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CrRAP2.2 reprograms transcriptional networks associated with vascular cell wall remodeling and stress responses in *Citrus*

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Declarations

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Abstract

The *Rap2.2* gene encodes a member of the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) transcription factor family and plays an important role in plant growth, development, and responses to biotic and abiotic stresses. Previous studies have demonstrated that Rap2.2 contributes to resistance against *Xylella fastidiosa* in both *Arabidopsis thaliana* and *Citrus* species. Here, we further investigated the molecular mechanisms of *Rap2.2* in *Citrus* that lead to *X. fastidiosa* resistance. Transgenic *Citrus sinensis* plants overexpressing *CrRap2.2* from *Citrus reticulata*, a species naturally resistant to *X. fastidiosa*, were generated and subjected to transcriptomic and phenotypic analyses. RNA sequencing revealed that *CrRap2.2* overexpression induces genes associated with cell wall remodeling, and both biotic and abiotic stress responses. Consistent with the transcriptomic data, histochemical analyses revealed enhanced lignin deposition in the xylem vessel elements of *CrRap2.2*-overexpressing plants compared with wild-type controls. Collectively, these findings demonstrate that CrRAP2.2 reprograms defense-related transcriptional networks and promotes vascular cell wall reinforcement, establishing a mechanistic link between transcriptional regulation and xylem structural modification that likely underlies the contribution of this transcription factor to disease resistance.

Key words: Lignin; Biotic; Abiotic; Xylem

Introduction

The gene *Rap2.2* (RELATED TO *AP2-2*) encodes an Ethylene Responsive Factor (ERF)-type transcription factor (TF) belonging to the plant-specific APETALA2/ERF (AP2/ERF) superfamily (Nakano et al. 2006; Hinz et al. 2010). Members of the ERF family are characterized by the presence of a single DNA-binding domain, known as the AP2 domain (Su et al. 2025). The transcription of *ERF* genes is hormone-dependent and occurs throughout plant growth and development, as well as in response to biotic and abiotic stresses (Marsch-Martinez et al. 2006; Zhao et al. 2012; Jin et al. 2018; Chen et al. 2022a). In addition, post-translational modifications of ERF proteins, including phosphorylation or ubiquitination, regulate ERF activation or repression, respectively (Licausi et al. 2013).

ERFs have long been recognized as important regulators of numerous developmental processes, including flower and seed development (Riechmann and Meyerowitz 1998) and abiotic and biotic stress responses (Hinz et al. 2010; Zhao et al. 2012; Pereira et al. 2020). Initial studies revealed that some ERFs act as direct transcriptional activators or repressors (Ohme-Takagi and Shinshi 1995). Subsequent research has shown that ERFs also participate in protein complexes that regulate their localization, stability, abundance, and ultimately their transcriptional activity (Licausi et al. 2013).

One of the main characterized functions of RAP2.2 is its involvement in low-oxygen stress responses, as *RAP2.2*-overexpressing plants show enhanced survival under hypoxic conditions (Hinz et al. 2010; Licausi et al. 2011). Hypoxia resulting from submergence or waterlogging is a major constraint on plant growth and productivity, and plant survival under these conditions relies on rapid and coordinated transcriptional reprogramming (Fukao and Bailey-Serres 2008). Among the TFs involved, ERFs play a central role in sensing and transducing hypoxic signals (Licausi et al. 2010; Hartman et al. 2018). *RAP2.2* expression is upregulated during submergence-induced hypoxia through the coordinated action of WRKY TFs, reinforcing its role as a key node in hypoxia-responsive regulatory networks (Tang et al. 2021).

The overexpression of RAP2.2, an ERF-family TF from *Citrus reticulata*, has been associated with resistance to *Xylella fastidiosa* (Rodrigues et al. 2013). Moreover, subsequent studies identified CrRAP2.2 as a putative ortholog of *Arabidopsis thaliana* AtRAP2.2, which is associated with plant defense responses against pathogens (Pereira et al. 2020; Zhao et al. 2012). Functional analyses supported a conserved role for these genes in plant immunity, as *Arabidopsis rap2.2* mutant plants complemented with *CrRAP2.2* displayed restored resistance to *X. fastidiosa* (Pereira et al. 2020). Moreover, overexpression of *CrRAP2.2* in *Citrus sinensis* was positively associated with resistance to Citrus Variegated Chlorosis (CVC), supporting its role as a positive regulator of defense response against *X. fastidiosa* (Pereira et al. 2020).

Plant growth-defense is fundamental for optimizing plant performance and maintenance in a changing biotic/abiotic environment. This process involves readjusting resource allocation to different pathways. It has been frequently observed modifications on secondary cell wall components, alteration in lignin biosynthesis resulting in changes in growth and defense in plants (Xie et al. 2018). Lignin is important for plants by providing structural support for plant growth, long-distance water transportation, and providing physical and chemical protection for plants against pathogens, forming a physical barrier to block pathogen invasion (Sattler and Funnell-Harris 2013). Indeed, previous studies have shown that the induction of genetic mechanisms involved in cell wall lignification is associated with impaired *X. fastidiosa* colonization in resistant or tolerant varieties (Rodrigues et al. 2013, Niza et al. 2015, Giampetruzzi et al. 2016; Sabella et al. 2018).

To gain insight into the molecular role of the RAP2.2 TF in *Citrus*, transcriptomic analyses were performed to identify the defense pathways and regulatory networks modulated by CrRAP2.2 that may explain the observed resistant phenotype (Pereira et al. 2020), while also expanding our understanding of its broader role in coordinating plant responses to biotic and abiotic stresses.

MATERIAL AND METHODS

Plant material

Transgenic plants of sweet orange cv. Pineapple were obtained in a previous study (Pereira et al. 2020) by *Agrobacterium*-mediated transformation with the A596p9ioGusi-FMV::CrRap2.2 construct (Pereira et al. 2020). Plants expressing the *CrRap2.2* gene (Ciclev10025816m.g) from *C. reticulata* were confirmed by β-glucuronidase assay and PCR by Pereira et al. (2020). These genetically modified plants were kept in a greenhouse for further experiments.

Sample collection, RNA extraction and sequencing

For transcriptome analysis, stems from 3 plants per treatment (transgenic plants overexpressing the *CrRap2.2* gene and non-transgenic wild-type plants - WT) were collected and immediately frozen in liquid nitrogen. Total RNA was extracted using Invitrogen's PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Residual DNA was removed by DNase I treatment (Thermo Fisher Scientific). The quality, integrity, and quantity of the extracted RNA were assessed by agarose gel electrophoresis and by NanoDrop spectrophotometry (ThermoFisher Scientific, Wilmington, DE, USA) for initial screening. RNA integrity was further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and only samples with an RNA integrity number (RIN) $\geq$ 7.0 were used for library construction. Libraries were prepared in biological triplicate and sequenced over two lanes using a 2 × 100 bp paired-end method on the Illumina NovaSeq 6000 system at NGS Soluções Genômicas.

Data preprocessing and differential gene expression analysis

The quality of the raw reads was assessed with FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Anders 2010), and the results were visualized using MultiQC v1.9 (Ewels et al. 2016). Reads were trimmed and filtered with Trimmomatic v0.39 (phred score threshold < 30, http://www.usadellab.org/cms/?page=trimmomatic) (Bolger; Lohse; Usadel 2014) using the parameters: TRAILING:5 AVGQUAL:30 SLIDINGWINDOW:5:30 MINLEN:100. After trimming, FastQC and MultiQC were run again to verify trimming efficiency. Reads representing ribosomal RNA gene fragments (rRNAs) were removed from the datasets using SortMeRNA v2.0 (Kopylova, Noé; Touzet 2012) with the SILVA SSU and LSU databases (release 119) based on multiple rRNA sequences (bacterial, archaeal, and eukaryotic) (Quast et al. 2012).

We performed RNA-seq analysis using Kallisto v0.46.2 (Bray et al. 2016) with 100 bootstrap samples to map reads to the *C. sinensis* transcriptome reference v3.0 (http://citrus.hzau.edu.cn/download.php) (Liu et al. 2022) and to calculate transcript abundances (TPM, transcripts per million). *CrRap2.2* transcript sequences (Ciclev10025816m.g) from the *C. clementina* genome v1.0, available at the Citrus Genome database (https://www.citrusgenomedb.org), were added to the reference file before quantification. In addition to the alignment-free quantification approach with Kallisto for the *C. sinensis* and *C. clementina* reference transcriptomes*, de novo* transcriptome assembly was performed. After quality assessment and data filtering, reads were pooled and assembled with Trinity v2.14.0 (Grabherr et al. 2011). To ensure quality and avoid potential assembly errors, transcripts with TPM values less than 1 were also removed. Candidate open reading frames (ORFs, at least 50 codons) within transcript sequences were predicted and translated using TransDecoder v5.5.0 (https://github.com/TransDecoder) (Haas et al. 2013).

Transcriptome completeness was assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO v5.3.2) tool (Manni et al. 2021) with the plant database *eudicots_odb10*. Downstream differential gene expression was performed using the Sleuth R package v0.30.0 (http://pachterlab.github.io/sleuth) (Pimentel et al. 2017) with the transform function = function (x) $\log_2(x+0.5)$ to convert the b (beta) value output of Sleuth into $\log_2$ fold change ($\log_2$FC). Genes considered differentially expressed (DEG) between the groups were identified using the Wald test and were presented with a *q-value* ≤ 0.05 and $| \log_2 FC | \geq 0.7$ (Supplementary Table 1). The DEGs were functionally annotated using Blast2GO (Conesa et al. 2005). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the TopGO package (Alexa et al. 2026). The RNA-Seq data generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) database under BioProject accession number PRJNA1023295.

Prediction of CrRAP2.2 binding sites

Given that CrRAP2.2 can function as both a transcriptional activator and a repressor, we investigated whether the DEGs identified in this study could be directly or indirectly regulated by the transcriptional regulator CrRAP2.2 using *in silico* approaches. The previously described CrRAP2.2 binding sites the ATCTA motif (Welsch et al. 2007; Gasch et al. 2016; Yin et al. 2019) were mapped within promoter regions extending up to 2,000 base pairs (bp) upstream of the ATG start codon, considering both sense and antisense strands. For this purpose, we performed a regulatory prediction analysis using the software PlantRegMap (Tian et al. 2019), which provides statistical predictions of interactions between TFs and DNA binding sites. Predicted TF–promoter

associations with positive log2(b) values indicate stronger association or enrichment, whereas negative values indicate weak or below-expected associations. For this analysis, promoter sequences from all DEGs were used as input.

Protein-protein interactions network analysis

To investigate DEG interactions, identify hub genes, and infer biological pathways, a protein–protein interaction (PPI) network was constructed. DEGs were mapped to their closest *A. thaliana* orthologs using STRING (v12.0). Only genes successfully mapped to *A. thaliana* proteins were retained for network analysis. A PPI network was generated using STRING with a high-confidence interaction score threshold of 0.7.

The STRING interaction table was imported into R (v4.6.0) and analyzed using the igraph (Csárdi and Nepusz 2006; Antonov et al. 2023; Csárdi et al. 2026), tidygraph (Pedersen 2025a), and ggraph (Pedersen 2025b) packages. A global PPI network encompassing all mapped genes was generated in R using the ggraph package (Pedersen 2025b). To facilitate biological interpretation, a refined network comprising only connected nodes (excluding isolated nodes) was generated and manually edited in Cytoscape v3.10.4.

Gene Ontology (GO) enrichment analysis was performed on the entire PPI network and on the EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) network. Enrichment analysis was conducted using the clusterProfiler package (Yu et al. 2012) and focused on GO Biological Process terms. GO terms with a false discovery rate (FDR) -p.adjust-value ≤ 0.05 were considered significantly enriched.

Microscopy analysis and lignin quantification

For qualitative assessment of lignin deposition, 18 petioles and 18 stems (Supplementary Fig. 1) were collected from 3 plants per treatment. From each sample, a segment located approximately 5 cm from the apical region was excised. To ensure structural comparability and minimize anatomical variation, branches with equivalent developmental stage were selected (Supplementary Fig. 1). Transverse sections (20 µm thick) were obtained using an automatic sliding microtome (Leica SM2010R) (Supplementary Fig. 1). Sections were stained with 0.1% (w/v) phloroglucinol (Sigma-Aldrich) for 20 min and subsequently examined to visualize lignin deposition, as previously described (Marques et al. 2018). For each sample, ten independent sections were analyzed under light microscopy using an Olympus BX61 microscope (Supplementary Fig. 1).

To assess differences in vessel morphology, fresh stem and petiole samples were collected (Supplementary Fig. 1) following the same sampling criteria as for lignin analysis. Immediately after sectioning,

small stem and petiole segments were mounted on aluminum stubs with carbon adhesive tape. Samples were analyzed directly, without coating, using an environmental scanning electron microscope (SEM) (TM4000 Plus; Hitachi, Chiyoda, Japan) (Supplementary Fig. 1) at an accelerating voltage of 5 kV. For quantitative assessment of vessel wall thickness, 15 randomly selected xylem vessels per image were measured using the measurement tool in ImageJ software (Schindelin et al. 2012). A total of 10 images per treatment were analyzed. Wall thickness measurements were averaged per image, and differences between treatments were evaluated using Student's t-test, with statistical significance set at $p \leq 0.05$.

Results

Global transcriptome analysis

In a previous study, we demonstrated that transgenic *C. sinensis* plants overexpressing *CrRAP2.2* exhibit enhanced resistance to *X. fastidiosa* (Pereira et al. 2020). To gain deeper insight into the transcriptional reprogramming mediated by *CrRap2.2* that may underline this resistance, we performed RNA-seq using the Illumina NovaSeq 6000 platform. An average of 56,399,687 and 45,733,626 raw reads were generated for the *CrRap2.2* transgenic and WT biological replicates, respectively. Following quality control and read filtering, an average of 53,156,612 reads (94.25%) for the *CrRap2.2* transgenic samples and 44,346,474 reads (96.97%) were retained for downstream analyses. Transcript abundance was evaluated across a total of 49,572 transcripts, comprising 49,567 predicted transcripts from the *C. sinensis* genome and five CrRAP2.2-related transcripts from the *C. clementina* genome. These transcripts corresponded to 29,876 genes, including 29,875 annotated *C. sinensis* genes and the *Rap2.2* gene.

Differential gene expression analysis was conducted using Sleuth with the *C. sinensis* reference genome annotation version 3.0. Among all expressed genes, 29,618 genes exhibited no significant changes in expression ($|\log2FC| \leq 0.7$), whereas 258 genes were significantly differentially expressed ($|\log2FC| \geq 0.7$; qvalue ≤ 0.05). Of these DEGs, 162 genes (62.79%) were upregulated, and 96 genes (37.21%) were downregulated in *CrRap2.2*-overexpressing plants compared with WT controls (Supplementary Table 1).

KEGG pathway analysis showed that the upregulated DEGs were significantly enriched in several pathways, most notably plant–pathogen interaction and phenylpropanoid biosynthesis (p.adjust < 0.001) (Fig. 1). In contrast, downregulated DEGs were enriched in pathways associated with primary carbon metabolism and photosynthesis, including glycolysis/gluconeogenesis, carotenoid biosynthesis, carbon fixation in photosynthetic organisms, and pyruvate metabolism (Fig. 1).

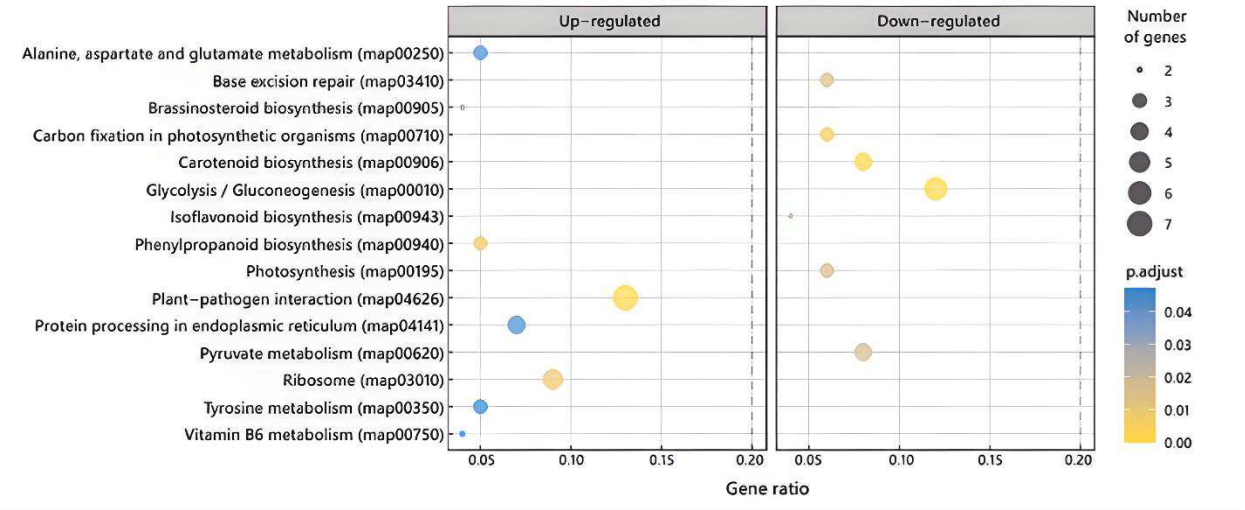


Fig. 1 Enriched KEGG pathways (p.adjust ≤ 0.05) among upregulated and downregulated differentially expressed genes (DEGs) between transgenic and non-transgenic plants.

Among the DEGs, a substantial number were associated with transcriptional regulation, cell wall remodeling, and plant responses to abiotic and biotic stresses (Table 1).

Table 1. Representative differentially expressed genes between transgenic and non-transgenic plants associated with transcriptional regulation, cell wall remodeling, lignin biosynthesis, and responses to biotic and abiotic stresses.

General function	Gene ID	Log2FC	Annotation	Reference
CrRap2.2 (Transgene)	*Ciclev10025816m*	3.15	*Ethylene-responsive transcription factor RAP2.2*	Pereira et al. (2020)
Transcription factors	*Cs_ont_9g025120*	3.73	*Dehydration-responsive element-binding 1D (DREB)*	Han et al. (2022a)
	Cs_ont_9g025130	2.80	*Ethylene-responsive transcription factor ERF027*	Feng et al. (2020)
	Cs_ont_5g036780	2.66	*LOB domain-containing 41 (LBD41)*	Wang et al. (2024b)
	Cs_ont_7g019820	1.60	*AP2 ERF and B3 domain-containing transcription repressor RAV2*	Feng et al. (2020)
	Cs_ont_6g012890	1.46	*Probable WRKY transcription factor 70 (WRKY70)*	Javed and Gao (2023)
	Cs_ont_8g028700	1.45	*NAC domain-containing*	Ng et al. (2018)
	Cs_ont_7g010660	1.03	*PLATZ transcription factor family*	Zhang et al. (2025)
Pectin/Cell-wall remodeling	*Cs_ont_3g019850*	4.55	*Pectin acetylesterase 8-like*	Shahin et al. (2023)
	Cs_ont_3g019840	3.23	*Pectin acetylesterase 8*	
Lignin metabolism/Cell-wall remodeling	*Cs_ont_1g022420*	2.61	*Cinnamyl alcohol dehydrogenase 8 (CAD8)*	Yoon et al. (2015)
	Cs_ont_1g024770	2.52	*Caffeoyl-CoA O-methyltransferase-like (CCOMT)*	
	Cs_ont_6g019320	2.39	*Caffeoyl-CoA O-methyltransferase-like (CCOMT)*	

	Cs_ont_5g044180	2.17	*4-coumarate--CoA ligase-like 9 (4CL)*	
Plant defense/Abiotic stress	*Cs_ont_1g029230*	10.20	*Phosphoserine aminotransferase chloroplastic-like (PSAT)*	Ma et al. (2024)
	Cs_ont_1g019190	3.10	*Cytochrome P450 71A1 (CYP71A1)*	Zhang et al. (2023)
	Cs_ont_5g043380	2.57	*AAI domain-containing*	Long et al. (2023)
	Cs_ont_3g029510	2.04	*Berberine bridge enzyme-like 18 (BBB18)*	Chen et al. (2022b)
	Cs_ont_3g007930	2.00	*Alcohol dehydrogenase 1 (ADH1)*	Shi et al. (2017)
	Cs_ont_2g013250	1.88	*MLP 43*	Wang et al. (2016)
	Cs_ont_3g000320	1.55	*MIZU-KUSSEI 1 (MIZ1)*	Miyazawa et al. (2012)
	Cs_ont_1g016000	1.09	*Coatomer subunit beta -2*	Sánchez-Simarro et al. (2020)
	Cs_ont_7g025620	1.82	*Glutathione S-transferase U17-like*	Kumar and Trivedi (2018)
	Cs_ont_7g025640	1.71	*Glutathione S-transferase U17 (GSTU17)*	
	Cs_ont_9g008250	1.23	*Glutathione S-transferase U8 (GSTU8)*	
	Cs_ont_3g033090	1.12	*Glutathione S-transferase Z1 (GSTZ1)*	
	Cs_ont_5g008630	1.04	*Glutathione S-transferase F6 (GSTF6)*	
Plant defense/Biotic stress	*Cs_ont_9g026580*	8.02	*SAR DEFICIENT 4 (SARD4)*	Hartmann and Zeier (2018)
	Cs_ont_2g026460	6.82	*F-box protein MAX2*	Piisilä et al. (2015)
	Cs_ont_8g011520	3.99	*DC1 domain-containing*	Hwang et al. (2014)
	Cs_ont_9g013950	2.06	*RPM1-interacting 4 (RIN4)*	Zhao et al. (2021)
	Cs_ont_3g029510	2.04	*Berberine bridge enzyme-like 18 (BBE18)*	Locci et al. (2019)
	Cs_ont_2g001420	2.01	*Chitin elicitor receptor kinase 1-like (CERK1)*	Desaki et al. (2019)
	Cs_ont_5g040980	1.78	*Subtilisin-like protease*	Figueiredo et al. (2014)
	Cs_ont_7g016880	1.71	*Aspartic ase CDR1*	Thomas and Van der Hoorn (2018)
	Cs_ont_3g018030	1.67	*Cysteine-rich receptor kinase 10*	Liu et al. (2024)
	Cs_ont_3g021130	1.64	*Cytochrome P450 CYP82D47*	Wang et al. (2024a)
	Cs_ont_5g031930	1.61	*Receptor kinase FERONIA*	Xie et al. (2022)
	Cs_ont_7g017320	1.54	*Benzyl alcohol O-benzoyltransferase*	Kotera et al. (2023)
	Cs_ont_7g003880	1.45	*Heat shock 70 kDa protein 1 (HSP70)*	Berka et al. (2022)
	Cs_ont_6g013510	1.44	*LURP-one-related 15*	Liu et al. (2026)
	Cs_ont_2g004030	1.38	*Anthranilate N-hydroxycinnamoyl benzoyltransferase*	Fang et al. (2022)
	Cs_ont_5g019880	1.37	*SOBIR1*	Liebrand et al. (2013)
	Cs_ont_5g046530	1.23	*Protein eds1-related (EDS1)*	Venugopal et al. (2009)
	Cs_ont_9g002090	1.19	*Major allergen Pru ar 1 (PR-10)*	Khanfir et al. (2024)
	Cs_ont_3g002140	1.19	*Heat shock protein 90 (HSP90)*	Wu et al. (2026)
	Cs_ont_5g031860	1.16	*Receptor-like protein kinase FERONIA*	Xie et al. (2022)

232
233

Collectively, these transcriptional changes indicate that *CrRAP2.2* overexpression promotes coordinated activation of pathways involved in cell wall remodeling, lignin biosynthesis, and immune responses, indicating a role for this TF in strengthening structural barriers while enhancing resistance to both biotic and abiotic stresses.

Promoter analysis identifies DEGs potentially directly regulated by CrRAP2.2

As CrRAP2.2 is a TF, we investigated whether it could directly regulate the DEGs by searching for putative CrRAP2.2-binding sites in their promoters. Among the 258 DEGs analyzed, only 15 contained multiple RAP2.2-binding motifs (ATCTA) within the 2-kb upstream promoter region (Fig. 2A), suggesting that these genes are potential direct targets of CrRAP2.2. Functional annotation indicated that these candidate targets include genes involved in transcriptional regulation, nutrient metabolism, and maintenance of mitochondrial function and homeostasis. Notably, six of the fifteen putative direct targets are associated with plant defense responses to both biotic and abiotic stresses, suggesting that CrRAP2.2 may directly coordinate stress-responsive pathways.

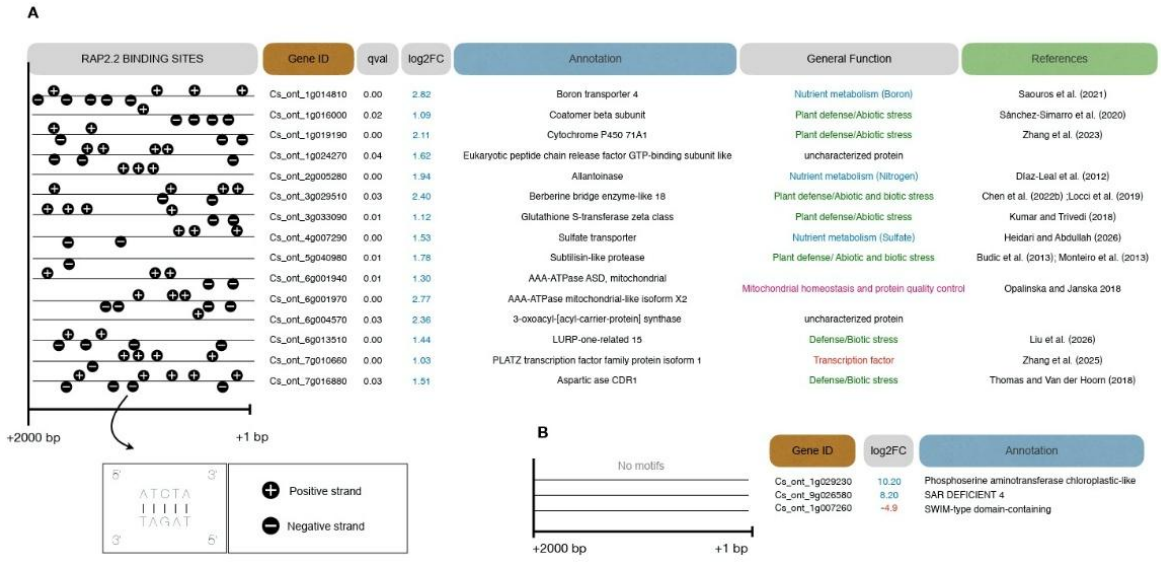


Fig. 2 Identification of putative CrRAP2.2 target genes through promoter motif analysis and regulatory prediction. The RAP2.2 binding motif (ATCTA) was identified in the 2-kb upstream promoter regions of putative CrRAP2.2 target genes predicted by PlantRegMap (A). Motif occurrences are indicated on both positive and negative DNA strands. In addition, the presence of this motif was manually inspected and mapped in selected differentially expressed genes (DEGs) to provide further support for the potential direct or indirect transcriptional regulation mediated by CrRAP2.2.

In contrast, some of the most strongly DEGs identified in the transcriptome analysis (log_2FC up to 10.2, Table 1) lacked the RAP2.2-binding motif in their promoters (Fig. 2B). This pattern suggests that the major transcriptional changes observed in the transgenic plant (Supplementary Table 1) do not result from direct binding of CrRAP2.2 to promoters. Instead, CrRAP2.2 can act upstream in a regulatory cascade, controlling the expression

of secondary TFs or signaling components that subsequently modulate downstream target genes. Therefore, the overall transcriptional response associated with CrRAP2.2 appears to be mediated predominantly by indirect regulatory mechanisms rather than by direct promoter binding.

Transgenic plants exhibit transcriptional activation of an immune-associated protein interaction network

To investigate whether the transcriptional changes induced by CrRAP2.2 converge on coordinated biological pathways, a PPI network was constructed. Network nodes represented the *A. thaliana* orthologs of differentially expressed *Citrus* genes, whereas edges represented predicted or experimentally supported functional protein associations retrieved from the STRING database (Fig. 3; Supplementary Table 2).

Community detection using the Louvain algorithm identified 101 modules in the PPI network (Fig. 3A; Supplementary Table 2). GO enrichment analysis of the entire network revealed significant overrepresentation of biological processes associated with defense response to bacterium, regulation of response to stress, defense response to other organism, immune response, innate immune response, response to hypoxia, response to decreased oxygen levels, and response to oxygen levels (Supplementary Fig. 2A; Supplementary Table 3). These results indicate that CrRAP2.2-mediated transcriptional reprogramming predominantly affects stress- and immunity-related pathways.

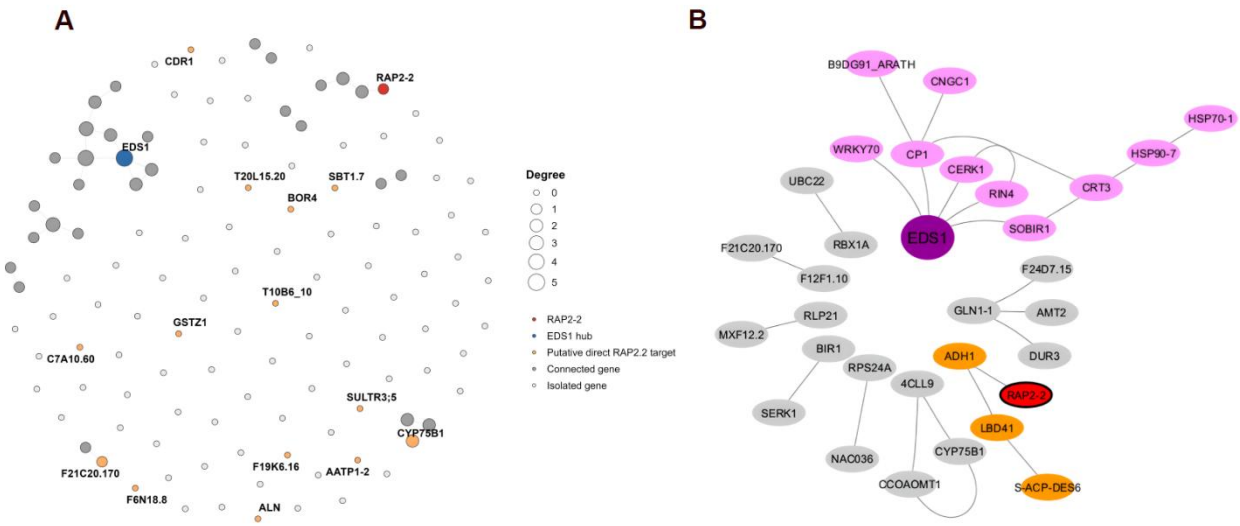


Fig. 3. Protein-protein interaction (PPI) network. (A) Global PPI constructed using the *Arabidopsis thaliana* orthologs of *Citrus* differentially expressed genes, The network highlights the EDS1- and RAP2-2-associated modules, as well as the putative direct targets of RAP2.2 identified by promoter analysis. (B) Refined PPI network showing the two biologically relevant modules identified by community detection, highlighting the EDS1-centered module and the RAP2.2-associated module.

Most modules consisted of small clusters or isolated proteins (Fig.3B). In contrast, the EDS1 exhibited the highest network connectivity (degree = 5, betweenness = 24.5, and PageRank = 0.0454), interconnecting ten proteins (Fig. 3B; Supplementary Table 2). Functional enrichment analysis of this module revealed a significant overrepresentation of biological processes related to defense response**,** stress response, and immune system processes (Table 2; Supplementary Fig. 2B; Supplementary Table 4), indicating that *CrRap2.2* overexpression promotes the activation of an EDS1-centered immune regulatory network.

Table 2 *Citrus* genes and their corresponding *Arabidopsis thaliana* orthologs belonging to the EDS1- and RAP2.2-associated protein–protein interaction modules.

Network module	***Citrus* protein ID**	**Arabidopsis gene symbol**	**Log2FC**	**Annotation**
EDS1-associated module	Cs_ont_5g046530.1	*EDS1*	1.23	Protein eds1-related
	Cs_ont_6g024400.1	*CRT3*	1.70	Calreticulin-3-like isoform X2
	Cs_ont_3g002140.1	*HSP90-7*	1.20	Heat shock protein 90
	Cs_ont_5g019880.1	*SOBIR1*	1.55	Leucine-rich repeat receptor-like serine threonine tyrosine- kinase
	Cs_ont_2g001420.1	*CERK1*	2.01	Chitin elicitor receptor kinase 1-like (CERK1)
	Cs_ont_9g013950.1	*RIN4*	2.06	RPM1-interacting 4
	Cs_ont_6g012890.1	*WRKY70*	1.46	WRKY transcription factor 70
	Cs_ont_7g003880.1	*HSP70-1*	1.45	Heat shock 70 kDa protein 1
	Cs_ont_9g001220.1	*CP1*	1.14	Calcium-binding CP1
	Cs_ont_2g021050.1	*CNGC1*	5.53	Cyclic nucleotide-gated ion channel 1
	Cs_ont_1g019570.1	*B9DG91_ARATH*	1.27	ANK REP REGION domain-containing
RAP2-2-associated module	Ciclev10025816m.1	*RAP2-2*	3.15	Ethylene-responsive transcription factor RAP2.2
	Cs_ont_3g007890.1	*ADH1*	2.00	Alcohol dehydrogenase 1 (ADH1)
	Cs_ont_5g036780.1	*LBD41*	2.66	LOB domain-containing 41
	Cs_ont_3g007870.1	*S-ACP-DES6*	5.79	stearoyl- [acyl-carrier-] 9-desaturase chloroplastic-like

CrRAP2.2 was directly connected to alcohol dehydrogenase class P (ADH1), which was further connected to LOB domain-containing protein 41 (LBD41) and a cytochrome P450 (S-ACP-DES6) (Fig. 3B; Table 2). Curiously, despite being overexpressed in the transgenic plants, CrRAP2.2 was not the major hub within the network, suggesting that it acts upstream of regulatory pathways that ultimately converge on the activation of EDS1, the central hub of the network (Fig. 3B; Table 2).

CrRAP2.2 transgenic plants show increased lignification of vessel elements

Given the differential expression of cell wall-related genes revealed by transcriptome analysis (Table 1), microscopy analyses were performed to investigate whether these transcriptional changes translated into structural

differences in vascular tissues. Anatomical analyses of petiole (Fig. 4A-D) and stem (Fig. 4E-H) cross-sections revealed differences between *CrRap2.2*-overexpressing plants and WT controls, particularly regarding vascular tissue organization and lignification patterns (Fig. 4C, D, G and H versus A, B, E and F). Overall, sections obtained 5 cm below the shoot apex showed comparable general tissue organization between genotypes, with clearly defined pith, vascular cambium, and xylem regions in both petiole and stem tissues (Fig. 4). However, despite the similar structure, *CrRap2.2* transgenic plants exhibited pronounced alterations in xylem development (Fig. 4C, D, E and F). In both petioles and stems, transgenic plants displayed a higher density of xylem vessel elements relative to WT plants (Fig. 4C, D, G and H versus A, B, E and F). This increase was evident as a greater number of vessel lumens per vascular bundle, suggesting enhanced xylem differentiation rather than changes in the proportional contribution of other xylem cell types, such as parenchyma or fibers.

In addition to the increased vessel number, xylem tissues of *CrRap2.2* transgenic plants showed markedly stronger phloroglucinol staining, indicating enhanced lignin deposition (Fig. 4C, D, G and H versus A, B, E and F). This intensified red coloration was consistently observed in vessel walls of both petiole (Fig. 4C and D) and stem sections (Fig. 4G and H), reflecting a higher degree of secondary cell wall lignification than in WT plants (Fig. 4A, B, E and F). Notably, this difference was more pronounced in regions adjacent to the vascular cambium (Fig. 4A-H), suggesting altered cambial activity that favors xylem maturation in Cr*Rap2.2*-overexpressing plants.

Furthermore, the vascular cambium in *CrRap2.2* transgenic plants appeared more active, as inferred from the broader xylem zones and tighter arrangement of newly formed vessels, particularly in stem cross-sections (Fig. 4G and H). These features are consistent with increased cambial output toward xylem formation, without evident disruption of overall vascular patterning. These results indicate that *CrRap2.2* overexpression promotes enhanced xylem vessel formation and lignification in *Citrus*, affecting both petiole and stem tissues, while preserving overall vascular organization. Such anatomical modifications may contribute to altered vascular functionality and are consistent with transcriptional changes associated with cell wall, and components of secondary metabolism as flavonoids, phenylpropanoids, and lignin observed in the transcriptomic analyses (Table 1; Supplementary Table 1).

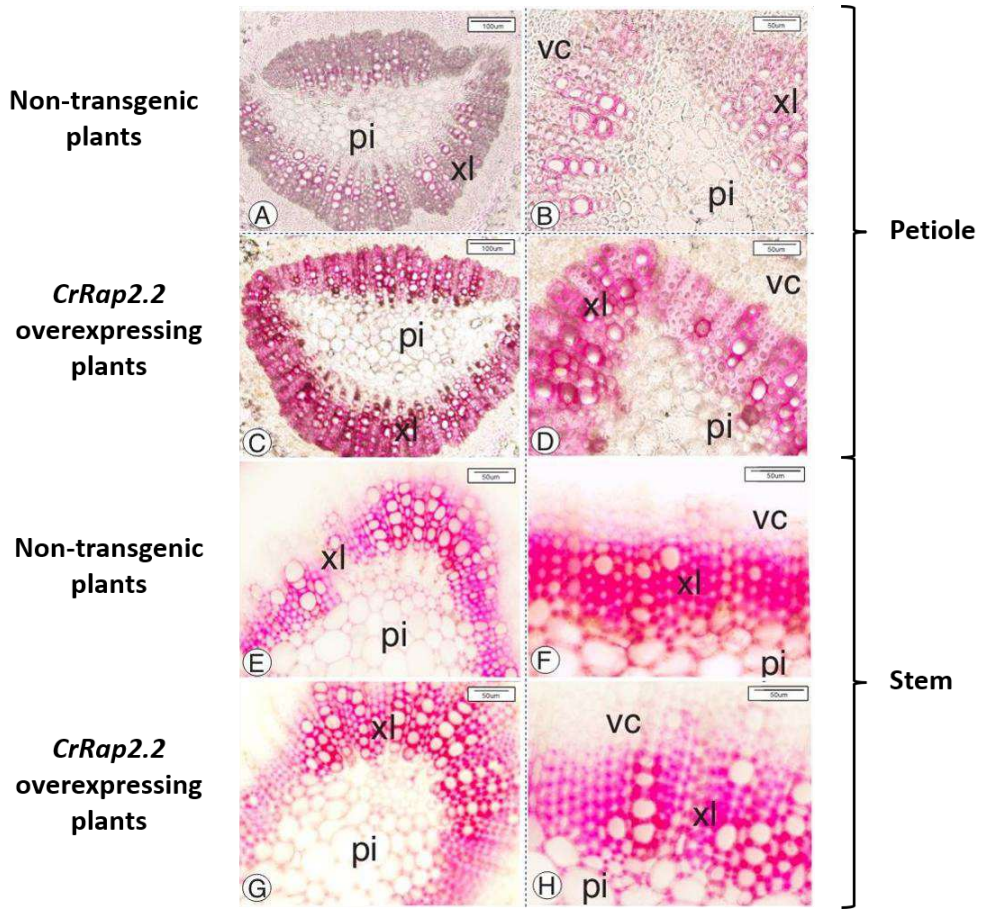


Fig. 4 Light microscopy images of petiole and stem cross-sections from non-transgenic and *CrRap2.2*-overexpressing *Citrus sinensis* plants. Sections were stained with 0.1% (w/v) phloroglucinol to visualize lignin deposition. The intensity of the pink/red staining is proportional to the amount of lignin deposited in the tissue. (A–D) Petiole cross-sections. (E–H) Stem cross-sections. (A, B, E, F) Non-transgenic plants. (C, D, G, H) *CrRap2.2*-overexpressing transgenic plants. (vc, vascular cambium; pi, pith; xl, xylem).

Transgenic plants exhibit increased xylem vessel wall thickness

SEM analysis revealed structural differences in the xylem of *CrRap2.2* transgenic plants compared with WT controls, in both petioles (Fig. 5A and B versus C and D) and stems (Fig. 5E and F versus G and H). In transgenic plants, xylem vessels displayed markedly thicker secondary cell walls and a more compact, homogeneous appearance (Fig. 5C, D, G and H), whereas WT vessels exhibited thinner walls and larger luminal areas (Fig. 5A, B, E and F). These differences were consistently observed across multiple sections and independent biological replicates.

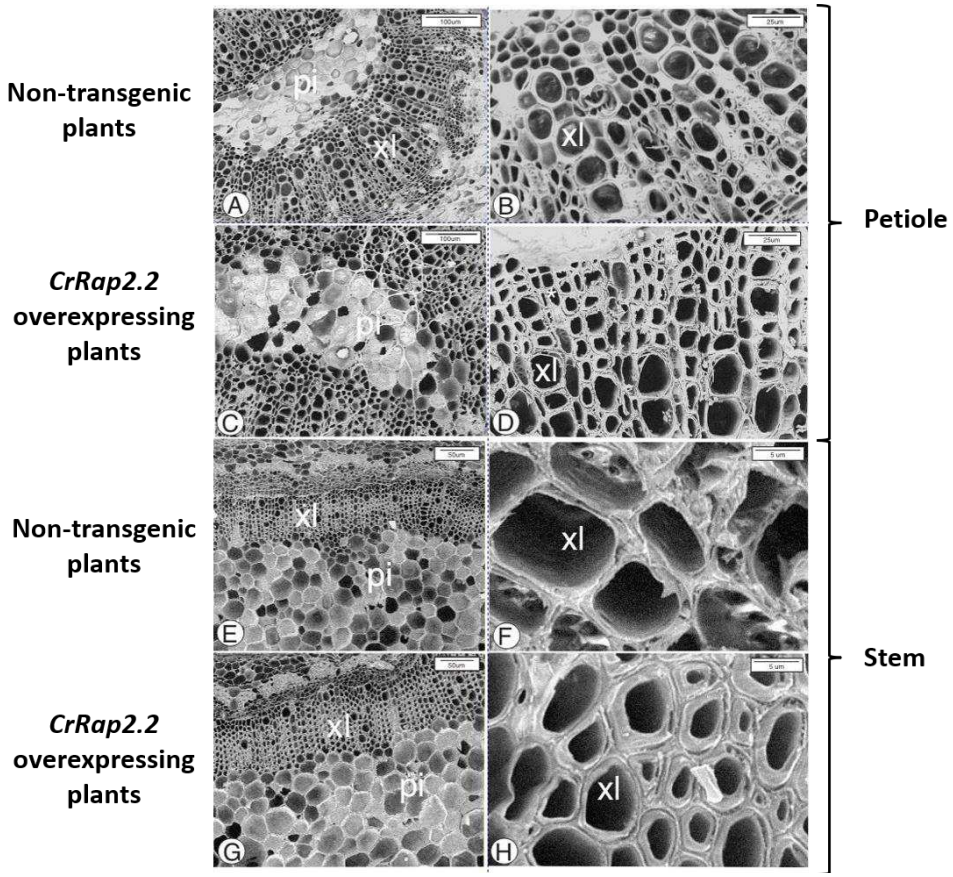


Fig. 5. Scanning electron micrographs (SEM) images of petiole and stem cross-sections from non-transgenic and *CrRap2.2*-overexpressing *Citrus sinensis* plants. (A–D) Petiole cross-sections. (E–H) Stem cross-sections. (A, B, E, F) Non-transgenic plants. (C, D, G, H) *CrRap2.2*-overexpressing transgenic plants. (vc, vascular cambium; pi, pith; xl, xylem).

Quantitative measurements confirmed a significant increase in vessel wall thickness in *CrRap2.2*-overexpressing plants, reaching values approximately threefold higher than in WT plants in both petioles and stems (Fig. 6A and B; $p \leq 0.005$). This thickening was not limited to specific vessel subsets but affected a broad population of xylem vessels (Fig. 5C, D, G, and H), indicating a global remodeling of vascular tissue associated with *CrRap2.2* overexpression.

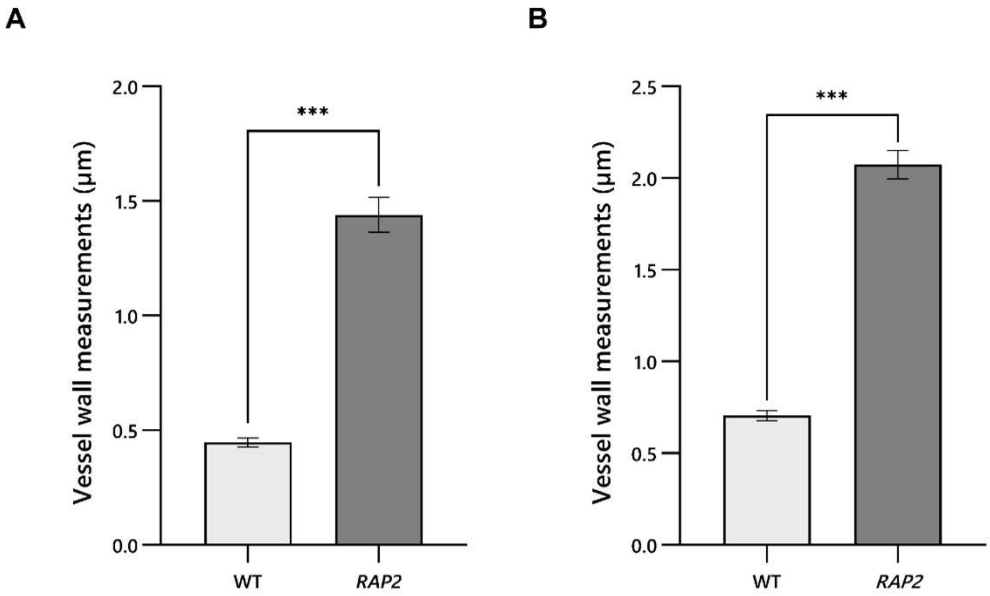


Fig. 6 Quantification of xylem vessel wall thickness in petioles (A) and stems (B) of non-transgenic (WT) and CrRap2.2-overexpressing plants (RAP2) using ImageJ. Data are presented as mean ± SE. Asterisks indicate statistically significant differences between groups according to Student's *t*-test ($p \leq 0.005$). (***$p \leq 0.001$).

The observed increase in vessel wall thickness (Fig. 6A and B) aligns with the transcriptional activation of cell wall–related pathways identified in the transcriptomic analysis (Table 1; Supplementary Table 1), and corroborates the enhanced lignification detected by histochemical staining in the transgenic plants (Fig. 4). Together, these structural modifications suggest that CrRAP2.2-mediated transcriptional reprogramming promotes vascular reinforcement as a key component of the enhanced resistance phenotype observed in *CrRap2.2* transgenic *Citrus* plants (Pereira et al. 2020).

Discussion

In a previous study, AtRAP2.2 and CrRAP2.2 were shown to share similar functions as TF, acting as positive regulators of defense responses against *X. fastidiosa* (Pereira et al. 2020). Here, using transcriptome analysis, we investigated the set of genes regulated by CrRAP2.2 in *C. sinensis* transgenic plants, aiming to elucidate the molecular basis underlying the CrRAP2.2-mediated resistance phenotype previously reported (Pereira et al. 2020; Rodrigues et al. 2013). In general, *CrRap2.2* overexpression was associated with the induction of several TFs families involved in biotic- and abiotic-stress responses, including ERF, DREB, RAV, WRKY, NAC, PLATZ, and LOB-domain proteins (Ng et al. 2018; Feng et al. 2020; Han et al. 2022a,b; Javed and Gao

2023; Wang et al. 2024b; Zhang et al. 2025) (Table 1). This pattern indicates that CrRAP2.2 may trigger a broader regulatory cascade by activating secondary TFs, thereby amplifying its effects across multiple stress-responsive pathways. To determine whether CrRAP2.2 regulates these genes directly or indirectly, we searched for putative CrRAP2.2-binding sites within the promoter regions of the DEGs (Fig. 2). Among the TFs, only PLATZ was predicted to be a direct target of CrRAP2.2. Beyond plant growth and development, PLATZ TFs have been associated with a range of stress responses, such as drought, salt, cold, waterlogging, heavy metal stress tolerance, and disease resistance (Kim et al. 2018; Han et al. 2022b; Liu et al. 2023; Rehman et al. 2024; Zhang et al. 2025; Zeng et al. 2025). Among the other predicted targets of CrRAP2.2, genes encoding CYP71A1, berberine bridge enzyme-like 18 (BBE18), subtilisin-like protease, LURP-one-related 15 (LURP15), and the extracellular aspartic protease (CDR1) (Fig. 2A), also have well-established roles in plant responses to biotic and abiotic stresses (Figueiredo et al. 2014; Benedetti et al. 2018; Dasaki et al. 2019; Locci et al. 2019; Zhang et al. 2023; Salvati et al. 2025; Liu et al. 2026).

CDR1 is an extracellular aspartic protease required for both local and systemic salicylic acid (SA)-mediated resistance (Xia et al. 2004; Thomas and Van der Hoorn 2018). Consistent with this prediction, the most highly connected defense-associated module in the PPI network was centered on EDS1 (Fig. 3), a master regulator of SA-dependent immunity (Dongus and Parker 2021; Khan et al. 2022). This module included proteins involved in pathogen perception (CERK1, SOBIR1) (Liebrand et al. 2014; Domazakis et al. 2018; Desaki et al. 2019), immune signaling (WRKY70, RIN4) (Li et al. 2004; Ray et al. 2019; Deng et al. 2020), and stabilization of immunity-related proteins (HSP90, HSP70) (Berka et al. 2022; Wu et al. 2026) (Fig. 3B; Table 2). Transcriptomic analyses further supported the activation of systemic acquired resistance, as evidenced by the induction of *SARD4*, *benzyl alcohol O-benzoyltransferase*, and *MAX2*, genes associated with pipecolic acid biosynthesis and SA-mediated defense (Ding et al. 2016; Kotera et al. 2023; Zheng et al. 2023; Kumari et al. 2025) (Table 1). Interestingly, Liu et al. (2026) demonstrated that SARD1 directly activates the expression of LURP1, a defense-related gene required for SA accumulation and disease resistance. Together, these results raise the possibility that CrRAP2.2 converges on a conserved regulatory module associated with SA-mediated immunity.

Although RAP2.2 is an ethylene (ET)-responsive TF (Hinz et al. 2010), our data suggest that constitutive overexpression of CrRAP2.2 in *C. sinensis* activates SA-mediated defense pathways (Table 1; Figs. 2 and 3). Even though SA- and ET-mediated signaling are frequently described as antagonistic (Tian et al. 2025), accumulating evidence indicates that these pathways can also act synergistically depending on the biological context. Wang et al. (2020) demonstrated that ET is required for SA-induced stomatal closure, highlighting a

positive interaction between these two phytohormones. Consistent with this concept, Rodrigues et al. (2013) reported that both CrRAP2.2 and PR1, a well-established marker of SA-dependent defense (Vlot et al. 2009), were more strongly induced in CVC-resistant *C. reticulata* than in susceptible *C. sinensis* following *X. fastidiosa* infection.

CrRAP2.2 was also predicted to directly regulate BBE18 (Fig. 2A), members of whose family have been implicated in plant immunity through the oxidation of damage-associated molecular patterns (DAMPs) (Scortica et al. 2022). In addition, several members of the BBE family function in monolignol oxidation (Daniel et al. 2015), a key step associated with lignin formation and cell wall remodeling (Tobimatsu and Schuetz 2019). Likewise, CYP71A1, another predicted direct target of CrRAP2.2 (Fig. 2A), has recently been linked to drought tolerance by stimulating lignin biosynthesis (Zhang et al. 2023). The predicted regulation of BBE18 and CYP71A1 therefore provides a plausible molecular mechanism linking CrRAP2.2 to the enhanced vascular lignification observed in the transgenic plants (Fig. 4). Consistent with this hypothesis, transcriptional modulation of lignin biosynthetic genes (Table 1) was accompanied by increased xylem lignification (Fig. 4C, D, G and H versus Fig. 4A, B, E and F) and thicker vessel walls (Fig. 6A and B) in CrRAP2.2-overexpressing plants compared with WT controls. These observations are consistent with the well-established role of AP2/ERF TFs in coordinating stress adaptation and defense responses (Xie et al. 2019; Feng et al. 2020; Su et al. 2025) and reinforce the importance of lignin as a structural barrier that limits pathogen invasion and vascular colonization (Sattler and Funnell-Harris 2013; Marques et al. 2018).

In citrus and olive, lignin accumulation has been recognized as an important resistance mechanism against *X. fastidiosa* (Rodrigues et al. 2013; Niza et al. 2015; Giampetruzzi et al. 2016; Sabella et al. 2018). In tolerant olive genotypes, activation of lignin biosynthesis is associated with cell wall thickening and reduced vessel diameter, thereby restricting bacterial movement and impairing biofilm formation (Niza et al. 2015; Sabella et al. 2018). Drought-tolerant *Cyamopsis tetragonoloba* genotypes exhibit coordinated upregulation of lignin biosynthetic genes, including *4CL*, *CCOMT*, and *CAD6*, together with increased vascular lignification (Narayan et al. 2023), a pattern consistent with the transcriptomic (Table 1) and anatomical (Fig. 4, 5 and 6) changes observed in the present study. This conserved response was further supported by the strong induction of *phosphoserine aminotransferase* (*PSAT*), the most highly upregulated gene in our dataset (Table 1). PSAT has been implicated in metabolic adaptation to oxygen deprivation and other abiotic stresses (Ho and Saito 2001; Ma et al. 2024), indicating that CrRAP2.2 activates not only structural reinforcement of the vascular tissue, but also metabolic pathways associated with stress adaptation. Because *X. fastidiosa* colonization progressively restricts

xylem sap flow, creating physiological conditions resembling drought and hypoxia within vascular tissues (Habermann et al. 2003; McElrone et al. 2003; Alves et al. 2009), the coordinated induction of *PSAT*, together with *DREB*, *ADH1*, *MIZ1*, *GSTs*, *MLP43*, and other abiotic stress-responsive genes (Miyazawa et al. 2012; Wang et al. 2016; Agarwal et al. 2017; Shi et al. 2017; Kumar and Trivedi 2018; Long et al. 2023) (Table 1), suggests that CrRAP2.2 activates a vascular stress-adaptive transcriptional program. Similar responses have been reported in previous transcriptomic studies of *X. fastidiosa*-infected plants, indicating that activation of abiotic stress pathways is an intrinsic component of the host response to vascular colonization (Choi et al. 2013; De Pascali et al. 2019; Ingel et al. 2021). This hypothesis is further supported by the PPI network, in which CrRAP2.2 was associated with ADH1 (Fig. 3B), a central component of anaerobic metabolism, and indirectly connected to LBD41, a core ERF-VII target involved in hypoxia responses (Fig. 3B).

Functional studies in *A. thaliana* demonstrated that RAP2.2 binds the LBD41 promoter directly and is required to induce both LBD41 and ADH1 under oxygen deprivation (Gasch et al. 2016), indicating conservation of this regulatory module. In *Citrus*, we suggest that CrRAP2.2 does not directly regulate LBD41, as this gene was not identified among the predicted targets from PlantRegMap following promoter motif screening (Fig. 2). Moreover, the AtLBD41 promoter exhibits substantial enrichment of ATCTA motifs, whereas the CsLBD41 promoter contains only three predicted sites (Supplementary Fig. 3).

Most studies on RAP2.2 have focused on its role in abiotic stress responses associated with vascular adaptation, particularly hypoxia (Hinz et al. 2010; Papdi et al. 2015; Gasch et al. 2016; Tang et al. 2021; Schippers et al. 2024), whereas its contribution to biotic stress responses remains poorly understood. Our findings expand the current understanding of RAP2.2 function by suggesting that this TF integrates responses to both biotic and abiotic stresses. Cell wall remodeling through lignification may represent a common mechanism underlying these responses, as lignin deposition reinforces vascular tissues during drought (Choi et al. 2023) while simultaneously restricting pathogen colonization (Rodrigues et al. 2013; Niza et al. 2015; Giampetruzzi et al. 2016; Sabella et al. 2018).

Our findings demonstrate that *CrRap2.2* overexpression promotes transcriptional reprogramming associated with lignin biosynthesis and responses to abiotic and biotic stresses, establishing a mechanistic link among transcriptional regulation, cell wall reinforcement, and immune activation (Supplementary Fig. 4). These coordinated responses position CrRAP2.2 as an important regulator of vascular stress adaptation, likely contributing to the enhanced resistance to *X. fastidiosa* previously reported in transgenic plants (Pereira et al.

2020). The relevance of AP2/ERF TFs in stress tolerance has also been highlighted by Tang et al. (2024), who emphasized their biotechnological potential for developing stress-resilient crop cultivars.

Supplementary Information: The online version contains supplementary material available

Author contributions: A. A. S. conceived and designed the study, supervised the research, and contributed to manuscript writing and proofreading. M. S. S. maintained the plants under greenhouse conditions, performed RNA extraction and microscopy analyses, and wrote the manuscript. N. V. S. and I. G. B. C. performed the transcriptomic analyses and contributed to manuscript writing. J. P. R. M. performed the microscopy analyses. V. S. S. performed the protein–protein interaction network analyses and contributed to manuscript writing. L. N. dos S. performed the promoter analyses and contributed to manuscript writing. M. A. T. contributed to manuscript writing and proofreading. All authors read and approved the final version of the manuscript.

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Declarations

Conflict of interest: The authors declare that they have no conflicts of interest

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