAK-RLKs. (B) The gene structure of MtWAK-RLKs. (C) The conserved domain of MtWAK-RLKs. (D) The top ten conserved motifs of MtWAK-RLKs.

2.4. Analysis of Cis-Acting Elements in the Promoter Region of MtWAK-RLKs

To understand the potential function of MtWAK-RLKs, the putative *cis*-acting elements were analyzed using PlantCARE software. Abundant phytohormone and stress responsive elements were observed in the promoter regions (Figure 5). Twenty-four MtWAK-RLKs possessed a larger number of ABA-responsive elements, ethylene-responsive elements (ERE) and MeJA responsive elements (CGTCA-motif, TGACG-motif). Twelve MtWAK-RLKs had salicylic-acid-responsive elements (TCA-element), while nine MtWAK-RLKs had auxin-responsive elements (TGA-element). Most MtWAK-RLKs had anaerobic induction elements (ARE) and stress-responsive elements (STRE) in the promoter region (Figure 5). A series of growth and development related *cis*-elements, such as meristem-expression element (CAT-box) and CCGTCC motif were observed in MtWAK-RLKs. The results suggested that MtWAK-RLKs might be involved in phytohormone regulation and stress response in *M. truncatula*.

2.5. Expression Analysis of MtWAK-RLKs across Different Tissues and Developmental Stages

To obtain insights into their temporal and spatial expression patterns, 12 MtWAK-RLKs having corresponding probesets in the gene expression database were prioritized for analysis (Table S3). MtWAK1, 4, 7, 8 and 18 were almost equally expressed in all tissues (Figure 6A). MtWAK10, 24 and 50 were exclusively and highly expressed in roots. MtWAK36 and 45 were highly expressed in roots, whereas MtWAK3 was highly expressed in stem. From base to apex of stem, MtWAK24 and MtWAK53 expression was increased gradually, while MtWAK3 exhibited the opposite trend with the lowest expression level in the top internode (Figure 6B). Interestingly, MtWAK24 and 53 were significantly up-regulated along with seed development (Figure 6C). In addition, most MtWAK-RLKs were down-regulated during nodulation, except that MtWAK1 was up-regulated greatly at 6 dpi and 20 dpi (Figure 6D).



To understand the expression of *MtWAK-RLKs* in response to pathogens, datasets of ‘Cell suspension_Yeast elicitor’, ‘Root_ *Macrophomina* infected’ and ‘Root Tip_A17_ *Ralstonia*’ from the MtGEA were used for analysis (Table S3). Transcripts of nine *MtWAK-RLKs* were induced greatly after 2 h of treatment with yeast elicitor (Figure 7A). Although most *MtWAK-RLKs* were not responsive to infection by *Macrophomina* or *Ralstonia*, *MtWAK7* transcript was induced greatly in roots at 36 and 48 hpi with *Macrophomina* (Figure 7B), and *MtWAK3*, 50 and 53 transcripts were up-regulated at 72 hpi with *Ralstonia* (Figure 7C). In addition, seven *MtWAK-RLKs* were up-regulated by infection with *Erysiphe pisi* (Figure 7D).

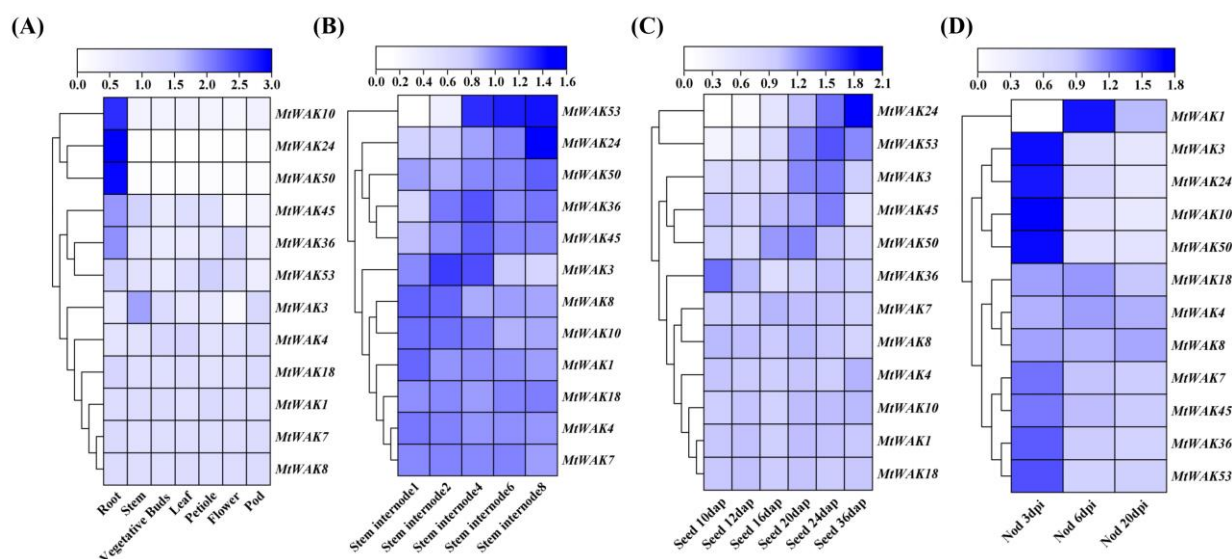


Figure 6. Clustered heatmap of the relative expression levels of *MtWAK*-RLKs in different tissues and developmental stages. (A) Different organs. (B) Developing seeds. (C) Developing internodes. (D) Developing nodules. Microarray data are normalized by the mean expression value of each gene in tissues. The clustered heatmap on log2 scale is drawn using TBtools software (Version 1.108). DAP, days after pollination. DPI, days post-incubation.

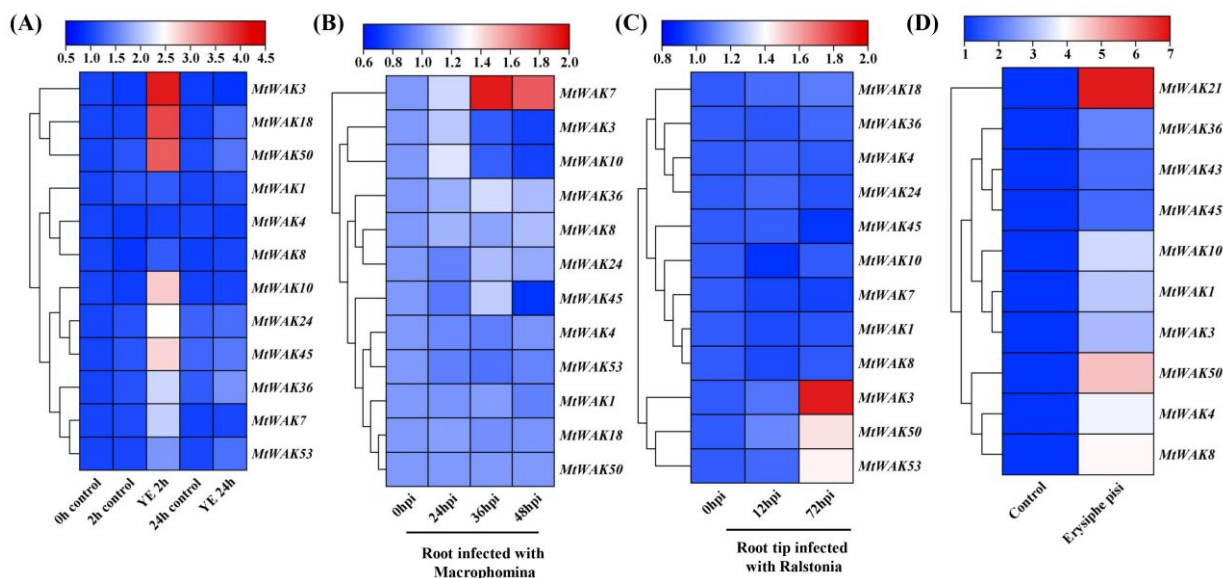


Figure 7. Clustered heatmap of the relative expression levels of *MtWAK*-RLKs involved in biotic stress response. (A) Yeast elicitor (YE) treatment. (B) *Macrophomina* infection. (C) *Ralstonia* infection. (D) *Erysiphe pisi* infection. The relative gene expression levels after treatments were calculated and compared with the untreated control or before treatment. The clustered heatmap on log2 scale was drawn using TBtools software (Version 1.108).

MtWAK-RLKs were responsive to abiotic stress (Figure 8, Table S3). *MtWAK*8, 21 and 43 were up-regulated at 2 h after drought stress, while *MtWAK*27 was down-regulated. *MtWAK*2, 5, 45 and 50 were continuously up-regulated after 2 h of drought stress, and *MtWAK*24 was not responsive to drought (Figure 8A). *MtWAK*21, 24, 43 and 53 were down-regulated in response to cold stress, while *MtWAK*10 was not responsive to cold. *MtWAK*3, 4, 8, 27, 36, 45 and 50 were up-regulated after cold treatment (Figure 8B). *MtWAK*8, 21

and 27 were up-regulated after 12 h of salt treatment, while *MtWAK3*, 10, 43 and 54 were down-regulated (Figure 8C).

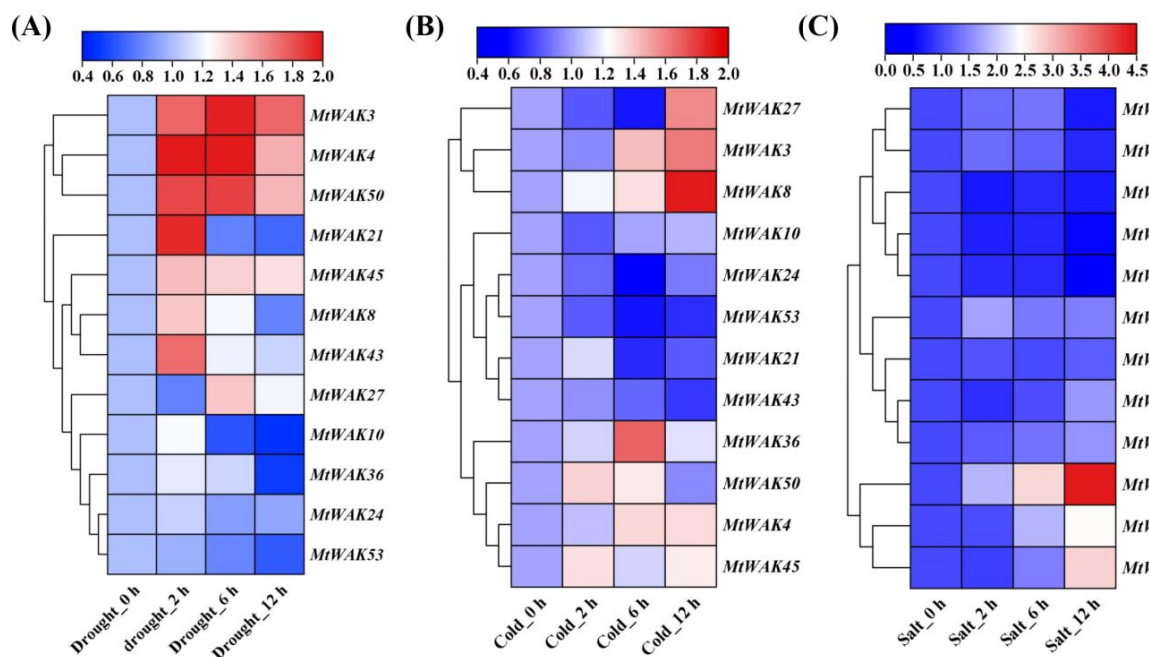


Figure 8. Clustered heatmap of the relative expression levels of *MtWAK*-RLKs involved in abiotic stress response. (A) Drought stress. (B) Cold stress (C) Salt stress. The relative gene expression levels after treatments were calculated and compared with the control untreated or before treatment. The clustered heatmap on log2 scale was drawn using TBtools software (Version 1.108).

2.7. Gene Expression Validation of *MtWAK*-RLKs by qRT-PCR

To verify the expression profiles obtained from microarray data, transcripts of five *MtWAK*-RLKs (*MtWAK1*, 3, 10, 24, and 53) in different tissues, including root, stem, leaf, flower, pod and seed, were detected using qRT-PCR. The data showed the expression patterns in different tissues were in consistence with the microarray data. For example, *MtWAK10*, 24 and 53 were highly expressed in roots (Figure 9A). The response of transcript levels in five *MtWAK*-RLKs (*MtWAK3*, 8, 10, 21, and 24) were also detected using qRT-PCR. *MtWAK3*, 8 and 21 were up-regulated after cold treatment, while *MtWAK10* was down-regulated (Figure 9B), which was consistent with the microarray data.

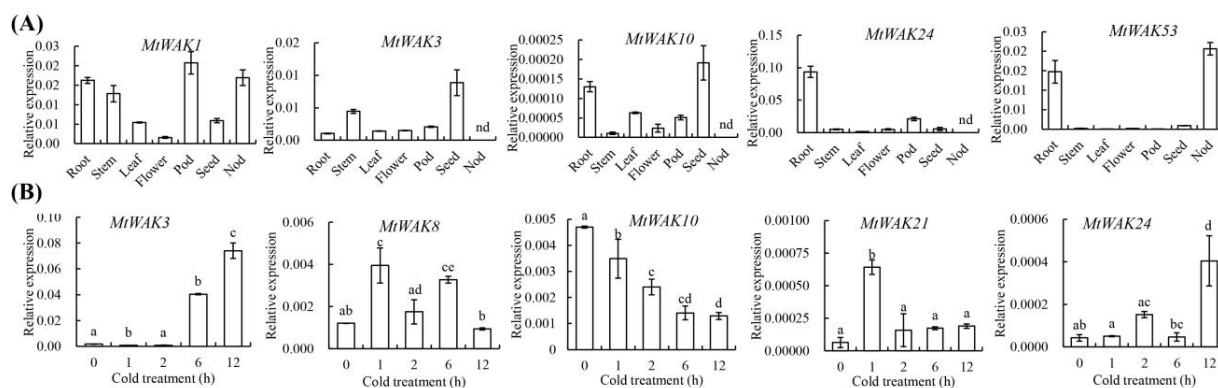


Figure 9. Expression analysis of selected *MtWAK*-RLK genes in different tissues and under cold stress using qRT-PCR. (A) Different tissues. (B) Cold stress. The error bars were obtained from three measurements. nd indicates not detected. Significant differences are indicated as different lowercase letters ($P \leq 0.05$, by one-way ANOVA).

2.8. Plasma Membrane Localized MtWAK24 Inhibited Phytophthora Infection in Tobacco

To explore the functions of some candidate MtWAKs in modulating plant immunity, we performed *P. parasitica* infection experiments in *N. benthamiana*. The lesion diameter, as well as relative biomass of *P. parasitica*, in leaves expressing MtWAK24, but not MtWAK36, was significantly decreased compared to the control leaves expressing empty vector, indicating that MtWAK24 could inhibit the pathogen infection by *P. parasitica* (Figure 10A,B).

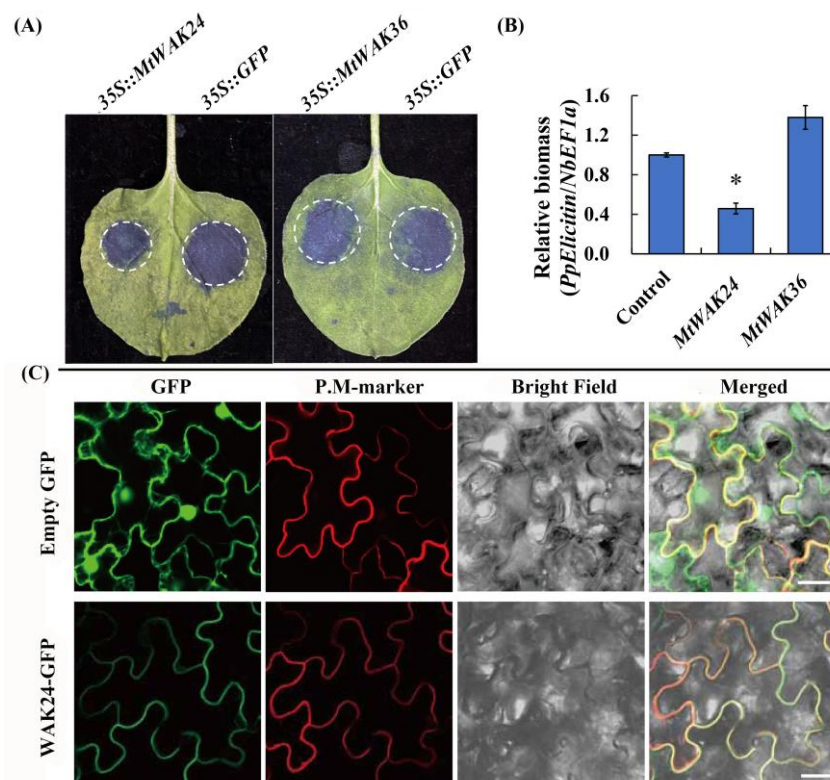


Figure 10. Plasma membrane localized MtWAK24 inhibited Phytophthora infection in tobacco. *N. benthamiana* leaves individually expressing 35S::MtWAK36, 35S::MtWAK24 or 35S::GFP were inoculated with *P. parasitica* mycelial plugs at 24 h after *Agrobacterium* infiltration. At 48 hpi, infected leaves were stained using Evans blue staining for lesion determination (A). Relative biomass of *P. parasitica* was determined by qPCR of *P. parasitica* genome DNA normalized to tobacco genome DNA (B). The results are indicated with means $\pm$ SE, $n = 6$. Asterisks indicate significant differences (* $p < 0.05$; Student's t test). The subcellular localization of MtWAK24-GFP proteins and a free GFP protein in tobacco (C). P. M-marker: a plasma membrane localization protein AtAKT1. Bars = 20 μ m.

Subcellular localization of MtWAK24 was further analyzed. The data showed that GFP protein was located in the cytoplasm and nucleus, while MtWAK24 protein was located in plasma membrane because the fluorescence was overlapped with that of AtAKT1, the plasma membrane marker protein (Figure 10C).

3. Discussion

The WAK gene family consists of a large number of members. The numbers of WAK proteins identified by the iTAK (<http://bioinfo.bti.cornell.edu/tool/itak>) program [19] enlarged from moss to dicot and monocot species, indicating a large expansion of WAK family genes during the evolutionary process (Table S4). There are 26, 29, 29, 96, 91, 125, 175, 27 and 320 WAK genes in the genomes of *Arabidopsis* [20], cotton [21], tomato [22], Chinese cabbage [23], barley [24], rice [25], *Populus* [26], *Juglans regia* [27] and *Triticum aestivum* [28], respectively. Through BLASTP and conserved domain searches, 54 MtWAK genes were identified in *M. truncatula*, including 26 MtWAK-RLK, 9 MtWAK-RLCK, 10 MtWAK-RLP

and 9 short protein types (Table S1). The majority of *MtWAK-RLK* and *MtWAK-RLCK* genes were clustered in chromosome 1 and 3, attributed mainly to tandem duplications, and the rest were distributed separately among chromosomes (Figure 1, Table S2). Compared to 11 pairs of orthologous WAKs between *Arabidopsis* and *M. truncatula*, 31 pairs between *M. truncatula* and *G. max* were found, indicating that the expansion of *MtWAK* family might appear before the separation of *G. max* (Figure 2). Phylogenetic analysis showed that *MtWAK25* and *MtWAK26* were close to *AtWAKs* (Figure 3), and *AtWAK4-MtWAK25* and *MtWAK25-MtWAK26* were orthologous and paralogous pairs, respectively (Table S2), indicating that *MtWAK25* and *MtWAK26* were probably involved in cell expansion like *AtWAK4* [3].

Multiple *cis*-acting elements responsive to phytohormones, stresses, growth and development were observed in the promoter regions of *MtWAK-RLKs*, indicating their potential roles in these processes. The *GhWAKs/WAKLs* having the above *cis*-elements in the promoter were responsive to multiple phytohormones and abiotic stresses [12]. Transcript levels of five *TaWAKs* in wheat (*Triticum aestivum*) were altered by treatments with GA, BR, IAA, JA and ABA [29]. Gene function is associated with its tissue specific expression pattern. *MtWAK10*, 24 and 50 showed a root-specific expression with extremely low expression in other organs. *MtWAK36* and *MtWAK45* were also mainly expressed in roots, whereas *MtWAK3* and *MtWAK53* were highly expressed in stem and petiole, respectively. *MtWAK1*, 4, 7, 8 and 18 were evenly expressed in each organ, but the transcript levels were lower than that of other genes (Figure 6A, Table S3). Diverse tissue expression patterns of *MtWAK-RLKs* implied that they might function broadly in plant tissues.

Plant cell expansion and elongation depend on turgor maintenance and cell wall modification, which is associated with the rigidity and elasticity of the cell wall [30]. WAK genes can monitor pectin and participate in both turgor maintenance and cell wall modification [31]. *AtWAK4* and *AtWAK2* positively regulate cell expansion [3,4], *HvWAK1* positively regulates root growth [32], while *OsWAK11* regulates both stem and seed elongation [5]. Transcript levels of *MtWAK24* and *MtWAK53* were increased gradually from bottom to top internodes and along with seed growth, while expressions of *MtWAK3* and *MtWAK45* increased, followed by a decrease from internode 1 to internode 8 and along with seed development (Figure 6B,C, Table S3), indicating that they are probably involved in the regulation of cell elongation.

Cell wall modification is involved in symbiosis between rhizobia and legume plants. Passing of an infection thread from cell to cell requires local cell wall degradation [33]. Modifications in the localization of high- and low-methylated homogalacturonans were detected in nodules [34]. The majority of *MtWAK-RLKs* were greatly down-regulated in the process of nodulation, while *MtWAK1* transcript was increased dramatically, implying that *MtWAKs* are probably associated with nodulation in leguminous plants (Figure 6D, Table S3).

A large body of investigations revealed that WAK genes regulate pathogen resistance. *AtWAK1* [7], *AtWAKL22* [35], *Htn1* (ZmWAK) [8,9], *Stb6* [10], *Xa4* [11], *OsWAK1* [36], *OsWAK90-92* [37], *OsWAK25* [38], *SiWAK1* [39], *TaWAK6* [40], *GhWAK7A* [41], *CsWAKL08* [42] and *Rlm4/7/9* [43] positively regulate resistance to various pathogens, and *OsWAK112d* and *Sbs1/2* negatively regulate the defense against pathogenic fungus [12,44]. *MtWAK3*, 18, 50, 10, 24, 45, 36, 7 and 53 transcripts were rapidly induced by yeast elicitor treatment (Figure 7A). Among them, *MtWAK7* was up-regulated by *Macrophomina* infection other than *Ralstonia*, and *MtWAK3*, 50 and 53 were up-regulated by *Ralstonia* other than *Macrophomina* infection (Figure 7B, C), indicating that these genes had specific roles in resistant to *Macrophomina* or *Ralstonia*. Powdery mildews caused by *Erysiphe pisi* are a serious disease that leads to great decreases in crop production worldwide [45]. Ten *MtWAK-RLK* genes were up-regulated greatly after incubation with *Erysiphe pisi*, indicating that they were probably involved in powdery mildew resistance (Figure 7D). To explore the functions of *MtWAK-RLKs* in plant immunity, we selected two yeast elicitor induced *MtWAKs*, *Mt-*

WAK24 and 36, for *P. parasitica* infection experiments in *N. benthamiana*. It was revealed that expressing *MtWAK24*, but not *MtWAK36*, inhibited infection by *P. parasitica* (Figure 10).

WAK genes are involved in metal, salt, drought and cold tolerance, although the mechanisms remain unknown. *OsWAK112* negatively regulates plant salt tolerance, possibly via direct binding with *OsSAMS1/2/3* [17]. The *Slwak1* null mutant exhibited disturbed osmotic homeostasis and source-sink balance under long term salinity and thereby reduced fruit yield [46]. The *CpGRP1-CpWAK1* complex regulates dehydration-induced morphological changes in *Craterostigma plantagineum* [47]. Twelve *MtWAK-RLK* genes were quickly responsive to drought, salt and cold (Figure 8). Ten *MtWAK-RLKs* transcripts were induced after 2 h of drought (Figure 8A). Seven and four *MtWAK-RLKs* were up-regulated or down-regulated by cold stress, respectively (Figure 8B), and three and four *MtWAK-RLKs* were up-regulated or down-regulated by salt stress, respectively (Figure 8C). The results indicate that *MtWAK* expression is probably involved in abiotic stress resistance.

4. Materials and Methods

4.1. Identification of *MtWAK* Genes

The genome sequences were obtained from *M. truncatula* genome database (<http://www.medicagogenome.org/>, Mt4.0v2). The gene information of WAK family in *Arabidopsis* and Rice were retrieved from the previous studies. The hidden Markov model (HMM) profiles of the WAKs were down-loaded from the Pfam database (<http://pfam.xfam.org/>, accessed on 1 August 2020). EGF_CA (PF07645), WAK_assoc (PF14380), GUB_WAK_bind (PF13947) and Pkinase_Tyr (PF07714) were used to identify *MtWAKs*. Firstly, BLASTP search was performed at *M. truncatula* genome database with an *e*-value of 1e-5 using previously reported sequences of *AtWAKs* and *OsWAKs* as query. Then, we searched EGF_CA (PF07645), WAK_assoc (PF14380), GUB_WAK_bind (PF13947) and Pkinase_Tyr (PF07714) domain from putative *MtWAKs* with *e*-value cut-off at 1.0 by using HMMER v3.1b2 software. The integrity of the four domains was verified by using the online program SMART (<http://smart.emblheidelberg.de/>, accessed on 1 August 2020) with an *e*-value < 0.1. Lastly, each candidate gene was assessed for its sequence similarity to other putative *MtWAKs*. Only genes that fit into one of the four *MtWAK* types (see Table S1) according to Zhang et al. [18] were defined as *MtWAKs*. Protein length, molecular weight (MW) and isoelectric point (PI) were predicted by ExPasy program (<http://www.expasy.org/tools/>, accessed on 10 August 2021).

4.2. Chromosomal Location and Synteny Correlation Analysis

The physical position of the *MtWAK* genes on the chromosome was mapped using TBtools software (Version 1.108). The synteny correlation analysis of WAK genes between the homologs in *M. truncatula* and *A. thaliana* or *G. max* were verified and visualized using TBtools software.

4.3. Phylogenetic Analysis and Gene Structure, Motif and Conserved Domain Analysis

Multiple alignments of protein sequences were performed using CLUSTALX software (Version 1.81). The phylogenetic tree was constructed by using MEGAX with the neighbor-joining method and 1000 bootstrap replications. The gene structure, conserved domain and conserved motifs were displayed using TBtools software. The conserved domains and conserved motifs of the 26 *MtWAK-RLKs* were analyzed by SMART program and MEME program (Version 5.4.1) (<http://meme-suite.org/tools/meme>, accessed on 20 July 2022).

4.4. Analysis of *cis*-Acting Regulatory Elements

The 2000 bp promoter sequences upstream from the initiation codon of *MtWAK-RLKs* were extracted from the genome of *M. truncatula* and analyzed using PlantCARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 1 August 2020). However, less than 300 bp promoter sequence of *MtWAK10* was available.

4.5. Expression Pattern Analysis

The genome-wide microarray data obtained from MtGEA (<https://mtgea.noble.org/v3/>, accessed on 1 August 2020) was used to analyze the expression of *MtWAK* genes in different tissues and developing stages. The expression data were gene-wise normalized. The clustered heatmap of expression pattern profile on log2 scale was portrayed using TBtools software. Analyses of WAK expression in response to biotic and abiotic stress were conducted on datasets: (i) ‘Cell suspension_Yeast elicitor’, ‘Root_Macrophomina infected’ and ‘Root Tip_A17_Ralstonia’ (microarray data obtained from MtGEA); (ii) powdery mildew *Erysiphe pisi* treatment [48] RNA-seq data were retrieved from NCBI Database (SRR7589436, SRR7589435, SRR7589438, and SRR7589437); (iii) drought, salt and cold treatment, data from NCBI GEO (dataset accession: GSE136739). The expression abundance was presented by the reads per kilobase per million (Table S3). The relative transcript level after treatments was calculated compared with the untreated control or before treatment (0 h). The clustered heatmap of relative expression pattern on log2 scale was analyzed by the TBtools.

4.6. Tissue Samples Collection and Cold and Salt Treatment

M. truncatula plants were grown in a growth chamber at 25 °C with 16 h of light. Root, stem, mature leave, flower, pod and seed were sampled from three-month-old plants. Six-week-old plants were exposed to low temperature (5 °C) as a cold stress treatment. Leaves were collected at time intervals of 0, 1, 2, 6 and 12 h.

4.7. RNA Extraction and qRT-PCR

Total RNA was extracted using the RNeasy pure Plant Kit (Qiagen, Beijing, China). cDNA synthesis was performed with HiScript III RT SuperMix for qPCR (+gDNA wiper) reagent kit with gDNA Eraser (Vazyme, Nanjing, China). qRT-PCR was performed following the instructions of ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The *MtActin7* (*Medtr3g095530*) gene was used as the controls. All primer sequences are shown in Supplementary Table S5. The relative gene expression level was calculated with $2^{-\Delta Ct}$ method.

4.8. Subcellular Localization

The full CDSs of *MtWAK24* without the terminator were cloned and ligated to pCambia 1305-GFP vector driven by *CaMV* 35S promoter. Primer sequences for constructions are shown in Supplementary Table S5. Positive *Agrobacterium* colonies were cultured in LB medium containing 50 µg/mL rifampicin and kanamycin at 28 °C overnight; the cells were harvested by centrifugation at 2500 × g at room temperature for 3–5 min and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES pH 5.6, and 100–200 µM acetosyringone). The cell density was adjusted to OD₆₀₀ = 0.2 before the cell suspensions were infiltrated into one-month-old *Nicotiana benthamiana* (*N. benthamiana*) leaves. After 48–72 h, fluorescence was observed using confocal laser scanning microscopy (Zeiss LSM800, Jena, Germany).

4.9. Phytophthora Infection in N. benthamiana

Vectors expressing selected *MtWAK*-RLKs under control of *CaMV* 35S promoter were constructed. The infection experiments were performed as described [49]. Briefly, the infiltrated leaves were inoculated with *P. parasitica* mycelium at 12 hpi, and leaf lesions were determined using the Evans blue method at 48 hpi. *P. parasitica* strain was obtained from Yuanchao Wang’s lab (Nanjing Agricultural University, Nanjing, China). Relative biomass of *P. parasitica* was determined by qPCR of *P. parasitica* genome DNA normalized to tobacco genome DNA at 48 hpi. All primer sequences are shown in Supplementary Table S5.

5. Conclusions

In summary, a comprehensive genome-wide analysis of WAK family was performed. A total of 54 *MtWAK*s were identified in *M. truncatula*, including 26 *MtWAK*-RLK, 9 *MtWAK*-RLCK, 10 *MtWAK*-RLP and 9 short protein type genes. *MtRLKs* and *MtRLCKs* were largely

tandem duplicated. Most *MtWAK-RLKs* had similar exon–intron organization and motif distribution. Multiple *cis*-acting elements responsible for phytohormones, stresses, growth and development were observed in the promoter regions. The expression patterns of *MtWAK-RLKs* varied in different plant tissues and developmental stages and biotic and abiotic stress conditions. The results suggest that *MtWAKs* might have multiple functions in *M. truncatula*. Furthermore, plasma-membrane-localized *MtWAK24* significantly inhibited *Phytophthora* infection in tobacco, indicating its role in pathogen resistance, which is worthy to be investigated in the future.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants12091849/s1>, Figure S1: The motif of *MtWAK-RLKs*; Table S1: The predicated information of *MtWAK* family genes in *M. truncatula*. Table S2: The synteny analysis of *WAK* homologs between *M. truncatula* and *A. thaliana* or *G. max*; Table S3: The gene expression profiles of *MtWAK-RLKs* in different tissues and developmental stages as well as biotic and abiotic stress; Table S4: The number of plant protein kinases and *WAKs* predicted via iTAK program; Table S5: Primers.

Author Contributions: Z.G., H.Z. and W.K. designed and wrote the article; H.Z., W.K., J.S., S.Y. and P.Z. collected data and performed most of the data analysis. B.Y. provided instructions in the *Phytophthora* infection experiments. All authors approved the submitted version. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (Grant numbers 32101439) and the Natural Science Foundation of Jiangsu Province (BK20190527).

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gish, L.A.; Clark, S.E. The RLK/Pelle family of kinases. *Plant J.* **2011**, *66*, 117–127. [CrossRef] [PubMed]
2. He, Z.H.; Cheeseman, I.; He, D.; Kohorn, B.D. A cluster of five cell wall-associated receptor kinase genes, *Wak1-5*, are expressed in specific organs of *Arabidopsis*. *Plant Mol. Biol.* **1999**, *39*, 1189–1196. [CrossRef] [PubMed]
3. Lally, D.; Ingmire, P.; Tong, H.Y.; He, Z.H. Antisense expression of a cell wall-associated protein kinase, *WAK4*, inhibits cell elongation and alters morphology. *Plant Cell* **2001**, *13*, 1317–1331. [CrossRef] [PubMed]
4. Kohorn, B.D.; Kobayashi, M.; Johansen, S.; Riese, J.; Huang, L.F.; Koch, K.; Fu, S.; Dotson, A.; Byers, N. An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* **2006**, *46*, 307–316. [CrossRef]
5. Yue, Z.L.; Liu, N.; Deng, Z.P.; Zhang, Y.; Wu, Z.M.; Zhao, J.L.; Sun, Y.; Wang, Z.Y.; Zhang, S.W. The receptor kinase OsWAK11 monitors cell wall pectin changes to fine-tune brassinosteroid signaling and regulate cell elongation in rice. *Curr. Biol.* **2022**, *32*, 2454–2466.e7. [CrossRef]
6. Yin, W.; Yang, H.; Wang, Y.; Feng, P.; Deng, Y.; Zhang, L.; He, G.; Wang, N. *Oryza sativa* PECTIN DEFECTIVE TAPETUM1 affects anther development through a pectin-mediated signaling pathway in rice. *Plant Physiol.* **2022**, *189*, 1570–1586. [CrossRef]
7. He, Z.H.; He, D.Z.; Kohorn, B.D. Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. *Plant J.* **1998**, *14*, 55–63. [CrossRef]
8. Hurni, S.; Scheuermann, D.; Krattinger, S.G.; Kessel, B.; Wicker, T.; Herren, G.; Fitze, M.N.; Breen, J.; Prestler, T.; Ouzunova, M.; et al. The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 8780–8785. [CrossRef] [PubMed]
9. Zuo, W.L.; Chao, Q.; Zhang, N.; Ye, J.R.; Tan, G.Q.; Li, B.L.; Xing, Y.X.; Zhang, B.Q.; Liu, H.J.; Fengler, K.A.; et al. A maize wall-associated kinase confers quantitative resistance to head smut. *Nat. Genet.* **2015**, *47*, 151–157. [CrossRef]
10. Saintenac, C.; Lee, W.S.; Cambon, F.; Rudd, J.J.; King, R.C.; Marande, W.; Powers, S.J.; Berges, H.; Phillips, A.L.; Uauy, C.; et al. Wheat receptor-kinase-like protein Stb6 controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nat. Genet.* **2018**, *50*, 368–374. [CrossRef]
11. Hu, K.; Cao, J.; Zhang, J.; Xia, F.; Ke, Y.; Zhang, H.; Xie, W.; Liu, H.; Cui, Y.; Cao, Y.; et al. Improvement of multiple agronomic traits by a disease resistance gene via cell wall reinforcement. *Nat. Plants* **2017**, *3*, 17009. [CrossRef] [PubMed]
12. Delteil, A.; Gobbato, E.; Cayrol, B.; Estevan, J.; Michel-Romiti, C.; Dievart, A.; Kroj, T.; Morel, J.B. Several wall-associated kinases participate positively and negatively in basal defense against rice blast fungus. *Bmc Plant Biol.* **2016**, *16*, 17. [CrossRef] [PubMed]
13. Denoux, C.; Galletti, R.; Mammarella, N.; Gopalan, S.; Werck, D.; De Lorenzo, G.; Ferrari, S.; Ausubel, F.M.; Dewdney, J. Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Mol. Plant.* **2008**, *1*, 423–445. [CrossRef]

14. Sivaguru, M.; Ezaki, B.; He, Z.H.; Tong, H.; Osawa, H.; Baluska, F.; Volkmann, D.; Matsumoto, H. Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in *Arabidopsis*. *Plant Physiol.* **2003**, *132*, 2256–2266. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Hou, X.; Tong, H.; Selby, J.; Dewitt, J.; Peng, X.; He, Z.H. Involvement of a cell wall-associated kinase, WAKL4, in *Arabidopsis* mineral responses. *Plant Physiol.* **2005**, *139*, 1704–1716. [\[CrossRef\]](#)
16. Zhang, H.; Lian, C.; Shen, Z. Proteomic identification of small, copper-responsive proteins in germinating embryos of *Oryza sativa*. *Ann. Bot.* **2009**, *103*, 923–930. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Lin, W.; Wang, Y.H.; Liu, X.Y.; Shang, J.X.; Zhao, L.Q. OsWAK112, a wall-associated kinase, negatively regulates salt stress responses by inhibiting ethylene production. *Front. Plant Sci.* **2021**, *12*, 751965. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Zhang, S.; Chen, C.; Li, L.; Meng, L.; Singh, J.; Jiang, N.; Deng, X.W.; He, Z.H.; Lemaux, P.G. Evolutionary expansion, gene structure, and expression of the rice wall-associated kinase gene family. *Plant Physiol.* **2005**, *139*, 1107–1124. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Zheng, Y.; Jiao, C.; Sun, H.H.; Rosli, H.G.; Pombo, M.A.; Zhang, P.F.; Banf, M.; Dai, X.B.; Martin, G.B.; Giovannoni, J.J.; et al. iTAK: A program for genome-wide prediction and classification of plant transcription factors, transcriptional regulators, and protein kinases. *Mol. Plant* **2016**, *9*, 1667–1670. [\[CrossRef\]](#)
20. Verica, J.A.; Chae, L.; Tong, H.Y.; Ingmire, P.; He, Z.H. Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in *Arabidopsis*. *Plant Physiol.* **2003**, *133*, 1732–1746. [\[CrossRef\]](#)
21. Dou, L.; Li, Z.; Shen, Q.; Shi, H.; Li, H.; Wang, W.; Zou, C.; Shang, H.; Li, H.; Xiao, G. Genome-wide characterization of the WAK gene family and expression analysis under plant hormone treatment in cotton. *BMC Genom.* **2021**, *22*, 85. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Sun, Z.Y.; Song, Y.P.; Chen, D.; Zang, Y.D.; Zhang, Q.L.; Yi, Y.T.; Qu, G.Q. Genome-wide identification, classification, characterization, and expression analysis of the wall-associated kinase family during fruit development and under wound stress in tomato (*Solanum lycopersicum* L.). *Genes* **2020**, *11*, 1186. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Zhang, B.; Li, P.; Su, T.B.; Li, P.R.; Xin, X.Y.; Wang, W.H.; Zhao, X.Y.; Yu, Y.J.; Zhang, D.S.; Yu, S.C.; et al. Comprehensive analysis of wall-associated kinase genes and their expression under abiotic and biotic stress in chinese cabbage (*Brassica rapa* ssp. *pekinensis*). *J. Plant Growth Regul.* **2020**, *39*, 72–86. [\[CrossRef\]](#)
24. Tripathi, R.K.; Aguirre, J.A.; Singh, J. Genome-wide analysis of wall associated kinase (WAK) gene family in barley. *Genomics* **2021**, *113*, 523–530. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Hu, W.; Lv, Y.Y.; Lei, W.R.; Li, X.; Chen, Y.H.; Zheng, L.Q.; Xia, Y.; Shen, Z.G. Cloning and characterization of the *Oryza sativa* wall-associated kinase gene *OsWAK11* and its transcriptional response to abiotic stresses. *Plant Soil* **2014**, *384*, 335–346. [\[CrossRef\]](#)
26. Tocquard, K.; Lafon-Placette, C.; Auguin, D.; Muries, B.; Bronner, G.; Lopez, D.; Fumanal, B.; Franchel, J.; Bourgerie, S.; Maury, S.; et al. *In silico* study of wall-associated kinase family reveals large-scale genomic expansion potentially connected with functional diversification in *Populus*. *Tree Genet. Genom.* **2014**, *10*, 1135–1147. [\[CrossRef\]](#)
27. Li, M.; Ma, J.; Liu, H.; Ou, M.; Ye, H.; Zhao, P. Identification and characterization of wall-associated kinase (WAK) and WAK-like (WAKL) gene family in *Juglans regia* and its wild related species *Juglans mandshurica*. *Genes* **2022**, *13*, 134. [\[CrossRef\]](#)
28. Xia, X.; Zhang, X.; Zhang, Y.; Wang, L.; An, Q.; Tu, Q.; Wu, L.; Jiang, P.; Zhang, P.; Yu, L.; et al. Characterization of the WAK gene family reveals genes for FHB resistance in bread wheat (*Triticum aestivum* L.). *Int. J. Mol. Sci.* **2022**, *23*, 7157. [\[CrossRef\]](#)
29. Liu, Y.; Liu, D.C.; Zhang, H.Y.; Gao, H.B.; Guo, X.L.; Fu, X.D.; Zhang, A.M. Isolation and characterisation of six putative wheat cell wall-associated kinases. *Funct. Plant Biol.* **2006**, *33*, 811–821. [\[CrossRef\]](#)
30. Cosgrove, D.J. Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 850–861. [\[CrossRef\]](#)
31. Cosgrove, D.J. Plant cell walls: Wall-associated kinases and cell expansion. *Curr. Biol.* **2001**, *11*, R558–R559. [\[CrossRef\]](#)
32. Kaur, R.; Singh, K.; Singh, J. A root-specific wall-associated kinase gene, *HvWAK1*, regulates root growth and is highly divergent in barley and other cereals. *Funct. Integr. Genom.* **2013**, *13*, 167–177. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Van Spronsen, P.C.; Bakhuizen, R.; van Brussel, A.A.; Kijne, J.W. Cell wall degradation during infection thread formation by the root nodule bacterium *Rhizobium leguminosarum* is a two-step process. *Eur. J. Cell Biol.* **1994**, *64*, 88–94. [\[PubMed\]](#)
34. Wilmowicz, E.; Kucko, A.; Alche, J.D.; Czeszewska-Rosiak, G.; Florkiewicz, A.B.; Kapusta, M.; Karwaszewski, J. Remodeling of cell wall components in root nodules and flower abscission zone under drought in yellow lupine. *Int. J. Mol. Sci.* **2022**, *23*, 1680. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Diener, A.C.; Ausubel, F.M. RESISTANCE TO FUSARIUM OXYSPORUM 1, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics* **2005**, *171*, 305–321. [\[CrossRef\]](#)
36. Li, H.; Zhou, S.Y.; Zhao, W.S.; Su, S.C.; Peng, Y.L. A novel wall-associated receptor-like protein kinase gene, *OsWAK1*, plays important roles in rice blast disease resistance. *Plant Mol. Biol.* **2009**, *69*, 337–346. [\[CrossRef\]](#)
37. Cayrol, B.; Delteil, A.; Gobbato, E.; Kroj, T.; Morel, J.B. Three wall-associated kinases required for rice basal immunity form protein complexes in the plasma membrane. *Plant Signal. Behav.* **2016**, *11*, e1149676. [\[CrossRef\]](#)
38. Harkenrider, M.; Sharma, R.; De Vleeschauwer, D.; Tsao, L.; Zhang, X.T.; Chern, M.; Canlas, P.; Zuo, S.M.; Ronald, P.C. Overexpression of rice wall-associated kinase 25 (*OsWAK25*) alters resistance to bacterial and fungal pathogens. *PLoS ONE* **2016**, *11*, e0147310. [\[CrossRef\]](#)
39. Rosli, H.G.; Zheng, Y.; Pombo, M.A.; Zhong, S.; Bombarely, A.; Fei, Z.; Collmer, A.; Martin, G.B. Transcriptomics-based screen for genes induced by flagellin and repressed by pathogen effectors identifies a cell wall-associated kinase involved in plant immunity. *Genom. Biol.* **2013**, *14*, R139. [\[CrossRef\]](#)

40. Dmochowska-Boguta, M.; Kloc, Y.; Zielezinski, A.; Werecki, P.; Nadolska-Orczyk, A.; Karlowski, W.M.; Orczyk, W. *TaWAK6* encoding wall-associated kinase is involved in wheat resistance to leaf rust similar to adult plant resistance. *PLoS ONE* **2020**, *15*, e0227713. [[CrossRef](#)]
41. Wang, P.; Zhou, L.; Jamieson, P.; Zhang, L.; Zhao, Z.X.; Babilonia, K.; Shao, W.Y.; Wu, L.Z.; Mustafa, R.; Amin, I.; et al. The Cotton Wall-associated kinase GhWAK7A mediates responses to fungal wilt pathogens by complexing with the chitin sensory receptors. *Plant Cell* **2020**, *32*, 3978–4001. [[CrossRef](#)] [[PubMed](#)]
42. Li, Q.; Hu, A.H.; Qi, J.J.; Dou, W.F.; Qin, X.J.; Zou, X.P.; Xu, L.Z.; Chen, S.C.; He, Y.R. CsWAKL08, a pathogen-induced wall-associated receptor-like kinase in sweet orange, confers resistance to citrus bacterial canker via ROS control and JA signaling. *Hortic. Res.* **2020**, *7*, 42. [[CrossRef](#)] [[PubMed](#)]
43. Haddadi, P.; Larkan, N.J.; Van DeWouw, A.; Zhang, Y.Q.; Neik, T.X.; Beynon, E.; Bayer, P.; Edwards, D.; Batley, J.; Borhan, M.H. *Brassica napus* genes *Rlm4* and *Rlm7*, conferring resistance to *Leptosphaeria maculans*, are alleles of the *Rlm9* wall-associated kinase-like resistance locus. *Plant Biotechnol. J.* **2022**, *20*, 1229–1231. [[CrossRef](#)]
44. Ameen, G.; Solanki, S.; Drader, T.; Sager-Bittara, L.; Steffenson, B.; Kleinhofs, A.; Vogiatzis, C.; Brueggeman, R.S. *rcs5*-mediated spot blotch resistance in barley is conferred by wall-associated kinases that resist pathogen manipulation. *BioRxiv* **2020**. [[CrossRef](#)]
45. Glawe, D.A. The powdery mildews: A review of the world's most familiar (yet poorly known) plant pathogens. *Annu. Rev. Phytopathol.* **2008**, *46*, 27–51. [[CrossRef](#)] [[PubMed](#)]
46. Meco, V.; Egea, I.; Ortiz-Atienza, A.; Drevensek, S.; Esch, E.; Yuste-Lisbona, F.J.; Barneche, F.; Vriezen, W.; Bolarin, M.C.; Lozano, R.; et al. The salt sensitivity induced by disruption of cell wall-associated kinase 1 (*SIWAK1*) tomato gene is linked to altered osmotic and metabolic homeostasis. *Int. J. Mol. Sci.* **2020**, *21*, 6308. [[CrossRef](#)] [[PubMed](#)]
47. Giarola, V.; Krey, S.; von den Driesch, B.; Bartels, D. The *Craterostigma plantagineum* glycine-rich protein CpGRP1 interacts with a cell wall-associated protein kinase 1 (CpWAK1) and accumulates in leaf cell walls during dehydration. *New Phytol.* **2016**, *210*, 535–550. [[CrossRef](#)] [[PubMed](#)]
48. Gupta, M.; Sharma, G.; Saxena, D.; Budhwar, R.; Vasudevan, M.; Gupta, V.; Gupta, A.; Gupta, R.; Chandran, D. Dual RNA-Seq analysis of *Medicago truncatula* and the pea powdery mildew *Erysiphe pisi* uncovers distinct host transcriptional signatures during incompatible and compatible interactions and pathogen effector candidates. *Genomics* **2020**, *112*, 2130–2145. [[CrossRef](#)]
49. Qiu, X.; Kong, L.; Chen, H.; Lin, Y.; Tu, S.; Wang, L.; Chen, Z.; Zeng, M.; Xiao, J.; Yuan, P.; et al. The *Phytophthora sojae* nuclear effector PsAvh110 targets a host transcriptional complex to modulate plant immunity. *Plant Cell* **2022**, *35*, 574–597. [[CrossRef](#)]

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