University of São Paulo
"Luiz de Queiroz" College of Agriculture

De novo transcriptome assembly, functional annotation and expression profiling of sweet passion fruit (*Passiflora alata*) in response to *Xanthomonas axonopodis* pv. *passiflorae* infection

Jéssica Luana Souza Cardoso

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
2022

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Bachelor in Biotechnology

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Cardoso, Jéssica Luana Souza

De novo transcriptome assembly, functional annotation and expression profiling of sweet passion fruit (*Passiflora alata*) in response to *Xanthomonas axonopodis* pv. *passiflorae* infection /Jéssica Luana Souza Cardoso. - - versão revisada de acordo com a Resolução CoPGr 6018 de 2011. - - Piracicaba, 2022.

83 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

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DEDICATORY

To my beloved parents, Maria José and Nivaldo

ACKNOWLEDGMENTS

I would like to start by thanking to the University of São Paulo, "Luiz de Queiroz" College of Agriculture for providing me with the structure and conditions, in addition to the professors who gave me knowledge that I could use for the development of this thesis. I am immensely proud to have had the opportunity to do my doctoral studies at this prestigious university.

I would also like to thank the National Council for Scientific and Technological Development - CNPQ (Grant number 142587/2018-4) for the doctoral scholarship.

To my advisor, Maria Lucia Carneiro Vieira, for giving me the opportunity to be part of her research group, and for having guided me during the development of my thesis. I am extremely grateful to you for providing me the conditions for the development of the project, for teaching me, and for helping me in my professional growth.

To Dr. Daniel Guariz Pinheiro, who have been willing to welcome me in his laboratory, introducing me in some bioinformatics' tools. I certainly would not have been able to complete this work without your help.

To Dr. Alessandra Alvez de Souza for her collaboration, offering her knowledge and patience during the review article elaboration.

To the laboratory technician Carlos Alberto de Oliveira and my laboratory colleagues who helped me in different ways during this period.

I would like to extend a very special thank you to my parents, Maria Jose and Nivaldo, two amazing human beings, who taught me important lessons for the formation of my character, for giving priority to my education, and for always being there when I need support to minimize the difficulties that I have to face.

To my fiancé, Sherlock F.A. Borges who also supported me in difficult moments, having a lot of patience with me, for understanding the importance of a having a doctoral degree and for making my days lighter and happier.

Finally, I would like to express my gratitude to all those who, in some way, allowed this thesis to be concretized. It is been a long and challenging journey and I wouldn't have made it without support.I would like to express my gratitude to all those who, in some way, allowed this thesis to be concretized. It is been a long and challenging journey and I wouldn't have made it without support.

EPIGRAPH

"If money is your hope for independence, you will never have it. The only real security that a man will have in this world is a reserve of knowledge, experience, and ability".

Henry Ford

SUMMARY

RESUMO

Montagem *de novo* do transcriptoma, anotação funcional e perfil de expressão do maracujá doce (*Passiflora alata*) em resposta à infecção por *Xanthomonas axonopodis* pv. *passiflorae*

Xanthomonas é um dos fitopatógenos mais importantes, atacando severamente mais de 500 hospedeiros diferentes ao redor do mundo. Por outro lado, as plantas desenvolveram mecanismos de defesa a fim de prevenir a proliferação de patógenos e, consequentemente, a doença. Assim, visando um melhor entendimento dos mecanismos de defesa em diferentes hospedeiros em resposta ao ataque de *Xanthomonas*, uma ampla revisão da literatura foi realizada e constitui o primeiro capítulo desta tese. Foi apresentado o conhecimento atual das bases moleculares do sistema imune em diferentes culturas suscetíveis à *Xanthomonas*. Entre os hospedeiros tropicais, os maracujazeiros cultivados são muito vulneráveis a este patógeno levando a graves perdas em pomares comerciais. A doença, conhecida como mancha bacteriana, é causada por *Xanthomonas axonopodis* pv. *passiflorae* (*Xap*). Não há informações moleculares sobre a interação de *Passiflora alata* (o maracujá doce) e *Xap*., tornando importante a condução de estudos para entender esse patossistema. Assim, nosso objetivo foi analisar o perfil do transcriptoma de *P. alata* em resposta à infecção por *Xap*. Os resultados desta análise constituem o segundo capítulo desta tese. Para este fim, o RNA total de folhas saudáveis e infectadas com *Xap* foi isolado, 5 dias após a inoculação, e sequenciado usando a plataforma NextSeq (Illumina), resultando em cerca de 50 milhões de leituras pareadas por amostra. Como não há genoma de *P. alata* disponível para ser usado como referência, foi realizada uma montagem *de novo*, seguida de anotação funcional dos transcritos. A análise de expressão diferencial revelou 638 transcritos induzidos e 604 reprimidos, considerando FDR $\leq$ 0,05 e fold change $\geq$ 1,5 e $\leq$ -1.5. Entre eles, foram detectados receptores de reconhecimento de padrões (PRRs) e genes de resistência que, após a percepção do patógeno, desencadeiam uma sinalização de resposta de defesa envolvendo um rápido aumento do influxo de cálcio e a produção de espécies reativas de oxigênio (ROS). Em seguida, quinases dependentes de cálcio ativam genes relacionados à patogênese e à produção de compostos voláteis (os terpenos germacrene D e nerolidol) que atuam como sinais para a produção de hormônios. É importante ressaltar que dois genes de suscetibilidade, LOB1 e SWEET10, foram identificados superexpressos em maracujá doce sob a infecção do patógeno. O primeiro é um fator de transcrição membro da família Lateral Organ Boundaries (LOB), e o segundo é um transportador de açúcar. Sugere-se que um nocaute desses genes pode resultar em aumento da tolerância ou mesmo resistência contra *Xap*, uma vez que também foram identificados dois genes de resistência contendo o domínio CC/TIR-NBS-LRR. A RT-PCR quantitativa de genes selecionados foi realizada para validar a análise de expressão gênica diferencial. Nossas descobertas não apenas fornecem uma primeira análise completa do transcriptoma da resposta do maracujá doce à infecção por *Xap*, mas também revelam uma informação valiosa sobre potenciais genes-alvo para edição de genes de plantas.

Palavras-chave: Maracujá doce, *Passiflora alata*, *Xanthomonas*, RNA-Seq, Análise de transcriptoma, Suscetibilidade de plantas

ABSTRACT

De novo transcriptome assembly, functional annotation and expression profiling of sweet passion fruit (*Passiflora alata*) in response to *Xanthomonas axonopodis* pv. *passiflorae* infection

Xanthomonas is one of the most important phytopathogens; it severely attacks over 500 different hosts around the world. Plants have evolved various defense mechanisms aimed at preventing pathogen proliferation and controlling disease. To improve our understanding of the defense mechanisms deployed by different hosts in response to *Xanthomonas* attack, a broad review of the literature was conducted and forms the first chapter of this thesis. We outline our current knowledge of the molecular basis of immunity systems in different crops susceptible to *Xanthomonas*. Among its tropical hosts, cultivated passion fruits are very vulnerable to this pathogen, leading to severe losses in commercial orchards. The disease, known as bacterial spot, is caused by *Xanthomonas axonopodis* pv. *passiflorae* (*Xap*). There is no molecular information on the interaction between *Passiflora alata* (sweet passion fruit) and *Xap*, and studies are therefore required to improve our understanding of this pathosystem. Thus, our aim was to analyze the transcriptome profile of *P. alata* in response to *Xap* infection. The results of this analysis are given in Chapter 2. Total RNA of healthy and *Xap*-infected leaves was isolated 5 days post inoculation and sequenced on the Illumina NextSeq platform, resulting in some 50 million paired-end reads per sample. Since there is no *P. alata* genome available for use as a reference, de *novo* assembly was performed, followed by functional annotation of sequence reads. Differential expression analysis revealed 638 upregulated and 604 downregulated transcripts, based on an FDR-adjusted *p*-value $\leq$ 0.05 and a fold change $\geq$ 1.5 and $\leq$ -1.5. Pattern recognition receptors (PRRs) and resistance genes were detected. On perceiving the pathogen, these receptors trigger defense response signaling entailing a rapid increase in calcium influx and the production of reactive oxygen species (ROS). Next, calcium-dependent kinases activate pathogenesis-related genes and result in the production of volatile compounds (germacrene D and nerolidol) to signal hormone production. Importantly, two susceptibility genes, LOB1 and SWEET10, were identified as upregulated in sweet passion fruit in the presence of pathogen infection. LOB1 is a member of the Lateral Organ Boundaries family of transcription factors, and SWEET10 is a sugar transporter. We suggest that knocking out these genes might result in increased tolerance or even resistance to *Xap*, since two resistance genes containing the CC/TIR-NBS-LRR domain were also identified. A quantitative RT-PCR of selected genes was performed to validate differential gene expression analysis. Our findings not only provide the first complete transcriptome analysis of the molecular mechanisms in the sweet passion fruit's response to *Xap* infection, but also supply valuable information on potential target genes for plant gene editing.

Keywords: Sweet passion fruit, *Passiflora alata*, *Xanthomonas*, RNA-Seq, Transcriptome analysis, Plant susceptibility

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1. GENERAL INTRODUCTION

The *Passiflora* genus is the largest of the Passifloraceae family, encompassing over 500 species distributed in tropical and subtropical regions, over 160 of which are native to Brazil (Bernacci et al., 2020). *Passiflora alata,* popularly known as the sweet passion fruit, is native to South America and ranks as the second largest species commercially produced in Brazil, surpassed only by *Passiflora edulis* (sour passion fruit), which is cropped in over 90% of orchards. Sweet passion fruit has great potential for commercialization because of its fruity flavor, typical aroma and exotic characteristics. It is also produced for the pharmaceutical and ornamental plant markets, in addition to the *in natura* consumption market (see Faleiro et al., 2017).

One of the main obstacles to boosting the production of both sour and sweet passion fruit is the incidence of diseases, such as the bacterial leaf spot. This disease devastated plantations in 1996 in several regions of the country (Castelões, 2017). Bacterial spot is caused by the gram-negative bacterium *Xanthomonas axonopodis* pv. *passiflorae* (*Xap*), which penetrates the plant through natural openings or wounds and spreads to all tissues, causing leaf chlorosis and necrosis, spots on the fruit, and finally plant death (Pereira, 1969). Some preventive methods are available to stem the disease, such as using disease-free seeds and seed treatments, and chemical compounds containing copper oxychloride. However, there is no effective method for controlling the disease after contamination with *Xap* (Fischer and Rezende, 2008). Therefore, a better understanding of this pathosystem is extremely important if we are to develop effective technologies for preventing the crop losses it causes.

The plant defense system generally works in two stages. First, cell surface pattern-recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs), and this triggers a defense response called plant triggered immunity (PTI). In the second stage, disease resistance (R) proteins recognize effectors (molecules secreted by the pathogen) which are able to penetrate host cells via secretion systems. This defense mechanism is called effector-triggered immunity (ETI). The two responses are similar, but ETI is faster and more intense than PTI (see Han et al., 2018; Zhou and Zhang, 2020).

In order to improve our fundamental understanding of the plant's defense mechanisms to combat *Xanthomonas* species, we conducted an extensive and comprehensive literature review on this subject. It describes our current knowledge of the molecular basis of the plant host-*Xap* interaction, providing useful information for plant breeders. The review forms the first chapter of this thesis.

Our research group has been developing studies to better understand the response of cultivated passion fruits to inoculation with *Xap*. First, we generated linkage maps to locate resistance genes in a sour passion fruit biparental population (Lopes et al. 2006). Along similar lines, Matta (2005) and Braga (2011) mapped quantitative resistance loci (QRL) in sour and sweet passion fruit, respectively, to dissect the genetic control of resistance. A few QRLs were detected, suggesting that resistance is based on oligogenic control. The group subsequently developed a PCR-based method for early detection of *Xap* in plant tissues, even before disease symptoms appear (Munhoz et al. 2011).

We also used suppression subtractive hybridization to construct two cDNA libraries enriched for transcripts induced and repressed by *Xap* 24 h post inoculation. The authors reported that expression profiles changed in 76% of the genes, and the differences in expression ratios ranged from 0.51-fold to 1.83-fold. RT-qPCR revealed that lipoxygenase 2 was strongly differentially expressed 5 days after infection (500-fold compared to control), suggesting that this gene plays an important role in sour passion fruit defence. Moreover, most of the genes

involved in pathogen recognition signaling pathways were repressed by *Xap* and it was suggested that there is a failure to activate the jasmonic acid signaling pathway during the first hours of interaction (Munhoz et al., 2015).

However, no studies focused on the molecular interaction between *P. alata* and *Xap* were found in the literature. To rectify this, we evaluated the transcriptome profiling of *P. alata* in response to *Xap* inoculation using RNA-seq technology. This involved extracting total RNA from non-inoculated and *Xap*-inoculated leaves 5 days after inoculation, and six RNA-libraries were constructed by sequencing on the Illumina NextSeq platform. Fifty million paired-end reads per sample were obtained, processed and evaluated for quality. Then, in the absence of a *P. alata* genome to serve as a reference, *de novo* assembly was performed and evaluated for quality and completeness. Functional annotation and expression profiling were carried out and some of the results validated by RT-qPCR analysis. This study constitutes the second chapter of this thesis. The main host response mechanisms to *Xap* are described and discussed.

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2. MOLECULAR BASIS FOR CROP HOST RESPONSES TO *XANTHOMONAS* INFECTION

Abstract

Xanthomonas is one of the most important genera of gram-negative phytopathogenic bacteria, severely affecting the productivity of economically important crops worldwide, colonizing either the vascular system or the mesophyll tissue of the host. Due to its rapid propagation, *Xanthomonas* poses an enormous challenge to farmers because it is usually controlled using huge quantities of copper-based chemicals, adversely impacting the environment. Thus, developing new ways of preventing colonization by these bacteria has become essential. Advances in genomic technologies has significantly elucidated interactions between various crops and *Xanthomonas* species. Understanding how these hosts respond to the infection is crucial if we are to exploit potential approaches for improving crop breeding and cutting productivity losses. This review focuses on our current knowledge of the defense response mechanisms in agricultural crops after *Xanthomonas* infection. We describe the molecular basis of host-*Xanthomonas* interactions over a broad spectrum with the aim of improving our fundamental understanding of which genes are involved and how they work in this interaction, providing information that can help to speed up plant breeding programs.

Keywords: *Xanthomonas*, Plant-immunity, Crop defense mechanisms, Signaling pathways

2.1. Introduction

Crops are susceptible to several pathogen attacks leading to huge losses in yield and productivity. In order to survive, plants have evolved and developed an elegant innate immune system capable of identifying systemic signals emanating from pathogens, and strategies for dealing with stress. Importantly, Delplace et al. (2021) reviewed the recent progress made to understand the network complexity of regulatory pathways leading to plant immunity.

During the plant-bacterial interactions, the bacteria have to tackle the plant's pathogen recognition mechanisms. Pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) are essential structures for protecting against invasive organisms, and are therefore broadly conserved within various classes of foreign organisms and can be recognized by host cell surface receptors, generally called pattern recognition receptors (PRRs). Recognition induces PAMP-triggered immunity (PTI), a complex set of signals that triggers a physiological change in the plant cell in an attempt to fend off the bacteria. As only pathogenic *Xanthomonas* species were addressed in this review, the term PAMPs will be used throughout.

The infection cycle of *Xanthomonas* species usually consists of two phases. The first is the epiphytic phase, associated with environmental stress, i.e., bacteria live on the surface of the host until they are able to penetrate through natural openings, such as hydathodes and stomata, or even wounds. In the second phase, the bacteria develop endophytically, either spreading systematically through the vascular system or colonizing the mesophyll parenchyma, which produces different symptoms (see An et al. 2020).

The second active plant defense response is effector-triggered immunity (ETI). This mechanism is specific and involves the recognition of molecules introduced by pathogens' effectors based on intracellular resistance proteins (R proteins). If the host does not have the cognate R protein, the effector inhibits ETI, resulting in effector-triggered susceptibility (ETS). PRRs are generally conserved over generations. In contrast, effectors and R proteins are widely variable, constantly coevolving to produce mutations in pathogen effectors and hosts receptors.

Both mechanisms of pathogen recognition induce a complex network of very similar signaling pathways, although ETIs are an amplified, stronger form of defense, which often leads to localized cell death, known as the hypersensitive response (HR) (see Peyraud et al. 2017; Tabassum and Blilou 2021).

Xanthomonas spp. are responsible for causing diseases in many crops, so an in-depth understanding of host-*Xanthomonas* interactions and which genes are involved is essential. Knowledge of how plants defend themselves can help to find possible approaches and avoid devastating losses in crop yield. Timilsina et al. (2020) have broadly reviewed the main aspects of the pathogenicity of the *Xanthomonas* genus, highlighting the secretion systems, the emergence of new strains, and how they infect specific hosts leading to pathogenicity. Here, we focus in the crop defense mechanisms against the respective *Xanthomonas* pathovars, including the signaling network and downstream responses occurring after the bacterium is recognized.

2.2. *Xanthomonas* spp.

The genus *Xanthomonas* belongs to the order Xanthomonadales and is a member of the Xanthomonadaceae family. Species belonging to this genus are gram-negative aerobes with an optimal growth temperature of between 25 and 30°C. Most of them are yellow-pigmented due the presence of xanthomonadin which plays important roles in the epiphytic survival of bacteria and also in host-pathogen interactions. They provide protection against UV radiation, as well as antioxidants to combat chemical compounds produced by the host, such as reactive oxygen species (ROS). The outer membrane of *Xanthomonas* consists of lipopolysaccharide (LPS) that also plays a key role in interactions, providing a barrier against adverse environmental factors. In addition, the Lipid A moiety of the structure is highly conserved, and can therefore act as a PAMP when recognized by host receptors, triggering plant defense responses (Di Lorenzo et al. 2017).

The species nomenclature has changed over the past few years and encompasses around 30 species, subdivided into subspecies or pathovars, with a high degree of host plant specificity (Parte et al. 2020), although there are non-pathogenic strains that can cohabit with pathogenic strains (Garita-Cambronero et al. 2016; Martins et al. 2020; Muñoz-Bodnar et al. 2014). *Xanthomonas* species are responsible for many diseases in more than 400 hosts, causing yield losses in economically important crops. There are over 1700 *Xanthomonas* genome assemblies available in the National Center for Biotechnology Information (NCBI) database. Their genomes usually contain over 4,000 genes and GC content of over 60%. They are around 5 Mb long, except for *Xanthomonas albilineans,* which has a shorter genome of around 3.7 Mb and 3115 putative protein-coding sequences (CDS). *X. albilineans* shows a lower G+C content which is associated with genome erosion, and many genomic features that may help its interaction with sugarcane (Pieretti et al. 2015). Comparative analysis of *Xanthomonas* spp. genomes suggests that some genes and gene clusters involved in the virulence of *Xanthomonas* are conserved, including 6 types of secretion system.

2.2.1. *Xanthomonas* secretion systems

Bacteria have evolved different secretion systems which are able to transfer a wide range of substrates with various functions (adhesion, degradation and virulence) to respond to environmental conditions and enhance survival. The secreted substrates can either remain associated with the bacterial outer membrane for delivery to the extracellular space, or be released inside a host target cell. In gram-negative bacteria, these systems can be mediated

by the general secretory pathway (Sec) or the twin argenine translocation (Tat) pathway. The substrate passes through the membranes by a two-stage process: first, it is directed to the periplasm using Sec or Tat secretion systems; it is subsequently delivered across the outer membrane using other systems. The main difference between the Sec- and Tat-mediated secretion systems is that Sec delivers unfolded proteins and Tat already folded proteins. The substrate can also be secreted through pathways that comprise both bacterial membranes, a mechanism known as Sec- or Tat-independent protein secretion. Costa et al. (2015) and Alvarez Martinez et al. (2020) articles broadly compile the role of secretion systems in gran-negative bacteria, including *Xanthomonas*.

Type I secretion systems (T1SS) secrete unfolded proteins, such as adhesins and antimicrobial compounds, from the cytoplasm either directly or in two stages through the periplasm to the extracellular medium (Spitz et al. 2019). In contrast, Type 2 secretion systems (T2SS) secrete folded proteins, including virulence factors, toxins and degradative enzymes, along the Sec or Tat pathway (Chernyatina and Low 2019). Although *Xanthomonas* generally has 6 types of secretion system (Figure 1), the type 3 secretion system (T3SS or injectisome) is the most important in terms of pathogenicity due to its ability to directly inject effector proteins from the pathogen into plant host cells. This system is Sec-independent and encoded by the hypersensitive response and pathogenicity (*hrp*) cluster, consisting of more than 20 genes (Bernal et al. 2019). T3SS is conserved in most species, except *Xanthomonas maliensis* (Triplett et al. 2015)*, Xanthomonas cannabis* (Jacobs et al. 2015) and *Xanthomonas sacchari (*Studholme et al. 2011). T3SS effectors play a very important part in pathogenicity, since target effectors suppress the host's defense and consequently induce disease progression. Most of them are transcription activation-like effectors (TALE) or Xops (*Xanthomonas* outer proteins). TALEs generally bind to promoters of specific genes in hosts, whereas *Xops* effectors promote disease by directly or indirectly targeting genes involved in the signaling responses, for instance by manipulating mitogen-activated protein kinase (MAPK) signaling by means of kinase phosphorylation (Teper et al. 2018; Teper and Wang 2021). The type IV secretion system (T4SS) is responsible for transferring proteins and/or protein-DNA complexes into target cells by Sec/Tat-independent means (Sgro et al. 2019). Unlike T4SS, type V secretion systems (T5SS) export toxins and exoenzymes from the cytoplasm to the periplasm through the Sec translocase pathway, and subsequently to the extracellular medium, or alternatively it can remain associated with the cell surface. In addition, T5SS have been reported to play a part in adhesion in pathogenic interactions (Fan et al 2016). Finally, Type 6 secretion systems (T6SS) translocate effector proteins directly into the host. T6SS are known to secrete potent weapons against rival bacterial cells, such as antibacterial proteins (e.g. peptidoglycan hydrolases) and toxins (e.g. DNases and phospholipases) (Coulthurst 2019). Virulence factors in *Xanthomonas* species are controlled by the diffusible signal factor quorum sensing system (He et al. 2010).

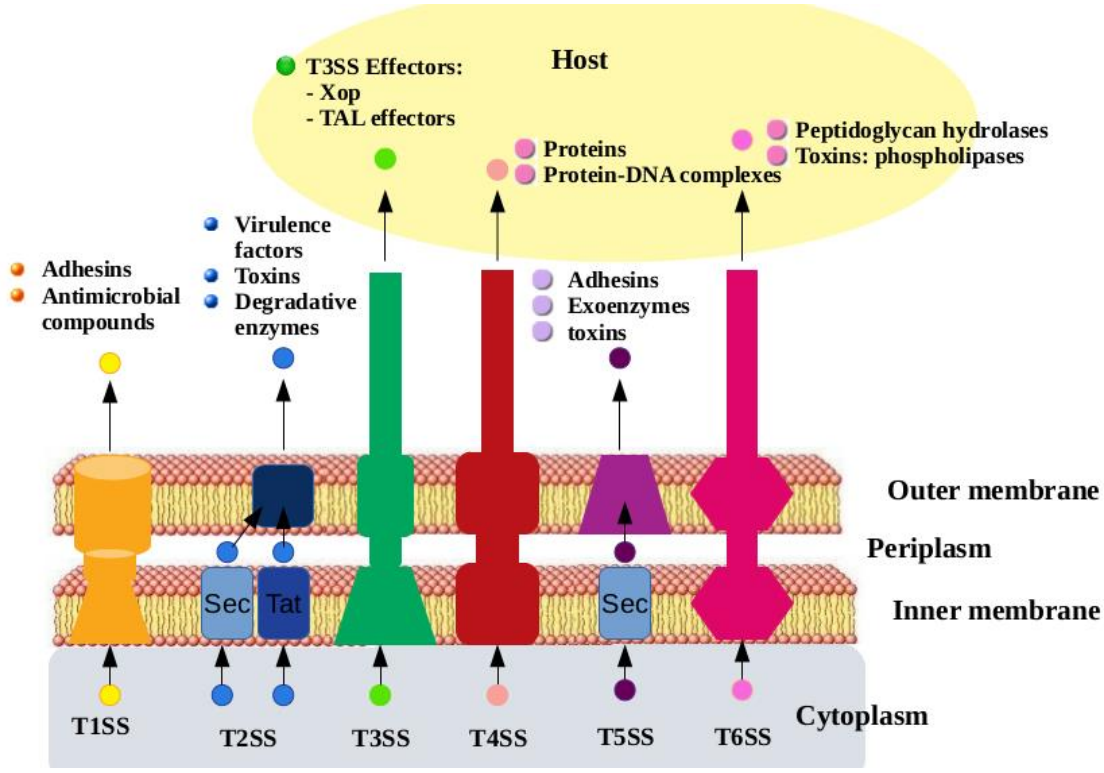

Figure 1. Schematic overview of the different secretion systems in *Xanthomonas* species. T1SS is Sec/Tat-independent and works by secreting adhesin and antimicrobial compounds, while T2SS is Sec/Tat-dependent and capable of secreting toxins and enzymes that degrade the host cell wall. Bacterial T3SS is Sec/Tat-independent, capable of delivering effector proteins into the cytoplasm of host target cells, and therefore the most important in pathogenesis. Similarly, T4SS is capable of translocating effectors into the host and is Sec/Tat-independent. In contrast, T5SS is Sec-dependent and responsible for adhesion and biofilm formation. T6SS is the most recently described, Sec/Tat-independent and associated with combating other microorganisms in competitive survival.

2.2.2. Quorum sensing and pathogenicity

Bacterial quorum sensing (QS) is defined as a cell-to-cell communication mechanism that directs bacterial behavior and controls several biological processes, including pathogenicity, producing chemical signal molecules (autoinducers) that modulate expression in specific genes. One extraordinary aspect of QS systems is that QS signals self-regulate their own biosynthesis by sensing their own population density. Diffusible signal factors (DSF) are reported to be QS signal molecules that modulate bacterial virulence and other biological activities in *Xanthomonas* species, such as regulation of motility; extracellular polysaccharide and extracellular enzyme production; and subsequent biofilm formation (Li et al. 2019; Zhang et al. 2019). DSFs have been structurally characterized as cis-2-unsaturated fatty acids which vary in length, double-bond configuration and side-chains (reviewed in Whiteley et al. 2017 and Baltenneck et al. 2021). These molecules are synthesized by *rpfF* and *rpfB* genes that respectively encode a putative enoyl-CoA hydratase and an acyl-CoA ligase in the fatty acid long chain. Several RPF proteins are involved in *Xanthomonas* species' quorum sensing (Li et al. 2019). However, the best system described so far handles DSF sensing and transduction in bacteria and comprises *rpfC*, a histidine kinase, and the *rpfG* regulator (Cai et al. 2017). The perception of DSF by *rpfC* induces a conformational change in *rpfC* that phosphorylates *rpfG*, activating its cyclic di-GMP phosphodiesterase function, reducing the level of cyclic di-GMP and releasing the *Clp* transcriptional activator that drives the expression of several genes, including those encoding virulence factor production (e.g., promotion of exopolysaccharide (EPS) synthesis), which are important to biofilm formation (Caicedo et al. 2017).

The synthesis of EPS, also known as xanthan, is encoded by the *gum* cluster, which comprises 12 genes (*gumB* to *gumM*), strongly conserved in the genus *Xanthomonas*, while biofilm formation is regulated by the *rpf* (regulation of pathogenicity factors) gene cluster (Katzen et al. 1998). In addition to protecting the bacterium against environmental stresses, xanthan can block water flow along xylem vessels in the host, causing wilting (Denny 1995); suppress callose deposition on the host cell wall (Yun et al. 2006); and contribute to the generation of a biofilm that provides defense against host antimicrobial compounds and to stress tolerance, enhancing epiphytic survival (Chatterjee et al. 2020). In fact, a recent work suggested that the *XanA* gene, which encodes phosphoglucomutase protein, necessary for the synthesis of expolysaccharides and lipopolysaccharides, plays a fundamental role during the epiphytic phase of the bacterial cycle, since leaves infected with mutant xanA transposon did not develop symptoms (Picchi et al. 2021).

This concludes our brief summary of virulence factors (e.g. EPS and LPS), types of secretion system and respective substrates, and quorum-sensing, all of which significantly contribute to bacterial survival. It shows the importance of understanding how these factors contribute to bacterial fitness, how the bacteria behave inside the host, and how they are recognized by the host, triggering subsequent defense strategies What follows is a description of how this pathogen is recognized by the host. We will then focus on the defense mechanisms based on our knowledge of the main host crops and their respective *Xanthomonas* pathogenic species (Table 1).

Table 1. List of *Xanthomonas* species and respective hosts discussed in this review.

Species	Host	Disease
Xanthomonas albilineans	Sugarcane	Leaf scald
Xanthomonas arboricola pv. *pruni*	Peach	Bacterial spot
Xanthomonas axonopodis pv. *manihotis*	Cassava	Bacteria blight
Xanthomonas campestris pv. *campestris*	Tomato, Crucifers	Bacterial spot, Black rot
Xanthomonas campestris pv. *musacearum*	Banana	Bacterial wilt
Xanthomonas campestris pv. *vesicatoria*	Pepper, Tomato	Bacterial leaf spot
Xanthomonas cannabis	Cannabis	Bacterial leaf spot
Xanthomonas citri subsp. *citri*	Citrus	Citrus canker
Xanthomonas citri subsp. malvacearum	Cotton	Angular leaf spot
Xanthomonas maliensis	Rice	Non-pathogenic
Xanthomonas cynarae pv. *gardneri*	Tomato	Bacterial leaf spot
Xanthomonas euvesicatoria pv. *perforans*	Tomato	Bacterial leaf spot
Xanthomonas oryzae pv. *oryzae*	Rice	Bacterial blight
Xanthomonas oryzae pv. *oryzicola*	Rice	Bacterial streak
Xanthomonas phaseoli pv. *phaseoli*	Bean	Bacterial blight
Xanthomonas sacchari	Sugarcane	Chlorotic streak disease

2.3. Pathogen recognition and immunity response

Since plants lack mobile defense cells and a somatic adaptive immune system, each cell needs to defend itself and emit signals from the infection site. Once the bacterial pathogens penetrate plant tissues, two classes of

receptors can recognize them and initiate a signaling cascade to try to stop the infection. When PAMPs are directly recognized by plant cell surface-localized pattern recognition receptors (PRR), they emit danger signals to initiate PTI. In many cases, PTI is not sufficient to suppress pathogen attacks, resulting in effector-triggered susceptibility (ETS). In some cases, TAL effectors secreted by *Xanthomonas* bind to susceptibility genes (S genes), impairing host resistance. These effectors can also be recognized by intracellular receptors (nucleotide-binding domain receptors and leucine-rich repeat-containing receptors (NLR), also called resistance (R) genes, triggering a second layer of plant protection, known as ETI. While PRRs detect apoplastic patterns from several microorganisms, NLRs are specific to a single cytoplasmic effector (reviewed in Zipfel 2014 and Boutrot and Zipfel 2017).

2.3.1. Pattern recognition receptors

Pattern recognition receptors are proteins capable of recognizing PAMPs and belonging to large families of receptor-like kinases (RLK) and receptor-like proteins (RLP). Both families have a ligand-binding ectodomain and a single-pass transmembrane domain, but RLKs also have an intracellular kinase domain, which RLPs lack. To compensate for this, RLPs require interaction with regulatory co-receptor kinases to transduce and activate downstream immune signaling, and the co-receptors are somatic embryogenesis receptor kinases (SERKs), such as the receptor-like cytoplasmic kinases (RLCKs) which, to date, are the most frequently reported (Zipfel 2014; Ma et al. 2016). PRRs can be divided into groups based on their ligand-binding ectodomains.

2.3.1.1. Leucine-rich repeat ectodomain receptors

The major PRR ligand-binding ectodomain is the leucine-rich repeat (LRR) that identifies plant- and pathogen-derived peptide epitopes (Belkhadir et al. 2014). The elongation factor-TU receptor (EFR) from *Arabidopsis thaliana*, which recognizes the conserved bacterial PAMP EF-Tu and derived elf peptides, was shown to increase resistance against *Xanthomonas citri* subsp *citri* in sweet orange transgenic plants (Mitre et al. 2021). A well-characterized example of recognition by LRR-RLK is the flagellin PAMP. The flagellin in *Xanthomonas* spp. has a conserved 22-amino acid epitope (*flg22* peptide) at the N terminal which is initially inaccessible to the receptor due to glycosylation (Sun et al. 2012). Inside the plant apoplast, plant glycosidase β galactosidase 1 (*BGAL1*) releases these epitope peptides so that they can be recognized by the plant's RLK receptor, FLS2 (Buscaill et al. 2019). Although the flagellin epitope is highly conserved, some *Xanthomonas campestris* pv. *campestris* strains incorporate a single amino acid polymorphism in *flg22* (Val-43/Asp) affecting recognition by the FLS2 receptor and increasing host susceptibility to the pathogen (Sun et al. 2006).

The rice genome contains several leucine-rich repeat (LRR) receptor kinases, being two well-characterized: *Xa3/Xa26* is responsible for inducing autophagy-like cell death in xylem parenchyma cells and possibly activating calcium ion signals sent to the defense response system (Cao et al. 2019); *Xa21* recognizes tyrosine-sulfated type 1–secreted protein, *RaxX,* highly conserved in *Xanthomonas* species and capable of mimicking the plant hormone regulating the host's biological process (Pruitt et al. 2015). The PSKR1 receptor has also been reported in rice and recognizes a phytosulfokine (PSK), which is a pentapeptide (Tyr–Ile–Tyr–Thr–Gln) and sulfate group in *Xanthomonas oryzae* pv. *oryzicola* triggering the activation of the SA pathway (Yang et al. 2019). A similar receptor with 45% identity to rice *Xa21*, called *RXam1* (Resistance to *Xam 1*), has been identified in cassava plants

on a QTL (called *XM5*) and seems to impair *Xanthomonas axonopodis* pv. *manihotis* growth (Díaz Tatis et al. 2018). Furthermore, three LRR, LRK10 and DUF26 RLKs have been identified in a comparative transcriptome analysis of resistant and susceptible banana genotypes (*Musa balbisiana).* The susceptible genotype ('Pisang Awak') defends against *Xanthomonas campestris* pv. *musacearum*, one of the stimuli for activating both PTI and ETI defense in the first genotype (Tripathi et al. 2019).

Brassinosteroid insensitive 1- associated kinase 1 – BAK1 is a receptor recruited to form a major phosphorylation complex between the kinase domains of the RLKs, including Botrytis-induced Kinase-1 (BIK1) attached to the plasma membrane. It is also a constituent of the FLS2 immune receptor complex (Lu et al. 2010; Liang et al. 2018). In fact, after recognition of flagellin, the OsBAK1 receptor is recruited to initiate a phosphorylation cascade in rice plants, inducing defense signaling molecules to combat *Xanthomonas oryzae* pv. *oryzae* (Liao et al. 2016). BIK1 is released from the complex (FLS2-BAK1) after being monoubiquitinated by an E3 ubiquitin ligase. BIK1 then directly phosphorylates RBOHD (NADPH oxidase), resulting in the accumulation of reactive oxygen species (ROS), an influx of calcium and stomatal closure to fend-off the pathogens (Li et al. 2014; Ma et al 2020).

RLPs lack kinase signaling domains and therefore one or several RLCKs initiate phosphorylation to transduce the signal sent to intracellular signaling networks, triggering a cellular response to a specific factor (Wu et al. 2018). In addition, BAK1, which suppresses BIR1-1 – SOBIR1 (a dual-specificity kinase), has been shown to play an essential role in forming complexes with LRR-RLPs and triggering the immune response. BAK1 generally interacts and phosphorylates BAK1-INTERACTING RLK-1 – BIR1, which acts as a negative regulator of defense-associated LRR-RLKs. SOBIR1 therefore inhibits BIR1 activity and acts positively in the defense response (Liebrand et al. 2014).

In a comparative analysis of a 'Citron C-05' genotype (the only citrus genotype that has shown strong resistance to *X. citri* subsp. *citri*) and a susceptible species, upregulation of receptors RLP12 (LRR receptor-like protein) and LRR8 (LRR receptor-like protein kinase) was observed. Since no inhibition of bacterial growth on the epidermal surface was observed, resistance to the phytobacteria must occur in mesophyll tissues after pathogen penetration, and these receptors could help enhance resistance in this genotype (Fu et al. 2020).

2.3.1.2. PRR alternative domains

Both receptors, RLKs and RLPs can also contain lysine motifs, Lysin motif receptor-like kinases, (LysM-RLKs) and lysin motif receptor-like proteins (LysM-RLPs), which have a highly conserved secondary structure with 40 amino acids (Buist et al. 2008), and could act either in hydrolysis or in the recognition of another bacterial PAMP, pathogenic peptidoglycan (PGN). PGN consists of alternating sugar components, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid cross-linked by oligopeptides (Gust et al. 2007). LysM-receptors have been reported to act in stimulating defense signaling, recognizing bacterial oligosaccharides and peptidoglycan (PGN), and driving the defense response (Buendia et al. 2018). Two LysM receptors (*LYP4* and *LYP6*) have been identified in rice under attack by *X. oryzae* pv. *oryzae.* (Liu et al. 2012), and more recently in a systematic annotation of 9 LysM receptors (*CsLYK 1-9*) in *Citrus sinensis*, 6 of which were upregulated during *X. citri* subsp *citri* infection (Li et al. 2021).

Furthermore, in addition to LysM-PRRs, S-lectin RLKs (LecRKs) have distinct extracellular lectin domains, a transmembrane domain and a kinase domain, suggesting that LecRLKs might recognize extracellular nucleotides and bacterial LPS leading to signaling transduction in the apoplast. A G-type LecRLK (*OslecRK*) interacts

directly with an actin-depolymerizing factor (OsADF) through its kinase domain and triggers downstream signaling responses to increase PR1 (Pathogenesis-related protein 1) and *lox2 (*lipoxygenase) gene levels in rice plants (Cheng et al. 2013). Similarly, an L-type lectin receptor kinase gene (CaLecRK-S.5) positively regulates plant defense at the transcriptional level to combat *Xanthomonas campestris* pv. *vesicatoria* in pepper, boosting MAPK (mitogen-activated protein kinase) activation and ROS accumulation upon infection (Woo et al. 2016).

2.3.2. Resistance genes

2.3.2.1. NBS-LRR receptors

Xanthomonas spp. have multiple secretion systems to deliver effectors directly inside host cells. As mentioned above, the best-characterized secretory system is T3SS. *Xanthomonas* effectors are known to have molecular or enzymatic functions to modify host targets specifically (Bernal et al. 2019). The Zig-Zag model, described by Jones and Dangl (2006), details the successive steps of the interaction between plant and pathogen: upon PTI, the invaders secrete effectors in an attempt to penetrate host cells, suppressing the initial host responses, culminating in effector-triggered susceptibility (ETS). Hosts have developed receptors to identify these effectors and trigger ETI, which is usually followed by hypersensitive responses to prevent pathogen infection. However, the bacteria continue in a constantly evolving race against the host, resulting in new effectors engendering new receptors. A Zig-Zag model adapted to *Xanthomonas*-host interaction is shown in Figure 2.

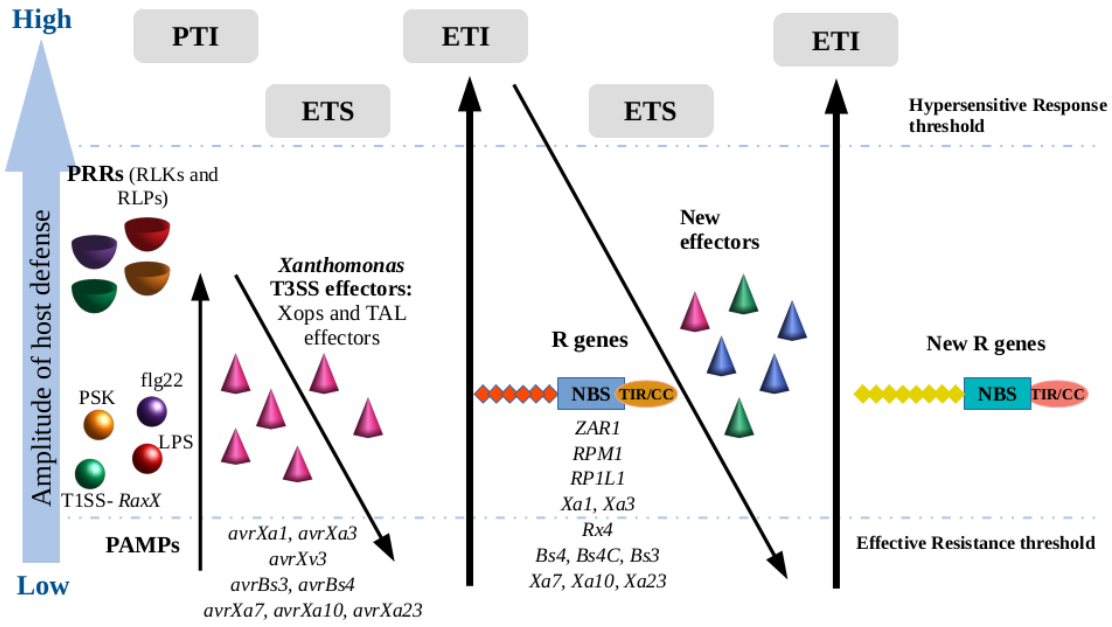

Figure 2. Zig-Zag model adapted to *Xanthomonas*-host interaction. First, *Xanthomonas* PAMPs (pathogen-associated molecular patterns) are recognized by PRRs (pattern recognition receptors); RLKs (receptor-like kinases) are the best known in this pathosystem. This recognition triggers an initial defense response called PTI (PAMP-triggered immunity). The bacteria then counterattack by releasing effector proteins inside the host, such as TAL effectors and Xops (Xanthomonas outer proteins), which leads to ETS (effector triggered susceptibility). In turn, these effectors can be recognized by NB-LRR receptors (R genes), or by executor genes, resulting in ETI (effector-triggered immunity). Both PTI and ETI are followed by similar defense mechanisms but at different intensities, which can lead to a hypersensitive response. In this model, some PAMPs, effectors and receptors described in this review are used as examples. This model was adapted from Jones and Dangl (2006).

Recognition of effector proteins is mostly mediated, directly or indirectly, by a class of receptors which contains nucleotide-binding (NB) domains that can bind to and hydrolyze ATP or GTP, switching on signal transduction and activating LRR domains involved in interactions between proteins, which cannot function without hetero- and homodimerization of their N terminal regions. NBS-LRR genes are classified in two subfamilies: NB-LRR proteins that comprise an N-terminal TIR (Toll, interleukin-1 receptor, resistance protein) domain, which acts in intracellular signaling, and NB-LRR proteins that comprise an N-terminal domain with a coiled-coil (CC) domain, or may have no conserved N-terminal region at all (Dubey and Singh 2018). Recently, R genes allelic to *Xa1*, such as *Xa1-2* and *Xa14*, were isolated in rice. They are atypical NLRs with unique central tandem repeats (CTR), comprising 93 highly conserved amino acids, but repeat numbers can vary from four to six. These CTR-NLRs are present only in Gramineae (Zhang B et al. 2020). The number and domain combinations of NB-LRR genes is highly diverse across different species, and even among different populations, indicating that they can be quickly gained or lost. Six cis-regulatory elements (*Myc, Stre, Myb, Abre, G-box,* and *AS-1*) were more frequent in the promoter regions of NB-LRR genes in rice subjected to *Xoo* infection. This suggests that their expression could potentially be regulated by certain transcription factors (Ding et al. 2020). Indeed, NB-LRR promoters bind to many sites in some transcription factors, such as WRKY, which is associated with the defense mechanisms in many plants. However, some NLRs regulate their own expression, while others are regulated by changes in the environment, such as the production of hormone SA (van Wersch et al. 2019).

The first structure of a plant's NLR protein (HOPZ-activated resistance1 - *ZAR1*) was fully described using cryogenic electron microscopy (cryo-EM) (Wang et al. 2019). In its inactive state, ZAR1 (CC-NBS-LRR) directly interacts with RKS1 (resistance related kinase) and remains in a monomeric form which sterically inhibits binding with ZAR1 adenosine diphosphate (ADP). *X. campestris* pv. *campestris* effector a*vrAC* first uridylates PBL2, an RLK receptor, to produce PBL2UMP, which then interacts with the RKS1–ZAR1 complex, inducing a conformational change in RKS1 and mediating the exchange of ADP and ATP. This results in the oligomerization of the ZAR1–RKS1–PUBL2UMP complex, generating a wheel-like pentamer (resistosome) that triggers cell death by impairing plasma membrane integrity (Wang G et al. 2015; Wang et al. 2019), creating pores in the membrane and inducing ion fluxes. The plant resistosome can be defined as an inflammasome-like molecular structure that initiates defense responses after pathogen recognition, leading to cell death (Pitsili et al. 2019).

Nine NBS-encoding R genes in cabbage, potentially involved resistance to black rot disease caused by *X. campestris* pv. *campestris,* were identified in a comparative analysis of the gene expression profiles of resistant and susceptible lines. Four of these genes were TIR-NBS types (*Bol003711*, *Bol022784, Bol040045* and *Bol042095*), two NBS-LRR (*Bol10135* and *Bol029866*), two NBS (*Bol010559* and *Bol031422*), and just one candidate gene (*Bol042121*) was TIR-NBS-LRR (Afrin et al. 2018). Similarly, NB-LRR genes *OsRP1L1* (*Oryza sativa Rp1-like 1*) (Wang et al. 2012), *Xa1* (Yoshimura et al. 1998) and *Xa3* (Liu F et al. 2020), involved in bacterial blight-resistance to some *X. oryzae* pv. *oryzae* strains, were isolated from rice. In addition to four disease resistance genes (RPM1) identified in peach (Socquet-Juglard et al. 2013), thirty NBS-LRR genes were identified in 14 QTLs with additive effects on resistance against *Xanthomonas arboricola* pv. p*runi* (Yang et al. 2013). Although the target effectors of these receptors are still unknown, when they are recognized, a robust immune response is triggered to try fend off the pathogen.

Specific interactions between these receptors and pathogen effectors have been studied in recent years. In tomato (*Lycopersicon esculentum)*, resistance gene *Bs4* TIR-NBS-LRR was identified. It recognizes TAL effector *avrBsP/avrBs4* in *X. campestris* pv. *vesicatoria* (Schornack et al. 2004). In *Solanum pimpinellifolium,* the *Rx4* gene (NBS-LRR) was also identified. It confers a hypersensitive response to *Xanthomonas euvesicatoria* pv. *perforans* race T3,

expressing the a*vrXv3* avirulence protein, but not directly. Rx4 interacts with SGT1-1 and RAR1, which interact with each other to form a complex and help HSP90 (chaperone) to stabilize the R proteins (Zhang X et al. 2020).

Receptors of NLR type are essential to plant immunity against *Xanthomonas* species. When the effectors are recognized by these receptors, an elaborate defense response is triggered. For this reason, understanding this interaction is very important for agricultural research in order develop resistant crop cultivars.

2.3.2.2. Executor genes

Executor genes (E) are resistance genes that are activated only when a *Xanthomonas* transcription activator-like effector (TALE) binds to their promoter regions. These R genes act as decoys for these effectors, initiating the transcription of defense genes. Specificity is not found in the R gene domain structure, but it is in the expression pattern of the R gene after identification of the pathogen effector. Thus, executor genes are expressed when the effector binds to a suitable EBE (effector binding element) in the respective promoter (Zhang J et al. 2015).

To date, few executor genes have been identified in *Xanthomonas* hosts. In pepper (*Capsicum annuum*), the *Bs3* gene provides resistance to the *avrBs3* effector in *X. campestris* pv. *vesicatoria* (Jordan et al. 2006), a*vrHah* in *Xanthomonas cynarae* pv. *gardneri* (Schornack et al. 2008; Timilsina et al. 2019) and Bs4C, a gene that is activated by TAL effector *avrBs4* (Strauss et al. 2012). Four pairs of TAL effectors and cognate E genes have been cloned from rice; they are *AvrXa27/Xa27*, where resistance is dependent on the developmental stage, increasing as the plant ages (Gu et al. 2005); *AvrXa10/Xa10* (Tian et al. 2014) and *AvrXa23/Xa23,* which share approximately 50% of amino acid sequence identity and 64% nucleotide sequence similarity (Wang C et al. 2015); and *AvrXa7/Xa7* or *PthXo3/Xa7* which also recognized EBEs in the *Xa7* promoter (Chen et al. 2021).

2.3.3. Susceptibility genes

In contrast to R genes that interact with effectors and, in some cases, activate ETI, the plant becomes diseased only if the host has a susceptibility gene (S) that matches a pathogen virulence effector and triggers ETS. Therefore, S genes are required for successful pathogen infection. S gene loss-of-function has been reported to be an effective genome editing technology for agricultural purposes and provides stronger resistance than that conferred by R genes, as emphasized by Engelhardt et al. (2018). The two well-known susceptibility family genes involved in interaction with *Xanthomonas* are SWEET (Sugars Will Eventually Be Exported Transporters) and LOB (Lateral Organ Boundary genes).

2.3.3.1. SWEET genes

SWEET genes are sugar transporters ubiquitous in plants and are able to transport sucrose across the plasma membrane. They mediate sucrose efflux from putative phloem parenchyma into the phloem apoplasm. As a sugar nutrition source, pathogens often use these host transporters. Mutations that block this sugar transporter mechanism have been found to confer pathogen resistance (Chen 2013; Gupta 2012; Gupta 2020). A review article (Boch et al. 2014) emphasized that the SWEET genes are activated by *Xanthomonas* TAL effectors binding in the EBE region of the promoter.

OsSWEET11 (Os8N3) (Yang et al. 2006) and *OsSWEET14* (*Os11N3)* are known bacterial blight susceptibility genes in rice, respectively induced by *pthXo1* and by both *avrXa7* and *pthXo3* effectors (Antony et al. 2010). An allele that contains a deletion of 18 bp in the promoter region (*xa41(t)*) confers resistance against several *X. oryzae* pv. *oryzae* species by preventing the binding of some TAL effectors known to target *OsSWEET14* (Hutin et al. 2015). The CRISPR/Cas9 genome editing system has been used to disrupt the function of *OsSWEET11* (Kim et al. 2019) and *OsSWEET14* in the rice cv. 'Zhonghua 11 (CR-S14)' genotype, improving resistance to the African *X. oryzae* pv. *oryzae* strain AXO1947 (Zeng et al. 2020). Similarly, natural variations in EBEs can alter the binding affinity of TALEs, and some of them have been identified in the *OsSWEET13* gene (a 2-bp deletion at the 5th and 6th positions, plus a substitution at the 17th position) and *OsSWEET14* gene (a single nucleotide replacement at position 10) (Zaka et al. 2018).

These sugar transporter genes have also been identified in cassava. *MeSWEET10* is targeted by an *X. axonopodis* pv. *manihotis* TAL effector known as TAL20 (Cohn et al. 2014). They have also been identified in cotton (*Gossypium hirsutum*), in which *GhSWEET10* is targeted by the *Xanthomonas citri* subsp. *malvacearum avrb6* effector (Cox et al. 2017). In both cases they were also associated with a drop in resistance and are therefore possible targets for silencing and genome editing technologies.

2.3.3.2. Lateral Organ Boundary (LOB) genes

LOB domain proteins (LBD) are transcription factors consisting of an N-terminal region, characterized by a highly conserved LOB domain and a variable C- terminal region that activates or represses target gene expression (Xu et al. 2016). These proteins are known to play key roles in the regulation of lateral organ development, as well as several biological processes, such nitrogen metabolism, pathogen defense, the metabolic process and hormone signaling (Zhang Y et al. 2020).

In citrus, a member of the LBD family has been identified as a lateral organ boundary. 1 - *CsLOB1* is a susceptibility gene (Hu et al. 2014) and its expression is activated by a T3S-secreted TAL effector (*PthA4*) in *X. citri* subsp *citri* (Li Z et al. 2014). Transcription factor CsWRKY22 binds to the W-boxes just upstream of the transcription start site in *CsLOB1*, also activating its expression. Upregulation of the cell wall remodeling and auxin and brassinosteroid hormonal pathways was also observed (Long et al. 2021). In fact, transcriptional analysis shows that activation of *CsLOB1* expression promotes cell proliferation by cell wall remodeling and via the brassinosteroid and cytokinini hormone pathways (Zou et al. 2021). No canker symptoms and improved resistance in citrus was observed when the CRISPR/Cas9 system was used to modify the EBE region of the CsLOB1 promoter (Peng et al. 2017). This was also the case when RNAi gene silencing was applied (Zou et al. 2021)

Two LOB genes (*Phvul.007G195100* and *Phvul.008G257400*) were upregulated in bean plants after inoculation with *Xanthomonas phaseoli* pv. *phaseoli*, and it has been suggested that they could act as a negative regulator of resistance to bacterial blight by induction of downstream genes involved in cell wall modification and resistance to the development of disease symptoms (Foucher et al. 2020). In other agricultural crops, LOB susceptibility genes remain unknown, but analyzing the information already reported for citrus plants, it can be concluded that these genes are potential targets for the development of resistant cultivars.

2.3.4. Signaling and downstream responses

Several coordinated cellular and physiological responses occur in plants upon pathogen recognition. Downstream responses and signaling in PTI and ETI after PRR and NB-LRR protein activation are very similar, but may vary in intensity, i.e., ETI elicits stronger and faster defense reactions than PTI (see Tabassum and Blilou 2021). A branched signaling cascade is activated to stimulate local and systemic defense responses in the host. Pathogen recognition triggers ion fluxes (including Ca2+) at the plasma membrane, phosphorylation events by MAPKs and Ca2+-dependent protein kinases (CDPKs), an oxidative burst, transcriptional reprogramming, and changes in hormone concentrations. These components together form a signaling network that coordinates and controls local and systemic immunity in an attempt to repress the infection (Figure 3). An understanding of the plant immunity signaling process is essential if we are to exploit host-pathogen interaction (Saijo et al. 2018; Noman et al. 2019). Some partial signaling pathways have been described in *Xanthomonas* crop hosts, but most of them are still elusive.

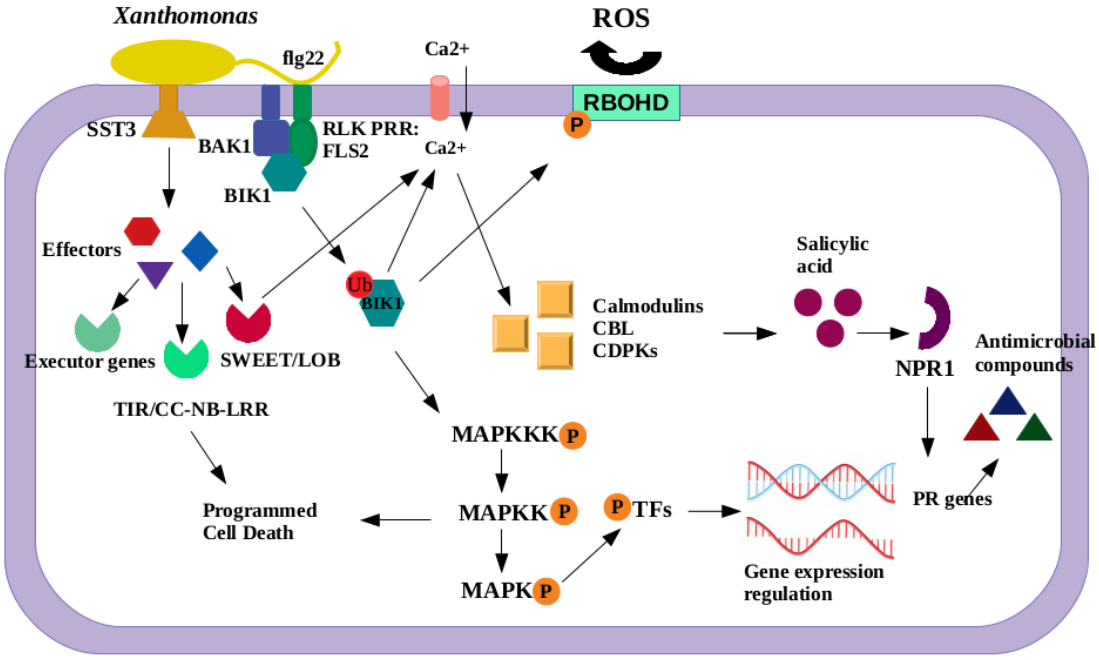

Figure 3. Molecular network interaction between *Xanthomonas* species and crop hosts. Briefly, pathogen-associated molecular patterns (PAMPs) are recognized by cell-surface pattern recognition receptors (PRRs), forming a phosphorylation complex with BAK1 and BIK1, which are released from the complex after ubiquitination and activate downstream responses, including ROS production, calcium influx and MAPK cascades. *Xanthomonas* effectors are detected by intracellular receptors (NB-LRR), which also activate downstream responses. The effector can also target executor genes which serve as decoys, and SWEET and LOB genes that lead to susceptibility. The calcium influx is perceived by calcium sensor proteins, including calmodulins and CBL proteins, and activates SA biosynthesis, which is recognized by NPR1 genes, leading to regulation of gene expression. Therefore, defense signaling outcomes include regulation of gene expression, activation of pathogenesis-related (PR) proteins, and biosynthesis of antimicrobial compounds. Programmed cell death also occurs in an attempt to fend off pathogen attack.

An increase in cytosolic Ca2+ concentration and calcium sensor proteins are network components of the downstream response

Importantly, Couto and Zipfel (2016) summarized the knowledge of the early signaling events that follow pattern recognition receptor activation in plants. For instance, fast ion-flux changes at the plasma membrane, together with an increase in cytosolic calcium levels, is required in the early stages of immune response activation, and is important to programmed cell death. There are two hypotheses as to how the Ca2+ ion can regulate multiple

signaling pathways. The first is that calcium may act as a 'binary switch' allowing a threshold Ca2+ concentration to trigger responses. The second is that different 'Ca2+ signatures', such as amplitude, duration and oscillation, may provide sufficient information to begin a signaling response (Moeder et al. 2019; Seybold et al. 2014). Alterations in calcium concentrations are usually perceived by sensor proteins, such as Ca2+-dependent protein kinases (CDPKs), calmodulin (CaM) and calcineurin B-like proteins (CBL) (Yu et al. 2017).

These proteins are very important calcium signaling regulatory proteins that have four EF-hand domains providing the structural basis for calcium binding, and also interacting with and activating a C-terminal regulatory domain in CBL-interacting protein kinases (CIPK). In cassava (*Manihot esculenta)*, MeCIPK23 interacts with MeCBL1/9, conferring an improved defense response, displayed activation of PR genes, ROS bursts and induction of callose depositions following *X. axonopodis pv. manihot* infection (Yan et al. 2018).

Similarly, calcium sensor proteins such as CDPKs and calmodulin were upregulated in a resistant line of tomato compared to a susceptible line, suggesting that these proteins are involved in a downstream response following *X. euvisicatoria* pv. *perforans* race T3 perception (Du et al. 2015). Calmodulins comprise two separate globular domains, each with a pair of EF-hand motifs that bind to calcium ions, changing the configuration and resulting in exposure of hydrophobic regions, responsible for forming strong affinity binding sites for downstream target proteins, such as CBP60g (CaM-binding protein 60g) (Zhang et al. 2014). In fact, after analyzing the plant transcriptome dynamics of *Brassica oleracea* infected by *X. campestris* pv. *campestris*, it was suggested that two CaM-binding proteins (CBP60g and SARD1) play a potential role in resistance. Both genes were upregulated and activated SA-independent immunity mechanisms by regulating the expression of transcription factor WRKY70. These genes are regulated in different ways. While CBP60g necessarily requires CML binding, SARD1 has a similar role, but is calcium-independent, indicating that they function in parallel (Tortosa et al. 2019). Calcium-sensing protein calmodulin CBP60g was also upregulated, as well as a calcium-dependent protein kinase (CDPK5), in a *Brassicas napus* cultivar after infection by *X. campestris* pv. *campestris* (Mamun et al. 2020).

2.3.4.1. Mitogen Activated Protein Kinases (MAPK) and their role in plant immunity

Mitogen Activated Protein Kinases (MAPK) are a class of enzymes that catalyze the phosphorylation of target proteins. MAPK activation is also the earliest signaling factor after pathogen perception, and MAPKs are responsible for sending transfer signals from the receptor that recognized the pathogen during PTI and ETI via signaling cascades that lead to the expression of genes involved in the defense mechanism. The signal is relayed and amplified in cascade via phosphorylation, in which an MAPK kinase kinase (MAPKKK or MEKK) phosphorylates an MAPK kinase (MAPKK or MKK) to phosphorylate an MAPK or MPK, which in turn phosphorylates other transcription factors or proteins, activating or repressing their functions (He et al. 2020).

The rice genome contains 74 MAPKKK genes, 8 MAPKK genes and 17 MAPK genes that act in different ways (Yang et al. 2015). OsMPK5 (Seo et al. 2011) and OsEDR1 (Shen et al. 2011) negatively regulate rice resistance to *Xoo,* whereas OsMPK12 positively regulates resistance to *Xoo,* interacting with OsEREBP1 (ethylene-responsive element-binding protein 1, AP2) (Seo et al. 2011). During *X. oryzae* pv. *oryzae* infection, OsMKK3 is induced to phosphorylate OsMPK7, which in turn targets OsWRKY30. This interaction inhibits disease symptoms. In contrast, silencing OsMPK7 confers disease susceptibility (Jalmi and Sinha 2016). Unlike OsMPK7, OsMPK6 negatively regulates resistance, and knock-out results in enhanced resistance to both *X. oryzae* pv. *oryzicola* (*Xoc*) and

X. oryzae pv. *oryzae*, leading to the accumulation of SA, and the expression of PR1a (systemic acquired resistance (SAR) marker gene) is induced (Shen et al. 2010).

MAPKs were also found be involved in the signaling networks of other *Xanthomonas* hosts. In *Citrus cinensis,* CsMAPK1 contributes to defense against *X. citri* subsp. *citri* (Oliveira et al. 2013) and *Xanthomonas axonopodis* pv. *aurantifolii* (Cernadas et al. 2008) by activating defense-related genes and producing ROS. Similarly, in tomato (*Solanum lycopersicum*), SlMKK2 and SlMPK2 are required for tomato resistance to *Xcv* strains and encode a positive cell death regulator (Melech-Bonfil and Sessa 2011). Recently some MAPK genes were identified in the genomes of *Saccharum* spp. hybrid (R570) and *S. spontaneum* (AP 85-441), and transcript levels were analyzed based on transcriptomes of sugarcane infected with *X. albilineans*. In the resistant cultivar (LCP85-384), three genes were identified (ShMAPKKK01/02 and ShMAPK07) with higher transcript levels than those of the susceptible cultivar (ROC20) (Ali et al. 2021). These examples of MAPKs that are upregulated in various hosts after *Xanthomonas* species infection suggest that they are essential to plant immunity.

2.3.4.2. Oxidative burst

Feedback regulation of calcium ions and ROS occurs because an increase in calcium concentration results in ROS accumulation and a second peak in calcium levels is triggered by H2O2 production. When stress occurs, intracellular levels of ROS can increase rapidly in a process known as an oxidative burst, reported for the first time in 1983 (Doke 1983). In plants, ROS exist in ionic and/or molecular states and the major forms are superoxide anion (O2−), hydrogen peroxide (H2O2) and hydroxyl radical. ROS can be generated in the plasma membrane, cell wall, mitochondria, chloroplasts, and peroxisomes through enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (also known as respiratory oxidase homologs (RBOHs), cell wall peroxidases, germin-like oxalate oxidases and amine oxidases. NADPH oxidases are proactive in converting electrons from cytoplasmic NADPH to molecular oxygen (O2), generating a superoxide anion (O2–) that dismutates to H_2O_2 (Lee et al. 2020; Qi et al. 2017).

Genes belonging to RBOH family have been reported to be involved in disease resistance, acting during the ROS burst in response to PAMPs, and also in ETI. Furthermore, these genes regulate stomatal closure and cell death in response to pathogen attacks (Wang et al. 2020). It was recently reported that *MeRbohF* and *MeRbohB* genes in cassava might act in the defense response against *X. axonopodis* pv. *manihot,* influencing the H2O2 signal transduction pathway, ROS production and callose deposition. A cis-acting element analysis of the promoter region of *MeRBohs* has shown that these genes consist of light, plant development and stress-related elements and can be regulated by mes-miR399 (Huang et al. 2021).

The accumulation of plant ROS at the pathogen attack site is known to be highly toxic to pathogens, and leads to a hypersensitive response that results in local cell death, preventing the spread of pathogens. The molecular mechanisms of peach resistance to *X. arboricola* pv. *pruni* during early leaf infection were investigated by comparing two *Prunus persica* cultivars, the resistant 'Redkist' and susceptible 'JH Hale'. *CuAO* (Copper amine oxidase) and *PAO* (Poly-amine oxidase) were over-expressed only in the resistant cultivar, but both are related to the hypersensitive response and ROS accumulation (Socquet-Juglard et al. 2013). Similarly, genes related to changes in the redox state, including glutaredoxins, peroxiredoxin, thioredoxins, ascorbate, glutathione and catalases, were identified in *Musa balbisiana* infected by *X. campestris* pv. *musacearum,* resulting in ROS accumulation that triggered a hypersensitive

response and induced lignification involving the cytochrome P450 and phenylpropanoid pathways (Tripathi et al. 2019).

Accumulation of ROS might be generally associated with an increase in pathogenesis-related (PR) gene expression. PR proteins are encoded by host plants upon pathogen recognition or related situations (Huang et a. 2019). In the resistant citrus genotype 'Citron C-05' mentioned above, the *GST1* (glutathione S-transferase) gene is associated with the production of ROS, the hypersensitive response and the PR1 gene (Fu et al. 2020). Upregulation of six defense-related genes (*PR-1, PR-2* and *PR-5* - pathogenesis-related genes, *Lox* – lipoxygenase, *PAL* - phenylalanine ammonia-lyase and *CAT* – catalase) was observed in tomato in response to infection by both *Xanthomonas axonopodis* pv. *phaseoli* as a non-host pathogen and *X. euvisicatoria* pv. *perforan* as a host pathogen, and was found to be stronger in the first case (Safaie-Farahani and Taghavi 2017). In pepper plants (*Capsicum annuum***),** *CaOSM1* (Osmotin-like protein 1 gene) was identified. It is a PR-5 family member that acts as a positive regulator for the cell death response and defense signaling (e.g. ROS accumulation). *CaXEGIP1* (xyloglucan-specific endo-b-1,4-glucanase inhibitor1 gene) was also identified and positively regulates cell death during *X. campestris* pv. *vesicatoria* infection (Choi HW et al. 2013; Choi du S et al. 2013). *FDH1* (formate dehydrogenase gene), which catalyzes the oxidation of formate into carbon dioxide, and *CaDC1* (cysteine/histidine-rich DC1 domain protein gene), acting as a positive regulator of cell death and in SA-dependent defense responses, were also identified (Choi et al. 2014; Hwang et al. 2014). Overexpression of *CaCYP450A* (cytochrome gene) was reported to cause milder symptoms, lower pathogen growth, ROS accumulation and the hypersensitive response (Hwang et al. 2010). The *CrsbohD* gene that encodes an NADPH oxidase could be involved in resistance to *Xanthomonas citri* subsp. *citri* in 2 citrus genotypes, promoting H_2O_2 accumulation by MAPK activation (Mei et al. 2019). An inverse correlation between H_2O_2 concentration and the growth of *X. citri* subsp. *citri* was found (i.e. as the concentration of H_2O_2 increases, bacterial growth decreases) (Mei et al. 2020).

Another compound involved in the hypersensitive response is nitric oxide (NO). It acts as a signal mediator in bacterial pathogen defense responses, promoting programmed cell death (Wang et al. 2013). An important factor that positively regulates cassava disease resistance against *X. axonopodis* pv. *manihot* is transcription factor *MeRAV5,* which coordinates nitric oxide (NO) and hydrogen peroxide (H_2O_2) levels. It has been suggested that *MeRAV5* binds to the CAACA motif in promoters of the *MeNR1/2* genes, which encode nitrate reductases and therefore are responsible for NO accumulation. In addition, *MeRAV5* interacts with the *MeCAT1* gene, which directly catalyzes the decomposition of H_2O_2, inhibiting its activity, and consequently increasing endogenous H_2O_2 levels (Yan et al. 2020). CaRAV1, an RAV protein containing the AP2/ERF and B3-like DNA-binding domain, was also upregulated in both host and non-host resistant responses to *Xanthomonas axonopodis* pv. *glycines* (*Xag*) in chili pepper (Kim et al. 2005). Both ROS and NO accumulate simultaneously as a downstream response to pathogen perception, leading to programmed cell death via the hypersensitive response in an attempt to fend off the pathogen.

2.3.4.3. Hormone signaling and transcription factors

Plant hormones are essential regulators of plant immunity, acting as signaling molecules to trigger extensive transcriptional reprogramming (Aerts et al. 2021). Defense mechanisms against *Xanthomonas* species usually require salycylic acid (SA) biosynthesis (Shigenaga et al. 2017), although jasmonic acid (JA) production has also been reported. For instance, in passion fruit (*Passiflora edulis*) jasmonate and a lipoxygenase, generally involved in the jasmonic acid synthesis pathway, were implicated in plant defense (Munhoz et al. 2015). In fact, SA pathway genes

were identified in a transcriptional analysis of kumquat plants (Fortunella spp) in response to *X. citri* subsp. *citri* infection (Giraldo-Gonzáles et al. 2021). Similarly, a comparative analysis of two common bean (*Phaseolus vulgaris L.*) genotypes, BAT93 and JaloEEP558, respectively resistant and susceptible to *X. axonopodis* pv. *phaseoli*, showed that the resistance mechanism was linked to upregulation of SA pathway genes and downregulation of photosynthesis and sugar metabolism genes, whereas susceptibility was related to the upregulation of ethylene pathway genes (Foucher et al. 2020).

SA can be synthesized via two pathways: the phenylpropanoid biosynthesis pathway, where phenylalanine ammonia-lyase (PAL) is responsible for converting Phe to trans-cinnamic acid, which acts as a precursor of SA (Zhang and Li et al. 2019), and the isochorismate pathway, on which isochorismate synthase (ICS) is activated by proteins such as CBP60g and SARD1 and controls SA biosynthesis (Wang et al. 2011). In a *Prunus persica* resistant cultivar, the expression of transcription factor WRKY28 was upregulated in the presence of *X. arboricola* pv. *pruni* infection. It activates isochorismate synthase to promote SA biosynthesis (Gervasi et al. 2018).

SA can undergo several modifications in the plant, most of which are aimed at rendering it inactive. One of these modifications is methylation of SA to form methyl salicylate (MeSA), which renders the SA more volatile (Lefevere et al. 2020). In sweet orange, CsMES1 catalyzes the hydrolysis of MeSA in SA, which is necessary for localized defense against *X. citri* subsp. *citri* (Lima Silva et al. 2019).

SA is detected by Nonexpressor of Pathogenesis-Related (NPR) receptor proteins, such as NPR1 and NPR4, which are reported to play a part in positive feedback amplification of SA biosynthesis and regulation homeostasis (Liu Y et al. 2020). NPR1 genes were upregulated in tomato (Liu et al. 2021) and pepper (Dutta et al. 2017) after infection by *X. euvesicatoria* pv. *perforans* and in the Brassica napus – *X. campestris* pv. *campestris* pathosystem (Islam et al. 2017). A novel NPR1 homolog regulated by SA has been identified in rice, and contains a genetic variation of single nucleotide polymorphisms compared to previous NPR1 homolog OsNH5 (Bai et al. 2011). This is the result of a single amino acid substitution of asparagine for serine at residue 16, and its overexpression is related to improved resistance against *X. oryzae* pv. *oryzae* by increasing PR gene expression (Son et al. 2021).

Although SA is the main hormone found in the response to *Xanthomonas* species, there are reports of other hormones upregulated under bacterial infection. Hormone metabolic pathways such as those of abscisic acid (callose deposition), auxin and jasmonate metabolism, and ethylene synthesis, were activated in banana resistant genotype *Musa balbisiana* infected by *X. campestris* pv. *musacearum* (Tripathi et al. 2019). The overexpression of MeAux/IAAs (Auxin/indole-3-acetic acid proteins), which play crucial roles in auxin signaling pathways, confers enhanced disease resistance in cassava in the presence of *X. axonopodis* pv. *manihot*. MeAux/IAAs regulate the transcript levels of pathogenesis-related genes (PR), ROS accumulation and callose deposition, and therefore could influence plant disease resistance (Fan et al. 2020).

Several families of transcription factor (TF) play important roles in the regulation of gene expression in plants, including genes related to plant immunity. One important family, basic helix–loop–helix (bHLH) transcription factors, are known to play a part in regulating several physiological, metabolic and developmental processes in eucaryotic organisms (Feller et al. 2021). Two bHLH transcription factors, OsbHLH034 (Onohata and Gomi 2020) and RERJ1 (Valea et al. 2021) have been identified in rice and found to regulate jasmonic acid signaling, boosting resistance to *X. oraze* pv. *oryzae*. Four bHLH genes (SsbHLH03/04/36/37) were also found to be upregulated in a cultivar of *Saccharum spontaneum* resistant to leaf scald after *Xanthomonas albilineans* infection, suggesting that these factors play a part in defense against these pathogens (Ali et al. 2021). Similarly, it was reported that putative bHLH transcription factor bHLH132 is required for resistance to *X. euvisicatoria* pv. *perforans* in tomato.

SUMO protease activity (non-TAL effector XopD*)* is required to activate it (Kim and Mudgett 2019). In particular, crosstalk between jasmonic acid and phosphate (Pi) starvation signaling, involving a bHLH transcription factor, has been reported to mediate resistance to *X. oryzae* pv. *oryzae* in rice. OsPHR2, a PHRs family gene known to control Pi deficiency by binding to P1BS (*cis*-element *PHR1* binding sequence) elements, binds to the promoter region of OsMYC2, a bHLH transcription factor regulating jasmonic acid signaling and consequently boosting defense against to *Xoo* in rice (Kong et al. 2021).

Proteins belonging to WRKY family are another TF family reported in rice and associated with the defense response against *Xanthomonas species*. In pepper, WRKY transcription factors are upregulated in defense against *X. campestris* pv. *vesicatoria* (Gao et al. 2021). OsWRKY114 (Son et al. 2020) and OsWRKY10 activate *OsPR1a* by binding to its promoter and by indirect activation through OsWRKY47. A second pathway has also been reported, in which OsWRKY47 acts downstream of OsWRKY10, and OsWRKY88 upstream (Choi et al. 2020). Similarly, OsWRKY51 binds to the W-box and WLE1 elements of the *OsPR10a* promoter, acting as a positive defense signaling transcription regulator (Hwang et al. 2016).

Transcription factors belonging to the basic domain-leucine zipper (bZIP) family have been reported to contribute to defense against *X. axonopodis* pv. *manihot* in cassava. MebZIP3 and MebZIP5 are regulated by the levels of H_2O_2 and SA and by MAMP *flg22*. Overexpression of these transcription factors conferred improved disease resistance against cassava bacterial blight by boosting callose deposition (Li et al. 2017). OsbZIP75 activates the *OsPR10a* gene and positively regulates defense against *X. oryzae* pv. *oryzae* in rice (Im et al. 2019). In addition, transcription factors such as MADS-box, the C2H2 zinc finger family, WRKY, AP2/EREBP, HSFC1, GRAS, ARF, C2C2, bHLH and homeobox families were upregulated in *Musa balbisiana*, indicating that they play a role in a cascade of defense genes (Tripathi et al. 2019).

2.3.5. Role of non-coding RNAs in plant immunity to *Xanthomonas* species

Recent advances in high-throughput gene sequencing technology, in addition to transcriptomic analysis, have revealed that plant immunity does not depend solely on protein-coding genes, but also on noncoding RNAs (ncRNAs) (functional RNA molecules that do not encode proteins but participate in the production of messenger RNA). They have been found to be key elements in regulating gene expression, epigenetic modification, and defense against exogen nucleic acids and transposable elements for maintaining genome integrity. NcRNAs can be classified based on length: small ncRNAs comprise around 18-30 nucleotides and long ncRNAs over 200 nucleotides (Song et al. 2021).

MicroRNAs are single stranded, polyadenylated RNA molecules, transcribed by Pol II, that usually contain 20-22 nucleotides folded into hairpin-like structures (Borges and Martienssen 2015). In A. thaliana, it has been found that when flg-22 is recognized, miR393 expression increases and transport inhibitor response 1 (TIR1) expression decreases, once it is specifically cleaved by miR393, which represses auxin signaling, boosting SA levels and plant resistance (Navarro et al. 2006). MiR393 and other miRNA families were found to be upregulated in cassava (*Manihot esculenta*) tissues infected with *X. axonopodis* pv. *manihot* (Pérez-Quintero et al. 2012).

Other microRNAs are reported to be involved in plant immunity, but the network signaling involved in this process remains elusive. The miR156, miR167, miR169 and miR390 have been shown to improve resistance in cabbage (Brassica oleracea var. capitata) to *X. campestris* pv. *campestris* (Santos et al. 2019). In citrus, miR159, miR167 and miR398 were found to be involved in combating infection by *X. citri* subsp. *citri* and *X. fuscans* subsp. *aurantifolii*.

(Alizadeh et al. 2017). Resistant rice genotypes showed higher constitutive levels of OsmiR1861k than genotypes susceptible to *Xoo* (Zhang S et al. 2015). Seventeen miRNAs were responsive to *X. oryzae* pv. *oryzae* infection in a resistant rice genotype, and function prediction revealed that they could be associated with plant hormone signal transduction, flavonoid synthesis and regulating reactive oxygen species (Lu et al. 2020).

The role of lncRNAs in plant immunity to *Xanthomonas* remains elusive. However, an RNA sequencing study of rice leaves infected by *X. oryzae* pv. *oryzae* identified 567 disease-responsive lncRNAs, highlighting *ALEX1*, which was found to be involved in the jasmonate pathway, suggesting involvement in plant defense mechanisms (Yu et al. 2020).

2.4 Conclusions and Perspectives

Over the past few years, as a result of the development of new approaches to genetics, genomics, bioinformatics, biochemistry and cell biology, many advances have been made in our understanding of plant immune activation by *Xanthomonas* species. Studies on PRR and NLR networks have highlighted the complexity of pathogen recognition, which involves integrating multiple signals simultaneously (e.g. phytohormones as signaling response amplifier molecules, a transcription factor that is involved in regulating gene expression, the influx of calcium, and reactive oxygen species that lead to a hypersensitive response). However, several questions remain to be answered as to how all these components are coordinated and activated during defense response against *Xanthomonas* and how this information can be used to combat this pathogen. Identifying key genes in this complex network of molecular plant defense responses poses a challenge and could provide valuable information on specific targets for use in disease control and plant breeding strategies.

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3 EXPRESSION PROFILING OF THE SWEET PASSION FRUIT (*Passiflora alata*) INOCULATED WITH *Xanthomonas axonopodis* pv. *passiflorae* REVEALS THE OVEREXPRESSION OF SUSCEPTIBILITY GENES

Abstract

The Brazilian production of the sweet passion fruit (*Passiflora alata*) has increased in recent years. The crop is appreciated for the typical aroma and characteristic flavor of its fruits, in addition to its traditional use in the pharmaceutical industry. However, crop production in tropical areas is constrained by diseases, which effects are exacerbated by high temperature and humidity. Among them, the bacterial spot caused by *Xanthomonas axonopodis* pv. *passiflorae* (*Xap*) can lead to severe losses in commercial orchards. Plants have different defense mechanisms to preventing pathogen proliferation, and a better understanding of this interaction is crucial for the development of disease-resistant cultivars. With this purpose in mind, we evaluated the transcriptome profiling of *P. alata* in response to *Xap* inoculation using the RNA-seq technology. For that, total RNA from mock and *Xap*-inoculated leaves was extracted 5 days after inoculation and 12 RNA-libraries were constructed sequenced using the Illumina NextSeq platform, resulting in about 50 million paired-end reads per sample. De novo transcriptome assembly, functional annotation, and expression profiling were carried out, which revealed 638 up-regulated and 604 down-regulated isoforms (*p*-value adjusted by FDR < 0.05 and a fold change ≥ 1.5 and ≤ 1.5). Pattern recognition receptors (PRRs) and resistance genes were detected which, after perceiving the pathogen, activated the plant's immune system. The first response is the rapid increase in calcium influx and the production of reactive oxygen species (ROS). Next, calcium-dependent kinases trigger a signaling cascade leading to the activation of pathogenesis-related genes, such as glucanases and chitinases, and the production of volatile compounds (the terpenes germacrene D and nerolidol) that act as signals for hormone production. Compounds of the salicilic acid pathway were identified. Importantly, two upregulated genes were detected in infected plants: the first is a sugar transporter, *SWEET10*, and the other is a member of the Lateral Organ Boundaries (LOB) domain transcription factor gene family. The activation of these genes suggests that they may be involved in plant susceptibility. For instance, *LBD20* is known to act as a repressor of a subset of jasmonate mediated defenses and in susceptibility to pathogens. Potentially, the silencing of these genes may result in tolerance or even resistance of sweet passion fruit to *Xap*, once two resistance genes containing CC/TIR-NBS-LRR domain were also identified in the host plant. Remarkable, we were able to validate certain gene's differential expression from RNA-seq data by RT-qPCR. Our study is the first report on the expression profiling of a cultivated species of *Passiflora* in response to *Xap* infection, providing a better understanding of this pathosystem. This was the first study that report the defense mechanism of *P.alata* against *Xap*.

Keywords: Sweet passion fruit, *Passiflora alata*, *Xanthomonas*, RNA-Seq, Plant susceptibility

3.1 Introduction

The genus *Xanthomonas* consists of some 30 plant-associated species, many of which cause important diseases of crops and ornamentals. The diseases, leaf spots and blights caused by this bacterium are ubiquitous worldwide, especially where conditions are warm and humid. Individual species (e.g., *X. axonopodis*) comprise multiple pathovars, characterized by distinctive host specificity or mode of infection. Most species and pathovars are widely distributed. Complete or draft genome sequences are available for several *Xanthomonas* strains, including vascular and nonvascular pathogens (See Nakayinga et al 2021).

Plant defense is conventionally described in two stages. Firstly, pattern recognition receptors (PRRs) recognize microbe associated molecular patterns (MAMPs), that are highly conserved regions. This recognition provokes a first defense response, called pathogen-triggered immunity – PTI. As counter-attack, such as the case of *Xanthomonas* spp., the pathogen injects effectors, for instance, transcription activator-like effectors (TALEs) and Xops (*Xanthomonas* outer proteins) into the host cells via type III secretion system (T3SS). These effectors increase plasticity in adaption of the bacteria to host plants. They have a rearrangeable repetitive domain that controls the ability to bind promoters of host susceptibility genes in a sequence specific manner (see Timilsina et al. 2020; Teper and Wang 2021). *Xanthomonas* spp. effectors can also activate resistance genes (R genes) and produce a second defense response called effector triggered immunity – ETI. The recognition of the pathogen by the host, either through PRRs or R genes, triggers a series of mechanisms to inhibiting the pathogen proliferation. Both responses are similar, however ETI is considered faster and more intense than PTI. Among these mechanisms, there is an increase in calcium influx, accumulation of reactive oxygen species (ROS), stimulation of a signaling cascade by MAP kinases or calcium-dependent sensor proteins, activation of transcription factors (TFs) that act in cellular reprogramming and production of hormones and antimicrobial compounds. Activation of these defense pathways leads to programmed cell death called hypersensitive response at the infection site (reviewed in Toruño et al. 2016; Timilsina et al. 2020).

Xanthomonas axonopodis pv. *passiflorae* (*Xap*) is the causal agent of bacterial spot disease in cultivated *Passiflora* species, popular known as passion fruits (Pereira 1969). *Xap* is one of the most important crop pathogens, decreasing the period of commercial exploitation. The bacterium can live both externally or internally in its host, penetrating into plant cells through natural openings, such as stomata, hydathodes, or by wounds. Once inside the plant, the bacterium can reach the vascular system and spread through all the plant tissues. In leaves, the pathogen induces chlorosis and necrosis, and lesions may coalesce causing leaf drop or even death in severe cases. Spots are also formed on fruits, making them undesirable for consumption (Ishida and Halfeld-Vieira 2009).

There is no effective control against the disease caused by *Xap*, although some induced-resistance has been reported (Boro et al. 2011). Preventive measures, such as the use of disease-free seeds and seed treatment with acibenzolar-S-methyl (ASM) or hairpin proteins, leaf treatment with ASM or glycoproteins from *Xanthomonas* spp. are inducers of passion fruit resistance against *Xap*, as well as the use of chemical compounds containing copper oxychloride. However, these approach are not completely effective and could be harmful to the environment.In this scenario, the development of disease-resistant cultivars is crucial (Fischer and Rezende 2008) and may include introduction from an outside source, selection, and generation of novel variation via gene editing. All three may be used at different stages in a continuous process.

Currently, molecular breeding techniques include identifying the molecular basis of defense mechanisms, aiming to recognize potential targets for the development of resistant cultivars. For instance, Munhoz et al. (2015) using a suppression subtractive hybridization constructed two cDNA libraries of *P. edulis* (the sour passion fruit) enriched for transcripts induced and repressed by *Xap*, respectively, 24 h post inoculation with a highly virulent strain. These authors reported that the expression profiles changed in response to the pathogen. In later stages of interaction (5- and 9-days post inoculation) when disease-associated symptoms were visible, qPCR analyses were performed for some genes selected from both libraries. The genes that responded most strongly to the pathogen attack encodes a lipoxygenase 2 and neomentol dehydrogenase. Moreover, they showed that most of the genes involved in well-known pathogen recognition signaling pathways were repressed by *Xap* and this lends support to the idea that the jasmonic acid signaling pathway fails to be activated during the first hours of interaction.

However, there were no studies focused on the molecular basis of the interaction between *P. alata* (the sweet passion fruit) and *Xap*. With this purpose in mind, we evaluated the transcriptome profiling of *P. alata* in response to *Xap* inoculation using the RNA-seq technology. Our results provide an overview of the pathways activated in the plant host under infection conditions. We have identified two susceptibility (S) genes that are upregulated in response to *Xap*. Typically, phytopathogens exploit plants' S genes to facilitate their proliferation, because of these genes have a considerable contribution to bacterial growth, cell-wall remodeling, and cellular reprogramming (Hu et al. 2014). We suggest that both S genes represent a potential target for gene editing techniques.

3.2 Materials and Methods

3.2.1.1 Bacterial inoculation and RNA isolation

Sweet passion fruit clones from the 'SV3' accession were kept under greenhouse conditions for 150 days at 29.09ºC ± 14.52. This accession is an indoor selection, and it was obtained from a commercial orchard located in the Southeast region of Brazil (22°17' S, 51°23' W). This is a vigorous accession that was used as a male parent of a segregating population on which promising genotypes were selected to improving fruit quality and yield (Pereira et al. 2013; Pereira et al. 2017; Chavarría-Perez et al. 2020). The Figure 1a shows the accession used and also the progression of lesions on the leaves at 20 and 30 days after inoculation, (Figure 4b and 4c), respectively.

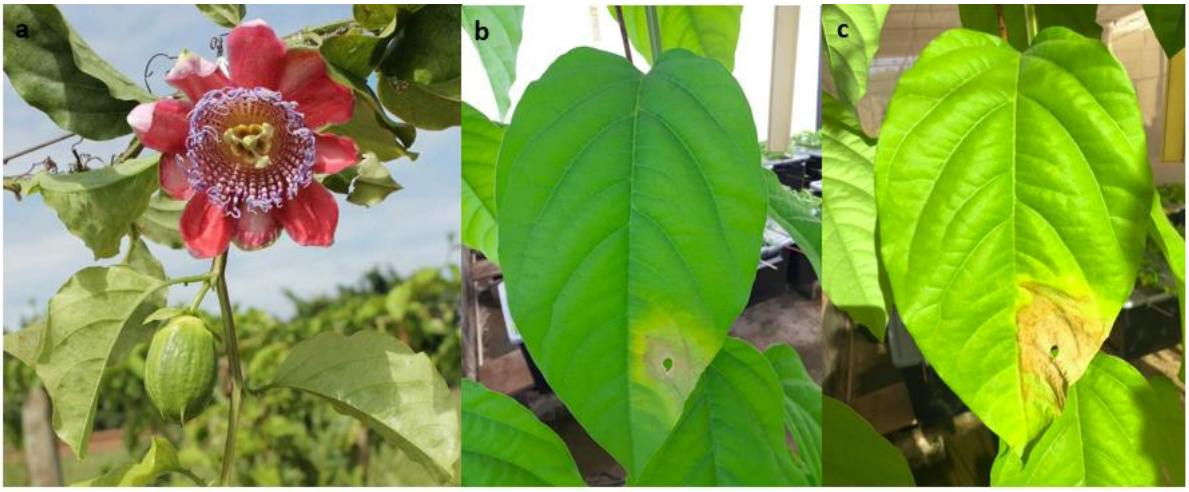

Figure 4. Passiflora alata. (a) A sweet passion fruit specimen under field conditions (22°17' S, 51°23' W). Photo credit: A. R. Benedetti (Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Brazil). Foliar lesions on sweet passion fruit leaves (b) 20 and (c) 30 days after inoculation with *Xanthomonas axonopodis* pv. *passiflorae*.

For leaf inoculation, a bacterial suspension at a concentration equivalent to 3 x 10^8 CFU/mL was made using a *Xap* virulent isolate (AP302) according to Munhoz et al. (2011). To prepare the inoculum, isolate AP302 was grown in liquid nutrient medium for 24 hours at 28ºC and 150 rpm. Then, 100 µL of the bacterial suspension was plated on nutrient agar medium and the plates were incubated for 24 hours at 28ºC. After the growth of the culture, 20 mL of saline solution (NaCl 0,85%) were added to suspend the colonies. The bacterial suspension was then

homogenized, diluted and standardized in a spectrophotometer for an optical density equal to 0.3 and a wavelength of 600nm, which is equivalent to a concentration of 10^8 CFU/mL.

A total of 6 plants were used in this experiment, 3 replicates were inoculated with *Xap* and in the other 3 replicates a 0,85% NaCl solution was used as a control (Figure 5). Holes were made in three leaves of each sweet passion fruit plant, using a paper punch. A cotton swab immersed in the respective solutions was passed through the orifices generated in order to have direct contact with the interior of the leaf tissue. The average temperature during the test was equal to 30.75ºC ± 13.7.

The virulence of this isolate was previously evaluated from a collection of ~90 *Xantomonas axonopodis* pv. *passiflorae* isolates maintained in our laboratory as described in Braga (2011). The leaves were collected five days after inoculation as in Munhoz et al. (2015) study. A small area (4 cm^2) around the wound was cut and immediately frozen in liquid nitrogen and then stored at −80°C for further extraction of total RNA. Total RNA was isolated from leaf samples using the TRIzol reagent (Invitrogen, USA) following to the manufacturer's protocol. The concentration of total RNA was determined by Qubit™ RNA BR Assay (Thermo Fisher Scientific, USA), and the RNA integrity value (RIN) was checked using RNA 6000 Pico LabChip of Agilent 2100 Bioanalyzer (Agilent, USA).

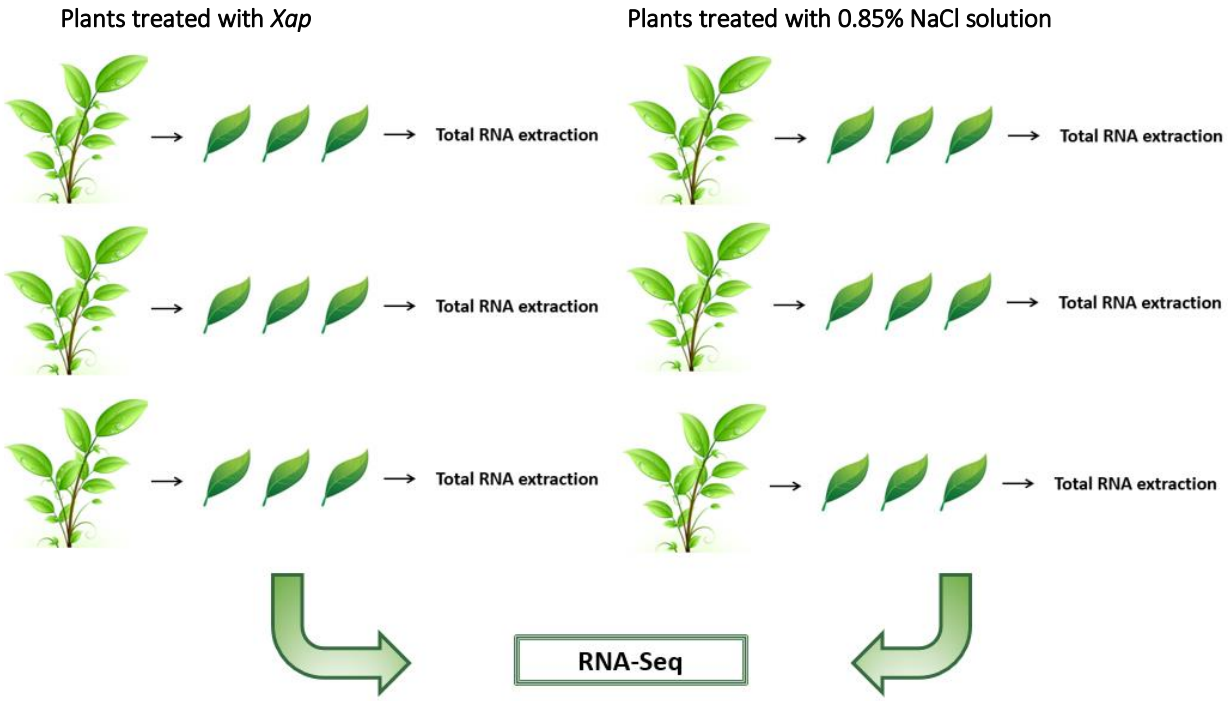

Figure 5. Schematic representation of the experiment in which leaf samples of *Passiflora alata* were inoculated with *Xanthomonas axonopodis* pv. *passiflorae*. Control samples were inoculated with a 0.85% NaCl solution. Total RNA was extracted 5 days after inoculation and sequenced by NextSeq 2000 (Illumina) plataform.

3.2.1.2 Library preparation and RNA Sequencing

Six sequencing libraries were generated using TruSeq Stranded mRNA Library Prep Kit (Illumina, USA), according to the manufacturer's instructions. Then, the libraries were sequenced with an Illumina NextSeq 2000 platform using the paired end strategy (2x100 pb), generating 50 million of reads.

3.2.1.3 Bioinformatic analysis

First, quality control of the reads was performed using the FastQC computational program (Andrews 2010), which is also repeated after the processing step. Then, sequence readings were submitted to Atropos v.1.1.24 (Didion et al. 2017) in order to remove remnants of adapter sequences from both ends of the reads.

Low-quality sequences (average Q-score less than 20), poly-A tail remnants (minimum of 5 consecutive bases at the 5' end), low-complexity readings (DUST score above 30) and those that do not have a minimum length of 20 bases were all eliminated by the PRINSEQ program (Schmieder and Edwards 2011).

Once the reads were checked for quality and processed, as the genome of *P. alata* is not yet available, a *de novo* assembly was performed using the default parameters of Trinity v2.11.0 (Grabherr et al. 2011). To evaluate the completeness and accuracy of the *de novo* assemblies, BUSCO v5.4.3 (Benchmarking Universal Single-Copy Orthologs) using embryophytes database (Manni et al. 2021) and TransRate v1.0.3 (Smith-Unna et al. 2016) analysis, respectively, were performed using the default mode. Next, protein-coding regions were predicted from the transcriptome reference assembly using TransDecoder v5.5.0 (Haas et al. 2013), which were then submitted to a further functional annotation based on pre-computed ortholog assignments from eggNOG database using the EggNOG-mapper v2 (Huerta-Cepas et al. 2017) tool.

We assessed changes in gene expression between uninoculated- (controls) and inoculated leaves using DESeq2 v1.37.6 (Love et al. 2014). The first step in the differential expression (DE) analysis workflow is read-count normalization, which is necessary to make accurate comparisons of gene expression between samples. First, for each gene, DESeq2 uses geometric mean across all samples. For every gene in a sample, the ratios (sample/geometric mean) are calculated. The median value of all ratios for a given sample is taken as the normalization factor for that sample. Then, the method calculates the normalized count values using the normalization factor. This is performed by dividing each raw count value in a given sample by that sample's normalization factor to generate normalized count values. Finally, the expression fold change for the genes is calculated. The isoforms with false discovery rate (FDR) values <0.05 and a fold change ≥ 1.5 and ≤ -1.5 were used as the criteria for identifying differentially expressed transcripts.

In order to identify which functions and pathways of transcripts are most represented, GO functional enrichment analysis was carried out by GOATOOLs software (Klopfenstein et al. 2018), considering FDR ≤ 0.05 to obtain the GO annotations on the categories "biological process" (BP), "molecular function" (MF) and "cellular component" (CC).

3.2.1.4 RT-qPCR validation

Some of the transcripts' expression reported in the present study was validated through RT-qPCR, quantitative real-time PCR. We selected six candidate genes on the basis of their biological function. For that, total RNA was extracted as above described that was quantified using Qubit™ RNA BR Assay (Thermo Fisher Scientific, USA) and 1 μg of RNA was used for cDNA synthesis using GoScript Reverse Transcription System (Promega, USA).

Gene-specific primers were designed using Primer3web version 4.1.0 (Table 2). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *Actin* were used as internal references. These two housekeeping genes were selected based on a study that demonstrated that these genes remain stable in different tissues and conditions and also based on the analysis of the generated transcriptome, where these two genes were shown to be stable in both conditions.

Amplifications were carried out in a 48-well reaction plate using the Step

One Plus PCR system (Applied Biosystem, CA, USA) and the Power up SYBR™ Green PCR Master Mix (Applied Biosystems, CA, USA). PCR cycling started by one denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, plus a melting curve. This melting curve analysis was used to verify the specificity of the amplification product and the possible presence of primer dimers by their unique melting peak behavior.

The amplification efficiency of each pair of primers was determined by the generation of a standard curve of 5 points, based on serial dilutions of cDNA samples. The efficiency was calculated from the slope of the standard curve generated based on the following equation E=10(–1/slope) and it was considered an acceptable amplification efficiency between 90 and 110% (Rutledge and Côté 2003). The relative expression levels of the selected genes by qRT-PCR were calculated using the 2-ΔΔCT method (Livak and Schmittgen 2001). qRT-PCR was performed using 3 biological replicates. Each of these biological replicates was run in 3 technical replicates.

Table 2. List of primers used for RNA-Seq validation.

Gene	Primer sequence (Forward 5' → 3')	Primer sequence (Reverse 3' → 5')
SWEET10	AGGACCCTAGCTTTCTTTGGTG	GGTTCCAGTCAGTTCCATACGT
LOB1	CTTTGGACGTGATTGAGTGCTG	AGGTTTCGTCTCGTCAACATGA
Germacrene D	CTAGCCTTGGGAAAGTTGGACT	CAAAGTACACTCCCACCATCCA
Nerolidol	GAATGGGGTGGTTAGTTCTGGT	TACCCATCCTGTTCCTCATCCT
CC-NBS-LRR	TGCGTCTGGAAAGCTACTGATT	AGCGCCATCAAAACCAGTACTA
TIR-NBS-LRR	TGAAAAACTGCCGTAGACTGAA	AAGCGGTGACCAGTACTTGAAT
Actin	TGTTATGGTAGGGATGGGTC	TTCTCTCTATTTGCCTTGGG
GAPDH	TGGCAGTGATGGAATGGACT	CCGATGTTTGTTGTGGGTGT

3.2.1.5 Expression profile of selected genes at 7 days after inoculation (DAI)

The expression of six selected genes was also evaluated at 7 DAI, in order to verify their expression profile two days later, due to the beginning of the appearance of leaf chlorosis, the main symptom of the disease. For

this, plant leaves were inoculated following the same protocol used for RNA extraction for sequencing, and the expression was evaluated following the protocol described above.

3.3 Results

3.3.1.1 Sequencing and *de novo* assembly

Approximately 300 million Illumina raw reads were generated from 6 RNA libraries, an average of 50 million (2x 100 bp) raw reads per library. After processing, a total of 323.737.390 clean reads, comprising 111 Gb of clean data, were retained. Quality checking with FASTQC indicated that the sequence data were of high quality, once a quality score above Q30 was delivered, meaning that 100% of the reads were perfect, with no errors or ambiguities.

Therefore, we proceeded with the next steps. The reads were *de novo* assembled into 132.698 contigs with an average length of 1.493 bp, and an N50 contig length of 2.498 bp, as measured by Transrate (Table 3). 95% of the fragments were mapped and only 0.04% were uncovered. The assembly presented a score equal to 0.42. This score, ranges between 0 and 1.0, and it is calculated using the geometric mean of all contig scores multiplied by the proportion of input reads that support the assembly (Smith-Unna et al. 2016).

Table 3. Quality of the assembly of *Passiflora alata* transcriptome without using a reference according to Transrate scores

Nº sequences (contigs)	132,698
smallest contig (bp)	194
Largest contig (bp)	22344
nº bases	198,217,177
mean length (bp)	1,493.74
n50	2,498
gc content	0.42
Nº Fragments	172,501,242
Nº Fragments mapped	163,069,356
% Fragments mapped	0.95
Good mappings	139,689,627
% Good mappings	0.81
Bad mappings	23,379,729
Nº Bases uncovered	18,434,500
% Bases uncovered	0.09
Nº Contigs uncovered	4,755
% Contigs uncovered	0.04
Contigs segmented	12,252
% Contigs segmented	0.09
Transrate assembly score	0.4187
Nº Good contigs	128,711
% Good contigs	0.97

Importantly, the evaluation of the quality of genomic "data products" such as genome assemblies or gene sets is critical in order to recognize possible issues and correct them during the generation of new data. BUSCO assessment of the quality of *P. alata* transcriptome assemblies revealed high completeness, in terms of expected gene content for a *de novo* transcriptome assembly, of them 1.329 were completed genes, 477 single copy genes and 852 are found to be duplicated. In other words, 96.65% of the conserved orthologs conserved across embryophytes were represented in our assembled transcriptome, that showed low fragmentation (2.32%), and low missing sequences (1.02%) (Figure 6).

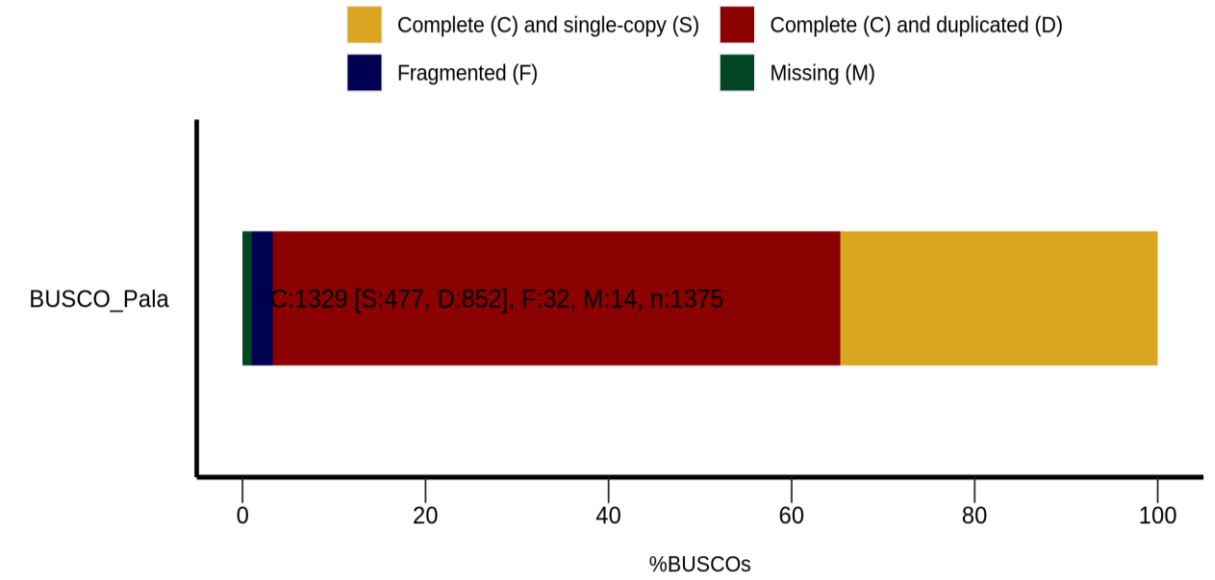

Figure 6. BUSCO assessment results. The assembly has 96.6% completeness and only 2.32% and 1.02% of fragmented and missing genes, respectively.

3.3.1.2 Differential gene expression analysis

A comparative analysis of the sweet passion fruit leaves transcript profiling in response to *Xap* inoculation was performed. A total of 1.242 transcripts were identified to be differentially expressed, considering an FDR-adjusted *p*-value < 0.05 and a fold change ≥ 1.5 and ≤ -1.5. Among them, 638 were upregulated and 604 were downregulated in the inoculated group compared with the control group as represented in the volcano plot below (Figure 7). The top 100 transcripts up and downregulated are represented in the tables 4 e 5 in appendix section. This plot shows significant genes (*p*-value) versus magnitude (fold change). It enables visual identification of genes with larger fold changes that are also statistically significant. The most upregulated and downregulated genes were plotted on the right (in red) and on the left (in blue), respectively. The most statistically significant genes are shown on the top of the graph.

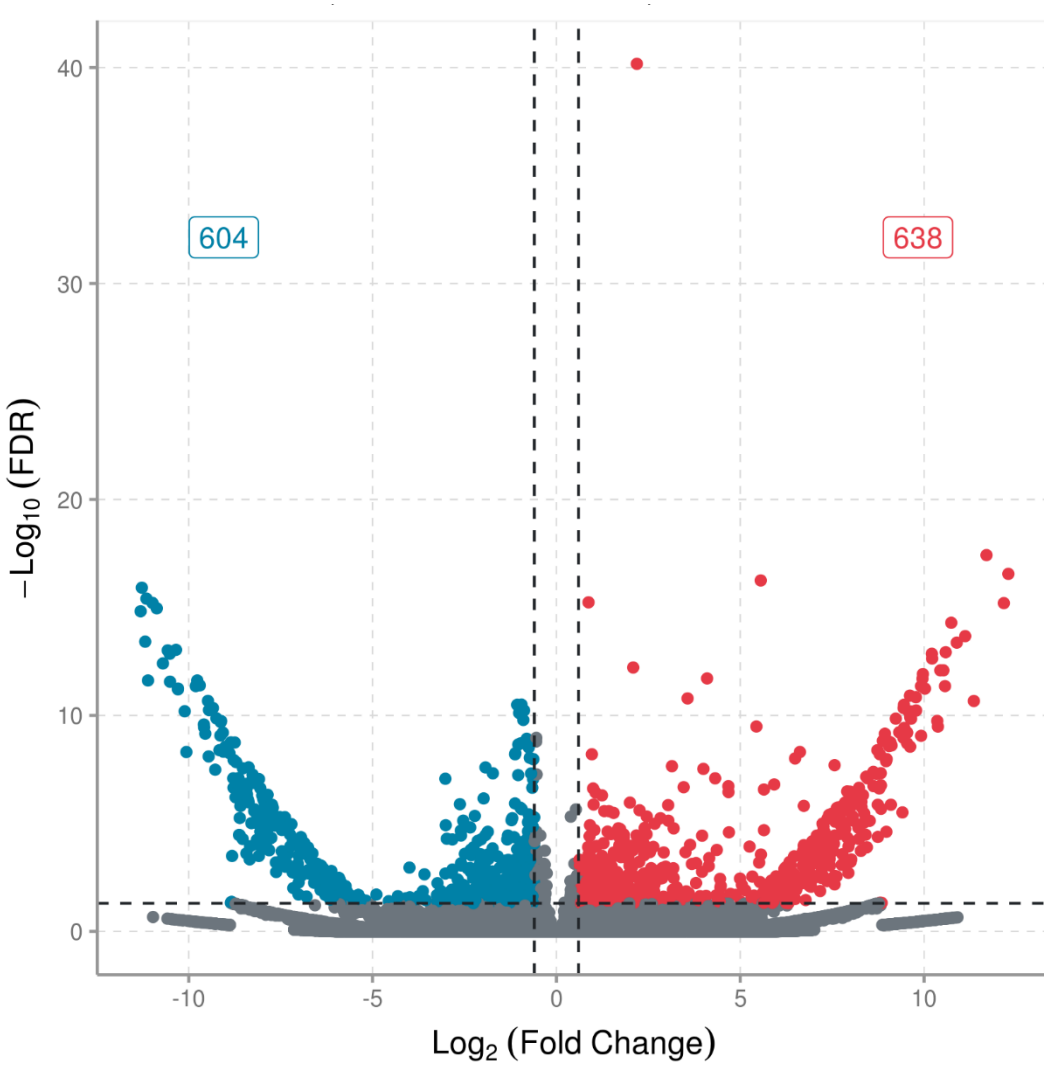

Figure 7. Volcano plot of transcript isoforms expression in *Passiflora alata* leaves. Blue and red points represent the downregulated and upregulated transcripts, respectively, in the inoculated group compared with the uninoculated (control) group. Grey points indicate the isoforms with no difference in expression between the two groups.

3.3.1.3 Coding regions' identification and functional enrichment analyses

De novo assembly approach refers to reconstructing a novel transcriptome of organisms where there is no reference sequence available for alignment. The transcripts newly assembled generally encode proteins to which it is not always possible to detect homology to known proteins. Therefore, in order to capture sequence-coding regions, we used the TransDecoder tool that predicts coding regions based on metrics tied to sequence composition (Haas et al. 2013). We first selected the best single open reading frame (ORF) per transcript. The total number of protein coding transcripts among the final non-redundant transcripts was 72.363. In the *P. alata* transcriptome assembly, 75% of the coding sequences were complete, approximately 7% were 5' partial, 12% were 3' partial, and 6% were internal regions (Figure 8).

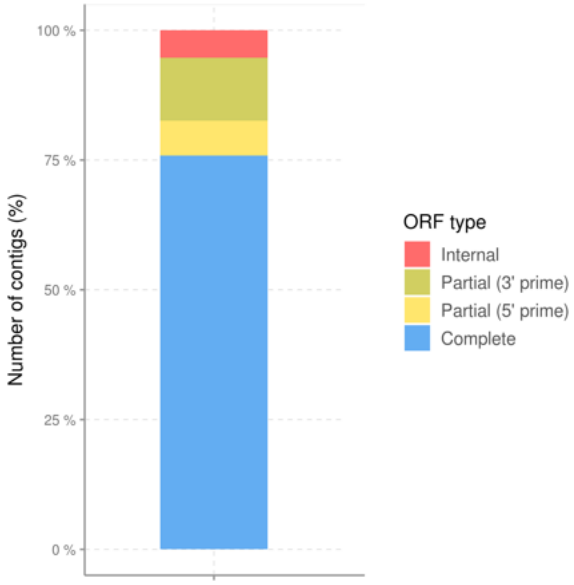

Figure 8. Percentage of transcripts identified as open reading frames in *Passiflora alata* transcriptome assembly using the TransDecoder tool. Shown in blue are the "complete ORFs", which are sequences in which the first and the stop codon were found. In yellow, are the "partial 5' prime", which refers to sequences that contain the start codon but lack the stop codon; in green, are the "partial 3' primer", which lacks the start codon, and in red, "internal ORFs that refers to sequences that lack both the codons.

Orthology assignment is suited for functional inference. Then, protein-coding transcripts were annotated trough eggNOG mapper, a tool for annotation of large sets of sequences using pre-computed clusters from the eggNOG database (Huerta-Cepas et al. 2017).

In addition, an enrichment analysis was performed on the basis of the Gene Ontology (GO) terms. The transcripts differentially expressed between the inoculated and non-inoculated groups were assigned to three categories: Cellular component, molecular function, and biological process. For that, we extracted the top 10 GO terms of each category that were up- (Figure 9a) and downregulated (Figure 9b).

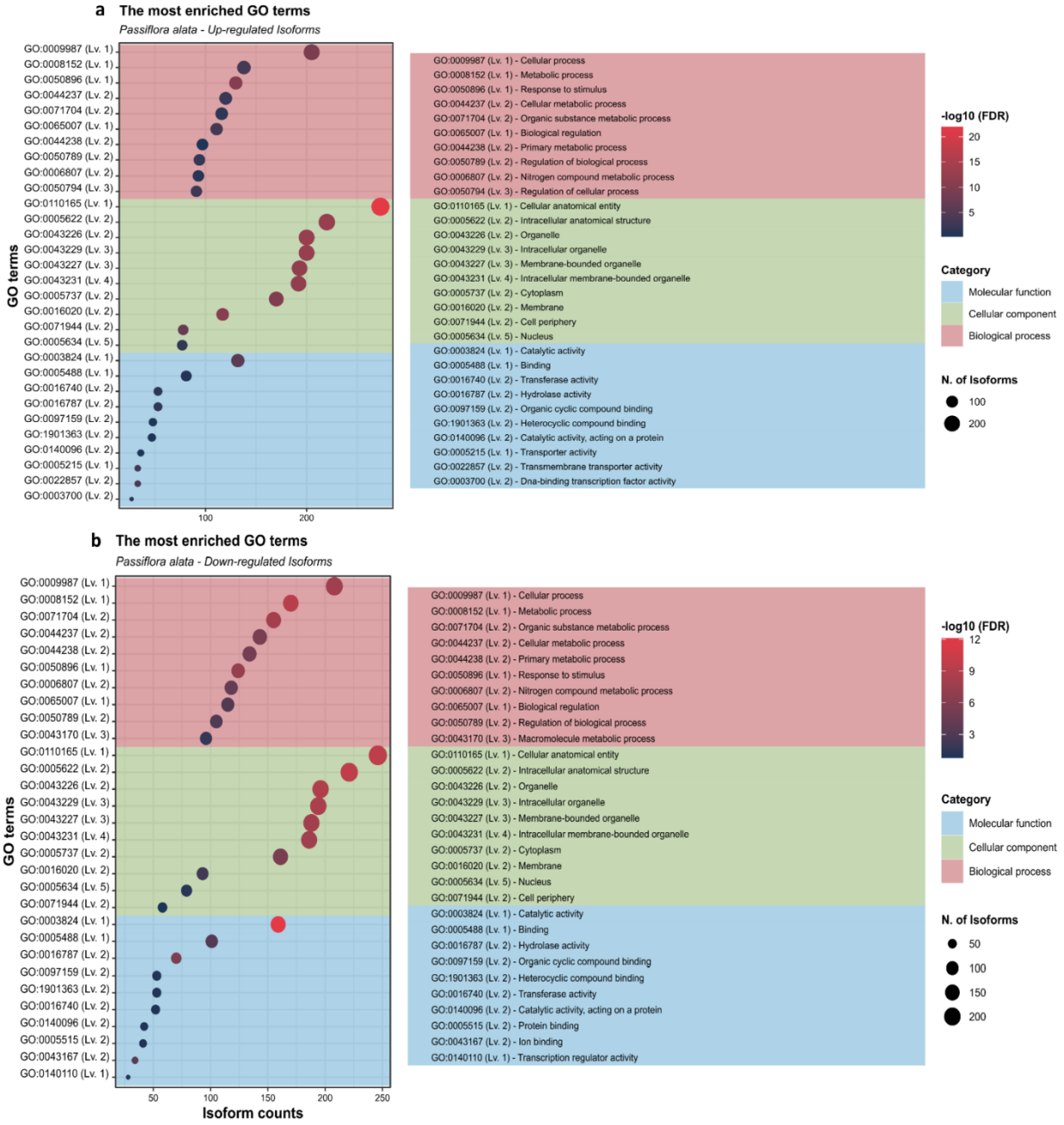

Figure 9. Bubble plot showing the enriched pathways of the top 10 GO terms. (a) The up- and (b) downregulated transcripts were classified into the three categories shown in pink (Molecular function), green (Cellular component) and blue (Biological process) rectangles. Only significantly enriched terms with p values < 0.05 were specified. The colors of each bubble represent the values of $-\log_{10}$ (FDR). The higher the $-\log_{10}$ (FDR) value, the higher the statistical significance. In a given pathway, bubble sizes are proportional to the number of differentially expressed transcripts.

3.3.1.4 Expression profiling in response to *Xap* infection

In an attempt to survive to *Xap* infection and stop bacteria propagation, the sweet passion fruit switches on several defense mechanisms. The expression analysis revealed transcripts from genes that were activated or repressed after the inoculation (Table 4), and a model of *P. alata* defense response to *Xap* infection, using heatmaps and literature information is proposed (Figure 10). According to Wang and Chai (2020), first, the host recognizes pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), through PRRs. Herein, 7 PRRs were identified

to be upregulated, all belonging to the large family of receptor-like kinases (RLK). They have a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain. Among them, 3 have a leucine-rich repeat (LRR) as a ligand-binding ectodomain that identifies plant- and pathogen-derived peptide epitopes, 3 have a G-Lectin ectodomain, which recognizes lipopolysaccharide (LPS), and one has a lysin ectodomain that acts in the recognition of pathogenic peptidoglycan (PGN). BAK1 (brassinosteroid insensitive 1- associated kinase 1), a kinase receptor is also activated, and it is generally recruited to form a major phosphorylation complex between the kinase domains of the RLKs to trigger a signaling cascade.

Once *Xap* has been recognized by these receptors, a defense response called "Plant triggered immunity" (PTI) is triggered leading to an increase in calcium influx and accumulation of ROS by the over-expression of the gene RBOHD (respiratory burst oxidase homolog protein D), which is a calcium-dependent NADPH - nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Kadota et al. 2014). Then, a signaling cascade was activated, such as hormone signaling, resulting in the activation of transcription factors that regulated de expression of target genes (Zhou and Zhang 2020). These responses were evidenced by our data.

Nine and 12 genes related, respectively, to hormone signaling and transcription factors (TFs) were identified. Genes involved in the hormonal pathway of salicylic acid, ethylene, and auxin were upregulated at 5 DAI. Among the TFs, the WRKY family was the most abundant. These TFs regulate the expression of other genes by binding to their promoter region. Three pathogenesis-related (PR) genes, two antimicrobial compounds, and two cell wall genes were also upregulated after *Xap* infection.

PTI was not enough to suppress the *Xap* attack, which secreted effectors that can be recognized by intracellular receptors (nucleotide-binding domain receptors and leucine-rich repeat-containing receptors (NLR), also called resistance (R) genes (see van Wersch S et al. 2019), triggering a second layer of plant protection, known as effector-triggered immunity (ETI). This response is similar to PTI, however faster and much more intense. Two R genes were identified, one with a TIR (Toll interleukin receptor) domain and other with a CC (coiled-coil) domain. Furthermore, *Xanthomonas spp.* effectors can bind to S genes, impairing host resistance (Teper et al. 2020). Two S genes were also shown to be over-expressed after infection: SWEET10 and LOB1, suggesting that these genes could be leading to host susceptibility.

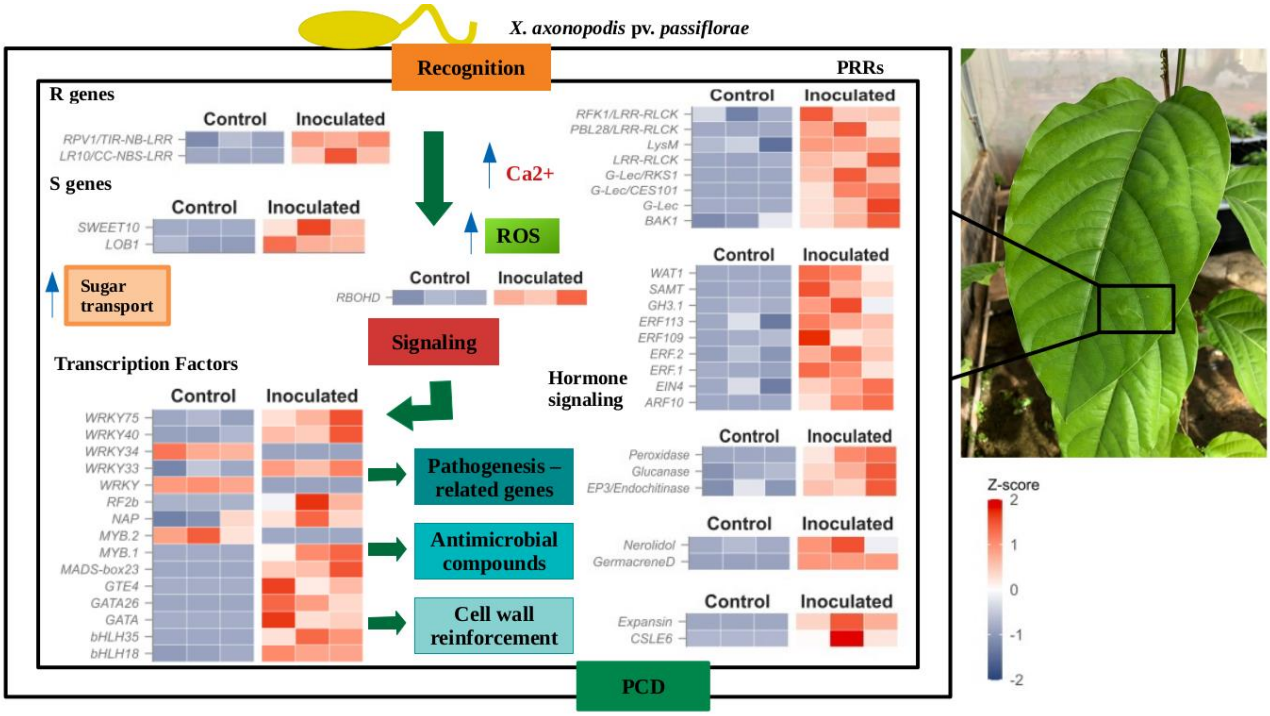

Figure 10. Expression profile of *Passiflora alata* in response to *Xap*. Once the pathogen was recognized by PRRs or R genes, there is an increase in calcium influx and ROS accumulation, triggering a signaling cascade, leading to the activation of hormone-related genes and transcription factors that act regulating genes related to pathogenesis, antimicrobial compounds, and cell wall expansion. Moreover, two S genes were upregulated after infection, suggesting that bacterial effectors activate them, leading to host susceptibility.

Table 4. Transcripts from genes that were activated or repressed after the inoculation of *Passiflora alata* leaves with *Xanthomonas axonopodis* pv. *passiflorae*).

Symbol	Description	LogFC	p-value
BAk1	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1-like	1.19995372377009	0.000423430476913283
lysM-RLK	lysM domain receptor-like kinase 3	0.839374533524309	0.000273669992871701
G-Lec	G-type lectin S-receptor-like serine/threonine-protein kinase	7.12290804534244	2.38923365929004e-07
G-Lec/CES1	G-type lectin S-receptor-like serine/threonine-protein kinase CES101	6.9453742200366	1.26302507796739e-05
G-Lec/RKS1	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1	6.38212426498823	4.09592564723631e-05
LRR/RFK1	LRR receptor-like serine/threonine-protein kinase RFK1	0.6521683546703	0.00042586001209681
LRR/RLK	LRR receptor-like serine/threonine-protein kinase	2.83055636018882	1.07977574537314e-08
LRR/RLK	LRR receptor-like serine/threonine-protein kinase	-6.20967814756505	1.78551079307389e-05
LRR/RLK	LRR receptor-like serine/threonine-protein kinase	-0.62107822176358	0.000502531925238616

PBL28-LRR	LRR receptor-like serine/threonine-protein kinase PBL28	7.58202619389946	1.64996445522062e-08
TIR-NB-LRR	R gene	1.02740967492438	0.027863378153449
CC-NBS-LRR	R gene	5.43308889724399	0.02558048693673
SWEET10	Susceptibility gene	8.03551945164662	1.46137767410355e-06
LOB1	Susceptibility gene	1.59357620897032	0.00270069072529042
RBOHD	NADPH adrenodoxin oxidoreductase	0.828425088653322	2.6120820995625e-05
EP3/Endochitinase	Endochitinase	1.68730735243121	0.0003261682945954
Peroxidase	Belongs to the peroxidase family	5.96975251917162	2.93972178643861e-0
Glucanase	1,4-beta-D-glucanase-like	2.06486136347498	4.04809404242022e-06
GermacreneD	(-)-germacrene D synthase-like	11.6971145381479	5.88742139225596e-23
Nerolidol	(3S,6E)-nerolidol synthase	4.87296433161147	0.00015575810686717
Expansin	expansin-like B1	8.2297457242818	2.6634995222482e-10
CSLE6	cellulose synthase-like protein E6	12.1684983977643	4.42486941374679e-20
SAMT	Salicylate carboxymethyltransferase	5.04249148603076	4.16345324023472e-05
EIN4	Protein EIN4	0.923502980388925	0.000453212192649248
ERF113	Ethylene-responsive transcription factor ERF113	0.83251159914744	0.000263887232933124
ERF109	Ethylene-responsive transcription factor ERF109-like	3.90707650523884	0.000186100491803284
ERF	Ethylene-responsive transcription factor	3.56749368017761	5.20498460921529e-15
ERF	Ethylene-responsive transcription factor	1.81811834756859	0.000114971935429913
ARF10	Auxin response factors (ARFs)	7.21129646559514	2.79134770694059e-06
GH3.1	Indole-3-acetic acid-amido synthetase GH3.1	4.15604106975368	1.43991432886329e-06
WRKY40	WRKY40 Transcription Factor	4.45834755558982	5.96381881202165e-05
WRKY75	WRKY75 Transcription Factor	1.94290496560596	1.21396391516257e-05
WRKY33	WRKY33 Transcription Factor	0.898043991622992	1.86739033510323e-06
WRKY34	WRKY34 Transcription Factor	-6.06461381583862	0.000187259690617813
WRKY	WRKY Transcription Factor	-8.8528197504692	0.000491720587509096
WRKY	WRKY Transcription Factor	-8.63172863690538	8.19943668118033e-08
bHLH35	Transcription factor bHLH35	10.0226002350337	1.65022140641892e-15
bHLH18	Transcription factor bHLH18	4.6927641654451	5.98313525333968e-08
MYB	MYB family transcription factor APL	6.83741771221823	1.32165233226577e-06
MYB	MYB family transcription factor	-6.50387926238602	2.93256272995849e-06
RF2b	transcription factor RF2b-like	6.86358173718873	4.73408673179809e-06
MADS-box23	MADS-box transcription factor 23-like	10.5877166438556	1.65692964371977e-17
NAP	NAC transcription factor	3.79916741764454	2.69404498381501e-06
GATA	GATA transcription factor	2.43745547948583	9.60848479673283e-06
GATA	GATA transcription factor	8.33678597808368	6.41672338103898e-10
GATA26	GATA transcription factor 26-like	8.05738924378241	6.16142095504508e-10

GTE4	Transcription factor GTE4	11.1175265989332	2.1610198141825e-18

3.3.1.5 Validation of RNA-Seq results by qPCR

Two susceptibility genes, two terpenes, and two resistance genes were selected for RNA-Seq validation. In order to perform the gene expression analysis through RT-qPCR, it is required correct data normalization by reference genes with stable expression. For this, we used GAPDH and Actin genes.

The *LOB1* (NCBI Gene ID: 778395) and *SWEET10* (NCBI Gene ID: 100817002) genes showed about 4.5 and 55 times more expression in inoculated and in non-inoculated leaves, respectively. With regard to the terpene coding genes, they showed 4.5 (*Germacrene D*- Gene ID: 114170534) and 6 (*Nerolidol* - Gene ID: 114288835) times more expression in inoculated leaves in comparison to non-inoculated ones. Finally, for the resistance genes with TIR (Gene ID: 126628761) and CC (Gene ID: 123059796) domains, these figures were 5 and 3.5 (Figure 11).

All these six genes showed statistically significant differences in expression, all of which were upregulated in leaves inoculated with *Xap*, when compared to non-inoculated ones, thus validating the sequencing results.

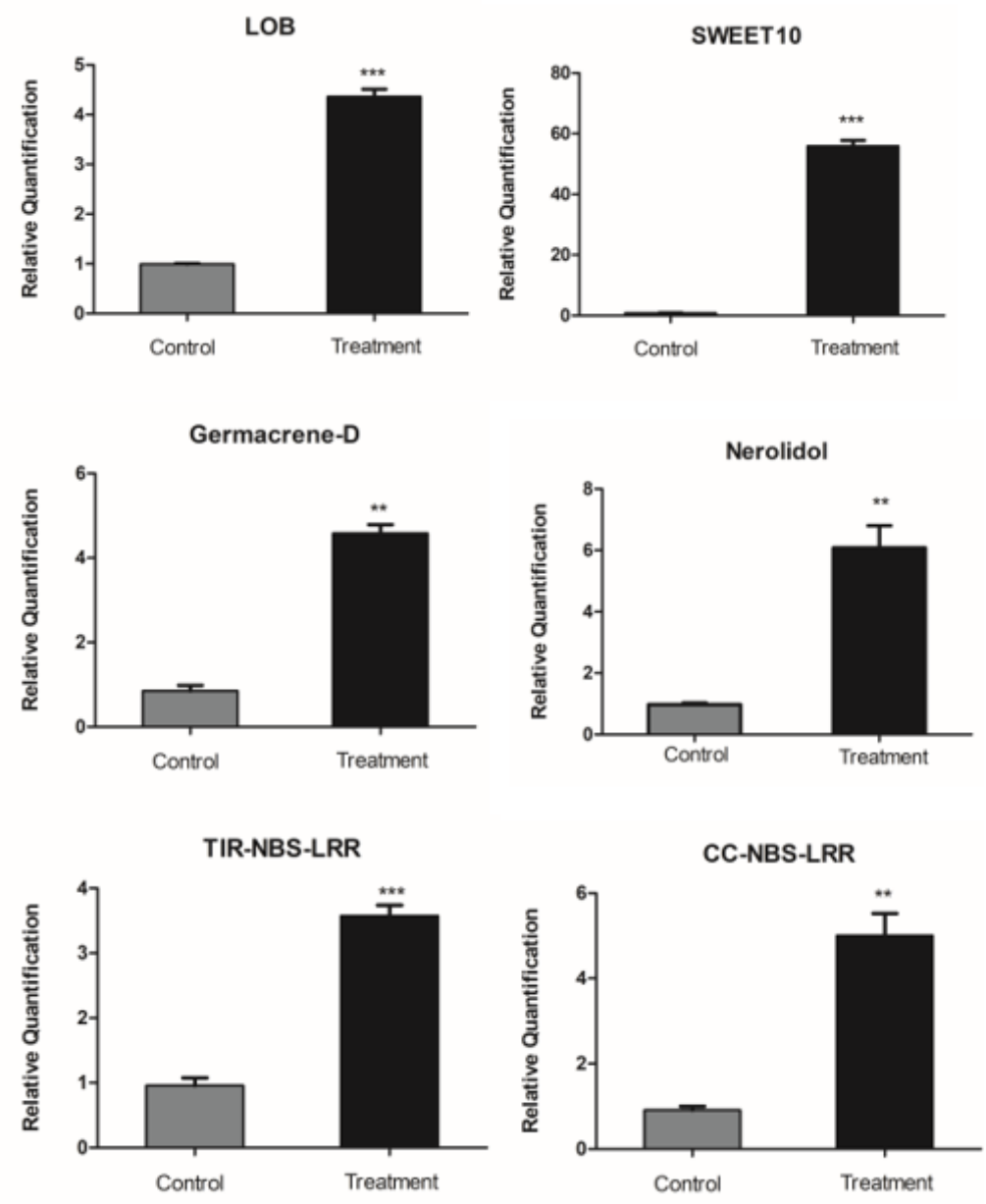

Figure 11. Validation of RNA-Seq results by Real-time PCR. Two susceptibility genes (*LOB* and *SWEET10*), two terpene coding genes (*Germacrene D* and *Nerolidol*) and two resistance genes (*TIR-NBS-LRR* and *CC-NBS-LRR*). All showed statistically significant differences in their expression level and were upregulated in leaves inoculated with *Xanthomonas axonopodis* pv. *passiflorae* in comparison to the controls.

3.3.1.6 Expression profiling of selected genes at 7 DAI

The measurement of the expression at 7 DAI of selected genes was also evaluated by RT-qPCR (Figure 12). Five genes showed a decreased expression at this period in comparison to that of 5 DAI, and just the *LOB* gene kept its level of expression steady, suggesting that these genes are expressed at very early stages of the response to *Xap*. Importantly, the *SWEET10* gene meaningfully decreased its expression in inoculated leaves at 7 DAI in comparison to its expression level at 5 DAI. At 7 DAI the expression rate was just 4 times more in inoculated leaves relatively to the control rate.

At 7 DAI, *Germacrene D* and *Nerolidol,* respectively, presented twice and 4.8 more the rate of expression in treated leaves compared to that of untreated ones. Finally, the two resistance genes showed no statistically significant difference in their expression at 7 DAI relatively to the controls rates.

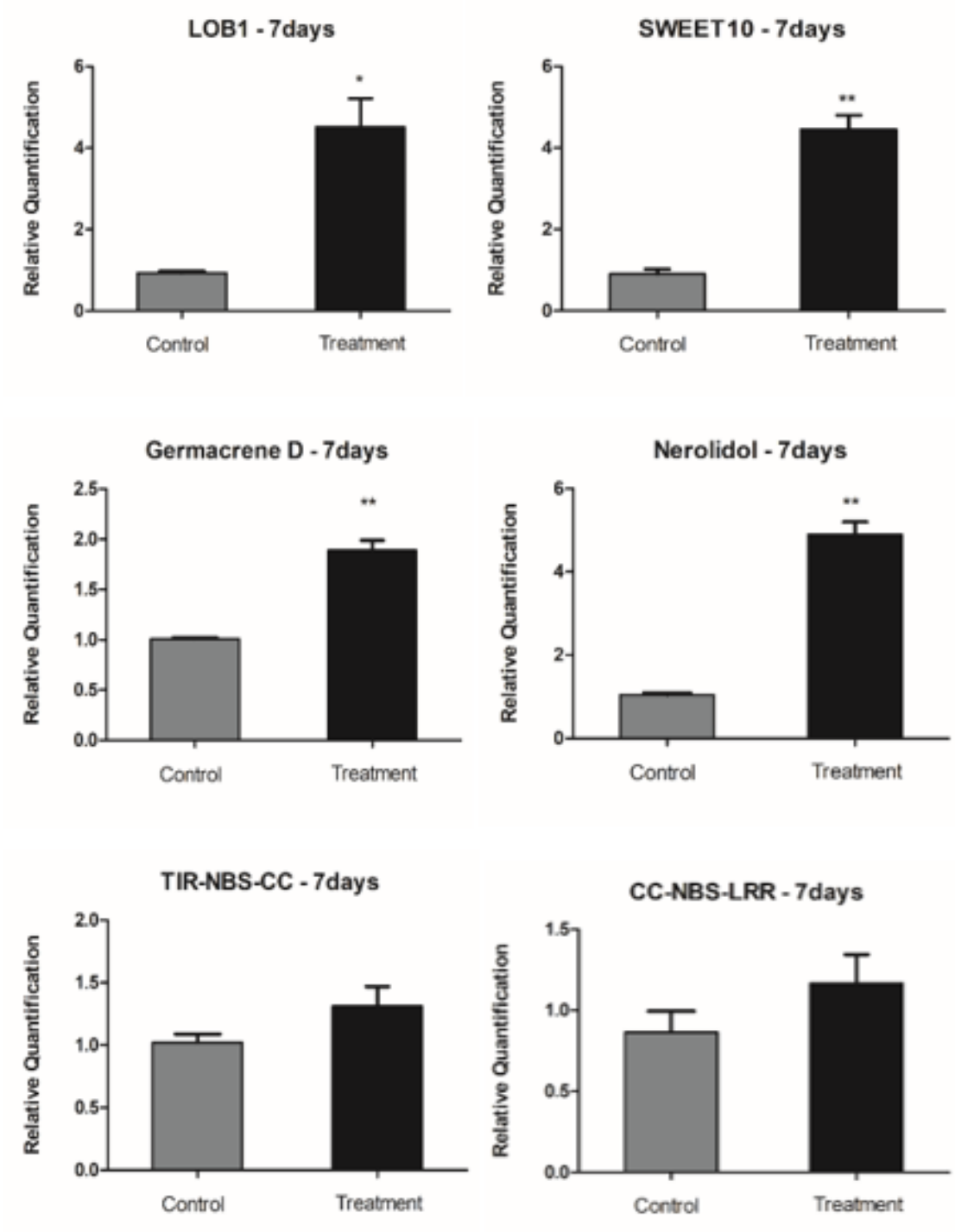

Figure 12. Expression profiling of two susceptibility genes (LOB and SWEET10), two terpene coding genes (Germacrene D and Nerolidol) and two resistance genes (TIR-NBS-LRR and CC-NBS-LRR) after 7 DAI. All genes decreased their expression levels relatively to those of 5 DAI, excepting the LOB gene that maintained the level of its expression. Both resistance genes showed no statistically significant differences in their expression levels.

3.4 Discussion

Plants have evolved a sophisticated immunity system to detect and try to stop pathogen proliferation and disease progression. Thus, by using a basic computational workflow of bioinformatics analysis of RNA-Seq data, we aimed to get a clear understanding of how the sweet passion fruit responds to the *Xap* infection.

The examination of the processed reads reveals a score over Q30, indicating that the sequence data were of high quality. The assembly evaluation by the TransRate tool was compared to other plant *de novo* transcriptome' assemblies (MacManes 2018). TransRate evaluates transcriptome assembly contiguity by producing a score based on length-based and mapping metrics. This tool has shown that *P. alata de novo* transcriptome assembly was of high contiguity and accuracy. The scores of *P. alata* (0.42) were higher than the ones of *Vigna angularis* (0.26), *Hevea brasiliensis* (0.16) and *Nicotiana tabacum* (0.30). Moreover, assembly quality is correlated with increased mapping rates. Herein, the percentage of read mapping (95%) was close, for instance, to the one of *Nicotiana tabacum* (93%) and higher than those of *Vigna angularis* (88%) and *Hevea brasiliensis* (89%).

When comparing BUSCO metrics, the majority were of high quality since 96.6% of the embryophytes genes were complete and present in *de novo* assemble from *P.alata* and only a small fraction was missing (1.02%) or fragmented (2.32%). Similarly, an assembly of the banana transcriptome in response to *Xanthomonas* infection, but using its genome as a reference, also revealed ~96% of complete genes (Tripathi et al. 2019). The BUSCO metrics assess transcriptome assembly and annotation completeness with single-copy orthologs. When applied to the above-mentioned transcriptomes it showed that 95% of the genes were complete in *Vigna angularis*, 93.8% in *Hevea brasiliensis* and 95.4% in *Nicotiana tabacum de novo* assemblies.

At the beginning of the infection process, the innate immune system constitutes the first line of defense against invading pathogens and relies on a large family of specific PRRs, which detect distinct evolutionarily conserved structures on pathogens, termed PAMPs. This process was identified in the sweet passion fruit against *Xap*. In our analysis, 7 PRRs with different domains were identified, being leucine-rich repeat (LRR) the most predominant, triggering a defense response in different ways. In rice, for instance, LRR receptors, *Xa3* and *Xa26*, were related to the induction of autophagy-like cell death and activation of calcium signaling and ROS production (Cao et al. 2019), whereas *Xa21* triggers a defense response after recognition of a secreted protein by *Xanthomonas*, called *tyrosine-sulfated type 1 – RaxX,* which is capable of mimicking plant hormones to regulate host responses (Pruitt et al. 2015). In cassava plants, the receptor *RXam1*(Resistance to *X. axonopodis* pv. *maninhotis 1*), which presents similarity to *Xa21*, decreases pathogen growth. This gene was found to be located in the Quantitative Trait Loci (QTL) called XM5. Its effect accounts for 13% of the cassava resistance (Díaz Tatis et al. 2018).

In addition to LRR-PRRs, receptors with lysin- and lectin motif domains were also identified in the sweet passion fruit. Similarly, 6 and 20 lysin receptors were upregulated in *Citrus sinensis* (Li et al. 2021) and in kumquat plants (Giraldo-Gonzáles et al. 2021), respectively, under infection with *X. citri* subsp *citri*. In addition, two lysin receptors (Liu et al. 2012) and one lectin receptor (Cheng et al. 2013) were upregulated in rice in response to *X. oryzae* pv. *oryzae*. The later ones were shown to interact with an actin-depolymerizing factor (OsADF) and resulted in the overexpression of PR1 (Pathogenesis-related protein 1) and *LOX2* (lipoxygenase) genes, while in pepper plants, an L-type lectin PRR activated the MAP kinases cascade and ROS accumulation, in an attempt to prevent the proliferation of *X. campestris* pv. *vesicatoria* (Woo et al. 2016).

The interaction process from the perception of the pathogen to the activation of a defense response was intensively reviewed by Delplace (2021). After the recognition of the pathogen by these receptors, a defense

response is triggered, and as a counterattack, the bacterium secretes effectors (Tal or Xops) inside the host cell. These effectors can be recognized by cytoplasmic receptors encoded by Resistance Genes (R), and thus a new defense response is triggered. The R genes are classified into two subfamilies according to the N terminal region: NB-LRR proteins that contain an N-terminal TIR (Toll, interleukin-1 receptor, resistance protein) domain, and NB-LRR proteins composed of an N-terminal domain with a coiled-coil (CC) domain, or having not conserved N-terminal regions at all (Dubey and Singh 2018).

Two resistance genes were found to be upregulated in *P. alata* 5 DAI, one of each subfamily. These genes are important for plant breeders, as they are crucial genes for host resistance. Indeed, in a comparative transcriptome analysis between two common bean genotypes, one susceptible and one resistant to *X. phaseoli* pv. *phaseoli*, 30 R genes were found to be repressed in the first genotype (Foucher et al. 2020). A similar analysis was performed in cabbage, and nine R genes were upregulated in the resistant genotype after *X. campestris* pv. *campestris* infection (Afrin et al. 2018), showing that this gene is crucial in acquiring the resistance phenotype.

In addition to R genes, effectors can also bind to promoter regions of susceptibility (S) genes, activating them. However, unlike the R genes, S genes are required for successful pathogen infection and are crucial for compatible plant-pathogen interactions, acting in pathogen recognition, contributing to pathogen proliferation and spread, and negatively regulating defense responses. Thus, they are potential targets for gene editing (See Zaidi et al. 2018) and manipulation for crop improvement.

We found two S genes that were upregulated in inoculated plants, which suggests that these genes lead to susceptibility, preventing resistance against *Xap*. One of the S genes was *SWEET10* (the transcript has 3054 bases, NCBI Gene ID: 100817002) that encodes sugar transporters and are used by pathogens to provide sugar as a source of nutrition, resulting in successful infection (Gupta 2020). In cotton (Cox et al. 2017) and cassava (Cohn et al. 2014), *SWEET10* was also identified to be upregulated under *X. citri* subsp. *malvacearum* and *X. axonopodis* pv. *manihotis* infection, respectively; they were activated by the corresponding TAL effector Avrb6 and TAL20. According to Cox et al. (2017), silencing of *SWEET10* reduces disease symptoms, supporting the involvement of this gene in cotton susceptibility. Similarly, in walnut (Jiang et al. 2020) and rice (Blanvillain-Baufumé et al. 2016), *SWEET* genes are shown to be activated by TAL effectors, promoting susceptibility to *Xanthomonas* infection.

The second S gene was *LOB1* (the transcript has 1062 bases, NCBI Gene ID: 778395), which belongs to the LBD proteins, a family of transcription factors characterized by a highly conserved LOB domain. These proteins are generally involved in lateral organ development, but also, act as a negative regulator of susceptibility (Xu et al. 2016). In Citrus, this gene is involved in susceptibility by inducing pustule formation and activating genes related do cellular expansion (Hu et al. 2014). Pustule formation in the infected tissues plays a vital role in Citrus canker development and pathogen spread.

Herein, we also found the expansin and cellulase synthase transcripts upregulated in inoculated leaves. The activation of *LOB1* supposedly leads, in turn, to the activation of cell wall-related genes as suggested by Hu et al. (2014). The knockout of this gene through gene editing mediated by the CRISPR-CAs9 system, revealed an increase in resistance against *X. citrus* subsp. *citrus*, with no formation of canker 12 DAI, supporting that *LOB1* is involved in Citrus susceptibility to canker (Peng et al. 2017).

After pathogen perception, there seems to be an increase in calcium influx indicated by the upregulation of genes encoding calcium-dependent kinases, which, in turn, phosphorylate the NADPH oxidase (Lee et al. 2020). These transcripts were also found to be upregulated in *P. alata*, resulting in ROS production. These compounds lead to cell death and act as signaling molecules that regulate normal responses to stress (see Huang et al. 2019).

Calcium-related proteins and NADPH oxidase are important for promoting an immune response against invaders as demonstrated in Brassica oleracea after *X. campestris* pv. *campestris* infection (Tortosa et al. 2019) and in two citrus species, where 6 *RBOHD* were found. Their silencing reduced the resistance and production of ROS, leading to increased *X. citri* subsp *citri* proliferation (Mei et al. 2019).

These changes in the cell can lead to hormone synthesis that can act as signaling molecules and induce transcriptional reprogramming by transcription factors activation (see Aerts et al. 2020). In this study, genes related to the salicylic acid and ethylene pathway were upregulated. In fact, salicylic acid pathway genes were upregulated in kumquat plants in response to *X. citri* subsp. *citri* infection (Giraldo-Gonzáles et al. 2021) and in a common bean resistant genotype compared to a susceptible one (Foucher et al. 2020).

In addition, salicylic acid can cause a range of modifications in gene expression, such as its methylation to form methyl salicylate (MeSA) that makes salicylic acid more volatile, triggering defense responses in uninfected plants (Lefevere et al. 2020). Herein, we found that the *SAMT* (salicylate carboxymethyl transferase) gene was upregulated.

Regarding the ethylene biosynthesis pathway, it is sensed by receptors of the endoplasmic reticulum membrane. Among them, there is the *EIN4* – Ethylene Insensitive 4, which was found upregulated in *P. alata*. Downstream components such as the Ethylene Response Factors (ERFs) were also identified to be upregulated. These elements are known to regulate the response to pathogen infection by binding to the GCC box of stress-responsive genes (Müller and Munné-Bosch 2015).

As mentioned above, transcription factors (TFs) are activated and act on transcriptional reprogramming of defense-related genes. In total, 15 TFs were found differentially expressed in sweet passion fruit after infection with *Xap,* confirming that they have an essential role in defense against these pathogens. Among the TF families, two stand out. The WRKY transcription factors were the most abundant. They are involved in the regulation of several biological processes, including responses to biotic stress. In rice, OsWRKY51 (Hwang et al. 2016) and WRKY114 (Son et al. 2020) bind to the promoter region of the pathogenesis-related genes PR10a and PR1a, respectively, regulating their transcription positively in defense against *X. oryzae* pv. *oryzae*.

The bHLH (basic helix–loop–helix) TF family, of which two (bHLH35 and bHLH75) were found upregulated in inoculated leaves of *P. alata*. Similarly, 4 genes of the same family (SsbHLH03/04/36/37) were found to be upregulated in a cultivar of *Saccharum spontaneum* resistant to leaf scald after *X. albilineans* infection (Ali et al. 2021). Relatedly, in tomato, the bHLH132 expression was induced after pathogen recognition and improves resistance against *X. euvisicatoria* pv. *perforans* (Kim and Mudgett 2019).

Finally, this entire signaling cascade leads to the expression of pathogenesis-related genes (PR), that are induced by phytopathogens attack, acting as defense-related signaling molecules and antimicrobial activities, such as chitinases, glucanases, peroxidases, and volatile compounds (Ali et al. 2018). In this study, we found transcripts encoding chitinases, glucanases and peroxidases, besides two terpenes' coding-transcripts (Nerolidol and Germacrene D). These later ones are synthesized by terpene synthases (TPSs) and are involved in plant defense responses, such as inhibition of bacterial growth. Upon pathogen perception, volatile terpenes can sensitize specific signaling pathways and trigger plant immunity by modulation of hormone signaling.

For example, the volatile compound, Nerolidol, was also found upregulated in rice, encoded by the gene *OsTPS18* in response to *X. oryzae* pv. *oryzae,* and its level was also increased after treatment with jasmonic acid (Kyriu et al. 2018). In *Citrus*, ectopic expression of the Tal effector, AvrXa7, from *X. oryzae* pv. *oryzae*, induced the

expression of *Germacrene D*, and also suppressed canker development (Sun et al. 2018), suggesting that these compounds are involved in pathogen response.

In *P. edulis* (the sour passion fruit), the genes that respond most strongly to *Xanthomonas* attack encodes the enzymes lipoxygenase 2 and (+)-neomenthol dehydrogenase, an antimicrobial protein involved in neomenthol biosynthesis, a monoterpene in the terpenoid family that is a volatile compound induced in response to pathogen attack. Extraordinary expression ratios of around 500-fold and 300-fold were reported for lipoxygenase 2 and 3.3-fold and 8.1-fold for the enzyme (+)-neomenthol dehydrogenase for the two evaluation periods (5 and 9 DAI) (Munhoz et al. 2015). These transcripts were not found differentially expressed in *P. alata* in response to *Xap*. Differences in the host responses to *Xap* may be attributed to infection conditions, evaluation periods (24 h, 5 and 9 DAI) and type of RNA-derived libraries. Munhoz et al. (2015) used the suppression substrative hybridization technique, cloning of transcripts recovered by each library (forward and reverse) and Sanger sequencing. Interestingly, both studies indicated a compatible interaction and passion fruit susceptibility to *Xap*.

Our RNA-Seq results were validated by real time RT-qPCR. A total of 6 six genes were selected for validation, two S genes, 2 resistance genes and 2 terpenes' coding genes. All showed statistically significant expression in inoculated plants in relation to non-inoculated ones, thus corroborating with our RNA-seq results. The expression profile of the same 6 genes were evaluated in other RT-qPCR assay in which leaves were collected at 7 DAI. Five genes showed a decrease in their expression, supporting that these genes manifest early its expression in response to *Xap*. Only the *LOB1* gene has kept its expression steady, and both resistance genes' expression was not substantially different from that of the first evaluation period i.e., 5 DAI.

3.5 Conclusion

In the present study, we performed an RNA-Seq-based transcriptome analysis, describing the expression response of the sweet passion fruit to *Xap*, 5 days after the bacterium inoculation. A total of 1.242 transcripts were differentially expressed. Genes involved in pathogen recognition such as PRRs and R genes and genes related do the signaling cascade (ROS accumulation, calcium and hormone signaling pathways), TFs and secondary metabolism were identified to be upregulated after infection, confirming their important roles in host defense response. On the other hand, however, two susceptibility genes were found upregulated, supporting their relevance in impairing the sweet passion fruit resistance to *Xap* and favoring the disease symptoms. Potentially, these genes could be targets for genome editing in order to obtain disease-resistant cultivars. Using the RNA-seq technology, our study provides the first transcriptional profiling of *P. alata* during infection with *Xap*, resulting in new information and perspectives to encourage breeders to produce resistant genotypes.

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APPENDICES

Table 5. Up regulated transcripts regulated in *Passiflora alata* leaves at 5 d.a.i. with *Xap*.

ID	logFC	FDR	Description
TRINITY_DN4004_c0_g1_i1	12,291134502534	2,79E-03	Belongs to the peptidase A1 family
TRINITY_DN456_c0_g1_i49	12,1684983977643	6,32E-02	Belongs to the glycosyltransferase 2 family
TRINITY_DN2906_c0_g1_i12	11,6971145381479	3,78E-04	(-)-germacrene D synthase-like
TRINITY_DN2906_c0_g1_i12	11,3534758531696	2,15E+03	assists the folding of proteins upon ATP hydrolysis
TRINITY_DN3741_c0_g1_i4	11,1175265989332	2,14E+00	transcription factor
TRINITY_DN944_c0_g1_i14	10,8887034243858	4,26E+00	chromosome transmission fidelity
TRINITY_DN7360_c0_g1_i34	10,7383393498779	5,13E-01	Belongs to the mitochondrial carrier (TC 2.A.29) family
TRINITY_DN2243_c0_g1_i52	10,5877166438556	1,18E+01	Unknow
TRINITY_DN7461_c0_g1_i9	10,5688707848506	4,39E+02	Asp/Glu/Hydantoin racemase
TRINITY_DN1965_c0_g3_i3	10,5239069008366	8,21E+01	Pentatricopeptide repeat-containing protein
TRINITY_DN244_c0_g1_i61	10,4425581055868	8,21E+01	ABC transporter C family member
TRINITY_DN4136_c1_g1_i1	10,3756433570872	3,29E+04	Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme
TRINITY_DN10288_c0_g1_i19	10,3573917214501	1,81E+04	Belongs to the RNase T2 family
TRINITY_DN5516_c0_g1_i1	10,2169585427804	2,33E+01	RAVE protein 1 C terminal
TRINITY_DN2514_c0_g1_i1	10,2097345421372	1,40E+00	PRLI-interacting factor
TRINITY_DN327_c1_g1_i1	10,0226002350337	5,73E+02	transcription factor bHLH35
TRINITY_DN6564_c0_g1_i6	9,96622748084146	1,22E+02	HAD-hyrolase-like
TRINITY_DN3817_c0_g1_i7	9,95437313577108	2,00E+02	NF-X1-type zinc finger protein
TRINITY_DN310_c0_g1_i5	9,91887275590637	8,82E+04	Rho GTPase-activating protein
TRINITY_DN8115_c1_g1_i2	9,91718091035298	4,39E+02	RNA-binding protein
TRINITY_DN3663_c0_g1_i2	9,78313900245829	5,90E+02	Mitochondrial fission protein
TRINITY_DN4654_c0_g1_i2	9,77672834348553	1,44E+03	Protein present in Fab1, YOTB, Vac1, and EEA1
TRINITY_DN1417_c0_g1_i1	9,64546643416936	1,42E+04	U3 small nucleolar RNA-associated protein
TRINITY_DN6982_c0_g1_i64	9,63735645908037	6,58E+03	CRT-like, chloroquine-resistance transporter-like
TRINITY_DN3259_c0_g1_i9	9,62016209809181	2,80E+05	Belongs to the short-chain dehydrogenases reductases (SDR) family
TRINITY_DN4911_c0_g2_i1	9,61868536338388	1,20E+04	Heat Stress Transcription Factor
TRINITY_DN4570_c0_g1_i45	9,61369405776473	1,22E+03	Mannosyltransferase involved in glycosylphosphatidylinositol-anchor biosynthesis
TRINITY_DN3165_c0_g1_i11	9,58895789340931	6,94E+03	Probable zinc-ribbon domain
TRINITY_DN33143_c0_g1_i10	9,54097956131326	6,25E+04	XS domain
TRINITY_DN4003_c0_g1_i16	9,53949984500819	1,97E+05	Mechanosensitive ion channel protein
TRINITY_DN5757_c0_g1_i23	9,46012991136638	3,80E+04	Pre-mRNA-splicing factor
TRINITY_DN1724_c0_g1_i10	9,45258049686654	3,23E+03	Glutamate-gated receptor that probably acts as non- selective cation channel
TRINITY_DN51138_c0_g1_i1	9,44215116835893	4,53E+03	Xyloglucan galactosyltransferase KATAMARI1
TRINITY_DN10188_c0_g1_i9	9,43485015370801	1,08E+05	21 kDa protein-like
TRINITY_DN7101_c0_g1_i10	9,41082491155803	3,10E+08	Calcium-dependent protein kinase/calmodulin-like protein 3

TRINITY_DN3817_c0_g1_i35	9,3248711258057	6,03E+04	NF-X1-type zinc finger protein
TRINITY_DN9292_c0_g2_i2	9,2289710235796	1,41E+04	RWP-RK domain
TRINITY_DN6013_c0_g1_i8	9,11236603940827	2,46E+05	AAA domain
TRINITY_DN7043_c0_g1_i47	9,09630948.576973	1,37E+08	ATP-dependent RNA helicase
TRINITY_DN8293_c0_g1_i3	9,08493757385143	1,78E+05	External alternative NAD(P)H-ubiquinone oxidoreductase B1
TRINITY_DN3602_c0_g1_i2	9,04609054417444	2,61E+05	zinc finger
TRINITY_DN5534_c1_g1_i22	9,03531191463543	1,61E+04	longifolia
TRINITY_DN3338_c0_g1_i25	8,98067299717382	1,06E+06	DCD
TRINITY_DN8151_c0_g1_i2	8,97435399315276	1,44E+05	subtilisin-like protease
TRINITY_DN1137_c0_g1_i24	8,97300616610225	2,46E+09	Unknow
TRINITY_DN3685_c0_g1_i5	8,97217445051475	1,08E+06	Bifunctional aspartokinase homoserine dehydrogenase
TRINITY_DN7490_c0_g2_i18	8,96580963241633	1,31E+06	MRNA-decapping enzyme-like protein
TRINITY_DN4056_c0_g1_i4	8,93448039764142	7,04E+04	Polyubiquitin-like
TRINITY_DN1607_c1_g1_i1	8,87841117810941	1,49E+05	Calmodulin-like protein
TRINITY_DN4900_c0_g1_i8	8,84510782268163	0.0478948 231599538	HPC2 and ubinuclein domain
TRINITY_DN6369_c0_g1_i6	8,82808087232017	2,11E+06	Catalyzes the reduction of fatty acyl-CoA to fatty alcohols
TRINITY_DN10113_c0_g1_i5	8,82714186930824	1,74E+07	Unknow
TRINITY_DN7490_c0_g2_i13	8,82245879011184	4,73E+05	mRNA-decapping enzyme-like
TRINITY_DN22405_c0_g1_i2	8,80740729683751	0.0466608 535043865	Pentatricopeptide repeat-containing protein
TRINITY_DN1842_c0_g1_i9	8,79612145671188	6,31E+05	Splicing regulatory glutamine lysine-rich protein 1 isoform X1
TRINITY_DN6527_c0_g1_i2	8,79323251693292	0.0488079 462615189	Methionine
TRINITY_DN19211_c0_g1_i14	8,78554273666131	0.0488649 361223602	ribonuclease H protein At1g65750
TRINITY_DN17370_c0_g1_i12	8,78100061988082	2,42E+07	RNA uridylyltransferase activity
TRINITY_DN9055_c0_g1_i11	8,77272816784975	2,14E+07	Encoded by
TRINITY_DN11264_c0_g1_i7	8,74225133544281	4,24E+09	Belongs to the helicase family. Dicer subfamily
TRINITY_DN3089_c0_g1_i2	8,74141014197364	1,37E+08	Unknow
TRINITY_DN10053_c0_g1_i45	8,73081748858108	4,08E+05	Lys-63-specific deubiquitinase
TRINITY_DN1701_c0_g1_i4	8,70877277621091	5,15E+06	Acyclic terpene utilisation family protein AtuA
TRINITY_DN5548_c0_g1_i10	8,62575524055084	1,89E+07	Cleavage and polyadenylation specificity factor subunit
TRINITY_DN9289_c0_g1_i17	8,61933265256844	4,13E+06	Sodium hydrogen exchanger
TRINITY_DN6389_c0_g1_i5	8,51943261270488	7,56E+08	ATP-dependent RNA helicase
TRINITY_DN34930_c0_g1_i2	8,46269080935348	8,19E+06	Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Kinesin family
TRINITY_DN7775_c0_g1_i28	8,44377693208325	0.0001274 722518609 76	Acyl-CoA N-acyltransferase with RING FYVE PHD-type zinc finger protein
TRINITY_DN3712_c0_g1_i20	8,42577233895025	6,87E+06	Cell division cycle and apoptosis regulator protein
TRINITY_DN700_c0_g1_i5	8,40923592792038	3,35E+09	UDP-N-acetylglucosamine transferase subunit ALG14
TRINITY_DN3610_c0_g2_i20	8,37023369507597	3,45E+08	Belongs to the glycosyl hydrolase 1 family
TRINITY_DN4212_c0_g1_i10	8,35856912985015	9,36E+07	SWR1 complex subunit
TRINITY_DN27090_c0_g1_i4	8,33678597808368	4,91E+07	GATA transcription factor
TRINITY_DN4314_c0_g1_i79	8,29913666008907	1,75E+08	Structural maintenance of

			chromosomes protein
TRINITY_DN8011_c0_g1_i7	8,29543457398685	1,00E+08	RNA recognition motif
TRINITY_DN24178_c0_g1_i1	8,28171724980861	0.0001882589342102 75	Protein of unknown function (DUF2985)
TRINITY_DN23078_c0_g1_i3	8,2297457242818	2,25E+07	Belongs to the expansin family
TRINITY_DN3665_c0_g1_i17	8,22706073319655	5,31E+09	Subtilase family
TRINITY_DN27891_c0_g2_i2	8,21271750925031	1,60E+09	Plant protein of unknown function (DUF863)
TRINITY_DN8743_c0_g1_i13	8,18067586016401	4,63E+07	isoform X1
TRINITY_DN2961_c0_g1_i3	8,17525595113943	1,18E+08	deSI-like protein
TRINITY_DN7474_c0_g1_i24	8,13189242723029	1,03E+08	adenosine deaminase-like
TRINITY_DN10027_c0_g1_i2	8,09629764722522	4,16E+07	Unknow
TRINITY_DN5731_c0_g1_i1	8,06936943710709	7,46E+08	F-box protein
TRINITY_DN27090_c0_g2_i4	8,05738924378241	4,74E+07	GATA transcription factor
TRINITY_DN6860_c0_g1_i3	8,05332536207782	4,65E+08	MACPF domain-containing protein
TRINITY_DN8348_c0_g3_i1	8,03925336654204	9,53E+07	Triose-phosphate Transporter family
TRINITY_DN8279_c0_g1_i11	8,03551945164662	1,46E+08	SWEET10
TRINITY_DN2699_c0_g1_i21	8,03534022587125	6,15E+08	protein modification by small protein removal
TRINITY_DN10183_c0_g1_i1	8,03213024989325	1,30E+08	DNA annealing helicase and endonuclease
TRINITY_DN4003_c0_g1_i6	8,01858148763271	0.0004649762897427 5	Mechanosensitive ion channel protein 10-like
TRINITY_DN2490_c1_g1_i4	8,00033033664681	0.0006028585314636 03	Lysine-specific histone demethylase 1 homolog
TRINITY_DN5466_c0_g1_i35	7,990947516593	3,40E+07	DUF21 domain-containing protein
TRINITY_DN10718_c0_g1_i2	7,98120880822388	7,00E+09	RNA polymerase II transcription regulator recruiting activity
TRINITY_DN8484_c0_g1_i41	7,97580587441363	1,56E+08	Belongs to the glycosyltransferase 8 family
TRINITY_DN5747_c0_g1_i3	7,9425873341528	0.0001188481055871 2	Gpi-anchor
TRINITY_DN7941_c0_g1_i2	7,93969980160685	0.0019687867679474	Protein of unknown function (DUF668)
TRINITY_DN5813_c0_g1_i8	7,91562063110496	4,66E+09	F-actin-capping protein subunit beta
TRINITY_DN6412_c0_g1_i5	7,90756408884236	3,31E+07	Belongs to the enoyl-CoA hydratase isomerase family

Table 6. Down regulated transcripts in *Passiflora alata* leaves at 5 d.a.i. with *Xap*.

ID	LogFC	FDR	Description
TRINITY_DN1376_c0_g1_i83	-11,3076	1,50569375422697E-15	DEAD-box ATP-dependent RNA helicase
TRINITY_DN2085_c0_g1_i5	-11,274	1,21805770734422E-16	Protein FAR1-RELATED SEQUENCE
TRINITY_DN10288_c0_g1_i1	-11,1837	3,84283142345541E-14	Belongs to the RNase T2 family
TRINITY_DN5516_c0_g1_i14	-11,1486	3,93260363160486E-16	RAVE protein 1 C terminal
TRINITY_DN2982_c0_g1_i15	-11,1069	2,41593070026528E-12	Involved in chlorophyll biosynthesis. Catalyzes the insertion of magnesium ion into protoporphyrin IX to yield Mg-protoporphyrin IX
TRINITY_DN2988_c0_g1_i8	-10,9846	6,19040941792307E-16	Ribonuclease H protein
TRINITY_DN202_c0_g1_i8	-10,8679	1,09826795400784E-15	sodium-coupled neutral amino acid transporter
TRINITY_DN4497_c0_g1_i51	-10,7009	3,92260358216581E-13	lipid binding
TRINITY_DN2243_c0_g1_i64	-10,5706	9,73064104809473E-14	Unknow
TRINITY_DN4003_c0_g1_i23	-10,515	1,3994926771795E-13	Mechanosensitive ion channel protein
TRINITY_DN3136_c0_g1_i55	-10,5073	2,74865240016963E-12	oligopeptide transporter
TRINITY_DN7360_c0_g1_i39	-10,345	9,19279541059888E-14	Belongs to the mitochondrial carrier (TC 2.A.29) family
TRINITY_DN3259_c0_g1_i2	-10,2918	6,17951203200781E-12	Belongs to the short-chain dehydrogenases reductases (SDR) family
TRINITY_DN639_c0_g1_i51	-10,2904	5,72912271730096E-12	beta-D-xylosidase
TRINITY_DN327_c1_g1_i2	-10,1084	6,43385982495234E-11	transcription factor
TRINITY_DN3718_c0_g1_i5	-10,0635	4,96461757940607E-09	Protein BPS1, chloroplastic-like
TRINITY_DN3602_c0_g1_i3	-9,80895	4,39412425452435E-12	zinc finger
TRINITY_DN6982_c0_g1_i59	-9,7667	2,41593070026528E-12	CRT-like, chloroquine-resistance transporter-like
TRINITY_DN33143_c0_g1_i25	-9,69749	4,06844119525916E-12	XS domain
TRINITY_DN6564_c0_g1_i34	-9,58667	2,62777305971176E-10	HAD-hyrolase-like
TRINITY_DN1973_c0_g1_i27	-9,57949	3,59234419557784E-10	HAD superfamily, subfamily IIIB (Acid phosphatase)
TRINITY_DN7101_c0_g1_i2	-9,54962	7,03564708280595E-10	Calcium-dependent protein kinase
TRINITY_DN8267_c0_g2_i6	-9,47691	2,12901116316812E-11	malate transporter
TRINITY_DN7865_c0_g1_i5	-9,45652	7,93008694450432E-09	Ubiquitin carboxyl-terminal hydrolase
TRINITY_DN5534_c1_g1_i33	-9,44837	5,64933816359501E-11	longifolia
TRINITY_DN10718_c0_g1_i9	-9,40917	4,54269085221151E-11	RNA polymerase II transcription regulator recruiting activity
TRINITY_DN3725_c0_g1_i8	-9,34221	4,54269085221151E-11	Regulator of Ty1 transposition protein 107 BRCT domain
TRINITY_DN8151_c0_g1_i6	-9,27927	3,26705962963704E-08	subtilisin-like protease

TRINITY_DN283_c0_g1_i27	-9,24634	1,36757833625576E-10	Cinnamyl alcohol dehydrogenase
TRINITY_DN4900_c0_g1_i6	-9,16495	4,08462580977344E-09	HPC2 and ubinuclein domain
TRINITY_DN7389_c0_g1_i25	-9,13804	8,5668096022697E-10	NatA auxiliary
TRINITY_DN3669_c0_g1_i93	-9,12867	2,02914939328054E-10	Peptidyl-tRNA hydrolase ICT1
TRINITY_DN9292_c0_g2_i1	-9,11661	1,8276454068717E-10	RWP-RK domain
TRINITY_DN8115_c1_g1_i1	-9,07222	6,24807748174348E-10	RNA-binding protein
TRINITY_DN7466_c0_g1_i18	-9,05475	4,74241005659097E-09	carboxy-terminal domain
TRINITY_DN7865_c0_g1_i8	-9,05009	4,74241005659097E-09	Ubiquitin carboxyl-terminal hydrolase
TRINITY_DN5948_c0_g1_i35	-9,0002	2,80178419414239E-09	Polycomb group protein EMBRYONIC FLOWER
TRINITY_DN3665_c0_g1_i15	-8,89411	5,53437741346343E-09	Subtilase family
TRINITY_DN8743_c0_g1_i10	-8,85291	1,77112703673425E-09	isoform X1
TRINITY_DN12077_c0_g1_i5	-8,85282	0,0478148950400404	WRKY transcription factor
TRINITY_DN6042_c0_g1_i13	-8,84126	0,0436691020901421	Belongs to the peptidase A1 family
TRINITY_DN16784_c0_g1_i4	-8,82823	0,0432765127640048	DNA polymerase
TRINITY_DN2796_c0_g1_i1	-8,82116	0,0488649361223602	belongs to the protein kinase superfamily
TRINITY_DN8663_c0_g1_i6	-8,82081	0,000322604626698081	Ribonuclease H protein
TRINITY_DN14752_c0_g1_i8	-8,78767	8,19034057199956E-08	Protein of unknown function (DUF632)
TRINITY_DN2059_c0_g1_i17	-8,78064	2,23291408455983E-07	deaminase
TRINITY_DN8276_c0_g1_i1	-8,77772	1,15783449491715E-08	domain-containing protein
TRINITY_DN3435_c0_g1_i33	-8,74894	1,79791917439048E-09	PWWP domain
TRINITY_DN17370_c0_g1_i24	-8,72515	6,17433685753817E-07	RNA uridylyltransferase activity
TRINITY_DN6326_c0_g2_i12	-8,70826	1,4635963254707E-08	Cysteine desulfurase 2
TRINITY_DN3895_c0_g1_i12	-8,6872	1,98156679221209E-07	Belongs to the Casparian strip membrane proteins (CASP) family
TRINITY_DN628_c0_g1_i2	-8,6725	9,86373794017644E-08	Unknow
TRINITY_DN1164_c2_g1_i11	-8,6459	8,01854209232208E-08	Belongs to the mitochondrial carrier (TC 2.A.29) family
TRINITY_DN5284_c0_g1_i4	-8,63173	3,4419949001449E-05	WRKY transcription factor
TRINITY_DN16421_c0_g1_i45	-8,62057	3,48878896410042E-07	inactive protein kinase
TRINITY_DN1607_c1_g1_i6	-8,61876	5,84897855231006E-07	Calmodulin-like protein
TRINITY_DN5284_c0_g1_i3	-8,61597	3,48594297585971E-05	WRKY transcription factor
TRINITY_DN4630_c0_g1_i24	-8,61118	1,25418484582068E-07	Belongs to the peptidase S10 family
TRINITY_DN2961_c0_g1_i35	-8,61097	5,62439589709612E-06	deSI-like protein
TRINITY_DN14304_c0_g1_i6	-8,59289	1,56715580364911E-06	Serine threonine-protein kinase
TRINITY_DN1701_c0_g1_i3	-8,53073	5,30890269564962E-05	Acyclic terpene utilisation family protein AtuA
TRINITY_DN10053_c0_g1_i38	-8,52462	2,79875607865431E-08	Lys-63-specific deubiquitinase

TRINITY_DN6767_c0_g1_i25	-8,45804	1,49282694494117E-07	COP1-interacting protein 7
TRINITY_DN6657_c0_g1_i13	-8,43272	5,8044821311322E-07	Amino acid permease
TRINITY_DN407_c1_g1_i3	-8,43135	0,00024560027183331	Leucine-rich repeat-containing protein
TRINITY_DN4705_c0_g1_i27	-8,43079	2,59033184858826E-07	Zinc phosphodiesterase ELAC protein
TRINITY_DN4560_c0_g1_i1	-8,41659	4,55244099442E-07	Unknow
TRINITY_DN8307_c0_g1_i19	-8,38388	8,17955639641478E-07	Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Kinesin family
TRINITY_DN9069_c0_g1_i12	-8,37259	1,0509016589529E-06	isoform X1
TRINITY_DN3516_c0_g1_i31	-8,37071	2,5283908498719E-08	Coiled-coil domain-containing protein 93
TRINITY_DN5155_c0_g1_i8	-8,34122	0,000463750557449433	Protein RETICULATA-related
TRINITY_DN312_c0_g1_i1	-8,33041	0,00011484507494824	Belongs to the NPH3 family
TRINITY_DN5690_c0_g1_i38	-8,30078	1,16753610694088E-07	post-GPI attachment to proteins factor
TRINITY_DN3744_c0_g1_i5	-8,2963	9,75537366649225E-06	Unknow
TRINITY_DN1316_c0_g1_i53	-8,26153	5,50676721439701E-08	Belongs to the major facilitator superfamily. Sugar transporter (TC 2.A.1.1) family
TRINITY_DN8996_c0_g1_i39	-8,23943	1,34118126448726E-07	Uncharacterized conserved protein (DUF2054)
TRINITY_DN9684_c1_g2_i9	-8,21781	2,69580783951686E-06	187-kDa microtubule-associated protein
TRINITY_DN5399_c0_g1_i8	-8,20841	2,22508602230228E-06	TBC1 domain family member
TRINITY_DN18238_c0_g1_i1	-8,20098	9,27218440790158E-06	Belongs to the protein kinase superfamily. Ser Thr protein kinase family
TRINITY_DN5459_c0_g1_i7	-8,17324	9,29280415250394E-05	DEAD-box ATP-dependent RNA helicase
TRINITY_DN4588_c0_g1_i54	-8,15405	2,85376317388399E-06	Unknow
TRINITY_DN4750_c0_g1_i12	-8,14473	5,75100634397624E-05	Nucleolar MIF4G domain-containing protein
TRINITY_DN15276_c0_g1_i20	-8,12527	5,19308614064783E-06	This magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium
TRINITY_DN8044_c0_g1_i17	-8,0945	2,70295754537538E-06	PhoD-like phosphatase
TRINITY_DN12899_c0_g1_i3	-8,09249	0,000322435630199797	Unknow
TRINITY_DN11912_c0_g1_i14	-8,08973	7,11791291016114E-06	Serine threonine-protein kinase
TRINITY_DN18846_c1_g1_i9	-8,08878	8,92749725941463E-08	Ribonuclease H protein
TRINITY_DN3618_c0_g1_i4	-8,06002	1,22862054629496E-05	Unknow
TRINITY_DN7599_c0_g1_i6	-8,05366	2,33403886159071E-07	Unknow
TRINITY_DN4003_c0_g1_i4	-8,05221	2,40545640131859E-05	Mechanosensitive ion channel protein 10-like

TRINITY_DN3712_c0_g1_i1	-8,04199	5,17846426973236E-05	Cell division cycle and apoptosis regulator protein
TRINITY_DN6529_c0_g1_i2	-8,00115	0,000134376633847374	Unknow
TRINITY_DN8931_c0_g1_i37	-7,99889	5,05681287580217E-07	alpha-N-acetylglucosaminidase-like
TRINITY_DN78080_c0_g1_i2	-7,98002	0,000127472251860976	Unknow
TRINITY_DN8288_c0_g1_i11	-7,97407	7,49670961034036E-07	Auxin response factors (ARFs) are transcriptional factors that bind specifically to the DNA sequence 5'-TGTCTC-3' found in the auxin-responsive promoter elements (AuxREs)
TRINITY_DN5885_c0_g1_i4	-7,94526	1,17830538870224E-05	Pentatricopeptide repeat-containing protein At4g35850
TRINITY_DN1522_c0_g1_i15	-7,94351	9,68488626099401E-07	SPX domain-containing membrane protein
TRINITY_DN5438_c0_g1_i6	-7,91976	2,42693123141974E-06	Forkhead associated domain
TRINITY_DN6564_c0_g1_i3	-7,91204	1,37280383329825E-06	HAD-hyrolase-like