University of São Paulo
“Luiz de Queiroz” College of Agriculture

A comparative study of raspberry structural and biochemical responses to late leaf rust

Márcia Gonçalves Dias

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

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BSc in Biology

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Advisors:
Prof. Dr. **BEATRIZ APPEZZATO DA GLÓRIA**
Prof. Dr. **MARCEL BELLATO SPÓSITO**

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RESUMO

Estudo comparativo das respostas estruturais e bioquímicas de framboeseiras a ferrugem tardia da folha

Rosaceae é uma família composta por culturas importantes como maçã, pêssego, morango e framboesa. Entre essas espécies, as framboesas vermelhas e pretas pertencem ao gênero *Rubus* e são culturas de alto valor com crescente demanda mundial. No entanto, essas plantas são acometidas por diversas doenças que impactam sua qualidade e produtividade. A ferrugem tardia da folha é uma doença fúngica em framboesas causada por *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart). Enquanto as framboesas vermelhas (*Rubus idaeus* L.) são suscetíveis, as framboesas pretas foram relatadas anteriormente como mais resistentes (*R. occidentalis* L.) e imunes (*R. niveus* Thunb.) a esse patógeno. Uma vez que a resistência genética é uma forma promissora de manejar esta doença, a hibridização de framboesas vermelhas e pretas pode fornecer características interessantes a novas cultivares. No entanto, é importante entender como as plantas respondem aos patógenos antes de desenvolver caros e longos programas de melhoramento. O objetivo desta tese foi investigar as respostas histopatológicas e bioquímicas de framboesas vermelhas e pretas e um híbrido entre elas à colonização por *A. americanum*. Observou-se que o patógeno germinou e colonizou todas as framboesas estudadas, entretanto, as framboesas pretas e o híbrido tiveram respostas distintas em comparação a *R. idaeus*. O mesofilo compacto, os compostos fenólicos pré e pós-formados, os compostos pécticos pós-formados e o colapso celular na área lesionada foram os principais mecanismos de defesa contra *A. americanum*. Adicionalmente aos estudos estruturais e bioquímicos das respostas de defesa da framboesa, foi realizada uma análise genômica comparativa para identificar as proteínas quinases dependentes de cálcio (CDPKs), com foco nos ortólogos CPK28 em framboesa preta (*R. occidentalis*) e morango (*Fragaria vesca* L.) como representante das Rosaceae. Ambos têm 26 CDPKs juntos. Os ortólogos FvCPK28 e RoCPK28 foram clonados e expressos em mutantes *Nicotiana benthamiana* e *Arabidopsis cpk28*. Foi encontrado que ambos os ortólogos CPK28 de Rosaceae são localizados na membrana plasmática e sua superexpressão amorteceu a explosão oxidativa após a elicitação. Esses resultados forneceram uma prova de conceito para investigar as semelhanças funcionais entre essas proteínas e *Arabidopsis* CPK28.

Palavras-chave: CPK28, Mecanismos de defesa, Compostos fenólicos, *Rubus idaeus*, *Rubus occidentalis, Rubus niveus*

ABSTRACT

A comparative study of raspberry structural and biochemical responses to late leaf rust

Rosaceae is a family composed of important crops such as apple, peach, strawberry, and raspberry. Among these species, red and black raspberries belong to the genus *Rubus* and are high-value crops with increasing worldwide demand. However, these plants are affected by several diseases that impact their quality and productivity. Late leaf rust is a fungal disease in raspberries caused by *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart). While red raspberries (*Rubus idaeus* L.) are susceptible, black raspberries were previously reported as more resistant (*R. occidentalis* L.) and immune (*R. niveus* Thunb.) to this pathogen. Since genetic resistance is a promising way to manage this disease, hybridizing red and black raspberries can provide new cultivars with interesting traits. However, it is important to understand how plants respond to pathogens before developing expensive and long breeding programs. The aim of this thesis was to investigate the histopathological and biochemical responses of red and black raspberries and a hybrid between them to *A. americanum* colonization. It was observed that the pathogen germinated and colonized all studied raspberries, however, black raspberries and the hybrid had distinctive responses compared to *R. idaeus*. The compact mesophyll, the pre- and post-formed phenolic compounds, post-formed pectic compounds, and cell collapse in the lesioned area were the main defense mechanisms against *A. americanum*. Additionally, to structural and biochemical studies of raspberries defense responses, it was performed a comparative genomics analysis to identify the calcium-dependent protein kinases (CDPKs), focusing on the CPK28 orthologs in black raspberry (*R. occidentalis*) and strawberry (*Fragaria vesca* L.) as representative of Rosaceae. Both have 26 CDPKs together. The orthologs FvCPK28 and RoCPK28 were cloned and expressed in *Nicotiana benthamiana* and *Arabidopsis cpk28* mutants. It was found that both Rosaceae CPK28 orthologs are plasma-membrane localized, and their overexpression dampened the oxidative burst upon elicitation. These results provided a proof-of-concept to investigate the functional similarities between these proteins and Arabidopsis CPK28.

Keywords: CPK28, Defense mechanisms, Phenolic compounds, *Rubus idaeus*, *Rubus occidentalis*, *Rubus niveus*

1. GENERAL INTRODUCTION

Raspberries belong to the genus *Rubus* L. and occur naturally in all continents except Antarctica (Martin et al. 2018; Funt and Hall 2013). They are included within the family Rosaceae, along with blackberries, apples, pears, strawberries and several other species (Martin et al. 2018; Jung et al. 2019). Raspberries have been cultivated for centuries because of their nutritious fruit (druplets), as well as their herbal raw material rich in bioactive compounds (Oszmiański et al. 2015; Chwil and Kostryco 2021). Indeed, the global production of red and black raspberries has increased during the last couple of years to reach 822,000 tonnes in 2021 (Foster et al. 2019; Klewicka et al. 2020; FAO 2021). For this reason, local and global agricultural economies are highly dependent on maintaining production quality.

Rubus can be affected by a wide variety of diseases caused by viruses, fungi, and bacteria. More than 30 viruses and phytoplasmas affecting *Rubus* have been characterized in the last few years, and many others have not yet been identified (Funt 2013; Martin et al. 2018). Fungal diseases such as rusts are common in cane, fruits and leaves, and have the potential to cause severe yield loss (Hall et al. 2009; Funt 2013; Dolan et al. 2018).

Late leaf rust is a disease caused by *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart), an heteroecious and macrocyclic rust. Besides producing telia and uredinia in raspberries, white spruce (*Picea glauca* [Moench.] Voss) hosts the spermogonial and aecial stages of the *A. americanum* life cycle (Martin et al. 2018; Scholler et al. 2022). Although late leaf rust was once considered a minor concern, outbreaks have been reported in many locations of raspberry cultivation (Martin et al. 2018; Delisle-Houde et al. 2020; Oliveira 2021). The urediniospore stage is the only rust spore that causes reinfection, which may occur within a few days after sporulation and reach the same tissue or the neighbour plants (Aime et al. 2018; Duplessis et al. 2021). Due to this, they are persistent while finding optimal environmental conditions and are harder to control. Not surprisingly, late leaf rust has been found in raspberries in regions far from the white spruce occurrence, apparently overwintering on raspberries' aerial tissues (Martin et al. 2018).

Red raspberries (*Rubus idaeus* L.) are susceptible to late leaf rust, while black raspberries are considered immune (Luffman and Buszard 1989; Nelson 2011; Martin et al. 2018). It has been demonstrated that susceptibility varies among cultivars of *R.*

idaeus (Luffman and Buszard 1989; Hall et al. 2009). Even the black raspberry *R. niveus* is considered immune to *A. americanum* further studies are necessary to confirm this information. Moreover, based on the literature, the black raspberry *R. occidentalis* L. appears to have a contradictory host status. Even classified as black raspberry by Bushakra et al. (2012) and Foster et al. (2019) and immune to *A. americanum* by Dodge (1923), the occurrence of late leaf rust was previously observed (Martin et al. 2018).

Histopathological studies have helped clarify several mechanisms plants use during interaction with microbes (Braga et al. 2019; Navarro et al. 2019; Rasera et al. 2019; Rincón-Barón et al. 2020; Marques et al. 2022). In addition, the plant basal defense activates a cascade of responses performed by robust cellular machinery in response to the recognition of 'non-self' molecules (DeFalco and Zipfel 2021; Dias et al. 2022).

In order to control diseases, it is important to understand how plants respond to pathogens. In this context, the objective of this thesis was to uncover the structural and biochemical pre- and post-formed defense mechanisms of raspberries interacting with *A. americanum* (Chapters 2 and 3). In addition, calcium-dependent protein kinase CPK28 orthologs were identified and examined for their function in black raspberry and strawberry immunity (Chapter 4).

Chapter 2: Investigating biochemical and histopathological responses between raspberries and *Aculeastrum americanum*

Chapter 3: A new hybrid between red and black raspberry and its response to late leaf rust from a histopathological view

Chapter 4: Initial characterization of the calcium-dependent protein kinase CPK28 in black raspberry and strawberry

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2. INVESTIGATING BIOCHEMICAL AND HISTOPATHOLOGICAL RESPONSES BETWEEN RASPBERRIES AND *ACULEASTRUM AMERICANUM*

Abstract

Late leaf rust is a fungal disease in raspberries caused by *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart) leading to early defoliation and yield losses. Red raspberries (*Rubus idaeus* L.) are susceptible to this pathogen, even though this susceptibility varies among cultivars. In contrast, black raspberries were previously reported as more resistant (*Rubus occidentalis* L.) and immune (*Rubus niveus* Thunb.) to this pathogen, raising their importance in plant breeding programs. However, what features make them respond differently to the same pathogen? In this study, we characterized pre- and post-formed structural and biochemical defense mechanisms of *R. idaeus* 'Autumn Bliss', *R. occidentalis* and *R. niveus*. Ultrastructural and histopathological analyses were employed to uncover the interactions between these raspberries and *A. americanum*. The ultrastructural results indicated that the pathogen germinates on both leaf surfaces but can form appressoria only on stomata. Although the three raspberry species were infected and colonized by *A. americanum*, a clear difference in susceptibility was observed between them. A compact mesophyll, pre and post formed phenolic compounds, and post formed pectic compounds were the main plant defense mechanisms against fungal colonization. These findings provide new information about raspberries' defense mechanisms in response to *A. americanum* and elucidate the interactions occurring on these pathosystems.

Keywords: late leaf rust, *Pucciniastrum americanum*, *Rubus idaeus*, *Rubus occidentalis*, *Rubus niveus.*

Introduction

Late leaf rust, caused by *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart) affects red and purple raspberries (Martin et al. 2018; Scholler et al. 2022). The disease is hard to control and has caught attention after outbreaks in North American orchards (Martin et al. 2018; Delisle-Houde et al. 2020). It has also been reported as a concern in Argentina, Brazil, Chile, and Portugal (Figueiredo et al. 2003; Raseira et al. 2004; Lucero et al. 2008; Pio 2014; Oliveira 2021).

The main symptoms are powdery yellow spots, which correspond to reproductive structures called uredinia and are found in all aerial parts of infected plants (Dolan et al. 2018; Martin et al. 2018). Infected fruits become unfit for sale, and leaves may drop prematurely, causing severe yield loss (Hall et al. 2009; Funt 2013; Martin et al. 2018). Those are some outcomes of plant tissue colonization that lead to structural and physiological responses during the plant-pathogen interaction. However, it is still unknown which defense mechanisms raspberries employ against the colonization process by *A. americanum*.

Red raspberries (*Rubus idaeus* L.) are classified as susceptible to late leaf rust, even though this susceptibility varies among cultivars (Luffman and Buszard 1990; Nelson 2011). In contrast, black raspberries such as *Rubus occidentalis* L. and *Rubus niveus* Thunb. are immune to this pathogen (Martin et al. 2018). Nevertheless, late leaf rust has been documented in *R. occidentalis* (Dodge 1923; Darker 1929; Nickerson 1991).

Because raspberries are economically relevant crops on almost all continents (Foster et al. 2019; FAO 2021) diseases affecting these plants can not be neglected. Histopathological and biochemical studies have allowed the understanding of important pathosystems (Braga et al. 2019; 2021; Primiano et al. 2019; Rasera et al. 2019; Alves et al. 2021, Marques et al. 2022) and shed light on disease cycle and epidemiology studies (Nogueira Júnior et al. 2017; Boufleur et al. 2022; Dias et al. 2022).

Since no histopathological investigations have been performed yet on the infection and colonization processes of raspberries by *A. americanum,* this work seeks to elucidate whether there are differences in leaf anatomical and biochemical traits among red and black raspberries which may hinder/delay the infection of *A. americanum* in black raspberries leaves. This study focuses on uncovering pre- and post-formed defense mechanisms in red and black raspberries.

Conclusion

In conclusion, a more compact mesophyll, pre and post formed phenolic compounds, and post formed pectic compounds are the main defense mechanisms found in raspberries that played a role against *A. americanum.* Although raspberries had both preformed and post formed defense mechanisms, they were not sufficient to totally contain an infection and colonization by A. *americanum*. Based on the results, we confirmed the susceptibility of *R. idaeus* ‘Autumn Bliss’ to *A. americanum* and showed the absence of immunity for *R. occidentalis* and *R. niveus.*

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3. A NEW HYBRID BETWEEN RED AND BLACK RASPBERRY AND ITS RESPONSE TO LATE LEAF RUST FROM A HISTOPATHOLOGICAL VIEW

Abstract

Rubus idaeus 'Heritage' is a red raspberry cultivated worldwide for fresh or processed consumption. However, 'Heritage' is susceptible de late leaf rust, a disease caused by *Aculeastrum americanum* (Farl.) M. Scholler & U. Braun (syn. *Thekopsora americana* (Farl.) Aime & McTaggart). Genetic resistance is a promising way to manage this disease. Hybridization between red and black raspberries is commonly used for their genetic improvement. The wild black raspberry *R. niveus* is a reported as hight-value progenitor resistant to diseases. In fact, *R. niveus* exhibited remarkable histochemical responses to *A. americanum*. In this study, we characterized the leaf anatomy of a hybrid between *R. niveus* and *R. idaeus* 'Heritage' and its biochemical and histopathological responses to *A. americanum*. Our findings showed that the hybrid leaves inherited the morphology phenotype from *R. idaeus* and leaf anatomical traits from *R. niveus*. Only small pustules per leaf were observed 20 days after inoculation with *A. americanum*. In the lesioned area, the epidermal and mesophyll cells underwent collapses and exhibited phenol accumulation. This work sheds light on the histopathological interactions in the pathosystem *A. americanum*-raspberry and highlights traits that can help plant breeders target late leaf rust resistance in future cultivars.

Keywords: *Aculeastrum americanum,* defense mechanisms, phenolic compounds, *Pucciniastrum americanum, Rubus idaeus, Rubus niveus*

Introduction

Raspberries belong to the genus *Rubus*, a member of the globally-important Rosaceae family (Graham et al. 2018; Chwil and Kostryco 2020). As a result of consumer demand and research into their bioactive compounds, global production of red and black raspberries has increased during the last couple of years to reach 822,000 tonnes in 2021 (Bushakra et al. 2015; Foster et al. 2019; Klewicka et al. 2020; FAO 2021).

Plant breeding programs around the world have developed new cultivars in order to meet this demand and ensure fruit quality. Raspberry breeding focus on several agronomic traits, including pest and disease resistance (Hall et al. 2009; Zasada and Moore 2014; Foster et al. 2019). Because red and black raspberries are in the same subgenus *Idaeobatus* they have been crossed to improve traits (Hall et al. 2009; Martin et al. 2018; Foster et al. 2019). Successful hybridization has been reported between *Rubus idaeus* L. cultivars and the black raspberry *R. occidentalis* L. (Bushakra et al. 2012; Foster et al. 2019). Despite this, further research using other species of black raspberries remains to be done.

R. niveus Thunb. was previously classified as a high-value progenitor in North American breeding programs due to its cane and leaf disease resistance, among other agronomic and physiological characters (Finn et al. 2002; Zasada and Moore 2014). Indeed, *R. niveus* has displayed structural and biochemical distinctive responses to *Aculeastrum americanum*, the causal agent of late leaf rust in raspberries (Chapter 2). Although *R. idaeus* 'Heritage' is susceptible to late leaf rust, it is cultivated worldwide, performing high-yielding and good fruit quality (Luffman and Buszard 1990; Volk et al. 2013; Chapter 2). Combining these traits could result in new cultivars that are more productive and resistance to diseases.

Current work in an associated research group has performed classical breeding between different raspberry cultivars (Barbosa 2022) and species, including one between *R. niveus* as female parent and *R. idaeus* 'Heritage'. In our study, we aim to characterize the leaf morphoanatomy of the F1 hybrid and to investigate its histopathological and biochemical responses to *A. americanum*.

Conclusion

NH, as a new raspberry cultivar, is still in its early stages since other agronomic traits need to be explored. Nevertheless, considering that resistance to disease is one target in plant breeding, our results documented novelties about ultrastructural, histopathological, and biochemical responses of a hybrid offspring between *Rubus niveus* x *Rubus idaeus* 'Heritage' with potential resistance to *A. americanum.*

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4. INITIAL CHARACTERIZATION OF THE CALCIUM-DEPENDENT PROTEIN KINASE CPK28 IN BLACK RASPBERRY AND STRAWBERRY

Abstract

Plant diseases are a threat to the maintenance of biodiversity and the production of food. Throughout their evolutionary history, plants have evolved several mechanisms for dealing with environmental and biotic stressors. When plants recognize microbes, it triggers signals and responses by different pathways, including those with Ca2+-dependent protein kinases (CDPKs). These kinases are evolutionarily conserved, however, only a few have been described in the Rosaceae family, which includes important species such as raspberry, and strawberry. In this work, we use comparative genomics to identify CDPK orthologs in black raspberry (*Rubus occidentalis*) and strawberry (*Fragaria vesca* L.) as representative of the globally important Rosaceae family. We found that *R. occidentalis* and *F. vesca* have together 26 CDPKs. We focused subsequent analysis on those in subgroup group IV, due to their roles in plant immunity already described in other species such as rice, cotton and tobacco. To determine orthology with well-studied *Arabidopsis* subgroup IV CDPK, CPK28, we cloned and expressed FvCPK28 and RoCPK28 in *Nicotiana benthamiana* and *Arabidopsis cpk28* mutants to assess genetic complementation. We found that both Rosaceae CPK28 orthologs are plasma-membrane localized and their overexpression dampened the oxidative burst upon elicitation. Further investigations must be conducted to ascertain the functional role of Rosaceae CPK28 orthologs. Nevertheless, this study provided a proof-of-concept to investigate the functional similarities between these proteins and *Arabidopsis* CPK28.

Keywords: *Arabidopsis*, CDPK, *Fragaria vesca*, PAMP-triggered immunity, oxidative burst, *Rubus occidentalis*

Introduction

Raspberries and strawberries, both members of the Rosaceae family, are widely cultivated horticultural crops (Foster et al. 2019; Chebotar et al. 2022). Due to their relevance, healthy plants are essential to local and global agricultural economies. However, a variety of diseases can affect these plants, including viruses, fungi and bacteria (Silva et al. 2017; Martin et al. 2018). The black raspberry *Rubus occidentalis* L., for example, is seriously impacted by anthracnose (*Elsinoë necator*) and is reported as not immune to *Aculeastrum americanum*, the causal agent of late leaf rust (Martin et al. 2018; Chapter 2). Likewise, the woodland strawberry *Fragaria vesca* L. is vulnerable to diseases such as angular leaf spot and grey mold, caused by the pathogens *Xanthomonas fragariae* and *Botrytis cinerea*, respectively (Silva et al. 2017; Badmi et al. 2022).

Although plants can succumb to disease, resistance is the rule rather than the exception (Staskawicz 2001; Ávila-Méndez and Romero 2017). Plants have evolved several pre and pos-formed structural, biochemical and molecular mechanisms for dealing with environmental and biotic stressors (Jones and Dangl 2006; DeFalco and Zipfel 2021; Chapter 2; Chapter 3). If these pre-formed defense mechanisms are not enough to stop a pathogen invasion, the plant-pathogen interaction is raised to another level (Jones and Dangl 2006; Kaur et al. 2022). When plants recognize a pathogen, a cascade of responses takes place, resulting in changes in biological processes that can confer resistance to the pathogen (Couto and Zipfel 2016; Bentham et al. 2020).

The plant basal defense is activated by the recognition of pathogen-associated molecular patterns (PAMPs), which are perceived by pattern recognition receptors (PRRs), located on the plasma membrane surface (Hogenhout et al. 2009; Bentham et al. 2020; Defalco and Zipfel 2021). Upon recognition, PRRs form complexes with other proteins and/or receptor-like cytoplasmic kinases (RLCKs; Saijo et al. 2018, Bentham et al. 2020; Defalco and Zipfel 2021). The BOTRYTIS-INDUCED KINASE 1 (BIK1) is an RLCK considered a key plant immune signalling protein since it mediates immune responses triggered by several PAMPs (Monaghan et al. 2014; Dias et al. 2022). For this, BIK1 activity is fine-tuned through post-translational modifications such as phosphorylation (Dias et al. 2022).

The Ca^{2+}-dependent protein kinases (CDPKs or CPKs) are signal transducers that play a crucial role as regulators in biological processes in plants, such as innate immune responses (Bredow and Monaghan 2019; Crizel et al. 2020). Although these proteins were identified for several species, information for Rosaceae remains scarce (Cheng et al. 2002; Wang et al. 2018; Crizel et al. 2020).

The CDPKs are divided into four main groups that are evolutionarily conserved in plants but not found in animals and fungi (Boudsocq et al. 2010; Monaghan et al. 2014; Valmonte et al. 2014; Crizel et al. 2020). In group IV CDPKs, the CPK28 has been described as a negative regulator of PAMP-induced signalling and indicated as a hub in plant immunity responses (Bredow and Monaghan 2019; Delormel and Boudsocq 2019). Indeed, *Arabidopsis* plants CPK28 loss-of-function enhance the stress and immune response. Furthermore, previous studies have shown the possibility of dynamic regulation between CPK28 and BIK1, demonstrating that cellular homeostasis requires tight regulation (Monaghan et al. 2014; 2015; Wang et al. 2018; Wu et al. 2021).

Genome-wide comparisons between *Rubus* and *Fragaria* have supported high collinearity between these species (Bushakra et al. 2012; Foster et al. 2019; Wight et al. 2019). Since none of these mechanisms mentioned above are well understood in *R. occidentalis* and *F. vesca*, we aim to identify and analyze the CDPK family across these two Rosaceae species. Additionally, we seek to assess the conservation of group IV CDPKs using a transgenic approach to characterize and ascertain the functional role of the black raspberry and strawberry CPK28 orthologs.

Concluding remarks and future directions

This study has identified for the first time the CDPKs across *R. occidentalis* and *F. vesca* and encountered the CPK28 orthologs for these species.

The subcellular localization of FvCPK28 and RoCPK28 in *N. benthamiana* was an efficient tool to indicate their presence at the plasma membrane. Additionally, the elicitor-triggered oxidative burst assay performed in tobacco tissues expressing CPK28 orthologs was a proof of concept indicating that these proteins play the same biological role in different plants. Nevertheless, determining whether CPK28 orthologs can complement the immune phenotype of *cpk28-1* mutants requires more investigation.

Further studies must be conducted to ascertain the functional role of the Rosaceae CPK28 orthologs because the function of a protein can only be accurately explored by biochemical and structural investigations (Fang et al. 2010). For example, the orthology inferences can be integrated with gene expression patterns analysis and the CPK28 interactions with well-known partners in the same protein complex.

In conclusion, this work shed light on the significance of CPK28 role in immune signaling for the studied Rosaceae species.

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