UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Agronomia Eliseu Maciel
Programa de Pós-Graduação em Agronomia
Área de Concentração Fitomelhoramento

Tese

Fatores de Transcrição WRKYs em Espécies Rosaceae

Winder Felipez Chiri

Pelotas, 2023

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Fatores de Transcrição WRKYs em Espécies Rosaceae

Tese apresentada ao Programa de Pós-Graduação em Agronomia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área do conhecimento: Fitomelhoramento)

Orientadores: Prof. Dr. Antonio Costa de Oliveira (UFPel)
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Prof ª. Dra. Camila Pegoraro (UFPEL)

Pelotas, 2023

Universidade Federal de Pelotas /
Sistema de Bibliotecas Catalogação na Publicação

C532f Chiri, Winder Felipez

Fatores de transcrição wrkys em espécies rosaceae / Winder Felipez Chiri ; Antonio Costa de Oliveira, Railson Schreinert dos Santos, orientadores ; Luciano Carlos da Maia, Camila Pegoraro, coorientadores. — Pelotas, 2023.

124 f.

Tese (Doutorado) — Agronomia, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, 2023.

1. Evolução. 2. Expressão diferencial. 3. Divergência. 4. Estresse. 5. Pomaceas. I. Oliveira, Antonio Costa de, orient. II. Santos, Railson Schreinert dos, orient. III. Maia, Luciano Carlos da, coorient. IV. Pegoraro, Camila, coorient. V. Título.

CDD : 634.717

Elaborada por Maria Beatriz Vaghetti Vieira CRB: 10/1032

Winder Felipez Chiri

Fatores de Transcrição WRKYs em Espécies Rosaceae

Tese aprovada, como requisito parcial, para obtenção do grau de Doutor em Agronomia, Programa de Pós-Graduação em Agronomia, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas.

Data da Defensa: 10/04/2023

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Para meu filho Steven Felipez

Dedico.

Agradecimentos

Primeiramente, gostaria de agradecer meu orientador, Prof. Dr. Antonio Costa de Oliveira, por seu apoio, orientação e paciência durante todo o processo de pesquisa e escrita da tese. Seu conhecimento e experiência foram fundamentais para o sucesso deste trabalho.

Também gostaria de agradecer aos membros da minha coorientação, Prof. Dr. Railson Schreinert dos Santos, Prof. Dr. Luciano Carlos da Maia, Prof ª. Dra Camila Pegoraro, Dra. Karine Janner Freitas e Dr. Luis William Pacheco Arge e por seus valiosos comentários e sugestões ao longo do caminho. Agradeço também aos meus colegas do CGF-UFPel pelo seu apoio durante todo o processo de pesquisa e escrita. Sem sua ajuda na realização de experimentos e coleta de dados, este trabalho não teria sido possível.

Agradeço ao Lic. Carlos Delgado e Andrea Gutierrez pelo apoio e incentivo na Bolivia, assim aos meus amigos e familiares pelo apoio e incentivo, especialmente durante os momentos mais desafiadores da pesquisa. Seu apoio emocional e compreensão foram essenciais para o meu sucesso. Também, gostaria de agradecer a Sra. Marisa (+), Sr. Herondino , Braz e Kelen A. Caino Vieira, por me permitirem ficar em sua casa. Fui recebido com calor e cuidado desde o momento em que cheguei. Sou muito grato por sua bondade e generosidade.

Gostaria de agradecer à Universidade Federal de Pelotas -UFPEL pelo apoio da bolsa na pesquisa, bem como à Centro de Genoma e Fitomelhoramento - pela sua assistência no laboratório de Bioinformática para a pesquisa. Também agradeço ao Instituto de Agroecologia y Seguridad Alimentaria IASA-USFX, Bolivia, pelo vinculo e respaldo institucional. Por fim, gostaria de expressar minha gratidão pelo aprendizado que esta pesquisa proporcionou. Aprendi muito sobre o assunto da minha pesquisa, bem como sobre mim mesmo e sobre a importância do trabalho em equipe e da perseverança. Obrigado a todos que contribuíram para este sucesso.

Meu muito obrigado

"Na bioinformática, eu encontrei um meio de aplicar minha paixão pela tecnologia em benefício da pesquisa e avanços na área da segurança alimentaria."

Winder Felipez

Resumo

CHIRI, Winder Felipez. **Fatores de Transcrição WRKYs em Espécies Rosaceae** 2023. 126f. Tese (Doutorado) - Programa de Pós-Graduação em Agronomia. Universidade Federal de Pelotas, Pelotas.

Os fatores de transcrição WRKY são conhecidos por estarem envolvidos na defesa de plantas contra patógenos, tolerância a diferentes estresses ambientais e em processos que determinam o desenvolvimento fenológico das plantas. A identificação e classificação da sua estrutura primária, baseada na conservação de aminoácidos e suas relações filogenéticas é de grande importância. Baseado nisso foi possível identificar 59 genes *RoWRKY* em framboesa preta (*Rubus occidentalis*), classificados em quatro grupos (I, II, III, IV) e oito subgrupos (I, IIa,IIb,IIc,IId,IIe, III, IV), localizados em sete cromossomos. A divergência genética das duplicações em tandem e segmentais parecem ter ocorrido há aproximadamente 29,02 milhões de anos. A análise da expressão de 10 amostras de dados de RNA-seq dos genes *RoWRKY* de framboesa preta demonstra comportamento diferencial destes genes. Além disso, um total de 667 genes *pomWRKY* (*Malus domestica, Malus baccata, Malus sieversii, Pyrus communis, Pyrus betulifolia, Pyrus pyrifolia*), classificados em quatro grupos e em nove subgrupos baseado na conservação de domínios e relações filogenéticas das sequencias de aminoácidos, 121 membros (18%) no grupo I, 434 (65%) no grupo II, 99 (15%) no grupo III e 13 (2%) no grupo IV. 560 foram mapeados nos 17 cromossomos de *M. domestica, M. sieverssii, P. betulifolia, P. communis e P. pyrifolia*, incluindo 52 pares de genes duplicados em tandem. A expressão diferencial dos genes pomWRKY em 20 amostras de RNA-seq processadas foi observada principalmente nos grupos I e II, com respostas ao estresse biótico e abiótico em diferentes órgãos de *Malus spp.* e *Pyrus* spp. A identificação da estrutura dos genes WRKY, bem como sua expressão diferencial nos bancos de dados genômicos e transcriptômicos, permite compreender a capacidade de resposta e tolerância aos estresses, fornecendo pistas sobre o provável papel que desempenham os genes WRKY nas plantas frutíferas de clima temperado.

Palavras-chave: evolução, expressão diferencial, divergência, estresse, pomaceas

Abstract

CHIRI, Winder Felipez. ***Fatores de Transcrição WRKYs em Espécies Rosaceae*** 2023. 126f. Thesis (Doctorate) - Graduate Program in Agronomy. Universidade Federal de Pelotas, Pelotas.

WRKY transcription factors are known to be involved in plant defense against pathogens, tolerance to different environmental stresses, and in different processes that determine plant phenological development. The identification and classification of their primary structure based on amino acid conservation and phylogenetic relationships, as well as their differential expression in response to stress tolerance in temperate fruit species, allowed for the identification of 59 *RoWRKY* genes in black raspberry (*Rubus occidentalis*), classified into four groups (I, II, III, IV) and eight subgroups (I, IIa, IIb, IIc, IId, IIe, III, IV), located on seven chromosomes, with genetic divergences of tandem and segmental duplications occurring 29.02 million years ago (Mya). Differential expression analysis of 10 RNA-seq data samples from black raspberry *RoWRKY* genes showed preferential or specific expression in tissue samples. In addition, a total of 667 pomWRKY genes (*Malus domestica, Malus baccata, Malus sieversii*, *Pyrus communis, Pyrus betulifolia, Pyrus pyrifolia*) were classified into four groups and nine subgroups based on domain conservation and phylogenetic relationships of amino acid sequences, with 121 members (18%) in group I, 434 (65%) in group II, 99 (15%) in group III, and 13 (2%) in group IV. Of these, 560 were mapped to the 17 chromosomes of *M. domestica, M. sieverssii, P. betulifolia, P. communis*, and *P. pyrifolia*, including 52 pairs of tandem duplicated genes. Differential expression of pomWRKY genes in 20 processed RNA-seq samples was mainly observed in groups I and II, with responses to biotic and abiotic stress in different organs of *Malus spp*. and *Pyrus spp*. These findings are important for understanding the role of WRKY family genes in plant-environment and plant-pathogen interactions, as well as for their genetic, transcriptomic, and phylogenetic importance. Therefore, identification of their structure and differential expression in genomic and transcriptomic databases allows for understanding the capacities of response and tolerance to stresses, providing the role played by WRKY genes in temperate fruit plants.

Keywords: evolution, differential expression, divergence, stress, pomaceous.

Sumário

1 INTRODUÇÃO GERAL

Os fatores de transcrição WRKY são uma família de proteínas específicas de plantas, desempenhando um papel crucial na regulação de vários processos fisiológicos, incluindo crescimento, desenvolvimento e respostas ao estresse (EULGEM *et al.*, 2000; FELIPEZ *et al.*, 2022). Em frutíferas de clima temperado, como macieira, pereira e framboeseira preta, estes fatores de transcrição foram extensivamente estudados por seu envolvimento nas respostas a estresses bióticos e abióticos, bem como no desenvolvimento e amadurecimento de frutos (FELIPEZ *et al.*, 2022).

As principais limitações cientificas de produção frutícola em espécies de *Malus spp., Pyrus spp.,* e *Rubus spp*. no Brasil e no mundo apontam a necessidade de pesquisas em melhoramento genético, manejo integrado de pragas e doenças, tecnologias de produção, qualidade e pós-colheita, e agricultura sustentável para melhorar a produtividade, a qualidade e a competitividade das frutas brasileiras e do mundo (APAGRI, 2019; SPENGLER, 2019; EFSA PANEL ON PLANT HEALTH (PLH) et al. 2020) Nesse contexto, a busca por novas abordagens de pesquisa em genética molecular e evolutiva pode auxiliar na produção frutícola, com foco principalmente nos genes que regulam a transcrição da expressão genética, como os Fatores de Transcrição WRKY.

A família WRKY nas frutíferas de clima temperado é relativamente grande, com vários membros caracterizados. Macieira, por exemplo, possui 127 genes *MdWRKY* já identificados (MENG *et al.*, 2016) e seus padrões de expressão foram estudados sob diversas condições, incluindo estresse hídrico, frio e infecção por patógenos. Da mesma forma, em pereira branca (*Pyrus bretschneideri*), 103 genes WRKY foram identificados no genoma e seus papéis na regulação sob estresse hídrico (LIU *et al.*, 2020). Além disso, há 64 genes *FvWRKY* no genoma do morango-silvestre (Fragaria vesca), alguns dos quais são expressos nas amostras transcriptômicas em

receptáculos e aquênios durante o processo de amadurecimento e em resposta a ataques por patógenos(GARRIDO-GALA *et al.*, 2022).

Os fatores de transcrição WRKY nas frutíferas de clima temperado estão envolvidos em diversas vias de sinalização que controlam as respostas das plantas a estresses bióticos e abióticos (FELIPEZ *et al.*, 2022; RINERSON *et al.*, 2015). Por exemplo, a expressão de alguns genes WRKY em macieira é induzida pela infecção por patógenos, tendo tais fatores de transcrição um papel crucial na regulação da expressão de genes relacionados à defesa. Da mesma forma, em macieira, pereira e framboeseira preta, os fatores de transcrição WRKY se mostraram capazes de regular a expressão de genes envolvidos na biossíntese e sinalização do etileno, tendo papel crítico no amadurecimento de frutas. Ainda assim a importância maior destes fatores de transcrição geralmente está na resposta a organismos causadores de doenças (interação planta-patógeno) e em mecanismos de interação das plantas com o ambiente (luz, sal entre outros).

Nesse contexto, os fatores de transcrição WRKY são um componente essencial da rede regulatória nas frutíferas de clima temperado. Seu envolvimento em vários processos fisiológicos os torna um alvo atraente para o melhoramento genético, a fim de melhorar a qualidade dos frutos e aumentar a resistência das plantas a estresses. Por isso, o foco da pesquisa apresenta abordagem de importância, genômica, transcritômica, metagenômica e filogenia nas espécies frutíferas de clima temperado e a expressão dos membros da superfamília de fatores de transcrição WRKY.

No entanto, a abordagem aqui utilizada foi inteiramente realizada a partir de dados de domínio público. A disponibilidade de informações genômicas e complementos já anotados foi crucial, sendo que o foco dos trabalhos aqui apresentados foram espécies de *Malus* spp. e *Pyrus* spp., com ênfase naquelas já mais exploradas economicamente (*Malus domestica* cv. Gala e *Pyrus communis cv. Bartlett)*. Além destes gêneros utilizou-se ainda outro arbusto frutífero, o *Rubus occidentalis conhecido como framboeseira preta*. Destaca-se ainda que a disponibilidade de dados

transcritômicos, além de permitir o uso de ferramentas da bioinformática, foram essenciais para compreender o papel dos fatores de transcrição WRKY nestes genomas.

As pesquisas aqui debatidas apresentam-se por capítulos de estudo no formato de artigos. O artigo 1, de revisão, aborda o papel dos fatores de transcrição WRKY em *Malus* spp. e *Pyrus* spp. contemplando tópicos de estresses bióticos e abióticos em pomáceas, descrevendo sua evolução, bem como estruturas primária e terciária. O artigo 2, de pesquisa, foca nos genes WRKY de framboeseira preta (*Rubus occidentalis* L.), por meio de filogenia, descrição de estrutura gênica, predição da localização subcelular e descrição da regulação da expressão transcricional. Por fim, o artigo 3, também de pesquisa, aborda detalhes da expressão de fatores de transcrição WRKY na resposta a estresses em *Malus* spp. e *Pyrus* spp., descrevendo tópicos de evolução dos domínios WRKYs nas espécies pomáceas, discutindo as duplicações em tandem e a divergência destes genes.

2 OBJETIVOS

2.1 Objetivo geral

Prover informações sobre a estrutura gênica, expressão diferencial e as relações filogenéticas dos fatores de transcrição WRKY em espécies da família Rosaceae dos gêneros *Malus* spp., *Pyrus* spp. e *Rubus* spp.

2.2 Objetivos específicos

- Fornecer uma visão geral sobre os avanços nos estudos de fatores de transcrição WRKY em espécies dos gêneros *Malus* spp. *e Pyrus* spp., destacando as respostas e impactos na tolerância aos estresses bióticos e abióticos.

- Identificar e caracterizar os genes WRKY no genoma completo da framboeseira preta (*Rubus occidentalis*), além de exibir suas relações filogenéticas e de expressão diferencial.

- Determinar a expressão dos fatores de transcrição WRKY na resposta a estresses em *Malus* spp e *Pyrus* spp., além de realizar sua identificação, caracterização e demonstração de relações filogenéticas em genomas completos.

3 ARTIGO 1: The roles of WRKY transcription factors in *Malus* spp. and *Pyrus* spp.

*Manuscrito submetido e aceito ao periódico Functional & Integrative Genomics (Qualis A2)

Received: 6 June 2022 / Revised: 11 July 2022 / Accepted: 12 July 2022

https://doi.org/10.1007/s10142-022-00886-0

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Abstract

The WRKY transcription factor gene family is known to be involved in plant defense against pathogens and in tolerance to different environmental stresses at different stages of development. The response mechanisms through which these genes act can be influenced by different phytohormones as well as by many trans- and cis-acting elements, what makes this network an important topic for analysis, but still, something complex to fully understand. According to available reports, these genes can also perform important roles in pome species (*Malus* spp. and *Pyrus* spp.) metabolism, especially in adaptation of these plants to stressful conditions. Here we present a quick review of what is known about WRKY genes in *Malus* and *Pyrus* genomes offering a simple way to understand what is already known about this topic. We also add information connecting the evolution of these transcription factors with others that can also be found in pomes.

Keywords Stresses · Pomes · Evolution · Rosaceae

3.1 Introduction

The fleshy pseudofruits, composed of five carpels of the pome type, characterize the pomaceae, a group of Rosaceae inside the Maloideae subfamily. This highly diverse group has approximately 173 species and its variation occurs in several traits, including flower architecture and pseudofruit carp texture. Its diversity and evolution are partially explained by the dispersion of seeds that have been adapting to different environments and situations (Rohrer et al., 1991). Regarding their genomes, it basically consists of 17 chromosomes, which arose from an aneuploidy of a previous 18-chromosome genome, an event that probably took place in North America (Evans and Campbell, 2002).

Apple (*Malus* spp.), pear (*Pyrus* spp.), and quince (*Cydonia* spp.) genera stand out as the most economically and ecologically important. Here, we will highlight the most cultivated species: *Malus domestica* (Velasco et al., 2010) and *Pyrus communis* (Chagné et al., 2014). These are the most studied pome species aiming to improve rootstocks, fruit quality, disease resistance, tolerance to water and saline stress, and dormancy adaptation, among other traits. Thus, molecular studies, including transcriptomics, proteomics, and metabolomics, have been carried out, allowing the compression of defense and response mechanisms that allow the interaction of these species with the environment. Here, we collected and discussed some of the available data on WRKYs from *Malus* spp. and *Pyrus* spp., which we will call pomWRKYs, with special attention to pathogen defense and response to biotic and abiotic stresses. WRKY structure and classification adopted here is based on the studies of Eulgem et al. (2000), Wang et al. (2014), Meng et al. (2016), Gu et al. (2015), and Huang et al. (2015). Also, we performed bioinformatics analyses to classify and understand the evolution of these TFs in *Malus* and *Pyrus* genomes. Finally, we provide a rich overview about the advances in the study of pomWRKYs highlighting the targets that need attention for more studies.

3.2 WRKY structure

WRKY proteins vary in their number of domains and zinc finger motifs, but preserve at least one domain that gives rise to the family's name, which contains the DNA-binding heptapeptide "WRKYGQK" that is linked to zinc finger motifs, forming approximately 60 amino acids that can be near to either N- or C-terminal regions (Eulgem et al., 2000; Rushton et al., 2010; Wang et al., 2011; Brand et al., 2013; Bakshi and Oelmüller, 2014). These regions can bind to the W-box element (TTG ACT /C) or other cis regulatory elements (CRE) in the target promoter region, allowing gene expression regulation and participating in cell signaling network through the conserved WRKY domain (DARSHAN et al., 2016; Phukan et al., 2016; Cai et al., 2019).

The primary structural classification of the WRKY superfamily described by Eulgem et al. (2000) is given by subfamilies, where subfamily I contains two WRKY domains, while subfamilies II and III have only one C-terminal WRKY domain. C-terminal WRKY domain seems binding preferentially to the W-box, while N-terminal WRKY domains are less understood (Duan et al., 2007; Chen et al., 2017). Approximately 61% of WRKYs of the subfamily II of *M. domestica* are expressed in calli under water stress (Meng et al., 2016), while 69% of subfamily II of *Pyrus bretschneideri* WRKYs are expressed in leaves of plants that are under water stress (Huang et al., 2015). The overexpression of the WRKYs subfamilies will depend on the type of stress that is acting on the plant since; for instance, the IIa–e subfamily has 68% representativeness of 119 WRKY genes that were differentially expressed in response to fungal pathogens and hormonal treatments of *M. domestica* (Lui et al., 2017). Also, in Chinese pear (*Pyrus ussuriensis*), we can notice that six genes of the IIb–d subfamily are differentially expressed during fruit ripening (Huang et al., 2014).

According to their secondary structure, WRKY proteins perform specific interactions with DNA bases, leading to the recognition of particular sequences and regulatory motifs (Jiang et al., 2017; Chen et al., 2019). However, the understanding about how the tertiary structure of WRKY proteins affects DNA recognition is still incipient.

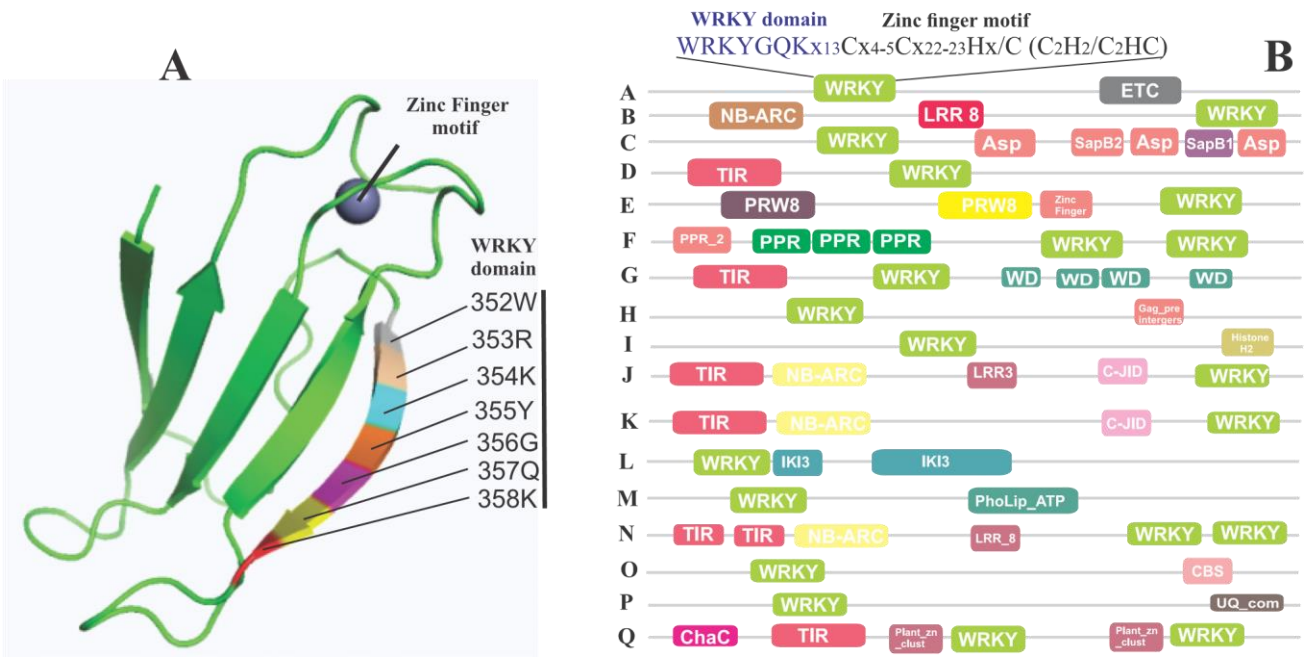

Fig. 1 WRKY transcription factors from different species of the Rosaceae family. **A** *PuWRKY31* 3D model. **B** Structure of the WRKY genes: (A) *PuWRKY27* domains (A0A5N5F198) followed by ETC_C1_NDUFA5 domains, (B) NB-ARC domain, followed by an LRR8 and a *PuWRKY* domain (A0A5N5GMZ0), (C) *MdWRKY* domain (A0A498HG15) followed of Asp (Aspartic Protease), SapB2 (Saposin Protein), Asp (Aspartic Protease), and SapB1 (Saposin Protein) domains and Asp (Aspartic Protease), (D) TIR domain followed by *MdWRKY* (0A498HM71), (E) RPW8 domain, NB domain ARC, Zinc domains Finger and *RcWRKY* (A0A2P6P401), (F) PPR_2 domain, two PPR domains and followed by one *MdWRKY* domain (A0A498K9W8), (G) TIR domains followed by *PuWRKY* (A0A5N5GXD3) and two WD domains, (H) *PuWRKY13* domain (A0A5N5H2 and followed by a domain (Gag_pre-integrs), (I) *MbWRKY* (A0A540MGR7) domain and followed by a Histone_ H2A_C domain, (J) TIR domain, an NB-ARC domain, an LRR_3 domain, a C-JID domain and followed by *RcWRKY* (A0A2P6SMR5), (K) TIR domain, one NB-ARC domain, one C-JID domain and followed by *RcWRKY* (A0A2P6SLT3), (L) *MbWRKY*(A0A540M2S4) domain followed by two of the IKI3 domain, (M) *MdWRKY* domain (A0A498ILB8) followed by one PhoLip_ATPase_C domain, (N) two TIR domains, one NB-ARC domain, one LRR_8 domain and followed by two *RcWRKY* domains (A0A2P6QMS4), (O) *MdWRKY* domain (A0A498HLR9) and a CBS domain, (P) *PyWRKY61* (A0A314YUI8) domains and followed by a domain (UQ_con), and finally (Q) ChaC domain, a TIR domain, two Plant_zn_clust domains, and two *PuWRKY* domains (A0A5N5GZ97).

The ten WRKY domain structures from model species *Arabidopsis thaliana* and *Oryza sativa* available on the Pfam database can be used for further investigations. The tertiary structure modeled for the *P. ussuriensis PuWRKY31* protein (A0A6G7PRG3) in SWISS-MODEL (https://swissmodel.expasy.org/expasy.org) Fig. 1A) presents general high-resolution quality patterns such as *A. thaliana* (*AtWRKY1*) from the study by Duan et al. (2007). Some structural features of WRKY proteins are showing isoform structures fused with two or more spliced isoforms that encode new domains such as ZF-SBP, CBS, kinase, PAH, ULP PROTEASE, FOR, MAC, LRR, ATP GRASP, and B3 (Chen et al., 2017). Pfam database version 34.0 (http://pfam.xfam.org/family/PF03106) presents 192 WRKY sequences, where we could find 17 amino acid sequences of five Rosaceae species, in addition to fused genes encoding new domains NB-ARC, LRR8, ETC, Asp, TIR, RPW8, Zinc Finger, PPR,

WD, Gag, Histone, LRR_3, C-JID, IKI3, PhoLip_ATPase_C, CBS, UQ_con, ChaC, and lant_zn_clust (Fig. 1B). Variants in the WRKYGQK conservative region are also being shown for having or not DNA-binding affinity (Chen et al., 2017; 2019), whereby the structure is changing and making the understanding of its structural and functional annotation of pome fruit tree proteomes.

Origin and classification of WRKYs

The first WRKY protein was found in sweet potato (*Ipomoea batatas*) by characterizing the Sweet Potato Factor1 (SPF1 is DNA-binding protein of 549 amino acids) cDNA that encodes a DNA-binding polypeptide. The amino acid sequence SPF1 represents the WRKY protein that binds to DNA, also recognizing oligonucleotide SP8 (SP8a=ACT GTG TA and SP8b= TAC TAT T) sequences in the 5' upstream regions of the genes, which allows coding for the sporamine protein and β-amylase enzyme in sweet potato roots (Ishiguro and Nakamura, 1994).

The WRKY proteins were initially considered to be plant specific (Eulgem et al., 2000). This is due to reports of parsley (*Petroselinum crispum*) (Rushton et al., 1996), tobacco (*Nicotiana tabacum*) (Yang et al., 1999), and others (Takatsuji, 1998; Rushton and Somssich, 1998). Afterwards, the WRKY superfamily was reported to have an early origin in eukaryotes being also present in fungi (Zhang and Wang, 2005). In addition, advances in genetics and bioinformatics provide valuable information about their possible roles in stress and hormone response, helping to understand the complexity of signaling networks (Rushton et al., 2010). The availability of sequenced genomes allowed the emergence of two alternative hypotheses on the evolution of WRKY genes: The “Group I hypothesis” considers that all WRKY genes have evolved from Group I members, that only had WRKY domains in the C-Terminal; “Separate Hypothesis IIa + b” considers that groups IIa and IIb evolved directly from an algae gene with unique domains separate from the Group I–derived lineage (Rinerson et al., 2015).

However, the origin of WRKYs presents unusual characteristics in flowering plants due to the existence of chimeric proteins comprising typical domains for intercellular type I resistance proteins (nucleotide binding site-leucine-rich repeat, NBS-LRR proteins) and WRKY transcription factors. These R protein-WRKY (RW) proteins are not found in all plant genomes but are in eight independent genomic rearrangements in NBS–LRR–WRKY (RW1-RW8) genes that contain domains from WRKY subgroups (Rinerson et al., 2016). Furthermore, the rapid progress of genomics and transcriptomics techniques allowed a better understanding of WRKY protein roles in different species (Chen et al., 2017). The accumulation of data on W-box binding proteins, as well as other cis elements in the WRKY protein structure and data on unusual chimeric R-WRKY proteins, allows us to understand plant resistance (Chen et al., 2019). Therefore, the origin of the WRKY was initially reported in eukaryotic plants and with variations in protein resistance of some flowering plants. In addition, these have three subfamilies with WRKY domains and zinc finger motif.

3.3 WRKYs: classification and functional domains

The first and most accepted classification of the WRKY superfamily is based on the characterization of the *Arabidopsis* genome, dividing these proteins into three groups: I, II, and III based on the number of WRKY domains and the characteristics of their zinc finger motifs. Group II is divided into five subgroups: II-a, II-b, II-c, II-d, and II-e, based on their relationships through molecular phylogeny (Eulgem et al., 2000; Chen et al., 2017). WRKY proteins contain variation in their domains and in their C-terminal regions. Group I is composed of proteins that have two WRKY domains; group II has proteins that contain only one WRKY domain; both groups I and II have a C2H2 ZINC finger motif (C–X4–5–C–X22–23–H–X1–H); group III has a WRKY domain and a C2HC zinc finger motif (C–X7–C–X23–H–X1–C) where X represents any amino acid (Eulgem et al., 2000; Cai et al., 2019). In the genomes of pome fruit species, we also have a fourth group (IV) that has a WRKY domain without a complete zinc finger structure in the C-terminal region (Wang et al., 2014; Meng et al., 2016).

The structural classification of the WRKY superfamily in Characteristics of the MdgWRKY sequences (supplementary S1). A phylogenetic tree was constructed to classify the MdgWRKY subfamilies or groups. Multiple alignment of WRKY protein sequences was performed with ClustalW-2.1 software using standard parameters. A phylogenetic tree was constructed using the MEGA X software (Kumar et al., 2018), with the Neighbor-Joining method. A bootstrap analysis was conducted using 1,000 replicates and the evolutionary distances calculated using the JTT matrix–based method (Jones et al., 1992) (supplementary S2). However, in the *Arabidopsis* model species, the classification of WRKY proteins evidenced domain fusion; four WRKYs were discovered with additional structural domains of Leucine-rich repeats (LRR) at the N-terminus (At4G12020, At5g45050, At5G45260) and BDP1 domain in the N-terminal (At1G55600), leading to the discovery of new subfamilies and new subfamily members (Chen et al., 2017). In *M. domestica*, it shows evidence of motif loss and heptapeptide structure changes (WRKYGQK), leading to a new subclassification of group IV and new members of the WRKY family (Meng et al., 2016). Therefore, the structural classification of WRKYs is evolving to four subfamilies or groups.

The subdivision of WRKY proteins according to their biological and molecular functions is still imprecise given the lack of curated information (Cai et al., 2019; Chen et al., 2019). The size of the groups varies according to the species: *M. domestica* has 17% (127) of the proteins in group I, which has two MdWRKY domains, while groups II, III and IV, which have only one MdWRKY domain, have 61, 11, and 10% in each group, respectively. *Arabidopsis* (group I, 18%; II, 63%; III, 19%) and *Vitis vinifera* (group I, 22%; II, 65%; III, 10%; IV, 3%) also have different distribution. *M. domestica* group IV has more members than other plants (Meng et al., 2016). In the analysis of *P. bretschneideri* genome, 103 PbWRKY TFs were identified (group I, 16%; II, 69%; III, 15%), but it does not have group IV (Huang et al., 2015). The classification of WRKY proteins changes according to the availability of genomic data sequences and their respective structural and functional annotations.

3.4 WRKYs and stress

The expression of WRKYs helps in temperature adaption, hormonal responses, and correct use of nutrients. It acts in signal transmission and post-translational responses, also impacting processes of senescence, dormancy, seed germination and interactions between organs and different environmental stimuli (Banerjee and Roychoudhury, 2015; Wang et al., 2017a). The overexpression of some WRKYs can mediate plant hormonal responses caused by abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA), and jasmonic acid (JA), possibly leading to an increase in disease tolerance (Mingyu et al., 2012). Evidence of the important roles of TFs in regulating plant development and response to many types of stress can provide transcriptional mechanisms for activating and deactivating a specific plant function (Jiang et al., 2017), providing a basis for compression of WRKYs evolution in species and in functional differentiation (Guo et al., 2019). WRKYs not only act in stress responses or development, but are also involved in specialized metabolic pathways, which further highlight the importance of the advances in biotic and abiotic stress research in *Malus* spp. and *Pyrus* spp. (Phukan et al., 2016).

3.5 Biotic stress

Biotic interactions between plant and disease-causing organisms (bacteria, viruses, nematodes, insects, and fungi) generate what we call biotic stress (Bhatla and A. Lal, 2018; Kar and Raichaudhuri, 2021). As a form of defense, plants can make use of phytohormones such as JAs, thus being able to fight infection by pathogens and even insect herbivory, activating several transcription factors (Zhao et al., 2019a). There are also microRNAs that accompany auxin signaling pathways in defense of pathogens in *Pyrus* spp. (Zhang et al. 2019b). In addition to plant growth–promoting microorganisms (PGPM) that trigger physiological and biochemical changes for tolerance to biotic stresses in plants (Thomas and Singh, 2020). However, there is evidence that TF *MdWRKY100* expression from *M. domestica* increases resistance to *Colletotrichum gloeosporioides* (Zhang et al., 2019a)**.**

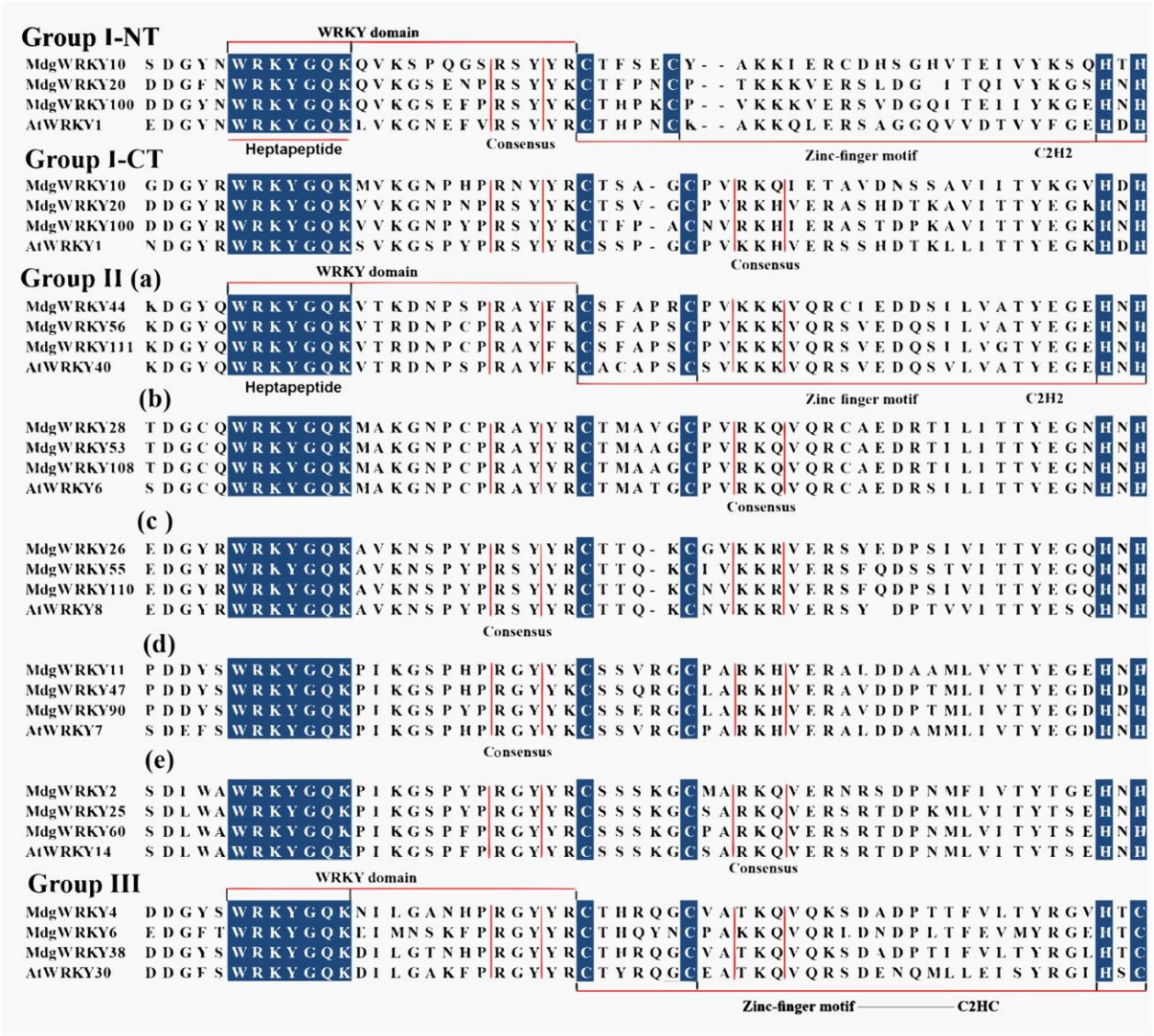

Fig. 2 The WRKY protein domain in *M. domestica*. The WRKY protein family is classified into subfamilies I (I N and I C), IIa, IIb, IIc, IId, IIe, and III, according to its zinc finger motif C2H2 and C2H

These plant defense mechanisms act against biotic stresses are help with the activation of many other TFs involved in tolerance to multiple pathogens (Phukan et al., 2016). Some functional mechanisms of resistance or response of WRKY to biotic stress in pome fruit species are shown in Table 1. WRKY responses to pathogens (viruses, fungi, bacteria) or disease-causing organisms in pome fruit trees are regulated activating signal to phytohormones (SA, JAs), in addition to interacting with

other families of TFs (Zhao et al., 2019b). The following describes some interactions and response mechanisms of WRKYs in pomes:

Alternaria alternata infection response

The leaf spot fungus (*A. alternata*) causes defoliation and reduced yields in apple trees. The response mechanism is the overexpression of *MdWRKY1*, something that can be mediated by SA and methyl jasmonate (MeJA), which are important signaling molecules (Fan et al., 2011). The overexpression of *MdWRKYN1* and *MdWRKY26* or the drop in the post-transcriptional activity of *Md-miR156ab* and *MdmiR395* helps in *Alternaria* tolerance in apple trees by being targeted by R proteins that recognize W-box elements in pathogenesis-related gene promoters (Zhang et al., 2017). The expression of *MdWRKY75d* and *MdWRKY75e* increases the *A. alternata* resistance in apple leaves; the same occurs when associated with jasmonate/ethylene signaling pathways and is not reduced by their treatment that represses SA (Hou et al., 2021a). Overexpression of *MdWRKY75e* increases resistance to infection by *A. alternata* by binding directly to the *MdLAC7* promoter regulating laccase biosynthesis and increasing lignin biosynthesis during infection (Hou et al., 2021b). The differential expression of 69 MdWRKYs in *M. domestica* is likely to increase the leaf tolerance to *A. alternata* infection through the interaction with phytohormones (SA, MeJA and ethephon) (Lui et al., 2017).

Responses to soil fungi

The root system of pome fruit species can be damaged by different fungi (e.g., *Cylindrocarpon spp., Rhizoctonia spp., Phytophthora spp.,* and *Pythium spp*.). These pathogens can reduce the growth of M. domestica trees right after its transplantation, negatively affecting its productivity and fruit quality. However, there are molecular mechanisms that help in defense against fungal infections by the expression of MdWRKY9 which is mediated by hormones like SA, JA and ethylene (ET) (Weiß and Winkelmann, 2017). *Fusarium solani*, a soil fungus, is also the main cause of the replanting disease caused by its attack to M. domestica rootstocks; still the

transcriptional expression of MdWRKY51 in the roots is moderate when compared to the performance of the enzyme UDP-glycosyltransferase and P450 protein as a single defense response (Xiang et al., 2021b).

The overexpression of MdWRKY40 in the root system of apple seedlings with arbuscular mycorrhizal fungi plays an important role in resistance to *F. solani* infection (Wang et al., 2021). Also, MdWRKY74 overexpression in apple calli improves resistance to *F. solani* infection (Xiang et al., 2021a). In this sense, some of the described soil pathogenic fungi can be repressed through the overexpression of apple WRKYs due to their interaction with phytohormones, enzymes, and its consequences to mycorrhizal fungi.

Responses to fungi infection in fruits

C. gloeosporioides causes bitter rot in *M. domestica*, leading to qualitative and quantitative losses on a large scale. However, the defense mechanism in which *MdWRKY100* is involved is in the activation of four pathogenesis-related genes (RPs), which code proteins MdPR3-1, MdPR101, and MdPR10-2; these genes included a gene related to salicylic acid (SA) signaling (PR2); endochitinase (PR3-1), which may provide protection against fungal pathogens, may provide protection against fungal pathogens, and two genes related to ribonuclease homology (*PR10-1,PR10-2*) (Zhang et al., 2017, 2019a). Also, there are beneficial fungi (*Antagonistic yeasts*) such as *Meyerozyma guilliermondii*, which significantly inhibits the natural decomposition of stored *P. pyrifolia* fruits through the activation of *PpWRKY9*, *PpWRKY31*, and other resistance genes (*Pyrc1*, *Pru ar-1like*, *Pru av-1like*) without adverse effects on storage quality (Yan et al., 2018).

Table 1 WRKYs transcription factors in *Malus* spp. and *Pyrus* spp. and their responses to biotic stress

Species	Protein	Group	Regulation	Mode of expression	Regulation function	ID/Access #	References
M. domestica	MdWRKY1	II-d	Positive	Overexpression	Resistance to the fungus *A. alternata* on the leaves	HM859901	Fan et al. (2011)
M. domestica	MdWRKY9	II-b	Probable	Overexpression	apple replanting disease (ARD)	MDP0000137704	Weiß and Winkelmann (2017)
M. domestica	MdWRKYN1	I-N	Positive	Overexpression	Resistance to the fungus *A. alternata* on the leaves	XM_008353805.1	Zhang et al. (2017)
M. domestica	MdWRKY26	I	Positive	Overexpression	Resistance to the fungus *A. alternata* on the leaves	XM_008386494.2	Zhang et al. (2017)
M. domestica	MdWRKY40	II-b	Positive	Overexpression	Resistance to bacterial pathogens	MDP0000263349	Qiu et al. (2017)
M. domestica	MdWRKY100	I	Positive	Overexpression	Resistance to the fungus *C. gloeosporioides*	MDP0000514115	Zhang et al. (2019)
M. domestica	MdWRKY40	II-a	Positive	Overexpression	Responses to injuries	XP_008342807.1	An et al. (2019)
P. pyrifolia	PpWRKY9	II-b	Positive	Overexpression	Resistance to antagonistic yeasts	Pbr001471	Yan et al. (2018)
P. pyrifolia	PpWRKY31	II-b	Positive	Overexpression	Resistance to antagonistic yeasts	Pbr023691	Yan et al. (2018)
M. domestica	MdWRKY40	II-b	Positive	Expression	Fungus and bacteria resistance	MDP0000794439	Balan et al. (2018)
M. domestica	MdWRKY6	II-b	Positive	Expression	Fungus and bacteria resistance	MDP0000935652	Balan et al. (2018)
M. domestica	MdWRKY18	II-a	Positive	Expression	Fungus and bacteria resistance	MDP0000418900	Balan et al. (2018)
M. domestica	MdWRKY33	I	Positive	Expression	Resistance to the bacterium *E. mylovora*	MDP0000935996	Balan et al. (2018)
M. domestica	MdWRKY51	II-c	Probable	Expression	Resistance to the fungus *F. solani*	MDP0000253189	Xiang et al. (2021b)
M. domestica	MdWRKY31	II-b	Positive	Expression	Resistance to the fungus *B. dothidea*	MD05G1349800	Zhao et al. (2019a)
M. domestica	MdWRKY46	II-a	Positive	Expression	Resistance to the fungus *B. dothidea*	MD07G1146900	Zhao et al. (2019b)
M. domestica	MdWRKY75	IV	Probable	Expression	Resistance to the bacterium *E. mylovora*	MD13G1122100	Singh et al. (2019)
M. sieversii	MsWRKY7	II-d I	Positive	Expression	Resistance to the fungus *V. mali*	MD15G1078200	Liu et al. (2021)
M. sieversii	MsWRKY33		Positive	Expression		MD03G1057400	
M. sieversii	MsWRKY6	II-b	Positive	Expression		MD05G1349800	
M. sieversii	MsWRKY19	I-N	Positive	Expression		MD05G1322700	
M. sieversii	MsWRKY40	II-a	Positive	Expression		MD00G1143500	
M. sieversii	MsWRKY45	II-c	Positive	Expression		MD14G1154500	
M. sieversii	MsWRKY51	II-c	Positive	Expression		MD16G1244300	
M. sieversii	MsWRKY61	II-b	Positive	Expression		MD13G1077900	
M. sieversii	MsWRKY75	IV	Positive	Expression		MD11G1059400	
M. robusta	MrWRKY53	II-b	Probable	Expression	Participates in bacterial resistance	MDP0000767097	Gardiner et al. (2012)
M. domestica	MdWRKY33		Probable	Expression	Participates in resistance to the fungus *M. coronaria*		Feng et al. (2019)
M. domestica	MdWRKY		Probable	Expression	Participates in fungal resistance		He et al. (2020)

Table 1 (continued)

Species	Protein	Group	Regulation	Mode of expression	Regulation function	ID/Access #	References
M. domestica	MdWRKY40		Probable	Overexpression	Resistance to the fungus *F. solani*	MD15G1039500	Wang et al. (2021)
M. domestica	MdWRKY75d	II-c	Positive	Expression	Resistance to the fungus *A. alternata*	MDP0000154734	Hou et al. (2021a)
M. domestica	MdWRKY75e	II-c	Positive	Expression	Resistance to the fungus *A. alternata*	MDP0000263768	Hou et al. (2021b)
M. domestica	MdWRKY75e	II-c	Positive	Expression	Resistance to the fungus *A. alternata*	MDP0000263768	Hou et al. (2021b)
M. domestica	MdWRKY74		Positive	Expression	Resistance to the fungus *F. solani*		Xiang et al. (2021a)
M. domestica	MdWRKY1-119		Probable	Differential expression	Resistance to the fungus *A. alternata*	69 genes	Lui et al. (2017)

Responses to bacteria and fungi pathogens

There are different networks of protein–protein and protein-phytohormone interactions in pome fruit species such as *M. domestica* that act in the regulation of different specific resistance responses to fungal and bacterial pathogens. *MdWRKY40* is involved in the response to fungi and/or bacteria, but its interaction with *MdWRKY6* and *MdWRKY18* is specific to fungal pathogens, while its interaction with *MdWRKY33* is related to *Erwinia amylovora*. Interaction of WRKYs with phytohormones (gibberellins and jasmonates) varies in gene signaling responses (Balan et al., 2018). *MdWRKY46* increases resistance to *Botryosphaeria dothidea* by activating the *MdPBS3.1* protein through the SA signaling pathway (Zhao et al., 2019a). *MdWRKY31* acts promoting resistance to *B. dothidea* by interacting with *MdHIR4*, repressing its expression, or causing responses in phytohormone signaling pathways (SA and JA), but in *Arabidopsis* and *Nicotiana benthamiana* species increases resistance to bacterial diseases (flg22 and Pst DC3000) (Zhao et al., 2019b).

Also, in *Malus robusta*, MrWRKY expression plays a likely role in resistance to fire blight (*E. amylovora*), which is associated with putative resistance genes inside a QTL (MDP0000767097) (Gardiner et al., 2012). The expression of *MdWRKY75* has a probable link to the resistance to the bacterial pathogen (*E. amylovora*) interacting with UDP-glycotransferase in the internal connectivity network, emitting a signal through MAPK and C a^{2+} sensors (Singh et al., 2019). Nine MdWRKYs participate in the defense to *Botryosphaeria berengeriana* and *A. alternata*, which are inhibited by the expression of the latex protein called *MdMLP423* (He et al., 2020). WRKYs and phytohormone protein interaction networks provide specific responses as defense mechanisms to disease-causing organisms in pome fruit trees.

Many functions of resistance to biotic stress caused by fungal pathogens, viruses (Apple stem grooving virus), and *E. amylovora* in pome fruit species are the signal emission of phytohormones or secondary metabolites that interact with and associated with WRKY PR genes (Zhang et al., 2017). The expression interactions between *MdWRKY40* and SA positively regulate the transcriptional activity of apple leaf in defense of bacterial pathogens (*Hyaloperonospora arabidopsidis*, *Botrytis cinerea*, *Pseudomonas syringae*.) but its overexpression inhibits its own transcription to signaling pathways (Qiu et al., 2017).

The same *MdWRKY40* will interact with phytohormone (ABA) and *MdBT2* and *MYB1* become a positive regulator by modulating anthocyanin biosynthesis as a treatment in response to injury induction of insect or other herbivory, and its degradation of anthocyanin accumulation is via the 26S proteosome pathway (An et al., 2019). The expression of *MdWRKY33* participates in the performance of important roles in the basal resistance to the infection by the fungi *Marssonina coronaria* (Feng et al., 2019).

Furthermore, the resistance to the fungus *Valsa mali*, which causes severe stem disease in *Malus sieversii*, is related to the expression of different WRKYs, where *MsWRKY7* and *MsWRKY33* are related to initial response while *MsWRKY6*, *MsWRKY7*, *MsWRKY19*, *MsWRKY33*, *MsWRKY40*, *MsWRKY45*, *MsWRKY51*, *MsWRKY61*, and *MsWRKY75* seem to act in the final stages of response, being JA and SA important hormones involved in these responses (Liu et al., 2021). Therefore, the resistance functions in biotic stress respond to protein-protein interactions, phytohormones or secondary metabolites, enzymes, and mycorrhizal fungi in defense of the mentioned pathogens in different plant organs of pome fruit species.

3.6 Abiotic stress

The interactions between plant and environment can result in stress by low or high temperature, high salinity, water deficiency or excess, heavy metals, and ultraviolet radiation, among other factors that generate what we call abiotic, also causing quantitative and qualitative losses (Saijo and Loo, 2020; Kar and Raichaudhuri, 2021). Plants have stress sensors that allow signal translation and consequent acclimatization, but early action sensors provide the initial signal for the stress response, where the signal translation by calcium and protein kinase/phosphatase helps dealing with reactive oxygen species (ROS), letting TFs and hormone induce not only mechanisms of acclimation, but also programmed

cell death (Mittler, 2017). The comprehension of WRKYs responses to abiotic stress and its application *in Malus spp.* and *Pyrus spp.* breeding are of great agricultural importance (Phukan et al., 2016; Han et al., 2018a). Some mechanisms of action of different WRKYs in response to abiotic stress in pome fruit species are presented in Table 2.

Water stress

Water stress (drought and flooding) affects plant growth and development, influencing photosynthesis, turgor loss, cuticle permeability of fruits, membrane composition and fluidity, solute and ion concentrations, protein–protein and protein-liquid interactions, and plant antioxidants, which interact with phytohormones (BRs, CKs, GAs, JAs, ABA, Eth, auxins, SA, SLs) in the regulatory defense network (Osakabe et al., 2014; Moustafa-Farag et al., 2020; Dalal, 2021). In pome fruit species, crop management techniques have been developed to reduce irrigation needs and save water through the shading effect (Mpelasoka et al., 2001; Lopez et al., 2018). However, there are mild, moderate, and severe water stress levels, which make it necessary to understand the mechanisms of resistance of plants to water stresses (Imadi et al., 2016). Since WRKYs not only regulate stress and developmental responses, but are also involved in specialized metabolic pathways, they interact in the network of TFs (ERF, MYB, MYC, and NAC) that regulate various responses (Phukan et al., 2016). In this review of water stress in Pome species, the term drought and flooding will be slightly different due to physiological and biochemical changes, but they will be indicated as water stress due to the different resistance or response functions of WRKYs (Table 2).

The response mechanisms of *M. domestica* gene expression when receiving an environmental signal due to water stress can be positively or negatively regulated, and are likely candidates for future complementary studies. In this case, the overexpression of *MdWRKY13* seems to act as a negative regulator in the response to water stress; this is because it can act as an activator of genes (*AtP5CS* and *AtProDH*) involved in proline degradation, acting as a repressor for plant resistance to water stress (Duan et al., 2014). On the other hand, the overexpression of *MdWRKY31* in roots and callus induced by ABA in response to drought protects plants against damage; this happens because *MdWRKY31* suppresses the expression of the *MdRAV1* gene promoter (Zhao et al., 2019c).

Table 2 WRKYs transcription factors in the regulation of abiotic stress expression in pome species (*Malus* spp. and *Pyrus* spp.)

Species	Protein	Group	Regulation	Mode of expression	Function	ID/Access #	References
M. baccata	*MbWRKY1*	II-a	Positive	Overexpression	Hydrical stress	XP_008392469.1	Han et al. (2018a)
M. baccata	*MbWRKY3*	II-a	Positive	Expression	Hydrical stress	MDP0000263961	Han et al. (2018e)
M. baccata	*MbWRKY2*	II-a	Positive	Expression	Hydrical stress	XP_008392469.1	Han et al. (2018d)
M. baccata	*MbWRKY5*	I	Positive	Overexpression	Hydrical stress	MDP0000514115	Han et al. (2018c)
M. domestica	*MdWRKY13*	II-c	Negative	Expression	Hydrical stress	HM122716.1	Duan et al. (2014)
M. domestica	*MdWRKY20*	IV				MDP0000145953	
M. domestica	*MdWRKY22*	IIb				MDP0000146390	
M. domestica	*MdWRKY40*	IV				MDP0000204606	
M. domestica	*MdWRKY47*	Iid	Probable	Expression	Hydrical stress	MDP0000231668	Meng et al. (2016)
M. domestica	*MdWRKY53*	Iib				MDP0000253189	
M. domestica	*MdWRKY77*	III				MDP0000299114	
M. domestica	*MdWRKY90*	Iic				MDP0000418900	
M. domestica	*MdWRKY91*	I				MDP0000431358	
M. domestica	*MdWRKY100*	I				MDP0000514115	
M. domestica	*MdWRKY125*	IIa				MDP0000935652	
M. domestica	*MdWRKY31*	I	Positive	Expression	Drought and saline stress	MD05G1349800	Zhao et al. (2019c)
P. bretschneideri	*PbWRKY81*	III	Positive	Expression	Hydrical stress	Pbr001425	Huang et al. (2015)
P. betulaefolia	*PbrWRKY53*	III	Positive	Expression	Hydrical stress	Pbr018725.1	Liu et al. (2019a, b)
M. baccata	*MbWRKY4*	I	Positive	Expression	Saline stress	MDP0000507805	Han et al. (2018b)
M. xiaojinensis	*MxWRKY55*	IIc	Positive	Expression	Saline stress	MDP0000119590	Han et al. (2020)
M. hupehensis	*MhWRKY*	II-c	Positive	Expression	Saline stress	MDP0000774288	Li et al. (2021)
M. domestica	*MdWRKY18*	II-b	Probable	Overexpression	Saline stress	MdWRKY18F	HaiFeng et al. (2018)
M. domestica	*MdWRKY40*	II-b	Probable	Overexpression	Saline stress	MdWRKY18R	HaiFeng et al. (2018)
M. domestica	*MdWRKY100*	I	Positive	Overexpression	Saline stress	DQ341381	Ma et al. (2020)
M. domestica	*MdWRKY30*	IIa	Positive	Overexpression	Saline stress	MH150910	Dong et al. (2020)
M. domestica	*MdWRKY70/23//40/30/3840like/51/61/61like*		Probable	Expression	Participates in drought tolerance		Gao et al. (2020)
M. xiaojinensis	*MxWRKY64*	II-c	Probable	Expression	Response to excess iron and salinity	MDP0000256514	Han et al. (2021)
M. domestica	*MdWRKY17*		Positive	Expression	Hydrical stress by water deficit	Md12G1181000	Shan et al. (2021)

However, the expression of 10 (20/22/40/47/53/77/90/91/100/125) of the 127 MdWRKYs denotes their possible involvement with water stress responses. These 10 MdWRKYs were identified in the transcriptional study of the entire genome of calli of the apple rootstock G-41 (Meng et al., 2016). Therefore, here we evidence promising uses for *M. domestica* WRKYs, especially as candidates in the increase to drought tolerance. In *Malus baccata*, the expression of WRKYs can help the adaption to water stress, since *MbWRKY1* is involved in drought tolerance by interacting with other factors in the promotion of the expression of responsive genes (*NtP5CS* and *NtLEA5*). In *M. baccata* seedlings, *MbWRKY1* can act in dehydration, salinity, and ABA treatment responses (Han et al., 2018a). *MbWRKY2* expression is also involved in drought tolerance, activating antioxidant enzymes related to ROS response, osmotic adjustment, and membrane protection, in addition its coordinated action with other factors (Han et al., 2018c). *MbWRKY3* responds

to drought, salt, and ABA treatments, being also correlated with activation of antioxidant enzymes (Han et al., 2018d). The expression level of *MbWRKY5* increases in response to salt, heat, cold, drought, and ABA, but it improves tolerance to drought and salt (Han et al., 2019). However, the overexpression of WRKYs in *M. baccata* plants did not affect their phenotypes, differently of what occurs with tobacco, which increases the expression levels of stress-related genes involved in the oxidative stress response (NtPOD, NtSOD, and NtCAT) and membrane protection (*NtLEA5*, *NtERD10D*, and *NtP5CS*).

In *M. domestica* tolerance to drought, dopamine can affect the expression of WRKYs (MdWR KY70/23/30/38/40/40like/51/61/61like), ERF, and NAC (Gao et al., 2020). Also, the regulatory pathway MdMEK2–MdMPK6–MdWRKY17–MdSUFB stabilizes chlorophyll levels under moderate water stress and could facilitate the cultivation of different apple varieties, maintaining high yields even when plants are under water stress (Shan et al., 2021). The expression of WRKYs from *P. bretschneideri* also shows positive responses to water stress. *PbrWRKY53* is highly regulated by drought and abscisic acid (ABA), but slightly induced by salt and cold, and performance is positive in drought tolerance which is in part promoted by vitamin C through regulation of *PbrNCED1* expression (Liu et al., 2019b).

However, there are 103 candidates of *P. bretschneideri* WRKYs that provide a solid basis for future work on functional experimentation with water stress, but the group III *PbWRKY81* (Pbr001425) highlights by presenting a positive and significant selection in the analysis of adaptive evolution of chromosome 12 (Huang et al., 2015). Therefore, the expressions of WRKYs in pome fruit species as response mechanisms to water stress provide evidence of adaptive signaling by plants when interacting with water stress.

Salt stress

Salt stress limits plant growth, development, and crop yields, causing high concentrations of NaCl and KCl salts in the soil. This accumulation is daily increasing in arable lands (Shrivastava and Kumar, 2015; Ma et al., 2021). Plant responses to NaCl stress are to reject sodium and absorb potassium to maintain the Na + /K + balance in the cytoplasm as a nutrient element in plants (Zhu, 2016). But in pome fruit trees, the application of fertilizers in large quantities for high productivity and quality generates salt stress, a phenomenon that damages the soil structure and limits the sustainable development of *M. domestica* (Jia et al., 2020). However, there are studies on the tolerance of apple calli to salinity and ABA due

to the overexpression of *MdCML3* responsive genes (Li et al., 2019a), in addition to transcriptomic analyzes that provide insights into the stress response due to high salinity in apple (Li et al., 2019b)**.** Also, in *Pyrus betulaefolia*, salt stress affects normal growth in production and quality due to osmotic effects and related to dehydration due to excess accumulation of Na^+ in plant cells (Li et al., 2017), which become evident in the leaves due to the concentration of salt (Yu et al., 2019).

Nevertheless, the overexpression of the *PbrNHX2* genes results in salt stress tolerance through modulation of ROS levels (Dong et al., 2019). In addition, expression profiles indicate that high salinity stress induces the expression of demethylase genes (DMEs) that change the methylation levels of salt-responsive genes (Liu et al., 2018). Therefore, there is evidence of tolerance to salt stress due to different transcriptional response mechanisms of the pome fruit trees. Thus, in this review, we will present the responses of WRKYs and how they interact with mineral salts, phytohormones, and proteins.

In *M. domestica*, *MdWRKY100* overexpression increases salt tolerance through microRNA modulation of target genes (miR156/SPL), since SPL genes play important roles in embryogenesis, morphogenesis, cycle stage modifications, flower formation, and other processes (Ma et al., 2021). Also, the overexpression of *MdWRKY30* induced by salt and water stress has positive effects on salt stress and osmotic tolerance of apple calli, but *MdWRKY30* interacts with *MdWRKY26* and *MdWRKY28* to form hetero- and homodimers (Dong et al., 2020). However, studies have been promising to help the discovery of WRKYs that act on toleration of high levels of stresses. The expression of *MdWRKY18* and *MdWRKY40* in *M. domestica* is likely to participate in salt stress, and the overexpression in the calli of cv. Orin can increase tolerance to salt stress (HaiFeng et al., 2018). On the contrary, treatments of low temperature (4 °C) and salt stress (100 μmol L^{-1} NaCl) suppressed the expression of *MdWRKY31* but caused the upregulation of *MdWRKY31* in roots and increased calli sensitivity to ABA, while the repression of *MdWRKY31* reduced the sensitivity to ABA in “Royal Gala” seedling roots; this happens by binding to the *MdRAV1* promoter, mediating ABA sensitivity (Zhao et al., 2019c).

In other pome fruit species, there are interesting information about WRKYs role in salt stress resistance: in *M. baccata* rootstocks, *MbWRKY4* expression is positive in response to high salinity, when induced in high salt concentration in comparison to drought and ABA treatments (Han et al., 2018b). In *Malus hupehensis* rootstock, which is widely used in

China, salt stress caused by potassium chloride (KCl) is relieved by the antioxidant resveratrol (RES), which regulates K^+/Na^+ homeostasis in seedlings, helping osmotic adjustment, and elimination of ROS (Li et al. 2021).

However, the expressions of *MhWRKY28* and *MhMYB39* have an important role in the KCl and RES signaling transduction pathway (Li et al., 2021). *MxWRKY55* (*Malus xiaojinensis*) expression is induced by salt and iron generating a positive effect in salt stress, as well as for low or high Fe content in soil (Han et al., 2020). *MxWRKY64* expression is affected by iron and salt stress in *M. xiaojinensis* seedlings. Also, the overexpression of *MxWRKY64* in transgenic *A. thaliana* improved its tolerance to iron and salt stress (Han et al., 2021). However, microsatellites or single sequence repeats (SSR) of the gene were identified and mapped for future genetic improvement studies in pome species (supplementary S3). Therefore, WRKYs have transcriptional responses to salt stress interacting with phytohormones, mineral salts, and proteins by tolerating the accumulation of salinity in pome fruit trees.

3.7 Other functions

The other functions of WRKY transcription factors that are involved in the biosynthesis of anthocyanins, flavonoids, oxidative stress, fruit flavor alteration, fruit ripening, rootstock dwarfism, and others are described in this topic; however, the complementary information is presented in the supplementary document (supplementary S4).

Oxidative stress

Abiotic stress can generate uncontrolled production of ROS and reactive nitrogen species (RNS), which oxidatively modify different biomolecules (proteins, lipids, and nucleic acids), causing different types of cell damage in a process we call nitro-oxidative stress (Chaki et al., 2020). However, for this review, oxidative stress will be defined not only as an excessive production of ROS that cannot be neutralized by the action of antioxidants, but also as a disturbance in the cellular redox balance, in addition to structural modifications and modulation of functions in acids nucleic acids, lipids, and proteins (Pisoschi and Pop, 2015).

There is evidence that pome fruits (*M. domestica*) suffer from oxidative stress when stored at normal environmental conditions, where there is a disturbance in the fruit surface due to the synthesis of α-farnesene (Whitaker, 2004). This oxidation occurs quickly after 6–8 weeks, but the biochemical and genetic mechanisms are still unknown (Whitaker, 2004).

Thus, some advances in the responses of WRKYs to oxidative stress of pome fruit species are presented.

In *M. domestica*, *MdWRKY33* and *MdWRKY23* participate in the ripening of the fruit, when induced by the lower concentration of oxygen (0.4 kPa), in addition to the expression of other WRKY genes (*MDP0000134105, MDP0000127976*) (Cukrov et al., 2016). Also, in *Malus prunifolia*, the overexpression of *MpWRKY6a* and *MpWRKY6b* homologs can help the fruit to handle oxidative stress through MeJA or ABA signaling; it happens after exposing the plant to low nitrogen (N), inorganic phosphorus (Pi), and methyl viologen (MV) which are detected in tissues of roots and leaves (Wang et al., 2017a). However, the expression of *PuWRKY56* in *P. ussuriensis* participates in the acceleration of fruit ripening, which is mainly promoted by methylcyclopropene (1-MCP) which is an inhibitor of ethylene action in plant cells, thus regulating oxidation of the fruits (Huang et al., 2014). *Cydonia oblonga* and *P. communis* rootstocks can activate the antioxidant defense system due incompatibility; it causes the transcription levels of six antioxidant genes, but no WRKY seems to be related with this process (Irisarri et al., 2015). Even though these genes seem not to be involved in antioxidant response due to rootstock incompatibility, WRKYs participate in response to oxidative stress through induction by low concentrations of oxygen, or nitrogen and use of ethylene inhibitors in pome fruits and other organs.

Biosynthesis of anthocyanins and flavonoids

Anthocyanins are plant pigments that contribute to color of leaves, flowers, and fruits, also serving as antioxidants with good effects on human health (Yang et al., 2018). Ultraviolet-B (UV-B) radiation promotes the synthesis of anthocyanins in many plants and several transcription factors respond to UV-B radiation. The expression of *MdWRKY72* in *M. domestica* increases the synthesis of anthocyanins through both direct and indirect mechanisms when induced by UV-B radiation (Hu et al., 2020). In *M. sylvestris*, the genes *MscWRKY12* and *MscWRKY19* are involved in leaf pigmentation during the development of this organ (Yang et al., 2018).

However, red skin *Pyrus* spp. fruits accumulate anthocyanin, and these genes can contribute to the improvement of their appearance. The interaction of *PyWRKY26* and *PybHLH3* could co-direct the *PyMYB114* promoter to generate anthocyanin accumulation in red pears (Li et al., 2020a). *MdWRKY11* overexpression plays a role in anthocyanin synthesis in red *M. domestica* fruits (Liu et al., 2019a). Eight PcWRKYs also participate in

the development of color in red *P. communis* fruits, and color fading in some fruits is due to the reduction of anthocyanin biosynthesis, increased anthocyanin degradation, and suppression of anthocyanin transport (Wang et al., 2017b). Furthermore, the participation of five WRKYs (PcWRKY9/33/17/51/40) together with an AP2 TF regulates anthocyanin biosynthesis of redskinned fruits, where the ANR (anthocyanidin reductase) and LAR (leucoanthocyanidin reductase) genes promote the proanthocyanidin (PA) biosynthesis contributing to the green skin variant (Yang et al., 2015). Thus, the accumulation of anthocyanins contributes to fruit coloration and its synthesis is induced through the interaction of trans and cis elements.

Flavonoids are important secondary metabolites that contribute to the nutritional quality of many foods (Wang et al., 2018) and to tolerance against different diseases (Li et al., 2020b). The overexpression of *MdWRKY11* upregulates other genes (F3H, FLS, DFR, ANS, and UFGT) that have influence over flavonoid biosynthesis and can be important for breeding of red *M. domestica* fruits, not only for its influence in fruit appearance but also for effective mitigation of impacts caused by ROS (Wang et al., 2018). The participation of *McWRKY6*, *McWRKY75*, and *McWRKY7* from *Malus crabapple* is associated in the regulatory network of flavonoid biosynthesis during fruit development (Li et al., 2020b). Also, the expression of five PcWRKYs participates in the transcriptional regulation of flavonoid biosynthesis induced by methyl jasmonate in pear calli (Premathilake et al., 2020).

Rootstock dwarfism

Dwarf apple (*M. domestica*) rootstocks are widely used due to their marked effects on precocity and growth control; still the genetic mechanisms acting on these traits are poorly understood (Yang et al., 2020). However, there are recent advances in the comprehension about the participation of pomWRKYs in rootstock responses. The overexpression of *MdWRKY9* stands out and is involved in dwarfism trait of M26 rootstock, directly inhibiting *MdDWF4* transcription and reducing brasinosteroid (BR) production, a common mechanism seen in woody plants (Zheng et al., 2018). Also, the expression of *MdWRKY2* found in the root creates an increase in the expression of *MdLBD3* (lateral organ boundaries domain 3) that indirectly refutes the level of rootstock dwarfism and the growth of the morphological formation of the plant (Yang et al., 2020). In dwarf pear (*P. communis*) rootstocks, the expression of four transcription factors *PcWRKY33/31/40/18* is related to root responses to moisture and abscisic acid signaling (Ou et al., 2015). However, in the vegetative propagation of rootstock, the expression of *MdWRKY1* is likely to participate in the

development of adventitious root and in the number of secondary roots of *M. domestic* (Moriya et al., 2015).

Fruit development

In the development of pome fruits, there are changes in flavor, sugar content, and ripening of the fruit. The expression of five TFs *MdWRKY23*, *MdWRKY17*, *MdWRKY46*, *MdWRKY48*, and *MdWRKY71* in *M. domestica* participates positively in the change of fruit flavor by the accumulation of hexanal and ethyl acetate by regulating gene expression via lipoxygenase that makes the biosynthesis of volatile organic compounds after 2 to 6 days of fruit storage with mechanical damage (Lin et al., 2021). Also, fruit ripening involves delicate changes in its metabolic and physiological traits through the wellorganized synchronization of various hormones and regulatory steps, but there is expression of *MdWRKY40*, *MdWRKY6*, and *MdWRKY19* which participate in pre- and post-ripening hormonal regulation and anthocyanin biosynthesis in ripening pseudofruits (Onik et al., 2018). Sucrose levels in *P. ussuriensis* fruits can be elevated by expression of *PuWRKY31* by binding to the *PUSEET15* promoter (Li et al., 2020d).

The ripening and senescence of *P. pyrifolia* fruits were regulated by salicylic acid treatment, where the expression of *PpWRKY18* was upregulated by 12 and 24 h of salicylic acid treatments (Shi et al., 2021). However, the expression of MdWRKY (GU013683) does not affect the development of the fruit of *M. domestica*, as it has a low correlation with the gene *MdAAT2* (*alcohol acyltransferase*) that plays an important role in the biogenesis of the ester in the ripening of fruits when being induced by phytohormones (ethylene, SA, methyl jasmonate) (Li et al., 2012). Thus, the participation of pomWRKYs in the regulation of changes in flavor, sugar content, and ripening occurs through interaction with other transcription factors and plant hormones.

The diversity of functions of WRKYs in pome fruit species also includes participation in development, defense, resistance to ozone stress, regulation in the vegetative phase, and in the absence of seeds in the fruit. In the structural analysis of MdVQ proteins from *M. domestica*, MdWRKY proteins from group I (*MdWRKY4* and *MdWRKY19*) and subgroup IIc (*MdWRKY52* and *MdWRKY68*) interact with MdVQ genes in the cellular nucleus of leaves, for their possible regulation in the apple development and defense mechanisms (Dong et al., 2018). *McWRKY75* expression participates positively in the stress of ozone (O_3) which is an oxidizing toxic atmospheric pollutant in *M. crabapple* leaves and exogenous methyl

jasmonate (MeJA) decreased the harmful effects of O_3, activating the flavonoid metabolic pathway in *Malus* spp., improving its resistance to O_3 stress (Wu et al., 2020). Differences in the expression and methylation of TFs determine the transition from juvenile to adult leaves in *Malus hupehensis*. However, the methylation levels of 22 WRKYs and other genes were higher in older leaves (Xing et al., 2020). The expression of 41 TFs, among them the WRKYs, seems to be determinant in seedless trait of *P. communis* fruits (Liu et al., 2020). In this sense, the diversity of WRKY roles depends on their interaction with other TFs and plant hormones.

3.8 Conclusions and future perspectives

Here, we provide a brief overview about pomWRKYs structure and classification in 14 species. The classification is based on the number of domains dividing these into four groups I, II (a, b, c, d, e), III, and IV, according to changes in the WRKYGQK heptapeptide and particular C_2H_2 C_2HC zinc finger features. The structural and functional annotation of pomWRKY presents oppositions on gene name and group classification regarding to what was found in *A. thaliana* by Eulgem et al. (2000). According to the observations, pomWRKYs are probably very important in the transcriptional regulation of genes involved in defense to pathogens, in abiotic stress adaption, organogenesis, and plant development. The expressions and overexpressions of pomWRKYs make positive, negative, or probable regulation when interacting with phytohormones and other trans and *cis* elements, in addition to being induced by microbiological treatments, mineral salts, and environmental stimuli at different stages of fruit and plant development.

Further studies aiming to identify and characterize these pomWRKYs in newly available genomes are an alternative to find answers about the evolution of this useful superfamily. Also, comparative genomic and epigenomic studies could provide inferences about the functionality of these genes, which will facilitate their application in response to many of the problems discussed here.

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4 ARTIGO 2: WRKY Genes in black raspberry (*Rubus occidentalis* L.): duplicate and conquer

*Manuscrito submetido ao periódico Functional & Integrative Genomics (Qualis A2)

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Abstract

WRKY transcription factors regulate several biological processes in plants, including responses to biotic stresses and tolerance to abiotic stresses, and are part of a gene superfamily in higher plants. There are many studies on the functions of WRKY proteins in several model species, aiming at identification and functional characterization, but there has not yet been a comprehensive analysis of the RoWRKY protein family in black raspberry (*Rubus occidentalis* L.) as shown here. In this study, the investigation of the complete genome of the black raspberry identified 62 *RoWRKY* genes that were evaluated and are unevenly distributed in all seven chromosomes. The proteins encoded by these genes were classified into four groups (I, II, III and IV), with those of group II divided into five subgroups (IIa - IIe) based on their conserved domains and zinc finger domain types. Motif analysis showed that all *RoWRKY*s contained one or two WRKY domains and that proteins from the same group had similar motif compositions. Five pairs of *RoWRKY* genes in segmental duplication and two pairs in tandem duplication were detected. The genetic divergence of the duplications occurs 29.02 million years ago. Analysis of the structure of *RoWRKY* genes showed that they have 1-11 introns, with most *RoWRKY* genes consisting of two introns and three exons. A cis element analysis showed that all promoters of the *RoWRKYs* genes contain at least one cis stress-response element. Differential expression analysis of 10 samples of RNA-seq data, reviewed *RoWRKY* genes from black raspberry, show preferential or specific expression in tissue samples. These findings provide a complete overview of the evolution and modification of the RoWRKYs protein family, which will help the functional characterization of these proteins in the response to biotic and abiotic stresses of black raspberry.

Keywords: phylogeny, gene structure, subcellular location, expression regulation, differential expression

4.1 Introduction

The black raspberry (*Rubus occidentalis* L.) is native to eastern North America. The species is diploid (2n = 2x = 14) of the subgenus Idaeobatus (VanBuren et al. 2018a). Breeding of this species began in the early 21st century at the USDA ARS New York State Agricultural Experiment Station in Corvallis, OR and in Beltsville, MD, and with New Zealand HortResearch Inc (Jennings 2018). Molecular improvement for fruit traits was hampered by the lack of a high quality reference genome (Jibran et al. 2018). However, an almost complete chromosome-scale assembly of the black raspberry genome is now available (VanBuren et al. 2018b). The updated high-quality reference genome in black raspberry allows for comparative genomic studies and gene analyses, including those encoding transcription factors (TF) (Jiang et al. 2021). It also enables the application of marker-assisted breeding and genomic selection.

The high content of anthocyanins and ellagitannins present in black raspberry are important in human health due to anticancer activity, which has led to an increase in the production of the species in the last 15 years (Kula and Krauze-Baranowska 2016; Bushakra et al. 2018). In commercial production of black raspberry, there are disease-causing organisms, such as the aphid *Amphorophora agathonica* Hottes, which is a vector of the raspberry mosaic virus complex (Bushakra et al. 2015). In addition to this biotic factor, abiotic factors impact the species, such as the high temperatures that occur in the natural temperate habitats of black raspberry, requiring it to tolerate or adapt to local environmental conditions of production (Bradish et al. 2016). The interactions of the multiple biotic and abiotic stresses affecting plants need to be understood, from the molecular mechanisms of stress response and tolerance, to identifying and characterizing the genetic components such as the transcription factors involved in these mechanisms, including WRKYs, which mediate the stress response in different species (Yoon et al. 2020).

The FT WRKYs superfamily plays a significant role in various biotic and abiotic stress responses (Meng et al. 2016). In apple (Malus x domestica) MdWRKY40a and MdWRKY54h play negative roles in the defense against infection by the B. dothidea pathogen. On the other hand, MdWRKY40, MdWRKY60 and MdWKRY33s may play important roles in the response to pathogens and are conserved in some angiosperm plants (Zhang et al. 2021). In the pear (*Pyrus ussuriensis*), PuWRKY31 induces sucrose levels in the fruit (Li et al. 2020b). WRKYs are considered defenders in *Oryza sativa* ssp. *japonica*,

Populus trichocarpa, *Arabidopsis thaliana*, and *Physcomitrella patens* (He et al., 2010). It is evident that the WRKY superfamily plays an important role in the plant responses to and tolerate stress through molecular signaling mechanisms.

The primary structure of FT WRKYs is composed of the WRKY domain and the zinc finger motif, a 60 amino acid region that is highly conserved by family members. These proteins are transcriptional regulatory factors with preferential binding to the cis W-box element and have the potential to regulate the expression of a variety of target genes (Eulgem et al. 2000). The classification of the superfamily depends on the number of domains and their characteristic zinc finger motif, leading to a subclassification of three subfamilies or groups. Group I is composed of TFs that have two WRKY domains, while group II (II-a, b, c, d, e) and III contain TFs with only one WRKY domain. However, group I and II TFs have the same zinc finger patterns C2 - H2 (C - X4–5 - C - X22–23 - H - X1 – H) and group III TFs have a zinc finger motif C2-HC binder (C - X7 - C - X23 - H - X1 – C) pattern (Eulgem et al. 2000; Li et al. 2020a).

The evolution of FT WRKYs in plants started from a structure similar to the zinc finger motif, and lateral gene transfer between plants and fungi may have taken them outside the plant kingdom. Also, it is possible to transfer WRKY genes from algae to a primitive fungus (Rinerson et al. 2015). By portions of the zinc finger motif, the proteins WRKY, GCm1, and flYWCH likely share a common ancestor in evolution (Rinerson et al. 2016).

The multiple functions of transcription factors, including WRKYs, can simultaneously control several pathways in plants subjected to stress conditions, becoming a potential tool for stress tolerance manipulation (Hrmova and Hussain 2021). Therefore, it is important to identify the WRKY proteins, as well as their subcellular and chromosomal gene location, in addition to the phylogenetic analysis, classification and identification of conserved motifs in the complete genome of the black raspberry (Rubus occidentalis). This characterization will provide a complete overview of the evolution and modification of the WRKY protein family and will help to determine the functional features of black raspberry *RoWRKY* genes in response to biotic and abiotic stresses.

4.2 Materials and methods

Identification of WRKY proteins in the black raspberry genome

The entire genome sequence of the black raspberry (Rubus occidentalis Whole Genome v3.0 Assembly & Annotation) was downloaded from the Rosaceae Genomes Database (GDR)) (VanBuren et al. 2018b). To assemble the possible candidate amino acid sequences of black raspberry WRKY, the HMM model of the WRKY domain (PF03106) was downloaded from Pfam (http://pfam.xfam.org/family/PF03106/hmm) (Finn et al. 2014). Hmmer software (Finn et al. 2011) was also used for similarity research in the annotated proteins in black raspberry using $1e\text{-}^{3}$ as the upper limit of the e-value. Also, BLASTp were performed with WRKY sequences from Arabidopsis thaliana (Rushton et al. 2010) with a value of e^{-10} on the black raspberry proteome. All protein sequences obtained were examined for the presence of the WRKY domain using the Web CD-Search Tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Lu et al. 2020). The ExPASY ProtParam (https://web. expasy.org/protparam/) (Gasteiger et al. 2003) was used to predict the isoelectric point (pI), molecular weight (MW) and grand mean hydrophobicity (GRAVY) of each RoWRKY.

Subcellular localization of WRKY family transcription factors

Subcellular localization was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/) (Horton et al. 2007) and TargetP-2.0 (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2007).

Analysis of cis elements in RoWRKY gene promoters

For each *RoWRKY* gene, a 2,000 bp sequence upstream of the start codon was retrieved from the black raspberry genome (*Robus occidentalis* Genome v3.0 Assembly) by applying integrative genomics visualization -IGV (Robinson et al. 2011). This sequence was submitted to the PlantCARE website to investigate cis-acting regulatory elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002).

Multiple alignment and phylogenetic analyses

A phylogenetic tree was constructed to compare black raspberry WRKY proteins. Multiple alignment of WRKY protein sequences was performed with ClustalW software using standard parameters (Larkin et al. 2007). The phylogenetic tree was constructed using BEAST v.2.5 software (Bouckaert et al. 2019), with the UPGMA clustering method (Sneath

and Sokal 1973). A bootstrap analysis was conducted using 10,000,000 replicates and evolutionary distances were calculated using the JTT matrix-based method (Jones et al. 1992).

Analysis of gene structure and identification of conservation of motifs

To investigate the diversity and structure of *RoWRKY* family members, genomic sequences for their exon/intron were used and plotted on TBtools (Chen et al. 2020a), based on black raspberry genome annotation information (*Rubus occidentalis* whole genome assembly v3.0) RoWRKY protein sequences were used to identify conserved motifs, Expectation Maximization Tool for Motive Elicitation MEME (https://meme-suite.org/meme/tools/meme) (Bailey et al. 2006). The parameters were as follows: number of repetitions: any; maximum number of reasons: 10; and optimal motif widths: 8 to 50 amino acid residues. The functions and locations of the reasons were queried in the databases MOTIF Search (https://www.genome.jp/tools/motif/) (Kanehisa and Goto 2000) and ELM (http://elm.eu.org/) (Kumar et al. 2022), a resource of the Eukaryotic Linear Motif for functional protein sites.

Chromosomal localization, gene duplication Ka/Ks calculation and divergence time estimation

The chromosomal location image of the RoWRKY genes was generated by the TBtools software (Chen et al. 2020a), according to chromosomal position information provided in the genomic database for Rosaceae-GDR (https://www. rosaceae.org). To identify tandem and segmental duplications, two genes of the same species, located in the same clade of the phylogenetic tree, were defined as co-paralogs. The GDR browser was consulted in order to detect segmental duplication of the target genes (VanBuren et al. 2018b). The local alignment of two protein sequences was calculated using the Smith-Waterman algorithm (http://www.ebi.ac .uk/Tools/psa/).

For the calculation of non-synonymous substitutions per site (Ka) and the number of synonymous substitutions per synonymous site (Ks), in addition to comparing the selection pressure, a Ka/Ks ratio greater than 1, less than 1 and equal to 1 represents selection positive, negative selection and neutral selection, respectively (Doerks et al., 2002). For each pair of genes, the value of Ks was used to estimate the time of divergence in millions of years based on a rate of 6.1×10^{-9} replacements per site per year, and the time of divergence (T) was calculated as $T = Ks/ (2 \times 6.1 \times 10^{-9}) \times 10^{-6}$ million years ago (Mya)

(Lynch and Conery, 2000). The bioinformatics tool used for genetic divergence was the simple Ka/Ks Calculator (NG) from TBtools -II (Chen et al., 2020).

Transcriptomic analysis of *RoWRKY* genes in black raspberry

The expression patterns of RoWRKYs genes were analyzed based on published RNA-seq data on NCBI BioProject ID PRJNA430858 (VanBuren et al. 2018b). For the analysis of differential expression, 10 samples of RNA-seq data were collected that were sequenced by the Illumina HiSeq4000, which are: canes (SRR7274864), root (SRR7274865), young leaf (SRR7274866), mature leaf (SRR7274867), red fruit (SRR7274868), ripened fruit (SRR7274869), flower bud (SRR7274870), green fruit (SRR7274871), leaf of post 24hr jasmonate spraying (SRR7274872), leaf of post 48hr jasmonate spraying (SRR7274873).

Data processing was performed in three steps. a) quality control and adjustment of samples: SRA toolkit (Leinonen et al. 2011) was used to download the data samples, FastQC (Wingett and Andrews 2018) was employed to analyze and visualize the quality of readings, Trimmomatic ver. 0.39 (Bolger et al. 2014) was applied to remove the low quality and library adaptors; b) The reads were mapped against the Black raspberry (*R. occidentalis* v3.0) reference genome (VanBuren et al. 2018b) using the software STAR (Dobin et al. 2013). In the next step, (c) for the counting of total reads aligned by gene in the different libraries, FeatureCounts (Liao et al. 2014) was used.

The quantification (d) was performed using the packages limma, EdgerR and DESeq2 in the R software (Law et al. 2018). In this protocol, a CPM normalization method (counts per million) was used, which are counts scaled by the total number of reads and the expression of *RoWRKYs* genes per library. A heatmap was produced using TBTools (Chen et al. 2020a), then a multidimensional scale graph (MDS) was generated to verify the repeatability of the sample and the general difference between the samples, a graph of sample mean variance (MeanVar) and Biological Variation Coefficient (BCV) as a function of gene abundance (in log2 counts per million).

4.3 Results

Identification of the WRKY protein family in black raspberry

The statistical alignment of the Hidden Markov Model (HMMsearch) in HMMER and the BLASTp local alignment of the black raspberry proteome, was used to identify a total of 62

WRKY transcription factors in this species, and 59 *RoWRKY* genes were characterized with complete heptapeptide (WRKYGQK) domains and based on their nomenclature of the WRKY protein family (PF03106) (Table 1). Three sequences without a complete heptapeptide domain (WRKYGQK), including variations in the heptapeptides (WGKYGQM, WGKYCQM and WGKYGVM) were discarded and not analyzed. The length of the peptide ranged from 170 amino acid residues (aa) (RoWRKY49) to 1,572 aa (RoWRKY24). The coding sequences (CDS) ranged from 513 to 4,887 nucleotides. The molecular weight of the predicted proteins ranged from 18,648.66 (RoWRKY49) to 179,205.2 kDA (RoWRKY24). The isoelectric point ranged from 4.75 (RoWRKY58) to 9.96 (*RoWRKY27*). The large mean hydrophobicity ranged from -0.26(RoWRKY55) to -1.18 (RoWRKY33).

Subcellular localization of the RoWRKY protein family

Wolf SORT evaluation revealed that within the 59 RoWRKY proteins, all have transport sequences targeting the nucleus, 26 the chloroplast, 15 the cytosol, 9 the plasma membrane, 8 the cytoskeleton, 7 the vacuolar membrane, 5 the mitochondria, 5 have extracellular targets, 3 the cytosol-nucleus, 2 the peroxisome, 1 the cytoskeleton-nucleus, 1 the endoplasmic reticulum and none the Golgi complex (Supplementary S1). TargetP analyses revealed that 4 RoWRKY proteins present transport sequences targeted to mitochondria (RoWRKY12=0.01; RoWRKY21=0.29; RoWRKY24 =0.08; RoWRKY55 = 0.029), 6 to chloroplast (RoWRKY6=0.02; RoWRKY12= 0.04; RoWRKY21 =0.01, RoWRKY24=0.12; RoWRKY55=0.0014; RoWRKY57=0.001), 3 to thylakoid luminal transit peptide (RoWRKY7=0.01; RoWRKY26=0.02; RoWRKY=0.001), 2 signal peptide in the secretory pathways of the cell (RoWRKY6=0.03; RoWRKY57=0.007) and 59 in other compartments, where values indicate score (0.00-1.00) and reliability class (1-5; the best class is 1).

Analysis of cis elements in *RoWRKY* gene promoters

In this study, a total of 91 types of cis regulatory elements were identified in the promoter regions of RoWRKYs (Supplementary S2). Among the cis elements found, the elements related to A-Box, CAAT-box (common in promoter and enhancer regions), TATA-box (around -30 from the beginning of transcription), HD-Zip 1 (element involved in the differentiation of palisade mesophyll cells), HD-Zip 3 (protein binding site) and W-box (WRKY transcription factor binding site), which was identified in 41 promoters of *RoWRKY* genes.

Table 1. Information on *RoWRKYs* genes in the black raspberry genome

Transcript ID	*Gene name*	*Group*	*Chr.*	*StartPos*	*EndPos*	*Strand*	*CDS*	*aa*	*MW*	*pI*	*GRAVY*
Ro01_G04143	*RoWRKY1*	IId	1	8004614	8006199	-	1361	320	34741.1584	9.650610924	-0.484375
Ro01_G05057	*RoWRKY2*	I	1	486462	491212	-	1954	521	57813.4867	5.52670536	-0.85431861
Ro01_G11245	*RoWRKY3*	IIb	1	21623505	21626419	-	1836	456	49593.0792	7.005485344	-0.57039473
Ro01_G11672	*RoWRKY4*	IIc	1	12667509	12669853	-	672	223	25066.263	5.71398983	-0.83856502
Ro01_G14731	*RoWRKY5*	IIc	1	13729330	13731052	-	1152	313	34833.4001	5.932592583	-0.82971246
Ro01_G29921	*RoWRKY6*	IIc	1	12864681	12868801	+	983	187	21736.4665	9.41781559	-0.80588235
Ro02_G00571	*RoWRKY7*	IId	2	3604411	3606236	-	1620	325	35382.8985	9.526380348	-0.52984615
Ro02_G00778	*RoWRKY8*	IId	2	2276033	2278868	-	2385	337	36622.19	9.510843468	-0.49732937
Ro02_G02477	*RoWRKY9*	IIa	2	201460	203052	-	1415	282	31663.0201	8.819355583	-0.77801418
Ro02_G03986	*RoWRKY10*	IId	2	9397448	9400257	+	1154	271	30042.7428	9.627789116	-0.72804428
Ro02_G34805	*RoWRKY11*	IIa	2	214719	216547	-	1437	355	38949.3513	6.94552021	-0.55042253
Ro02_G35659	*RoWRKY12*	III	2	30494983	30497277	-	1896	631	70604.8016	5.874503136	-0.39334389
Ro02_G35662	*RoWRKY13*	III	2	30492753	30493624	-	585	194	22223.8089	6.515703773	-0.80927835
Ro03_G05285	*RoWRKY14*	IIe	3	40023145	40025358	-	2024	276	30439.28	5.251832008	-0.77246376
Ro03_G10658	*RoWRKY15*	I	3	3721286	3724842	+	2124	693	76270.1518	8.919861794	-0.80634920
Ro03_G12091	*RoWRKY16*	I	3	2733850	2738731	-	2991	723	77939.6323	5.872741127	-0.79391424
Ro03_G13413	*RoWRKY17*	IIc	3	39334276	39336694	-	1842	350	38586.0963	6.975815392	-0.84714285
Ro03_G13495	*RoWRKY18*	IIb	3	42815055	42817810	-	2150	570	61799.6277	6.874641991	-0.72421052
Ro03_G15815	*RoWRKY19*	IIe	3	37586963	37590391	-	1817	433	46181.6653	8.063786125	-0.62979214
Ro03_G17200	*RoWRKY20*	IIb	3	27707338	27710148	+	1888	545	59648.1746	6.195529747	-0.55504587
Ro03_G17983	*RoWRKY21*	I	3	26713247	26721585	-	3642	503	56039.149	8.066300392	-0.92783300
Ro04_G00011	*RoWRKY22*	I	4	3328160	3331146	-	2180	592	65356.0227	8.263122368	-0.98023648
Ro04_G00111	*RoWRKY23*	IIb	4	2758434	2761817	+	1803	600	65399.3516	5.967889595	-0.7185
Ro04_G02811	*RoWRKY24*	I	4	5449926	5456804	-	4719	1572	179205.2	5.528524208	-0.39860050
Ro04_G07106	*RoWRKY25*	IIc	4	6800803	6802632	-	798	188	21409.737	9.517677116	-0.88776595
Ro04_G15164	*RoWRKY26*	IIc	4	3463308	3467140	+	2390	330	35681.5731	5.915370369	-0.77757575
Ro04_G17961	*RoWRKY27*	IId	4	27718283	27719905	-	1423	300	32684.5354	9.965538979	-0.67633333
Ro04_G19918	*RoWRKY28*	IIc	4	8611791	8614860	-	1426	420	46246.6665	6.710604668	-0.84119047
Ro05_G08626	*RoWRKY29*	IId	5	9635829	9637625	+	1379	311	34817.1475	9.299129295	-0.55659164
Ro05_G10257	*RoWRKY30*	IIb	5	30858965	30861918	+	1920	545	60508.7653	5.344138527	-0.90311926
Ro05_G16431	*RoWRKY31*	IIb	5	1618499	1620470	+	1311	436	47880.6458	8.442795753	-0.85344036
Ro05_G16799	*RoWRKY32*	IIe	5	7328837	7329918	-	918	305	34813.7713	5.158559227	-0.69836065
Ro05_G19154	*RoWRKY33*	IIe	5	40543638	40545702	-	972	281	32458.202	4.758867455	-1.18612099
Ro05_G22566	*RoWRKY34*	III	5	6701342	6705195	+	3492	359	40457.2833	5.121784401	-0.77493036
Ro06_G05663	*RoWRKY35*	I	6	29647066	29649427	+	1950	512	56672.2893	7.647709084	-0.92636718
Ro06_G06104	*RoWRKY36*	IV	6	31287949	31290314	+	1131	290	32426.7799	5.372558022	-0.76586206
Ro06_G06729	*RoWRKY37*	IIb	6	21395792	21398705	+	1964	547	59853.4546	5.125251579	-0.78939670
Ro06_G09223	*RoWRKY38*	I	6	24597005	24601526	+	3559	697	76828.2192	5.698643303	-0.71764705
Ro06_G14746	*RoWRKY39*	III	6	29132792	29133812	+	624	207	24062.7768	6.304603767	-0.60772946
Ro06_G14747	*RoWRKY40*	III	6	29142106	29144027	+	1390	327	37108.7447	5.788051033	-0.78501529
Ro06_G16328	*RoWRKY41*	IIb	6	219293	222133	-	1920	542	59131.3352	5.996252251	-0.59188191
Ro06_G16671	*RoWRKY42*	IIa	6	9075470	9078186	-	1922	321	35597.4632	8.715174675	-0.79968847
Ro06_G17302	*RoWRKY43*	I	6	20699892	20703187	-	2033	471	51291.2415	6.417258644	-0.91104034
Ro06_G17579	*RoWRKY44*	I	6	2626048	2629135	-	1605	534	58259.8364	8.345384026	-0.87378277
Ro06_G24980	*RoWRKY45*	IIe	6	7384224	7389857	+	4887	261	28526.367	5.052952385	-0.69578544
Ro06_G28717	*RoWRKY46*	I	6	23986840	23989927	+	1605	534	58259.8364	8.345384026	-0.87378277

Table 1. Information on *RoWRKYs* genes in the black raspberry genome (continued).

Transcript ID	*Gene name*	*Group*	*Chr.*	*StartPos*	*EndPos*	*Strand*	*CDS*	*(aa)*	*MW*	*pI*	*GRAVY*
Ro06_G29026	*RoWRKY47*	I	6	32246811	32249876	-	1653	550	60624.6455	8.95589962	-0.802
Ro07_G04379	*RoWRKY48*	IIe	7	2159617	2161306	-	1442	338	36994.7226	8.400568962	-0.70414201
Ro07_G04383	*RoWRKY49*	IV	7	2144733	2146053	-	513	170	18648.7	7.986939812	-0.76882352
Ro07_G04678	*RoWRKY50*	III	7	37004358	37007280	-	1506	349	39329.0047	5.839490318	-0.85587392
Ro07_G04679	*RoWRKY51*	III	7	37001846	37004086	+	1050	349	39070.9602	6.004550743	-0.74670487
Ro07_G06810	*RoWRKY52*	III	7	38645734	38647993	+	1961	378	41617.712	6.172964668	-0.62883597
Ro07_G14025	*RoWRKY53*	IIe	7	38958476	38960678	-	2025	428	46046.8154	5.270759392	-0.73271028
Ro07_G14616	*RoWRKY54*	IIc	7	6272668	6276945	-	1247	245	28144.4658	8.533244896	-1.08897959
Ro07_G17080	*RoWRKY55*	III	7	16277554	16284077	+	4795	1467	165921.3356	5.741954613	-0.26952965
Ro07_G18694	*RoWRKY56*	III	7	1633346	1636563	+	2290	353	38941.6711	5.376309395	-0.71529745
Ro07_G33471	*RoWRKY57*	III	7	5076284	5084214	+	846	281	31792.4618	4.893234825	-0.40711743
Ro07_G33472	*RoWRKY58*	III	7	5053673	5055956	+	1185	394	44060.196	4.752046776	-0.68959390
Ro07_G33475	*RoWRKY59*	III	7	5100454	5110794	+	3180	1059	119778.1416	4.926940346	-0.38696883
Ro02_G04519			*2*	*4715117*	*4725115*	*-*	*7086*	*965*	*107885.98*	*5.567743*	*-0.636477*
Ro06_G09963			*6*	*19044339*	*19046664*	*+*	*1314*	*320*	*35747.761*	*5.551771*	*-0.835*
Ro06_G28795			*6*	*25843863*	*25847599*	*-*	*3004*	*306*	*33939.584*	*9.27605*	*-0.51634*

aa = amino acid, pI= isoelectric point MW = molecular weight, GRAVY = large average hydrophobicity

Phytohormone responsive elements were found: TGA (auxin responsive), TATC-box (gibberellin response), TCA (salicylic acid response), ABRE (abscisic acid response), AuxRR-core (auxin response), CGTCA motif (MeJA response), TGACG motif (MeJA response), GARE motif (gibberellin responsive), P-box (gibberellin responsive element), AuxRE (auxin responsive) and TGA-box (auxin responsive). Cis elements responsive to different stresses were also found: TC-rich repeats (defense and stress response), LTR (low temperature response), ARE (anaerobic induction), GC motif (potentiator-like element - induction specific in anoxia), MBS (MYB binding site - drought induction), WUN motif (injury responsive element).

Phylogeny, gene structure and motif analysis of WRKY protein in black raspberry

Based on the unrooted phylogenetic tree of the 59 protein sequences, the black raspberry RoWRKY gene family was categorized into four subfamilies or groups (I, II, III, IV). Group I (containing 12 members) was the largest group, followed by group IIc (8), group IIb (8), group IIe (7), group IId (6) and group IIa (3). Group III contains 13 members and group IV without the zinc finger motif feature (Fig. 1A).

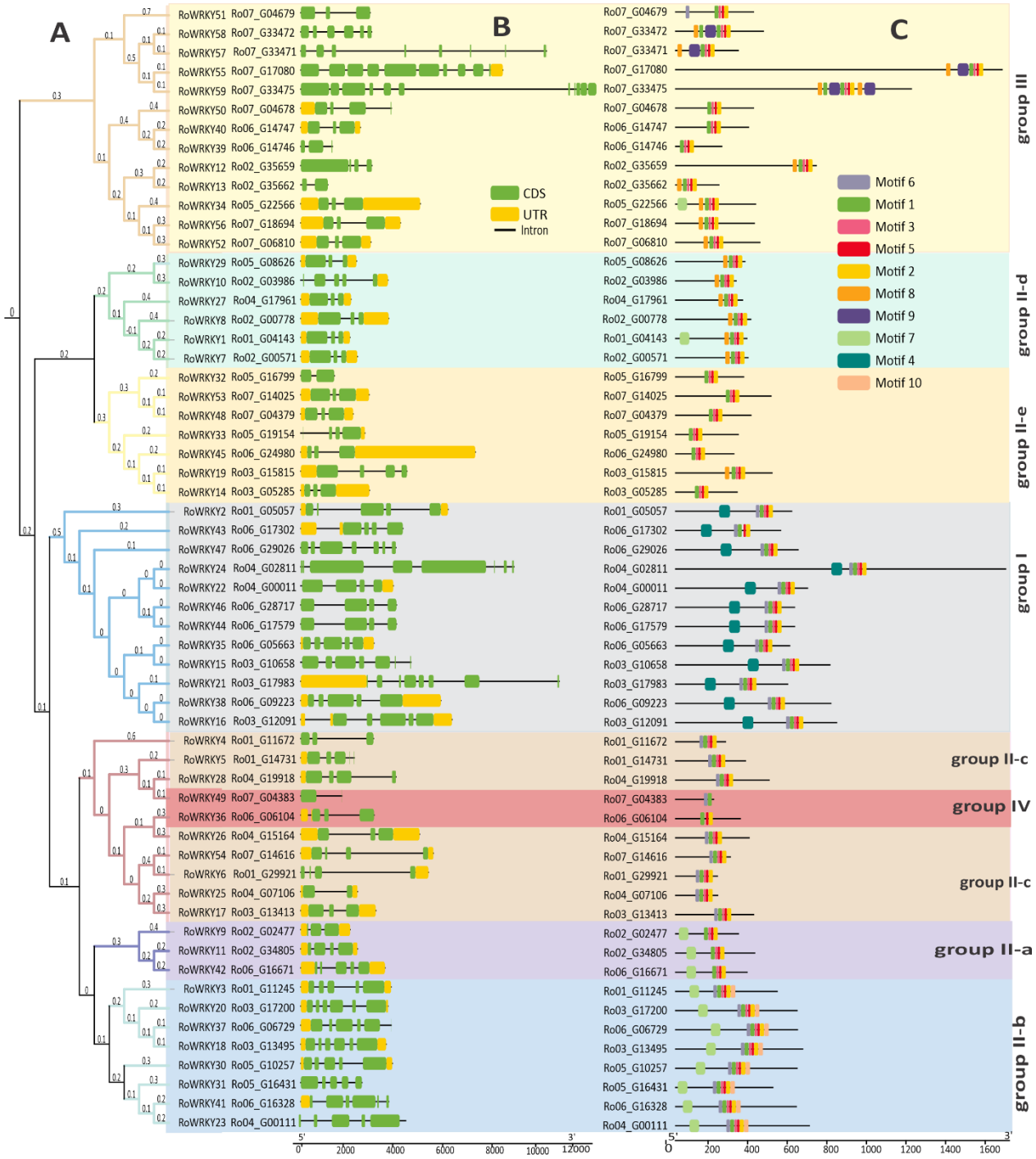

Fig. 1 Phylogenetic relationships and structure of genes encoding the black raspberry RoWRKY proteins: (**A**) The unrooted tree was generated with the BEAST program using the full-length amino acid sequences of the 59 black raspberry RoWRKY proteins by the UPGMA method, with 10,000,000 bootstrap replications. RoWRKY protein subfamilies (I, IIa, IIb, IIc, IId, IIe, III and IV); (**B**) Exon/intron organization of raspberry *RoWRKY* genes. Yellow boxes represent exons and black lines represent introns. Untranslated regions (UTRs) are indicated by green boxes. The sizes of exons and introns can be estimated using the scale at the bottom; (**C**) Schematic representation of conserved motifs in black raspberry RoWRKY proteins, elucidated from publicly available data (NCBI CDD Domain – Pfam –18271 PSSMs). Each colored rectangular box represents a motif with the given name and motif consensus

Fig. 1B provides a detailed illustration of the relative intron lengths and conservation of exon sequences within each black raspberry *RoWRKY* gene. The number of introns in all these genes ranged from 1 to 11. The conserved motifs of the 59 RoWRKY proteins were also analyzed (Fig. 1C). Results reveal conserved domains or motifs shared between related proteins and identified 10 conserved motifs. The type, order, and number of motifs are similar in proteins with the same subfamily or group, but differed from proteins in other subfamilies.

Chromosomal localization, *RoWRKY* gene duplication, Ka/Ks calculation and divergence time estimation

The 59 *RoWRKY* genes were distributed in seven chromosomes of the black raspberry genome. Among them, chromosome 6 had the highest density of *RoWRKY* genes with 13 members; chromosome 7 had the second highest density with 12 genes; eight *RoWRKY* genes were located on chromosome 3; seven genes were located on each of chromosomes 2 and 4, and six genes were located on each of chromosomes 1 and 5 (Fig. 2). The *RoWRKY* family showed two types of gene duplication (Supplementary S3). The *RoWRKY* family showed two types of gene duplication (Supplementary S3). Segmental duplication shows 20 pairs of paralogous genes detected, of which, five pairs of RoWRKY genes contain more than 70% similarity (RoWRKY40-RoWRKY39=100%; RoWRKY12- RoWRKY13 = 82.0%; RoWRKY24-RoWRKY22= 75,5%; RoWRKY46-RoWRKY44=100%; RoWRKY54-RoWRKY6 =73,2%). The tandem duplication presents 12 pairs of paralogous genes, of which, two pairs of RoWRKY genes were detected at a distance of less than 100kb on chromosome 2 (RoWRKY57-RoWRKY59= 75.2% similarity) and on chromosome 3 (RoWRKY12-RoWRKY13 = 82%).

Segmental duplication shows 20 pairs of paralogous genes detected, of which, five pairs of *RoWRKY* protein contain more than 70% similarity (*RoWRKY40-RoWRKY39*=100%; *RoWRKY12- RoWRKY13* = 82.0%; *RoWRKY24-RoWRKY22*= 75,5%; *RoWRKY46-RoWRKY44*=100%; *RoWRKY54-RoWRKY6* =73,2%). The tandem duplication presents 12 pairs of paralogous genes, of which, two pairs of *RoWRKY* genes were detected at a

distance of less than 100kb on chromosome 2 (*RoWRKY57-RoWRKY59*= 75.2% similarity) and on chromosome 3 (*RoWRKY12-RoWRKY13* = 82%).

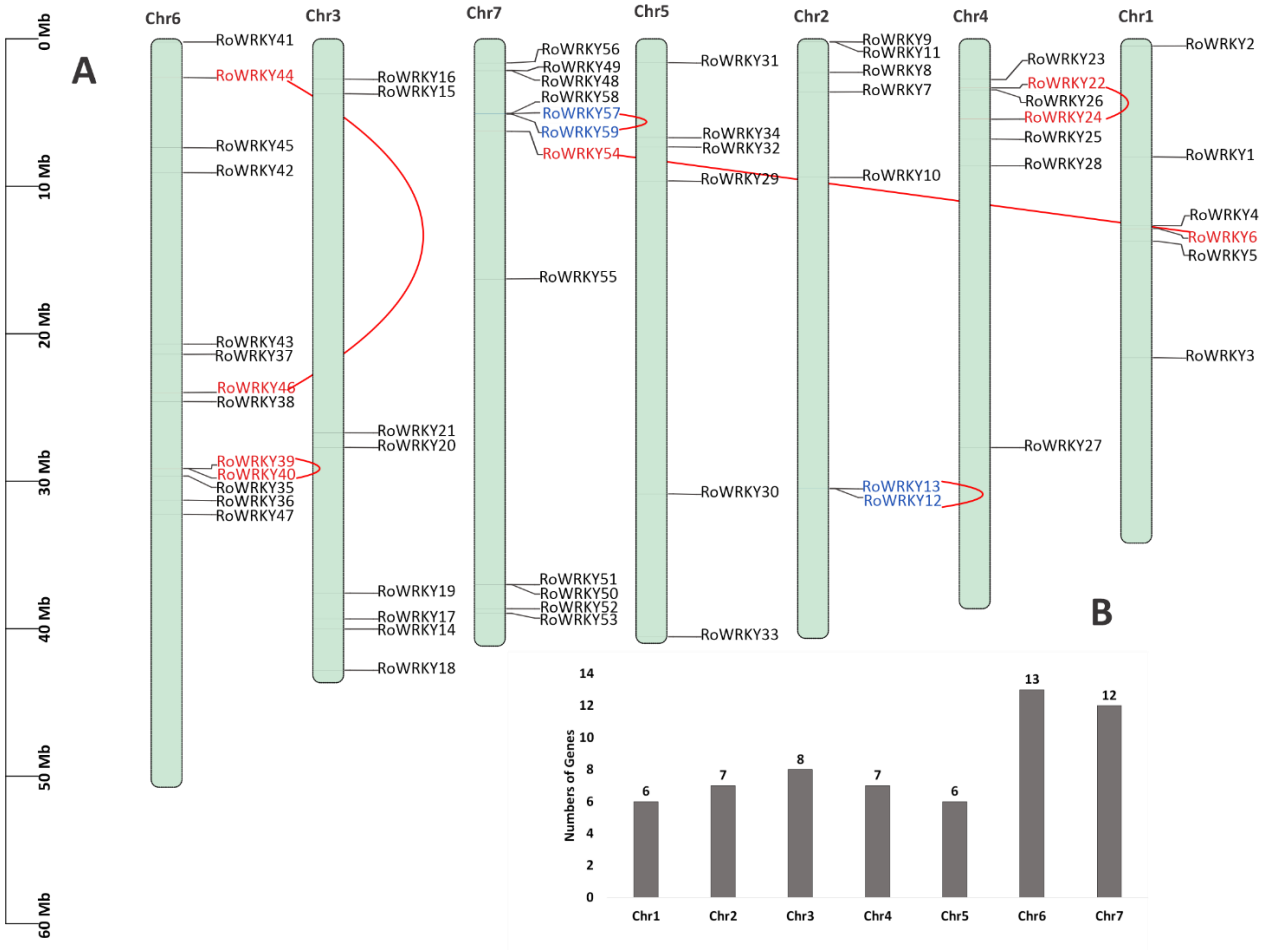

Fig. 2 Chromosomal map and coordinates of *RoWRKY* gene duplication events: (**A**) The identity of each linkage group is indicated in the central part of each bar. The possible Segmental duplicated genes are connected by red color lines and the duplicated gene pair in tandem in blue color on chromosome. (**B**) The number of *RoWRKY* genes in each chromosome.

The synonymous (Ks) and non-synonym rates (Ka), and Ka/Ks of these duplicates were calculated, and the time of duplication was estimated using Ks values (Table 2). The Ks of four segment duplications range from 0 to 0.3. As a result, the divergent time ranges from 17.97 Mya to 25.82 Mya. The Ka/Ks values of the segment's duplicates are less than one, indicating that they were subjected to purifying selection. The duplications occurred as early as 29.02 Mya and as recent as 9.55 Mya. The values of Ka/Ks in all of these

duplicates are less than one, indicating that purifying selection occurred in these duplicates.

Table 2. Ks, Ka, Ka/Ks calculation and divergent time of the duplicated black raspberry WRKY gene pairs

Duplicated Gene Pairs	Ks	ka	Ka/Ks	Purifying	Duplication Type	Time (Mya)*
RoWRKY57/RoWRKY59	0.4	0.4	0.9	Yes	Tandem	29.02
RoWRKY12/RoWRKY13	0.1	0.2	0.7	Yes	Tandem	9.55
RoWRKY40/RoWRKY39	0.3	0.5	0.7	Yes	Segmental	25.82
RoWRKY24/RoWRKY22	0.2	0.4	0.6	Yes	Segmental	17.97
RoWRKY46/RoWRKY44	0	0	**NaN**	**NaN**	Segmental	
RoWRKY54/RoWRKY6	0.3	**NaN**	**NaN**	**NaN**	Segmental	22.78

**Mya, million years ago*
NaN = Not a Number (Refers to an undefined value or a result that cannot be calculated)

Number of transcripts and expression patterns of *RoWRKY* genes in various tissues

Of the total of 33,252 genes annotated in the black raspberry genome, the transcriptional expression of genes for each sample was: canes (10,161 =31%), flower bud (9,390 =28%), green leaf (10,166=31%), mature leaf (11,603 = 35%), leaf MJ24(10,548=32%), leaf MJ48 (10,872=33%), red fruit (11,603=35%), ripened fruit (11,916=36%), root (10,161=31%) and young leaf (10,391=31%). However, the numbers of expressed of *RoWRKY* genes for the samples were: canes (44 =0.43%), flower bud (42 =0.45%), green leaf (41=0.40%), mature leaf (41 = 0.40%), leaf MJ24 (41=0.39%), leaf MJ48 (42=0.39%), red fruit (42=0.36%), ripened fruit (41=0.34%), root (44=0.43%) and young leaf (43=0.41%) (Supplementary S4).

Fig. 3A, shows the relationship between samples and treatments using multidimensional scaling (MDS). It shows the robustness of the data regarding number and types of samples, i.e., mature leaf, red fruit and ripened fruit, regarding expression when treatments MJ48 leaf and MJ24 leaf are compared, in addition to young leaf sample results. In Fig. 3B, a visual representation of the mean-variance relationship using the plotMeanVar was generated as indicated from the ten samples.

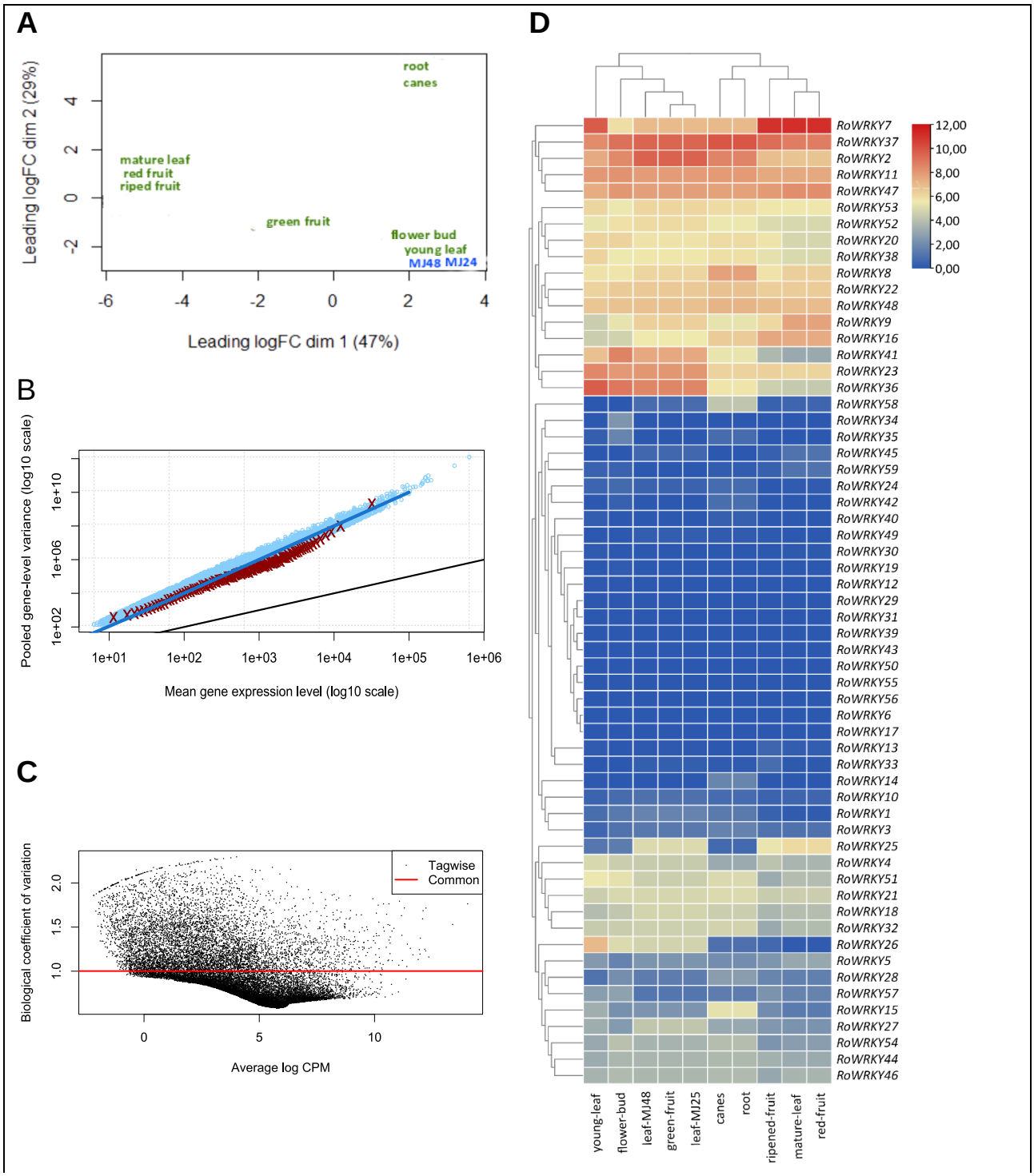

Fig.3. Analysis of gene expression in 10 samples of black raspberry: (**A**) Multidimensional scaling (MDS), indicating the dispersion of treatments and replications, as a function of the general pattern of gene expression of each sample, (**B**) mean-variance relationship and dispersion were plotted using edgeR's plotMeanVar function to explore the mean-variance relationship. Each point represents the estimated mean and variance for each gene, with pooled variances, as well as the common scatter of overlapping trend (untreated and treated samples), (**C**) Scatter plots, plotBCV illustrates the relationship of the biological coefficient of variation with the average log counts per million, (**D**) RoWRKY heat map (the values are presented in log2 from 0.00 to 12.00), the red color indicates the greater the number of reads or genes expressed according to the sequencing depth and blue indicates smaller and read or genes expressed according to sequencing depth.

Differential expression analysis using Expression analysis of digital gene expression data (edgeR) calculates whether there is evidence that counts for a transcript or exon are significantly different across experimental conditions (Fig. 3C). Finally, a heat map displaying the expression of *RoWRKY* genes of the ten samples is shown (Fig. 3D).

4.4 Discussion

Identification of the WRKY protein family in black raspberry

WRKY FTs are key regulators of many processes in plants, exhibiting specific responses to biotic stresses and involvement in tolerance to abiotic stresses (Pandey and Somssich 2009; Ramegowda et al. 2020). In model species such as *Arabidopsis thaliana* (Eulgem et al. 2000) as well as in non-model as those of the botanical family Rosaceae (Qiao et al. 2015), WRKY TFs and their involvement to promote resistance and tolerance to different stresses were identified and functionally characterized (Li et al. 2020a). The availability of assembly and annotation of the complete genome of the black raspberry in its version V3.0 (VanBuren et al. 2018b), made it possible to analyze the entire genome of the *RoWRKY* family. The identification of 59 *RoWRKY* genes in black raspberry corresponds to a slightly lower number than in other species, for example, there are 75 *AtWRKY* genes in Arabidopsis (Eulgem et al. 2000), 103 *PbWRKY* genes in pear (*Pyrus bretschneideri*) (Huang et al. 2015) and 127 *MdWRKY* genes in apple (*Malus domestica*) (Meng et al. 2016). In contrast, the rose (*Rosa chinensis*) contains 56 *RcWRKY* (Liu et al. 2019a), and 59 *FvWRKY* genes in wild strawberry (*Fragaria vesca*) (Zhou et al. 2016), and 61 *PpWRKY* genes in peach (*Prunus persica*) (Yanbing et al. 2016) were identified. The present findings in black raspberry, an important polydrupe fruit and a plant resistant to the biotic stress caused by the fungus *Verticillium dahliae* (Bushakra et al. 2016), could become a model plant of the species of the genus *Rubus* and have an agricultural and economic potential (Fuks 1984; Graham and Brennan 2018; Moreno–medina et al. 2020). *RoWRKY* transcription factors contribute to the recent identification of *WRKY* genes in Rosaceae plant species such as apple, pear, strawberry and peach.

Subcellular localization of the RoWRKY protein Family

WRKY proteins are part of a multigene family and are involved in many responses to adverse environmental effects (Eulgem and Somssich 2007; Rushton et al. 2012). Several members of the family are present in different cellular compartments as observed in Arabidopsis (*A. thaliana*), millet (*P. glaucum*), sorghum (*S. bicolor*) and pear (*P. betulaefolia*) species (Van Aken et al. 2013; Liu et al. 2019b; Baillo et al. 2020; Chanwala et al. 2020). In this study, analyzing the subcellular localization of RoWRKY proteins, it was found that several members present signaling for transport to many cellular compartments of the black raspberry`s cell. For example, all 59 RoWRKY proteins have nuclear localization sequences, similarly to peanuts (*Arachis hypogaea*), which out of 158 AhWRKY, 157 have nuclear localization sequences (Zhao et al. 2020). Specific WRKY proteins were also located in the nucleus such as PbWRKY53 from pear (*P. betulaefolia*) (Liu et al. 2019b), MbWRKY1 of the apple (*M. domestica*) (Han et al. 2018) and AtWRKY13 and AtWRKY57 of Arabipdosis (*A. thaliana*) (Van Aken et al. 2013). In addition, there are studies of functional categorization of cellular components of WRKY in the complete genomes of millet (*P. glaucum*) (Chanwala et al. 2020) and sorghum (*S. bicolor*) (Baillo et al. 2020), that could be used to complement the analysis of RoWRKY.

The subcellular localization of WRKY proteins in the nucleus was expected, as translation in the cytoplasm must return to the nucleus to bind to the promoter region of genes (W-Box) to regulate transcription. This action is aided by the signal peptides which are short proteins that act as a signal fragment or transport from the cytoplasm to the nucleus(Lu et al. 2021). We also detected signaling to mitochondria and chloroplast. Some genes are located in other cellular compartments such as chloroplasts and mitochondria that require transcription factors encoded by the nucleus (Lee et al. 2019). On the other hand, organelles send signals to the nucleus to coordinate nuclear and organelle activities(Woodson and Chory 2008).

Cis elements in the promoters of *RoWRKY* genes

Gene promoters have cis-acting elements, which act as the control center for gene transcription. The promoter regions of 59 *RoWRKY* genes exhibited several conserved cis-acting regulatory elements involved in various functions, such as abiotic and biotic stress responses (MBS, LTR, ARE, TC-rich repeat and GC motif), light-responsive elements (G -box, GT1-motif, Box4 and TCT-motif) and phytohormone regulation (ABRE, TCA element, TGA element, TGACG motif, CGTCA element, AuxRR nucleus and GARE motif). The presence of many cis-acting elements that are involved in environmental stress responses, light-responsive elements and phytohormones indicates the involvement of WRKYs in different biological processes. The expression of *WRKYs* genes can occur through the binding of a WRKY TF to the W-box or by the binding of another TF to a different cis element along the *WRKY* promoters, making an up- or down-regulation, by activating or repressing transcription (Phukan et al. 2016). Induced WRKYs rapidly bind to promoters of *WRKY* genes, inducing them by self-regulation and cross-regulation, building a regulatory network of WRKY TFs expression (Birkenbihl et al. 2018). In apple (*M. domestica*) the cis-acting elements W-box, GARE and ERE were found in the region of the *MdWRKY100* promoter, which may play a response to fungal and environmental stresses and in the developmental pathways (Zhang et al. 2019).

Similar to this study, the promoters of 17 *MdWRKYs* contain a cis methyl jasmonate (MeJA) response element, 12 *MdWRKY* promoters contain a salicylic acid response element, and 15 *MdWRKY* promoters contain cis elements G-Box, ABRE, CAAT-box and TATA-box, which are elements related to defense and stress (Zhang et al. 2021). *MdWRKY31* has been shown to bind to the promoters of the *MdRAV1* and *MdABIs* genes via the cis element TTGACC to mediate abscisic acid (ABA) sensitivity (Zhao et al. 2019). Therefore, cis elements present in promoter regions of WRKYs are key to respond to plant growth, development, defense, and stress tolerance.

Phylogeny, gene structure and motif analysis of RoWRKY protein in black raspberry

The structure of WRKY transcription factors is composed of the conservation of the WRKY domain number and the features of its zinc finger motif, which binds to the cis W-box element in the promoters of its target genes, the most essential feature of this superfamily (Eulgem et al. 2000; Zhang and Wang 2005). The classification of *RoWRKY* genes was performed according to the approach used in other species, based on the generated phylogenetic tree topology. The divisions of subfamilies or groups described in Arabidopsis were also adapted: groups I, II and III according to the number of WRKY domains and the type of zinc finger motif, together with the subdivision of group II into subgroups IIa, IIb, IIc, IId and IIe (Eulgem et al. 2000). In this study, 12, 32 and 13 *RoWRKY* genes were classified in groups I, II and III respectively, however, two *RoWRKY* genes were classified in group IV because they did not belong to any group. Group II was larger with 32 members and represented 54% of all *RoWRKY* genes.

These results are consistent with the approximations of the highest percentage size of WRKY representation in group II in Arabidopsis (63%), apple (62%), vine (65%) and rice (47%) (Meng et al. 2016). Among the subgroups, subgroup IIb and IIc have the highest number of genes, each with eight *RoWRKY* genes or 27% of the genes attributing to group II. In apple subgroup IIb (25%), Arabidopsis IIc (40%), vine IIc (41%) and rice IIc (33%) have the highest number of WRKY genes of group II (Meng et al. 2016).

Considering that most RoWRKY proteins contained the conserved WRKYGQK domain, four genes (*RoWRKY4, RoWRKY59, RoWRKY57 and RoWRKY55*) were identified with the WRKYGKK and WGKYGQM variant and three sequences were removed for having incomplete variants of the WRKY domain. Similar variations were identified in the heptapeptide of 13 SbWRKY proteins from sorghum (*S. bicolor*) (Baillo et al. 2020). There are also similar variations identified in the MdWRKY heptapeptide from apple (*M. domestica*), in the proteins MdWRKY78, MdWRKY53, MdWRKY92 and MdWRKY96 (WRKYGKK) and other variations of MdWRKY51 and MdWRKY38 (WRKYG*K), MdWRKY114 (WRKYARS), MdWRKY20 (WRKYGKI), MdWRKY42 (WRKYGKS) (Meng

et al. 2016). Furthermore, identical variations of the heptapeptide were found in peach (*P. persica*), PpWRKY33 (WRKYGKK) and PpWRKY35 (WRKYGMK) proteins (Yanbing et al. 2016). However, WRKYGKK variations in soybean (*G. max*), in proteins GmWRKY6 and GmWRKY21 do not allow binding to the cis W-box element (Zhou et al. 2008). The NtWRKY12 protein in tobacco with the WRKYGKK variant recognizes other binding sequences (TTTCCAC) instead of the normal W-box (van Verk et al. 2008). Therefore, further investigation is needed to identify the DNA-binding sequences of the variants that are part of the heptapeptide WRKYGQK.

Within the primary structure of the *RoWRKY* genes, a similar exon-intron pattern was detected. The number of introns in *RoWRKY* genes ranged from one to 11, which is almost similar to that reported in cucumber (*C. sativus*), which ranged from zero to 13 (Chen et al. 2020b). Also, these values range from zero to 17 introns in willow (*S. suchowensis*) (Bi et al. 2016) and one to 20 introns in strawberry (Zhou et al. 2016). The *RoWRKY* genes did not show absence of introns, indicating that a process of evolution of the gene family may be taking place. If there were no introns, it could be explained by the mechanisms of retrotransposition of genes without introns (retroduplicated genes), duplication of genes without existing introns and horizontal gene transfer (He et al. 2011). Variations in intron sizes within and between *RoWRKY* groups may have resulted from inversion, fusion or duplication events (Li et al. 2016). The similarity of exon-intron pattern provides important clues about the evolution of *RoWRKY* genes.

Of the 10 functional motifs identified in RoWRKY proteins, 1, 2, 3, 4 and 5 corresponded to the WRKY domain (Fig. 1C). Zinc finger domains are present in most RoWRKY members. Motif 6 (KTVREPRVVVQTRSE) is likely to represent the nuclear localization signal and histone methyltransferase complex (TVREPRV/EPRV) distributed mainly in groups I and II (IIe and IIc). In Arabidopsis, epigenetic regulation by histone methyltransferase is attributed to *AtWRKY53*, a senescence regulator (Banerjee and Roychoudhury 2015). In motif 7, 8 submotifs were identified, where the initiating motif of vegetative septum formation (FtsL/DivIC) (Villanelo et al. 2011) and the leucine zipper motif (bZIP_2) (Jakoby et al. 2002), are motifs that are distributed in subgroups IIa and

IIe of RoWRKY proteins, having specific functions of process regulation, pathogen defense and, among others, of stress signaling. However, motif 9 (WRKY-GCM1 zinc fingers) is a plant zinc finger domain (Babu et al. 2006), that has functional diversity through expression divergence rather than protein sequence divergence. This motif is uniquely distributed in proteins RoWRKY30, RoWRKY24, RoWRKY31, RoWRKY18, RoWRKY29, RoWRKY20 and RoWRKY33. Therefore, not all identified motifs of RoWRKY proteins are functional in their predicted structure, but some motifs are conforming to submotifs have specific functions.

Genomic Distribution and Gene Duplication of Black raspberry *RoWRKY* Members

The chromosomal location of the 59 *RoWRKY* genes on the seven chromosomes of the black raspberry genome is almost similar to the strawberry (*Fragaria vesca*), which contains seven chromosomes and 59 *FvWRKY* genes (Zhou et al. 2016), changing their distribution intensity in both genomes. The presence of segmental and tandem duplication in the black raspberry genome differs by the number of copies and their distribution, suggesting that duplication events occurred after the divergence between black raspberry and strawberry, ca. 75 million years ago (Xiang et al. 2017). Gene duplication events affect genome expansion, family size, and the distribution of genes in chromosomes, which are important factors for functional prediction (Zhou et al. 2016). However, whole genome, segmental, and tandem duplication were major contributors to the expansion of the *WRKY* gene family (Chen et al. 2019).

The tandem duplication of chromosomal regions leads to the expansion and evolution of the gene family, through structural and functional divergence over time (De Grassi et al. 2008; Chen et al. 2019). In this analysis, two pairs of genes were identified in tandem duplication (*RoWRKY57-RoWRKY59=75.2%* and *RoWRKY12-RoWRKY13* = 82.0%, with > 70% similarity), which is a lower number than the reported for strawberry (Zhou et al. 2016), Arabidopsis (Cannon et al. 2004) and similar to sorghum (Baillo et al. 2020). Despite these differences, tandem duplications may have shaped the evolution of the *RoWRKY* gene family in the black raspberry. A segmental duplication is the result of large-scale genomic events such as duplication of large chromosomal regions (Cannon et al.

2004). In this analysis, 20 pairs of paralogous genes were identified, of which five show similar segmental duplicates (*RoWRKY40-RoWRKY39*= 100%; *RoWRKY12-RoWRKY13*=82%; *RoWRKY24-RoWRKY22* =75,7%; *RoWRKY46-RoWRKY44*=100%; *RoWRKY54-RoWRKY6*=73.2%) and the comparison of promoter regions of segmental duplicate (*RoWRKY40-RoWRKY39*= 45.9%; *RoWRKY12-RoWRKY13*=53.9%; *RoWRKY24-RoWRKY22*=45.0%; *RoWRKY46*-RoWRKY44 = 45.9% ; *RoWRKY54-RoWRKY6*=46.2%) and tandem duplicate (*RoWRKY57-RoWRKY59*=47.3% and *RoWRKY12-RoWRKY13* = 53.9%). These results showed a higher divergence in the regulatory regions than in the coding region, suggesting events of neofunctionalization and/or subfunctionalization. The strawberry has the same segmental and tandem duplication of *RoWRKY* genes, suggesting that the duplication events occurred before the formation of these species from a common ancestor.

Tandem duplications and segmental duplications have played essential roles in the evolution and diversification of the *WRKY* gene family in plant species (Zhu et al. 2014). Duplication events are significant for *WRKY* diversification, as duplicated genes can acquire new functions (Baillo et al. 2020). Gene duplication can result in sub-functionalization. For example, expansion of functions among wheat *WRKY* gene family members has occurred through tandem and whole genome duplication (Hassan et al. 2019). Therefore, the location, distribution, and tandem and segmental duplications provide evidence for the evolution and prediction of functions of black raspberry *RoWRKY* genes. The genetic divergence of tandem duplication pairs (RoWRKY57/RoWRKY59; RoWRKY12/RoWRKY13) is part of Group III (9.55 a 29.02 Mya). However, members of the Group III of the *A. thaliana* and *O. sativa* species showed genetic divergence after the dicotiledôneas and monocotiledôneas between 120 and 200 million years ago (Mya) (Wu et al. 2005).

The presence of WRKY transcription factors in this species was reported in the mapping of quantitative traits at the -QTL locus (S0141:9310-97411: ID=G14616) of the whole genome and v1.0 annotation of the black raspberry (*R. occidentalis*) (VanBuren et al. 2016). The functional evidence of WRKY in black raspberry remains to be explored,

although the presence of chromosomal locations was identified, their functions could be computationally inferred, but could also be experimentally corroborated.

Differential expression of *RoWRKY* genes

Our heat map data showed that most of the *RoWRKY* genes were expressed in the black raspberry tissue and organ samples. These results indicate that they can participate in growth and development. For example, the genes *RoWRKY7*, *RoWRKY37* and *RoWRKY2* showed strong expression in all tissue types. However, nine *RoWRKY* genes (*RoWRKY6, RoWRKY17, RoWRKY29, RoWRKY31, RoWRKY39, RoWRKY43, RoWRKY50, RoWRKY55, RoWRKY56*) were not expressed in the 10 samples studied. In addition, some genes (*RoWRKY19* and *RoWRKY34*) were only expressed in the flower bud sample.

According to the results and information obtained in this study, the importance of WRKY in the growth, development and response to biotic and abiotic stresses in plants is evident. There are report of differential RNA-seq expression, for example, 13 *RiWRKY* genes out of 75 genes expressed in red raspberry (*Rubus ideaus* L.) provide information on the genetics of thorn development (Khadgi and Weber 2020). In addition, *ReWRKY35* and *ReWRKY31* expression annotation participates in the genetic variability of *Rubus ellipticus* (Sharma et al. 2021). Also, in the root tissue sample, the expression of *WRKY* genes in *Phytophthora rubi* rot resistance in red raspberry 'Latham (*Rubus idaeus*) was evidenced (Ward and Weber 2012).

Differential expression analyzes of strawberry (*Fragaria* × *ananassa*) RNA-seq data processing, show for receptacle tissues during specific stages of ripening (74 *FaWRKY* genes for White_R, Turning_R and Red_R) and achene (102 *FaWRKY* genes for White_A, Turning_A and Red_A). Also, it shows differential expression in response to infection by the fungus *Colletotrichum fructicola* (54 *FaWRKY* genes for Inf_24h, Inf_72h, Inf_96h)(Garrido-Gala et al. 2022). Therefore, gene expression studies provide functional information about WRKY transcription factors in Rosaceae species.

4.5 Conclusion

The complete genome of the black raspberry (*Rubus occidentalis*) contains 62 identified and 59 characterized members of the *RoWRKY* gene family, and these are located on seven chromosomes. The subcellular location of RoWRKY members is in many cellular compartments. Segmental and tandem duplications may be associated with paused fusion events of the black raspberry genome. The genome distribution, organization and gene structure suggest a complex evolutionary history of this family in the black raspberry. The cis-acting elements in the promoters of *RoWRKY* genes are involved in various biotic and abiotic stress response functions and biological processes. Also, the motifs and submotifs of RoWRKY proteins have specific functions of process regulation, pathogen defense and other stress signaling functions. Duplications show higher divergence on the promoter regions, indicating events of neo/subfunctionalization in the family. Differential expression of *RoWRKY* genes in tissue samples analyzed shows preferential or specific expression in tissue samples from plant organs. In addition, *RoWRKY* genes have a stress response, when sprayed with phytohormone and also without treatment.

4.6 Reference

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5 ARTIGO 3: The expression of WRKY transcription factors on stress response and tolerance in *Malus* spp. and *Pyrus* spp.

*Manuscrito por submissão ao periódico Frontiers in Plant Science (Qualis A1)

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ABSTRACT

WRKY transcription factors play a role in response and tolerance to biotic and abiotic stresses and in plant development. This family needs further studies allowing their identification and the characterization of their transcriptional expression using complete genomes and transcriptomic datasets that are currently available for species such as *Malus domestica, Malus baccata, Malus sieversii, Pyrus communis, Pyrus betulifolia and Pyrus pyrifolia*. In this study, a total of 667 *pomWRKY* genes were identified using the genomes of *Malus* and *Pyrus* spp. Phylogenetic analysis revealed that *pomWRKY* proteins can be divided into four groups based on the conservation of the WRKY domain obtained through comparison of their amino acid sequences. There are 121 members (18%) in group I, 434 (65%) in group II, 99 (15%) in group III and 13 (2%) in group IV. This split into four groups is further supported by conserved motif compositions and intron/exon structures. The 560 *pomWRKY* genes were mapped on the 17 chromosomes of the genomes of *M. domestica, M. sieverssii, P. betulifolia, P. communis* and *P. pyrifolia*, among which there were 52 pairs of duplicated genes in tandem. Differential expression of *pomWRKY* genes in 20 processed RNA-seq samples were mostly from group I and II, with responses to biotic and abiotic stresses in different organs of *Malus and Pyrus* spp. These discoveries not only expand our understanding on the phylogeny and transcriptional behavior of the WRKY family, but also provides valuable information on their responses in plant-environment and plant-pathogen interaction.

Keywords: domain evolution, genetic divergence, differential expression, stress, pome.

5.1 Introduction

WRKY is among the largest families of transcription factors (TFs) in higher plants (Jiang et al., 2017). This superfamily is involved in plant immunity, responding to biotic and abiotic stress, and has other transcriptional roles in regulating plant organs and development (Pandey and Somssich, 2009; Shuhao and Diqiu., 2016; Li et al., 2020a; Zhang et al., 2021). Its main structure is composed of a WRKY domain and a zinc finger motif, which contains a 60-amino acid region that is highly conserved by members of the family. These proteins are regulatory TFs with preferential binding to the cis element W-box and have the potential to regulate the expression of a variety of target genes (Eulgem et al., 2000; Rushton et al., 2010). Their classification depends on the WRKY domain number and their characteristic zinc finger motif, leading into a subclassification of three subfamilies or groups. Group I is composed of two WRKY domains and group II (IIa,b,c,d,e) and group III contain only one WRKY domain. However, groups I and II have the same C2 - H2 ligand pattern zinc finger motif (C - X4–5 - C - X22–23 - H - X1 – H) and group III has a standard zinc finger motif of the C2-HC ligand (C - X7 - C - X23 - H - X1 – C) (Eulgem et al., 2000; Li et al., 2020a).

The first WRKY protein discovered was in sweet potato (*Ipomoea batatas*) (Ishiguro and Nakamura, 1994). Afterwards, 74 *AtWRKY* genes were reported in *Arabidopsis thaliana* defining the structure and classification of WRKYs that is accepted until now (Eulgem et al., 2000). Also, 102 *OsWRKY* genes were found in rice (*Oryza sativa* ssp. *indica*) (Ross et al., 2007), 197 WRKY genes in soybean (*Glycine max*) (Schmutz et al., 2010) and such high numbers in other WRKYs reported in model species (Rushton et al., 2010). In some species of Rosaceae, 59 *FvWRKY* genes in wild strawberry (*Fragaria vesca*) at different stages of fruit development were identified and characterized in the complete genomes (Zhou et al., 2016) and 56 RcWRKY genes were found in rose (*Rosa chinensis*) acting in defense from the fungus *Botrytis cinerea* (Liu et al., 2019a). There are also reports of 58 *PpWRKY* genes in peaches (*Prunus persica*) expressed during bud dormancy (Chen et al., 2016) and 61 *PpWRKY* genes in studies of the same peach (Yanbing et al., 2016). In addition, 58 *PmWRKY* genes in *Prunus mume* in its response to cold stress (Bao et al., 2019), 139 *PyWRKY* genes in *Prunus yedoensis*, 262 *PdWRKY* genes in

Prunus domestica, 53 *PaWRKY* genes in *Prunus avium*, 56 PdWRKY genes in *Prunus dulcis* and 77 PyWRKY genes in *Prunus yendoensis* var. Nudiflora (Zhong et al., 2021).

The WRKY genes are also present in the subfamily of Rosaceae called Maloideae, where we can find a group of fleshy pseudofruits, composed of five carpels and pome-like structure, reason why we call them pome fruits. Its diversity is of approximately 173 species, being apple (*Malus domestica*), quince (*Cydonia oblonga*) and pear (*Pyrus communis*) the largest representatives of pome fruit species (Rohrer et al., 1991). Regarding the genome, it basically consists of 17 chromosomes (2n =2x = 34) that emerged from an aneuploidy of a genome of 18 chromosomes, with probable origin from North America (Evans and Campbell, 2002). Having a similarity in genome size *Malus* (1.57 pg/2C) and *Pyrus* (1.11 pg/2C), both genomes could be co-linear and closely related (Arumuganathan and Earle, 1991; Tatum et al., 2005; Morillo-coronado and Morillo-coronado, 2016).

The expression of WRKYs genes in pome fruit species (*Malus* and *Pyrus* ssp.), employ regulatory functions in defense of disease-causing organisms, tolerance to environmental stimuli and participation in plant organs and development. These functions are differential expressions or overexpression to treatments or response signals, which are involved in resistance to the bacterial pathogen *E. amylovora* (Gardiner et al., 2012) to the fungi *B. dothidea* (Zhao et al., 2019a), *F. solani* (Wang et al., 2021), *A. alternata* (Hou et al., 2021), *V. mali* (Liu et al., 2021) and among others. Abiotic stress also becomes evident in tolerance to water stress (Han et al., 2018; Xing et al., 2019) and salt stress (Zhao et al., 2019b; Ma et al., 2021). Other functions involved include the participation of anthocyanin and flavonoid biosynthesis (Liu et al., 2019a; Hu et al., 2020; Premathilake et al., 2020) in fruit sucrose levels (Li et al., 2020b), on fruit ripening (Huang et al., 2014; Nham et al., 2015; SHI et al., 2021), on rootstock dwarfism (Ou et al., 2015), red and green fruit skin color (Yang et al., 2015), oxidative stress (Cukrov et al., 2016) and among other functions based on protein-protein interaction, phytohormones and specific cis elements mainly.

Advances that were generated from the differential expressions of RNA-Seq, comparative genomics, genomic selection and among others the identification and characterization of the WRKY genes in the complete genome, as well as 103 *PbrWRKY* genes in the white pear (*Pyrus bretschneideri*) with patterns under stress (Huang et al., 2015) and *112 PbrWRKY* genes associated with bud dormancy and responses to abiotic stress (salt, drought, copper and waterlogging) (Shangguan et al., 2020). In apple (*Malus domestica*) there are 127 *MdWRKY* genes in response to flooding and water stress (Meng et al., 2016), 119 *MdWRKY* genes in response to fungal pathogens (Lui et al., 2017), 141 *MdWRKY* genes associated with dormancy of buds and responses to abiotic stress (Shangguan et al., 2020) and 113 MdWRKY genes in response to biotic stress (Zhang et al., 2021). The multiple functions of discovered and yet to be discovered WRKY genes were and are limited by the availability of complete reference genomes in pome fruit species. The identification and characterization of WRKYs genes in available complete genomes, could provide even more information for functional inferences, discover new specific functions and support differential transcriptomic expressions, epigenetic regulations, among others.

Although much more WRKY TFs have been reported in various model plants, this gene superfamily has not yet been exhaustively studied for its identification, characterization, and genomic comparison in the pome fruit group. Here, we conducted a comprehensive and systematic analysis of the WRKY genes of six pome fruit species and cultivars, including gala apple (*M. domestica*), wild apple (*M. sieversii*), siberian apple (*M. baccata*), Japanese pear (*P. pyrifolia*), wild pear (*P. betulifolia*) and european pear (*P. communis*). Finally, we report the evolutionary relationship of *pomWRKY* proteins, their divergence of tandem duplicated genes, and differential gene expression in stress response and tolerance. Our results provided precise information on the *pomWRKYs* genes that will support future research on this important superfamily of genes in fruit plants.

5.2 Materials and Methods

Identification of the WRKY superfamily in the complete genomes of *M. domestica cv. Gala, M. baccata, M. sieversii, P. communis, P. betulifolia and P. pyrifolia*

Data from six complete genomes were collected from the Rosaceae Genome Database - GDR (https://www.rosaceae.org/) and from the BioProject Database of the National Center for Biotechnology Information -NCBI (http://www.rosaceae.org/). /www.ncbi.nlm.nih.gov/bioproject). For *Malus* spp. there were three species: gala apple (*M. domestica* cv. Gala haploid v1.0), wild apple (*M. sieversii* v1.0) under accession number PRJNA591623 and siberian apple (*M. baccata* v1.0) under accession number access code PRJNA428857. For *Pyrus* spp. there were also three species: European pear (*P. communis* v2.0) under accession PRJNA555057, wild pear (*P. betulifolia* v1.0) under accession PRJNA529328 and Japanese pear (*P. pyrifolia* cv Cuiguan v1.0) on access PRJNA669907. To identify WRKY proteins in pome fruit species, a Hidden Markov Model (HMM) of the WRKY domain (PF03106) in pome fruit species was used using HMMER software with an E-cutoff value of 0.001 (E 1e-3). Afterwards, a thorough examination of all sequences was performed using Pfam tools, InterProScan database, NCBI database and SMART (Letunic and Bork, 2018; Sharma et al., 2018; Blum et al., 2021; Mistry et al., 2021). For chemical characteristics of protein sequences, ExPASY ProtParam (https://web.expasy.org/protparam/) (Gasteiger et al., 2003) was used to predict isoelectric point (pI), molecular weight (MW) and overall average hydrophobicity (GRAVY) of each *pomWRKY*.

Multiple alignment and phylogenetic analyses

For global or multiple alignment ClustalX2 was used with default settings (Larkin et al., 2007). All aligned sequences were rigorously cured in Gblocks 0.91b (Castresana, 2000) on the NGPhylogeny.fr website (Lemoine et al., 2019). MEGA XI (Tamura et al., 2021) was used to correct gaps, align columns and generate tree topologies by the Neighbor-Joining -NJ method with 1000 bootstrap and applying the JTT matrix (Jones et al., 1992). The BEAST v.2.5 software (Bouckaert et al., 2019) was also used, with the maximum probability UPGMA clustering method (Moulton et al., 2018), with 10,000,000 bootstrap and JTT replacement matrix replicas to generate a single tree topology of all kinds of groups and subgroups or domains of gene sequences.

Analysis of gene structure and identification of conservation of motifs

To investigate the diversity and structure of *pomWRKY* family members, genomic sequences for their exon/intron were used and plotted in TBtools-II (Chen et al., 2020), based on annotation information from the six genomes of *Malus* and *Pyrus* spp. Protein sequences from *pomWRKY* were used to identify conserved motifs, Expectation Maximization Tool for Motive Elicitation MEME (https://meme-suite.org/meme/tools/meme) (Bailey et al., 2006). The parameters were the following: number of repetitions: any; maximum number of reasons: 10; and ideal motif widths: 8 to 50 amino acid residues.

Chromosomal localization, pomWRKY gene duplication, Ka/Ks calculation and divergence time estimation

The chromosomal locations of the *pomWRKY* genes were generated by the TBtools-II software (Chen et al., 2020), according to the chromosomal position information provided in the Rosaceae-GDR genomic database (https://www.rosaceae.org). To identify species tandem duplications of pomWRKYs, the following criteria were used: genes within a 100 kbp region on an individual chromosome with a sequence similarity of ≥ 70% (Hu et al., 2010). The pairwise local alignment calculation of two protein sequences was performed by the Smith-Waterman algorithm of EMBOSS Water (http://www.ebi.ac.uk/Tools/psa/)(Madeira et al., 2022).

For the calculation of non-synonymous substitutions per site (Ka) and the number of synonymous substitutions per synonymous site (Ks), in addition to comparing the selection pressure, a Ka/Ks ratio greater than 1, less than 1 and equal to 1 represents selection positive, negative selection and neutral selection, respectively (Doerks et al., 2002). For each pair of genes, the value of Ks was used to estimate the time of divergence in millions of years based on a rate of 6.1×10^{-9} replacements per site per year, and the time of divergence (T) was calculated as $T = Ks/ (2 \times 6.1 \times 10^{-9}) \times 10^{-6}$ million years ago (Mya) (Lynch and Conery, 2000). The bioinformatics tool used for genetic divergence was the simple Ka/Ks Calculator (NG) from TBtools -II (Chen et al., 2020).

Transcriptomic analysis of *pomWRKY* genes in *Malus* and *Pyrus* spp.

Expression patterns of the *pomWRKY* genes were analyzed based on published data from 48 plant organ tissue samples from RNA-seq in the NCBI bioProject. For *M. domestica*, nine samples were collected from ID PRJNA756786 (Song et al., 2019), for *M. baccata*, nine samples from ID PRJNA801073 (Ge et al., 2022), *P. pyrifolia*, six samples from ID PRJNA669907 (Gao et al., 2021), *P. betulifolia* 12 samples of ID PRJNA829646 (Gao et al., 2021) and for *P. communis* 12 samples of ID PRJNA527142 (Hewitt et al., 2020). Sample treatments were performed in four steps: SRA toolkit (Leinonen et al., 2011) was used to download the data samples, FastQC (Wingett and Andrews, 2018) was employed to analyze and visualize the quality of the readings, Trimmomatic ver. 0.39 (Bolger et al., 2014) was applied to remove low-quality and library adapters; b) The reads were mapped against the reference genome of *M. domestica* cv. Gala (Sun et al., 2020), *M. baccata* (Chen et al., 2019b), *P. pyrifolia (*Gao et al., 2021), *P. betulifolia* (Zhao et al., 2022) and *P. communis* (Hewitt et al., 2020), using the HISAT2 software (Pertea et al., 2016). In the next step, (c) for the count of the total aligned reads per gene in the different libraries, FeatureCounts (Liao et al., 2014).

Quantification (d) was performed using the limma, EdgerR and DESeq2 packages in the R software (Law et al., 2018). In this protocol, a FPKM (Fragments Per Kilobase Million) normalization method was used, which means fragments per kilobase of exon per million mapped fragments, which evaluates the sequencing depth and length of the sequences and the expression of *pomWRKYs* genes per library. A heat map was produced using TBTools (Chen et al. 2020a), then a multidimensional scale plot (MDS) was generated to check sample repeatability and overall difference between samples, a sample mean variance plot (MeanVar) and Biological Coefficient of Variation (BCV) as a function of gene abundance (in log2 counts per million).

5.3 Results

Identification of the WRKY superfamily in the complete genomes of *M. domestica cv. Gala, M. baccata, M. sieversii, P. communis, P. betulifolia and P. pyrifolia*

The statistical alignment of the Hidden Markov Model (HMMsearch) in HMMER and the local BLASTp alignment of the proteome of *M. domestica cv. Gala, M. domestica cv. Gala,*

M. baccata, M. sieversii, P. communis, P. betulifolia and *P. pyrifolia*, was used to identify a total of 667 WRKY TFs in six species (Table 1). The genes of *Malus* and *Pyrus* spp., were identified and characterized with complete heptapeptide (WRKYGQK) and based on their WRKY protein family nomenclature (PF03106) (Supplementary 1). Nineteen sequences without complete heptapeptide domain (WRKYGQK) were discarded and 32 sequences with variations in heptapeptides (WCKYGQK, WRKYGKK, WRKYGHK, WRKYGNK, WKNCGQD, WRKYGQN, WRKYGMK, WQKYGQK, WSKYGQK, WHKYGQK and WRKYGNK) were included in the analysis. The length of the peptide ranged from 65 amino acid residues (aa) (MbWRKY3) to 2,032 aa (MdgWRKY32). Coding sequences (CDS) ranged from 198 to 6,099 nucleotides. The molecular weight of predicted proteins ranged from 7,388.79 to 225,671.83 kDA. The isoelectric point ranged from 4.68 (PcWRKY14) to 10.28 (PcWRKY16). The mean large hydrophobicity ranged from -1.28 (MdgWRKY69) to 0.21 (MbWRKY3).

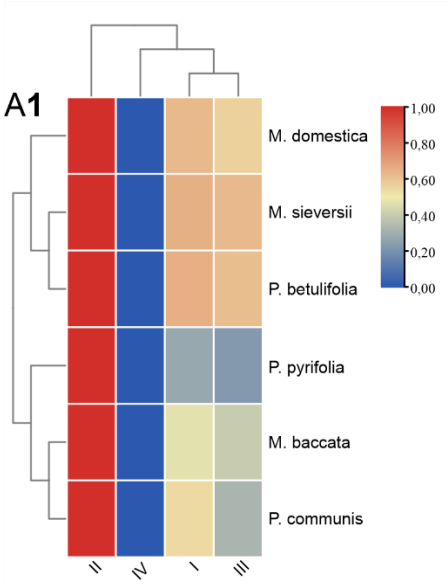
Phylogeny, gene structure and motif analysis of the WRKY protein in the complete genomes of *Malus spp. and Pyrus spp.*

Based on the unrooted maximum probability phylogenetic tree of the 667 protein sequences, the *pomWRKY* gene family from *Malus* and *Pyrus* spp was categorized into four groups I, II, III and IV (supplementary 2 Fig. 1). Of the total of 667 WRKY genes classified by groups and by species, group II contains the largest number of WRKY members (434 genes) and the smallest is group IV with 13 members of (Fig. 1A). Six maximum probability unrooted phylogenetic trees of each species with their genetic structure and conservation reasons were generated (supplementary 2 Fig. 2-7). In addition, a maximum probability unrooted phylogenetic tree was generated with nine subgroups of the WRKY domains of *Malus* spp. And *Pyrus spp.* Which are: I-NT, I-CT, Iia, Iib, Iic, Iid, Iie, III, IV (Fig.2). The group I (containing 119 members) and Iic (155) are the largest groups, followed by group III (100), group Iid (87), group Iie (80), group Iib (78) and group Iia (41). Then to the fourth group (IV) with 10 members without the feature of the zinc finger motif (Fig.1B). The phylogenetic tree of the subgroups presents an evolution of the complete and incomplete domain based on the conservation and variation of the heptapeptide (WRKYGQK), also on the presence, absence and variation of the zinc

finger motif in the subgroups. And three sequences PcWRKY79, PcWRKY88 and MbWRKY16 in the I-NT group. Also, seven group IV sequences (PcWRKY14, PcWRKY33, PcWRKY64, PbWRKY84, MbWRKY3, MbWRKY14, MsiWRKY46) were aligned and grouped into different groups (I-CT, III, II-b, II-c).

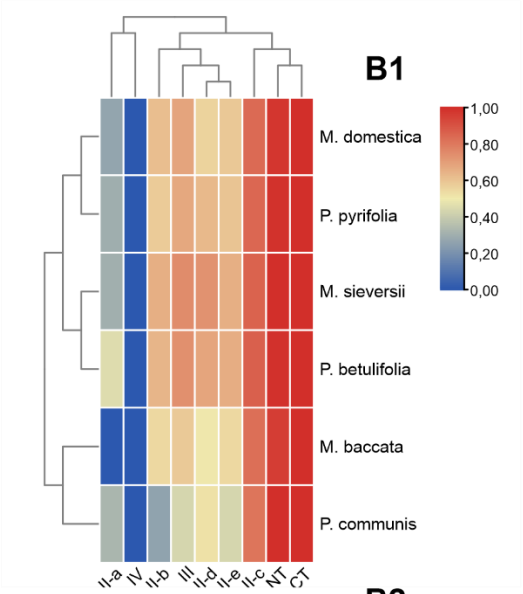
Table 1. Genomic information and WRKY gene numbers identified in six species of *Malus spp*. And *Pyrus spp.*

Common name	Scientific name	Chrosome number	Release version	Genome gene number	Identified WRKY genes	Incomplete domain
Gala apple	*Malus x domestica cv. Gala*	17	Haploid v1.0	45352	110	2
Asian wild apple	*Malus sieversii*	17	Haploid v1.0	45210	118	5
Wild apple	*Malus baccata*	Scaffold	Whole Genome v1.0	45931	107	3
European pear	*Pyrus communis*	17	BartlettDHv2.0	37445	90	3
Chinese wild pear	*Pyrus betulifolia*	17	Shanxi v1.0	59552	116	3
Cuiguan' pear	*Pyrus pyrifolia*	17	Whole Genome v1.0	42559	126	3



A2

Species	I	II	III	IV
M. baccata	18	69	15	5
M. domestica	21	70	17	2
M. sieversii	21	77	19	1
P. betulifolia	21	76	18	1
P. communis	18	58	10	4
P. pyrifolia	22	84	20	0
Total	121	434	99	13



B2

Species	I-CT	I-NT	II-a	II-b	II-c	II-d	II-e	III	IV
M. baccata	17	15	5	14	23	13	14	15	5
M. domestica	21	19	5	14	26	12	13	17	2
M. sieversii	21	21	4	14	27	18	14	19	1
P. betulifolia	21	20	7	13	27	15	14	18	1
P. communis	16	16	8	7	21	12	10	10	4
P. pyrifolia	22	20	8	16	29	17	14	20	0
Total	118	111	37	78	153	87	79	99	13

Figure 1: *(A1-2)* Number of WRKY genes and group in *M. domestica cv. Gala, M. baccata, M. sieversii, P. communis, P. betulifolia and P. pyrifolia, (B1-2)* Subgroup

distribution of WRKY members *M. domestica cv. Gala, M. baccata, M. sieversii, P. communis, P. betulifolia and P. pyrifolia.*

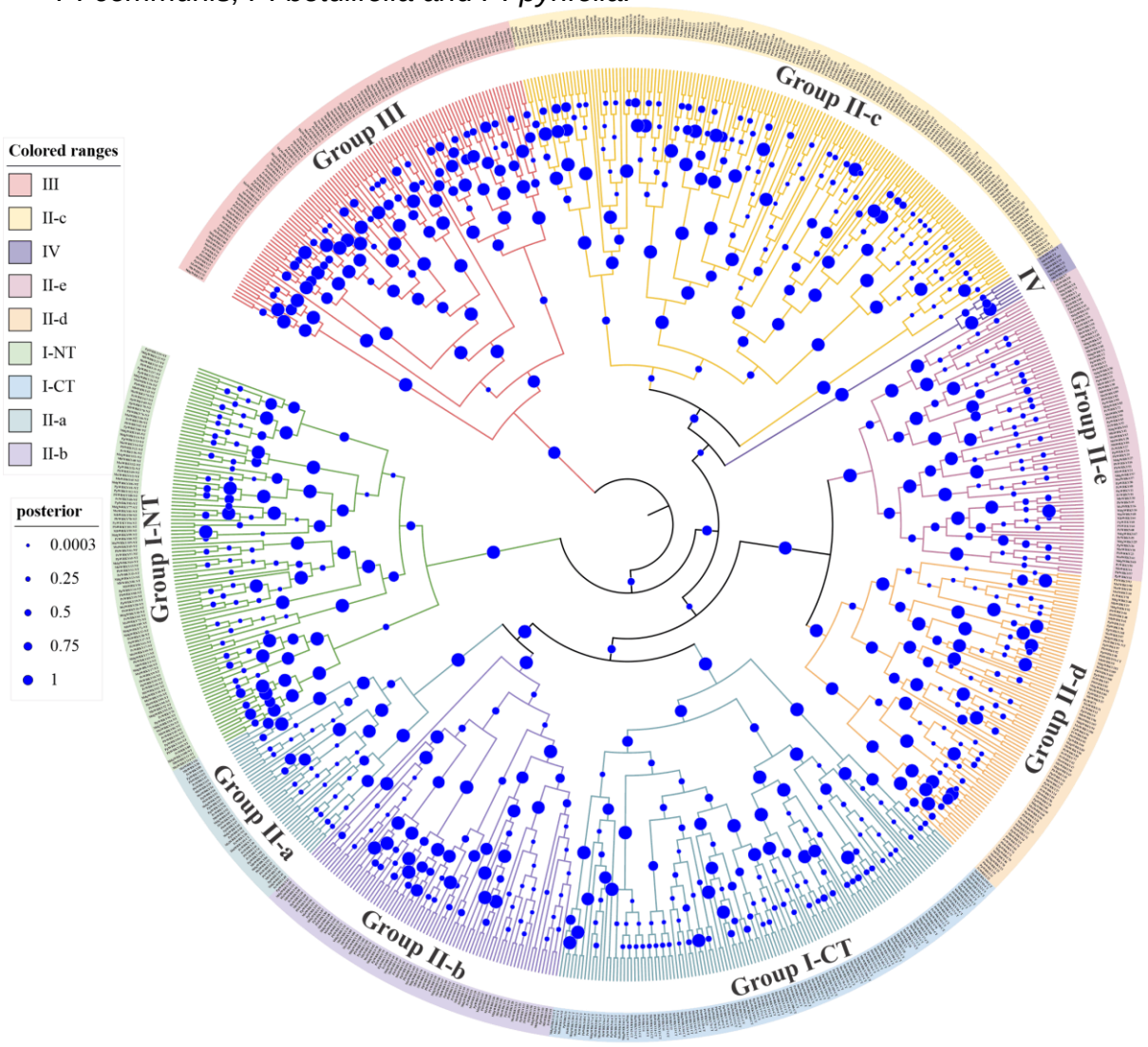


Figure 2: Superfamily of pomWRKY genes: The unrooted maximum likelihood phylogenetic tree was generated with the BEAST program using the complete and non-complete amino acid sequences of 777 pomWRKY from *M. domestica cv. Gala, M. baccata, M. sieversii, P. communis, P. betulifolia and P. pyrifolia* by the UPGMA method, with 10,000,000 bootstrap replications. The subgroups of pomWRKY proteins (I-NT, I-CT, Iia, Iib, Iic, Iid, Iie, III and IV).

Chromosomal localization, pomWRKY gene duplication and Ka/Ks calculation and divergence time estimation

Of the total of 667 *pomWRKY* genes, 560 were mapped to the 17 chromosomes of the genomes of *M. domestica, M. sieverssii, P. betulifolia*, *P.* communis and *P. pyrifolia* (supplementary 3A-E). 107 *M. baccata* genes were not mapped because the genome is

at the level of Scaffolds. The largest number of pomWRKYs genes are distributed on chromosomes chr15 (57) and chr12 (48), also the smallest number of genes are distributed on chromosomes chr14 (21) and chr11 (20). Wild species have a greater number of *pomWRKY* genes than *M. sieversii* (*118 MsiWRKY*) and *P. pyrifolia* (126 PpWRKY). Commercial species contain fewer *pomWRKY* genes than *M. domestica* (*110 MdgWRKY*) and *P. communis* (*90 PcWRKY*). The number of tandem duplicated genes per species were identified based on closeness of chromosome mapping, out of a total of 112 pairs of pomWRKYs identified by proximity and 52 genes were identified as tandem duplicated genes (supplementary4.Table1). In *M. domestica*, five pairs of *MdgWRKY* genes were identified, in *M. sieversii* 12 pairs of *MsiWRKY* genes, 16 pairs of PbWRKY genes from *P. betulifolia*, seven pairs of PcWRKY genes from *P. communis* and 12 pairs of PpWRKY genes from *P. pyrifolia*.

Calculation of Ka/Ks and estimation of divergence time of tandem duplicated gene pairs, according to non-synonymous (Ka) and synonymous (Ks) substitutions (supplementary4. Table2). In total, we obtained the values of Ks, Ka and Ka/Ks and selected the types of 51 pairs of orthologous genes. Results revealed that most orthologous gene pairs (50/51) had Ka/Ks ratios < 1, indicating that purification and selection acted on these orthologous genes. A pair of *MsiWRKY88/MsiWRKY89* genes could not be calculated with the Ks value, indicating that there may be more sequence divergence between these two genes. Finally, we estimated the divergence time of orthologous pomWRKY gene pairs according to their synonymous replacement rates. The results revealed that the divergence time interval ranged from 0.10 to 46.36 million years for the orthologous gene pairs *PpWRKY75/PpWRKY77* and *PbWRKY91/PbWRKY93*.

Expression patterns of WRKYs genes in different tissues

To explore the expression patterns of pomWRKY family genes in *Malus* (*M. domestica, M. baccata)* and *Pyrus* spp. *(P. communis, P. betilifolia, P. pyrifolia*), an analysis of gene expression of the pomWRKY family in different organs of each species was performed (Supplementary 5 Fig. A-E). Furthermore, the highly expressed pomWRKY genes and also the overlap between the ellipses represents the intersection of the set of genes from

each sample that are involved in response and tolerance to biotic and abiotic stresses (Fig. 3A1-5; 3B1-5). Of the total of 45,352 genes annotated in the genome of *M. domestica cv.* Gala, the transcriptional expression of genes and *MdgWRKY* genes for each sample to abiotic stress by low temperatures was: leaves with 30 days of treatment 0 hours (29,559=65.18%; 95 MdgWRKY = 0.32%), three days of treatment (30,437=67.11%; 101 MdgWRKY = 0.33%), seven days of treatment (30,942= 68.23%; 102 MdgWRKY = 0.33%). In the *M. baccata of* the total of 45,930 annotated genes, the transcriptional expression of the genes and MbWRKY genes of the branched samples to the environmental signals: in the wild species dormant bud (31,821=69.28%; 91 MbWRKY = 0.29%), in the waiting of the budding of the mutant species (32028= 69.73%; 93 MbWRKY = 0.29%) and in the dormancy of the bud of the mutant species (31,909 = 69.47%; 95 MbWRKY =0.30%).

In *P. communis* of the total of 37,445 annotated genes, the transcriptional expression of genes and PcWRKY genes in the regulation of cold-induced ripening were expressed in four physiological times: 0% conditioned (27,543=73.55%; 73 PcWRKY = 0.27 %), 50% conditioned (26,369=70.42%; PcWRKY70=0.27%), 100% conditioned (25,680 = 68.58%; 71 PcWRKY= 0.28%) and 100% matured (25,498=68.09% ;73 PcWRKY=0.28%). In *P. betulifolia* of the total of 59,552 annotated genes, the transcriptional expression of the genes and *PbWRKY* genes in response to the inoculum exposure time by *Valsa pyri* metabolites – VpM: day 0 with VPM inoculum (30,532 = 51.27%; 101 *PbWRKY* = 0 .33%), day 1 with VPM inoculum (30,697 = 51.55%; 100 *PbWRKY* = 0.33%), day 2 with VPM inoculum (30,670 = 51.50%; 103 *PbWRKY* = 0.34%), day 3 with VPM inoculum (30,003 = 50.38%; 97 *PbWRKY*= 0.32%). In *P. pyrifolia* of a total of 42,622 annotated genes, transcriptional expression of genes and *PpWRKY* genes in response to artificial cooling accumulation (ACA) in bud dormancy: day 0 with ACA (32,294 =75.77%; 108 *PpWRKY*= 0.25%), day 10 with ACA (32,267 = 75.71%; 104 *PpWRKY* = 0.24%), day 20 with ACA (31,974 = 75.02%; 96 *PpWRKY* = 0.23%), day 30 with ACA (32,073 = 75.25%; 104 *PpWRKY* = 0.24%), day 40 with ACA (31,544 = 74.01%; 103 *PpWRKY* = 0.24%) and day 50 with ACA (31,739 = 74.47%; 97=0.23%).

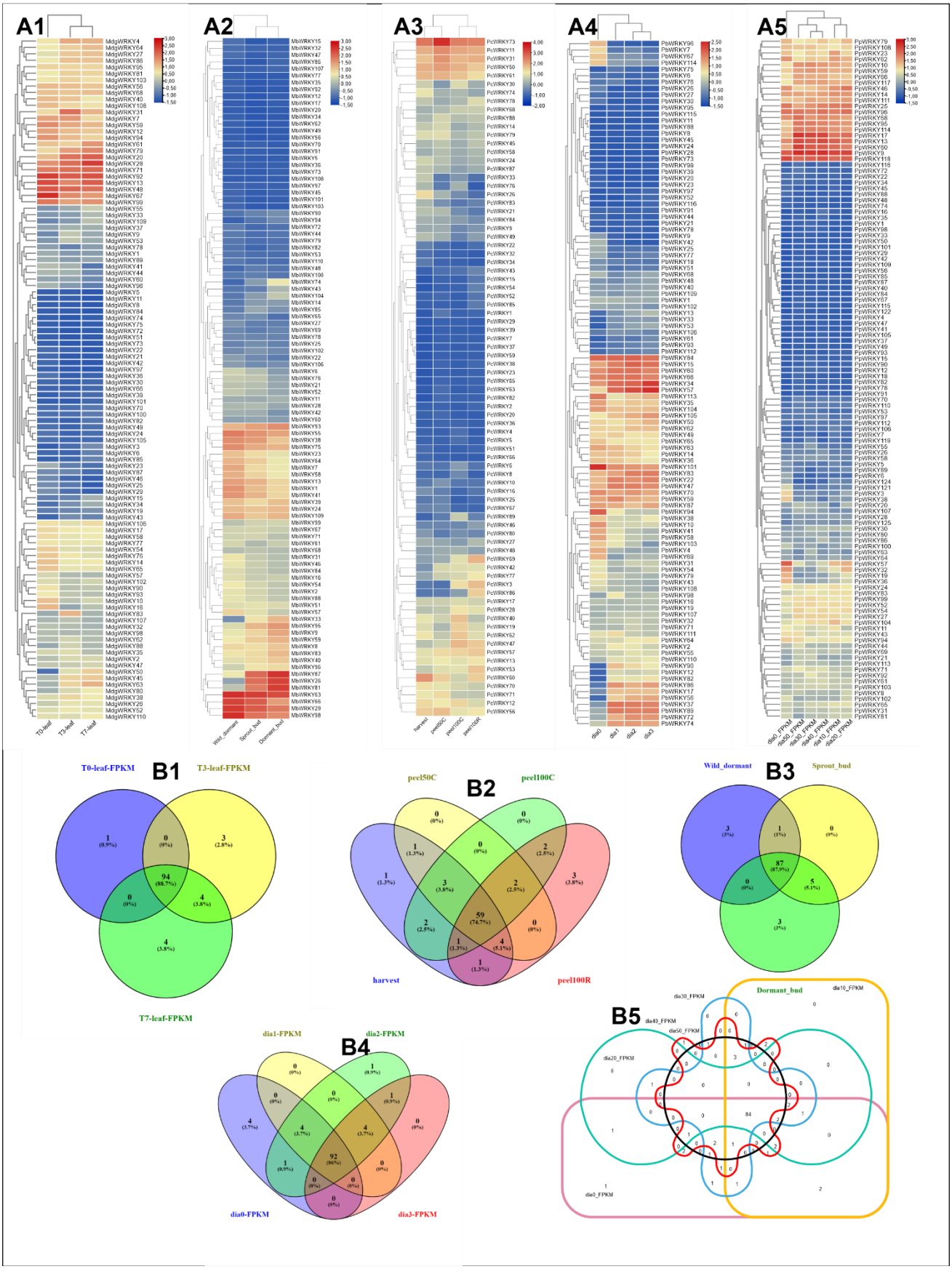

A1
A2
A3
A4
A5
T0-leaf-FPKM
T3-leaf-FPKM
T7-leaf-FPKM
B1
peel50C
peel100C
harvest
peel100R
B2
Wild_dormant
Sprout_bud
B3
dia1-FPKM
dia2-FPKM
dia0-FPKM
dia3-FPKM
B4
Dormant_bud
B5

Figure 3. Gene expression analysis of 48 samples processed and presented in 20 pomWRKY consensus samples: A1) heat map of MdWRKY genes (*M. domestica*), A2) heat map of MbWRKY genes (*M. baccata),* A3) heatmap of PcWRKY genes (*P.communis*), A4) heatmap of PbWRKY genes (*P. betulifolia*) and A5) heatmap of PpWRKY genes (*P. pyrifolia*), all values are presented in log2 of - 2 to 4.00 range), red color indicates the highest number of reads or genes expressed according to sequencing depth and blue indicates lowest and read or genes expressed according to sequencing depth and length. B1) Venn diagram of MdWRKY (*M. domestica*), B2) Venn diagram of MbWRKY genes (M. baccata), B3) Venn diagram of PcWRKY genes (*P. communis*), B4) Venn diagram of PbWRKY genes (*P. betulifolia*) and B5) Venn diagram of PpWRKY genes (*P. pyrifolia*).

5.4 Discussion

Identification of the WRKY protein family in *Malus spp. and Pyrus spp.*

WRKY TFs are key regulators of many processes in plants, exhibiting specific responses to biotic stresses and involvement in tolerance to abiotic stresses (Cheng et al., 2021; Felipez et al., 2022). In model species such as *Arabidopsis thaliana* (Rushton et al., 2010; Wang et al., 2011) and also in non-model species such as those of the botanical family Rosaceae (Jiang et al., 2021), WRKY TFs and their involvement for promoting resistance and tolerance to different stresses were functionally characterized (Wani et al., 2021).
The availability of assembly and annotation of the complete genome of *Malus spp*.: in gala apple (*M. domestica* cv. Gala Haploid v1.0), wild apple (*M. sieversii* Haploid Consensus Whole Genome v1.0) (Sun et al., 2020) and siberian apple (*M. baccata* Genome v1.0) (Chen et al., 2019b). In *Pyrus spp*.: in european pear (*P. communis* Bartlett DH Genome v2.0) (Linsmith et al., 2019), Chinese wild pear (*P. betulifolia* Genome v1.0) (Dong et al., 2020) and pear cultivar Cuiguan from China (*P. pyrifolia* Cuiguan Whole Genome v1.0) (Gao et al., 2021), enabled genome-wide analysis in the six species of the WRKY transcription factor family.

The identification of 667 pomWRKY genes in *Malus spp.* and *Pyrus spp*. corresponds to a number of 111 pomWRKY genes with an average slightly higher and lower than that of other species, for example, there are 103 PbWRKY genes in pear (*P. bretschneideri*) (Huang et al., 2015), 127 *MdWRKY* genes in apple (*M. domestica*) (Meng et al., 2016) and 113 *MdWRKY* genes in apple (*M. domestica*) (Zhang et al., 2021). In contrast to 75

AtWRKY genes in Arabidopsis (Eulgem et al., 2000), rose (*Rosa chinensis*) contains 56 *RcWRKY* genes (Liu et al., 2019b) and 59 *FvWRKY* genes in wild strawberry (*F. vesca*) (Garrido-Gala et al., 2022) and 61 *PpWRKY* genes in peach (*P. persica*) (Yanbing et al., 2016) were identified.

The pomWRKYs currently found in *Malus spp.* and *Pyrus spp*. has specific responses and tolerances to abiotic and biotic stresses, as mentioned in studies of the roles of WRKY in water stress, salinity, in addition to fungi and bacteria in *Malus spp.* and *Pyrus spp*. (Felipez et al., 2022), may become a model species group for the species of the genera and subfamily Maloideae. The species *Malus spp.* and *Pyrus spp.* have agricultural and economic potential (Bell et al., 2019; Qu et al., 2021). The pomWRKY TFs contribute to the recent identification of WRKY genes in Rosaceae plant species such as apple, pear, strawberry and peach and among others.

Phylogeny, gene structure and motif analysis of the pomWRKY protein

The structure of the WRKY TFs is compounded by the conservation of the number of the WRKY domain and the characteristics of its zinc finger motif, which binds to the cis W-box element in the promoters of its target genes, the most essential feature of this superfamily (Eulgem et al., 2000; Zhang and Wang, 2005; Jang et al., 2010; Felipez et al., 2022). The classification of *pomWRKY* genes was performed according to the approach used in other species, based on the topology of the generated phylogenetic tree. The divisions of were also adapted into groups and subgroups described in Arabidopsis and *Malus domestica*: groups I, II and III according to the number of WRKY domains and the type of zinc finger motif, along with the subdivision of group II into subgroups IIa, IIb, IIc, IId and IIe (Eulgem et al., 2000; Felipez et al., 2022).

In this study, 121, 434 and 99 *pomWRKY* genes were classified in groups I, II and III respectively, however, 13 *pomWRKY* genes were classified in group IV because they did not belong to any group and also did not have the zinc finger motif (supplementary2 fig.1). Group II was larger with 434 members and represented 65% of all *pomWRKY* genes. These results are consistent with approximations of the largest percentage size of WRKY

representation in group II in Arabidopsis (63%), apple (62%), grapevine (65%) and rice (47%) species (Meng et al., 2016) in response to abiotic stress and biotic stress 58% (113 = 65 members) in litter (Zhang et al., 2021). Of the total of 434 members of group II, 153 members of genes that are part of subgroup II-c, being a 23% of the total of 667 identified genes. In model species that have already been identified and classified, they report that also the largest number of WRKY gene members are present in group II and in subgroup II-c, among them are Arabidopsis IIc (40%), vine IIc (41%) and IIc rice (33%) have the highest number of groups II WRKY genes (Meng et al., 2016).

The presence of WRKYGQK heptapeptide domains in each protein sequence generated nine subgroups, I-CT (118 WRKY members), I-NT (111), IIa (37), IIb (78), IIc(153), IId(87), IIe(79), III(99) and IV(13). The breakdown of group I into subgroups of I-NT and I-CT was done to compress the evolution of the WRKYs genes. The conservation variability of amino acids of the I-CT subgroup, indicates that they are responsible for generating the rest of the subgroups of domains, as mentioned in the study on the evolution of TFs WRKY (Rinerson et al., 2015). However, in the hypotheses generated from the WRKY superfamily in plants and eukaryotes, it makes reference to the domain or subgroup I-CT of generating the domain of group II (IIa, IIb, IIc, IId, IIe) (Zhang and Wang, 2005). Also, in the hypothetical evolution of the WRKY domain (WD), the I-CT diversified into IIc and IIb, in addition to the WRKY domain IIa and IIe were deduced from IIb and IId, in addition to the I-NT domain that were obtained by domain duplication, having a subfunctionalization of the domain (Chen et al., 2019a).

Our study presented the same characteristics of the pomWRKYs domains, however, it presented 10 protein sequences of only one domain with the characteristics of the subgroup of the I-CT domains (PpWRKY16, PpWRKY31, PcWRKY12, PcWRKY14, PcWRKY23, PbWRKY66, MdgWRKY18, MdgWRKY69, MbWRKY3, MbWRKY24, MbWRKY36, MbWRKY37) and five other sequences with different alignment of amino acids (PcWRKY14, MbWRKY3, MsiWRKY18, MsiWRKY34-NT, MsiWRKY70) are phylogenetically part of the I-CT subgroup. In addition, single domain sequences are part of the I-NT (PcWRKY79, PcWRKY88, MbWRKY16) and also single domain sequences

without five WRKY group IV fingers (PcWRKY14, PcWRKY33, PcWRKY64, PbWRKY84, MbWRKY3, MbWRKY14, MsiWRKY46), some of them are phylogenetically part of other subgroups (I-CT, III, IIb, IIa) and among other variations the MsiWRKY18 and MsiWRKY70 sequences, due to their structural characteristics, are part of the II-d group and phylogenetically they are part of the I-CT group . Evidence from some pomWRKY genomic sequences indicates that there are still duplicated domains and amino acid structural variations that are evolving.

In addition to the conservation of the heptapeptide (WRKYGQK) in WRKY proteins, amino acid variants were identified in 32 genomic sequences of the pomWRKY domains (WCKYGQK, WRKYGQK, WRKYGHK, WRKYGNK, WKNCGQD, WRKYGQN, WRKYGMK, WQKYGQK, WSKYGQK, WHKYGQK and WRKYGQK). These variants are some similar to the heptapeptide of the 13 SbWRKY proteins of sorghum (*S. bicolor*) (Baillo et al., 2020), of the 12 legumes in the Q site in WRKYGQK, while W, K and Y are conserved (Song et al., 2018), also from two legumes with variations from R to K to give WKKYGQK, or from Q to E or K to give WRKYGEK or WRKYGKK (Srivastava et al., 2018). The heptapeptide variations in apple (*M. domestica*) of nine proteins MdWRKY78, MdWRKY53, MdWRKY92 and MdWRKY96 (WRKYGKK) and other variations of MdWRKY51 and MdWRKY38 (WRKYG*K), MdWRKY114 (WRKYARS), MdWRKY20 (WRKYGWKKI4, MdWRKYGKKI) are considered within the analysis and differentially expressed their functions to stress by flooding and water stress (Meng et al., 2016). Furthermore, identical variations are reported in peach (*P. persica*), PpWRKY33 (WRKYGKK) and PpWRKY35(WRKYGMK) (Yanbing et al., 2016). In contrast to variations without loss of function, there are loss of function of the WRKY genes, due to variations of the heptapeptide in soy (*G. max*), for example, the changes in the proteins GmWRKY6 and GmWRKY21 do not allow binding to the cis element W-box (Zhou et al. al., 2008). In tobacco the NtWRKY12 protein variant WRKYGKK recognizes other binding sequences (TTTCCAC) instead of the normal W-box (van Verk et al., 2008). Therefore, further investigations are needed to identify the DNA binding sequences of the variants that are part of the WRKYGQK heptapeptide.

Of the 20 functional motifs identified in the pomWRKY proteins, 1, 2, 3, 4 and 5 correspond to the WRKY domain (supplementary 2 Fig. 2-7). Zinc finger domains are present in most members of pomWRKY. The rest of the motifs identified in the amino acid sequences of the proteins represent the nuclear localization signal and the histone methyltransferase complex distributed mainly in groups I and II (IIe and IIc). In Arabidopsis, epigenetic regulation by histone methyltransferase is attributed to AtWRKY53, a senescence regulator (Banerjee and Roychoudhury, 2015). Some motifs have the role of initiating the formation of the vegetative septum (FtsL/DivIC) (Villanelo et al., 2011) and the leucine zipper motif (bZIP_2) (Jakoby et al., 2002), are motifs that are distributed in the subgroups IIa and IIe of the pomWRKY proteins, having specific roles in regulating processes, defending against pathogens and, among others, in signaling stress. However, the rest of the motifs identified are secondary structures of the plant zinc finger (Babu et al., 2006), which have functional diversity through expression divergence rather than protein sequence divergence. In the amino acid sequences of WRKY TFs, identifying motifs and pomWRKY domains are functional in their predicted structure, but some submotif-conforming motifs have specific functions that require further study.

Chromosomal location, duplication and divergence of *pomWRKY* genes

The chromosomal location of the five species were carried out and had the genome mutated at the chromosomal level (*M. domestica cv. Gala, M. sieversii, P. communis, P. betulifolia, P. pyrifolia*) of 560 pomWRKY genes on the 17 haploid chromosomes of the genome of *Malus spp.* and *Pyrus spp*. (Evans and Campbell, 2002), with changes in the intensity of its distribution in the genomes of *Malus spp* and *Pyrus spp*. The presence of tandem duplication in genomes differs by the number of copies and their distribution, suggesting that the duplication events occurred after the divergence between species, probably happened 30 to 35 million years ago (Mya) (Xiang et al., 2017). Gene duplication events affect genome expansion, family size and the distribution of genes on chromosomes, which are important factors for functional prediction (Zhou et al., 2016). However, whole-genome, segmental, and tandem duplication were major contributors to the expansion of the WRKY gene family (Chen et al., 2019a).

The tandem duplication of chromosomal regions leads to the expansion and evolution of the gene family, through structural and functional divergence over time (Xiang et al., 2017; Chen et al., 2019a). In this analysis, five pairs of genes were identified in tandem duplication from *M. domestica* cv Gala, 12 pairs of genes from *M. sieversii,* seven pairs of genes from *P. communis*, 16 pairs of genes from *P. betulifolia* and 12 pairs of genes from *P. communis*. of genes in *P. pyrifolia* with >70% similarity), which is nearly similar and sometimes higher than the other model species, reporting for strawberry (Zhou et al., 2016), Arabidopsis (Cannon et al., 2004), sorghum (Baillo et al., 2020) and *Prunus mume* (Bao et al., 2019) Despite these differences, tandem duplications may have shaped the evolution of the *pomWRKY* gene family from *Malus spp.* and *Pyrus spp*. However, segmental duplication is the result of large-scale genomic events such as polyploidy or duplication of large chromosomal regions that were not considered in the study.

The divergence of gene pairs identified in millions of years (Mya), show a variation of each studied species of *pomWRKY* genes. In *M. domestica* it is 2 to 63 Mya (MdgWRKY68/MdgWRKY69, MdgWRKY21/MdgWRKY22), *M. sieversii* is 1 to 10 Mya (MsiWRKY17/MsiWRKY18, MsiWRKY73/MsiWRKY75), *P. communis* is 2 to 6 Mya (PcWRKY3KY,3KY8 PcWRKY63/PcWRKY65), *P. betulifolia* is 1 to 46 Mya (PbWRKY74/PbWRKY75, PbWRKY91/PbWRKY93) and *P. pyrifolia* is 0.1 to 9 Mya. The greatest divergence of gene pairs in millions of years is the wild species *Malus ssp.* and *Pyrus spp*. Duplication events are significant for *pomWRKY* diversification, as duplicated genes can acquire new functions (Baillo et al., 2020). Therefore, whole genome duplications, tandem and also segmental duplications played essential roles in the evolution and diversification of the WRKY gene family in plant species (Zhu et al., 2014). In wheat it occurred through whole genome duplication and in tandem, the functions were expanded from members of the WRKY gene family (Hassan et al., 2019). In *O. sativa ssp. japonica* and *O. nivara*, group III of WRKYs emerged by tandem duplication (Wu et al., 2005; Xu et al., 2016) and the diversification of WRKY genes occurred by the divergence of monocots and dicots (Chen et al., 2019a). Therefore, the location, distribution, and tandem duplications provide evidence for the evolution and predicted functions of the *Malus and Pyrus* pomWRKY genes. Functional evidence of TF WRKY in pome fruit trees

still needs to be explored, although the presence of chromosomal localizations has been identified, its functions could be inferred computationally, but could also be confirmed experimentally.

Differential expression of genes *pomWRKY*

Our heat map data showed that the majority of pomWRKY genes were expressed in tissue from *M. domestica, M. baccata, P. communis, P. pyrifolia and P. betulifolia*. These results indicate that they can participate in growth and development. For example, in *M. domestica* cv Gala, genes *MdgWRKY20, MdgWRKY28, MdgWRKY71, MdgWRKY92, MdgWRKY13, MdgWRKY48, MdgWRKY67 and MdgWRKY99* showed strong expression in all types of tissue, in addition to four *MdgWRKY* genes not being expressed in the three studied samples of the species at low temperatures as a treatment in plant pigment anthocyanin biosynthesis. Reports from other studies on anthocyanin biosynthesis report the expression of *MdWRKY11* in *M. domestica*, participating in the accumulation of anthocyanin in red-fleshed apples, affecting the TF MYB and the photoresponse factor MdHY5 (Liu et al., 2019a) and found no WRKY expression in the transcriptomic profile of anthocyanin biosynthesis in the peel of 'Granny Smith' apples (*M. domestica*) after removal from the bag (Ma et al., 2019). The genes *MbWRKY87, MbWRKY26, MbWRKY81, MbWRKY63, MbWRKY66, MbWRKY29* and *MbWRKY98* from *M. baccata,* show strong expression in all types of tissues, however, 11 genes were not expressed in the three samples dormant bud, bud to sprout bud, more branched branches with dormant bud by plant hormone treatments on signal translation (Ge et al., 2022). The plant hormone is regulated by phytohormone signaling pathways, which play an important role in response to the pathogen (*V. mali*) in *M.sieversii*, where the genes MsWRKY7 and MsWRKY33 had a high response in the initial stage (Liu et al., 2021). Also plant hormone signal transduction including ABA, JA and SA, the special autoregulation of WRKY TFs is also described (Erpen et al., 2018).

The analysis of differential expression of *PcWRKY* genes in *P. communis* in response to treatment induced by cold on the fruit peel, indicated that the genes with the highest expression were *PcWRKY73, PcWRKY11, PcWRKY31, PcWRKY50* and *PcWRKY61* in

the four samples evaluated by cold stress, 11 *PcWRKY* genes did not were expressed by chilling stress in the fruit peel (Hewitt et al., 2020). The response of the WRKY genes in tolerance to cold stress in other species has a favorable response from the gene *GmWRKY21* (Soybean), *TaWRKY19* (Wheat), *MusaWRKY71* (Banana)(Tripathi et al., 2014) and *HvWRKY38* (barley) (Marè et al., 2004), however, in *M. baccata* the MbWRKY5 gene can regulate antioxidant responses to stress when treated by salt, heat, cold and drought and having high expression levels of MbWRKY5 in young leaves (Han et al., 2019). Also, reports of genes *AtWRKY34, VvWRKY24, OsWRKY76, GmWRKY21, BcWRKY46, PvWRKY2, TcWRKY53, JrWRKY2/7, VbWRKY32*, show tolerance responses to cold stress (Li et al., 2020a). The differential expression in *P. betulifolia*, the genes that had the highest expression were *PbWRKY84, PbWRKY15, PbWRKY60, PbWRKY66, PbWRKY34* and *PbWRKY57* in response to biotic stress by *Valsa pyri*, the samples were processed were cells in in vitro suspension, 11 genes were not expressed. The response of the *WRKY* genes to biotic stress by fungal pathogens in *M. domestica* was regulated by the *WRKY6*, *WRKY18* and *WRKY40* genes, when interacting with other molecules (Balan et al., 2018), and also the protein-protein interaction analysis (PPI) refers that WRKY18 and WRKY40 respond to the organism *E. amylovora* and fungal pathogens (Balan et al., 2017). Furthermore, reporting on the roles of WRKY TFs in *Malus spp.* and *Pyrus spp.* presents some responses to biotic stress (Felipez et al., 2022).

The differential expression of samples of *P. pyrifolia* bud tissues in response to stress by the accumulation of cooling (ACA) (Gao et al., 2021), had greater expression of the genes *PpWRKY118, PpWRKY9, PpWRKY60, PpWRKY13* and *PpWRKY17* in all samples, 10 *PpWRKY* genes were not expressed. The presence of the *WRKY* genes in the cold accumulation has not been exhaustively studied in the dormancy period in Japanese pears (Marafon et al., 2011), however, the approach in the regulation of buds by phytochrome (PHYA-PHYE) in *Malus spp*., have functions distinct from growth cessation (and endodormancy) is under photoperiodic regulation (Arora et al., 2003). However, studies of bud dormancy in deciduous fruit trees, such as the peach (*P. persica*), show an important adaptive mechanism for its survival in cold climates, where the expression of six *PpWRKY* genes (*Prupe.6G286000, Prupe.1G393000, Prupe .1G114800,*

Prupe.1G071400, Prupe.2G185100, and Prupe.2G307400) in peach buds during dormancy could help to manipulate programmed dormancy (Chen et al., 2016). In buds of grapevines treated with exo-ABA, it protects against damage caused by the cold, with a response from the raffinose and galactinol synthase genes (Wang et al., 2020). Therefore, gene expression studies provide functional information about two WRKY TFs in Rosaceae species.

5.5 Conclusion

A total of 667 pomWRKY genes were identified in six genomes studied in *Malus spp.* and *Pyrus spp*., classification, phylogenetic relationships, gene structure, conserved motif composition, chromosomal localization, duplication and tandem, and divergence of gene pairs were systematically analyzed. In addition, pomWRKY expression patterns of the analyzed tissues were obtained from the available RNA-seq data processing database, providing useful information for future functional investigations in response to biotic and biotic stress in *Malus spp.* and *Pyrus spp*. The members of group II of the pomWRKY genes show a higher differential expression in response to the abiotic stress of the samples analyzed in relation to the rest of the groups. In summary, our study may help researchers to better understand the gene expression response and tolerance function of pomWRKY in *Malus spp. and Pyrus spp*. during the stress response.

5.6 Reference

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6 CONCLUSÃO GERAL

Desde os primeiros relatos do papel dos fatores de transcrição WRKY na resposta e tolerância a estresses bióticos e abióticos, além de participarem do desenvolvimento vegetal, sua importância de estudo foi reconhecida em 14 espécies de *Malus spp.* e *Pyrus* spp., enfocando interações com organismos patogênicos, participação ambiental e específica na biossíntese de metabólitos. A classificação estrutural primária desta superfamília genética é em quatro grupos (I, II, III, IV) e nove subgrupos com base na conservação de domínios e relações filogenéticas (I-NT, I-CT, IIa, IIb, IIc, IId, IIe, III, IV). Entretanto, novos relatos de WRKYs em espécies de clima temperado foram pesquisados por serem comercialmente importantes e promissores, além de terem disponibilizado bancos de dados genômicos e transcriptômicos para aplicação de estudos de bioinformática. O relato pesquisado de 59 genes RoWRKY em framboeseira preta (*R. occidentalis*), localizados em sete cromossomos, com duplicações em tandem e segmentares e cálculo de divergência genética, apresenta a evolução dos genes RoWRKY nesta espécie. A expressão diferencial dos genes RoWRKY nas amostras transcriptômicas processadas de 10 tecidos faz referência à expressão em cada órgão da planta e aos tratamentos de hormônios vegetais aplicados. O relato de 667 genes pomWRKY em seis espécies de *Malus spp.* e *Pyrus spp*. também apresenta quatro grupos e nove subgrupos da estrutura primária, com base na conservação de aminoácidos e relações filogenéticas, além da localização em 17 cromossomos e cálculo da divergência genética. Sua expressão diferencial em 20 amostras transcriptômicas faz referência à resposta e tolerância a estresses bióticos e abióticos. Portanto, as funções dos pomWRKYs estudadas em resposta e tolerância ao estresse fornecem o papel desempenhado pelos genes pomWRKY nas espécies *Malus spp. e Pyrus spp*.

7 **CONSIDERAÇÕES FINAIS**

Com base nas informações apresentadas, algumas perspectivas futuras para os estudos de WRKYs em *Malus spp., Pyrus spp.* e *Rubus spp*. podem incluir: Na análise bioinformática, a disponibilidade de bancos de dados genômicos e transcriptômicos para espécies Rosaceae pode ajudar a identificar novos WRKYs e entender melhor suas funções em diferentes processos biológicos. A análise filogenética pode ser usada para classificar novos WRKYs em grupos e subgrupos com base na conservação de aminoácidos e relações filogenéticas. Também, na genética clássica, a edição gênica, particularmente a tecnologia CRISPR-Cas9, pode ser usada para elucidar a função de genes específicos em espécies de interesse comercial. Por exemplo, a edição genética pode ser usada para modificar a expressão dos genes WRKY em plantas cultivadas para aumentar sua tolerância a estresses bióticos e abióticos. Além disso, na genética molecular, é importante explorar os mecanismos moleculares pelos quais os WRKYs regulam a resposta e tolerância a estresses bióticos e abióticos. O estudo de proteínas interativas, vias de sinalização e reguladores transcricionais associados aos WRKYs pode fornecer informações valiosas sobre as redes de regulação gênica que controlam esses processos. Assi mesmo, na genética evolutiva, a evolução dos genes WRKY em diferentes espécies pode fornecer insights sobre como esses genes se diversificaram e se adaptaram a diferentes ambientes ao longo do tempo.

Em resumo, as perspectivas futuras para os estudos de WRKYs em *Malus spp*., *Pyrus spp*. e *Rubus spp*. incluem a aplicação de técnicas de bioinformática para identificar novos genes, a edição gênica para elucidar a função desses genes em espécies de interesse comercial, o estudo de mecanismos moleculares para entender melhor como esses genes regulam a resposta e tolerância a estresses bióticos e abióticos, e a análise da evolução desses genes em diferentes espécies. Tudo isso pode levar a uma melhor compreensão do papel dos WRKYs nas espécies Rosaceae e como eles podem ser usados para melhorar a produção agrícola e a segurança alimentar.

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9 ANEXOS

Anexo A – Disciplinas cursadas durante o doutorado.

DISCIPLINA	CONCEITO
BIOTECNOLOGIA APLICADA AO MELHORAMENTO	A
PESQUISA ORIENTADA EM FITOMELHORAMENTO	A
ESTÁGIO DE DOCÊNCIA ORIENTADA EM FITOMELHORAMENTO	A
SEMINÁRIOS	A
BIOINFORMÁTICA	A
GENÉTICA QUANTITATIVA I	A
MARCADORES MOLECULARES EM FRUTICULTURA	B
PRODUÇÃO DE POMÁCEAS	A
ATUALIDADES EM GENÉTICA	A
ESTATÍSTICA APLICADA AO MELHORAMENTO	A
INTRODUÇÃO À BIOINFORMÁTICA	A
MELHORAMENTO GENÉTICO DE PLANTAS	A
LEITURA E PRODUÇÃO DE TEXTOS ACADÊMICOS	B
GENÉTICA APLICADA AO MELHORAMENTO DE PLANTAS	B
ESTATÍSTICA EXPERIMENTAL II	A

Anexo B– Artigos publicados/submetidos durante o período de doutorado.

Nome	Revista	Condição Atual
The roles of WRKY transcription factors in *Malus* spp. and *Pyrus* spp.	Functional & Integrative Genomics	Aceito (2022)
WRKY genes in black raspberry (*Rubus occidentalis* L.): duplicate and conquer	Functional & Integrative Genomics	Submetido (2023)
The expression of WRKY transcription factors on stress response and tolerance in *Malus* and *Pyrus* spp.	Plant science	Por submissão