

Coumarins: versatile secondary metabolites with diverse functions

Master writing assignment

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Abstract

Coumarins are plant secondary metabolites that have been found to play a role in a variety of plant processes. These functions include improving plant defence, assisting in iron uptake in alkaline soils, and having the ability to reduce radical oxygen species (ROS), reducing the amount of plant oxidative stress. The functions in defence and iron uptake in particular have been well characterised. The ROS mitigation function of coumarins has also been intensively studied, albeit often not in plants. This creates opportunities for plant coumarin research, as parts of that research can be extrapolated to plants. Application of coumarin research could in the future lead to crops that are better adapted to the environment they are in, by employing coumarin-based strategies to increase plant defence, reduce iron deficiency-based stress, and reduce damage from ROS produced by various stress types. In this review, the three main functions of coumarins will be extensively discussed, as well as the interplay that the mechanisms behind these functions may have.

Lay Summary

Coumarins are a group of substances that are released by plants when they experience certain environmental conditions. Coumarins have a variety of different functions, and are a popular field of study within plant science. For example, coumarins are released during an infection by harmful microbes (for example bacteria), in which case they function like a natural antibiotic. Coumarins also interact with beneficial microbes in a more positive matter, promoting their presence and providing various health benefits for the plant. In addition, they have also been shown to actively help with the uptake of iron by plants from the soil, which is a limiting factor for plant growth in many types of soil. Lastly, different antioxidant effects in both plants and animals have also been attributed to coumarins, making them not only interesting for plant science, but also for medical research. The great diversity of coumarin functions makes them an interesting topic of study for a variety of plant-related fields, and in the future these studies could help to make plants in agriculture more healthy and allow them to take up resources more efficiently, potentially allowing for higher and more stable food production.

Introduction

Secondary metabolites are metabolites that are produced by plants and have functions outside of the primary metabolic pathways that drive growth, development and reproduction. Nevertheless, many of these metabolites are still important for a number of essential processes that allow a plant to acclimate to its environment. Secondary metabolites can fulfil a range of different functions, including but not limited to plant defence, resource acquisition, communication and mitigating the damage of oxidative stress (Theis & Lerda, 2003).

Coumarins are a broad group of aromatic secondary metabolites in plants that play an important role in many of these processes (Stringlis et al., 2019). Coumarins are a compound family of benzopyrones that have a backbone structure that consists of a typical benzene ring fused to an α -pyrone ring (fig. 1) (Venugopala et al., 2013). Over 1300 coumarins and coumarin derivatives exist in nature, of which the simple coumarin group has been most extensively researched in a plant context. Simple coumarins are distinguished from complex coumarins, which include heterocyclic additions, and include the subgroups furanocoumarins, pyranocoumarins, phenylcoumarins, dihydrofurocoumarins and biscoumarins (Medina et al., 2015). Several coumarins from the simple coumarin group have been extensively studied in a plant context, including umbelliferone, esculetin and scopolin (fig. 1). The simple coumarins will be the main focus of this review, although several other coumarins may be mentioned if relevant in a specific context. A lot of research into coumarins has been performed in *Arabidopsis thaliana* (hereafter: Arabidopsis), but some other plants have also been used as model systems.

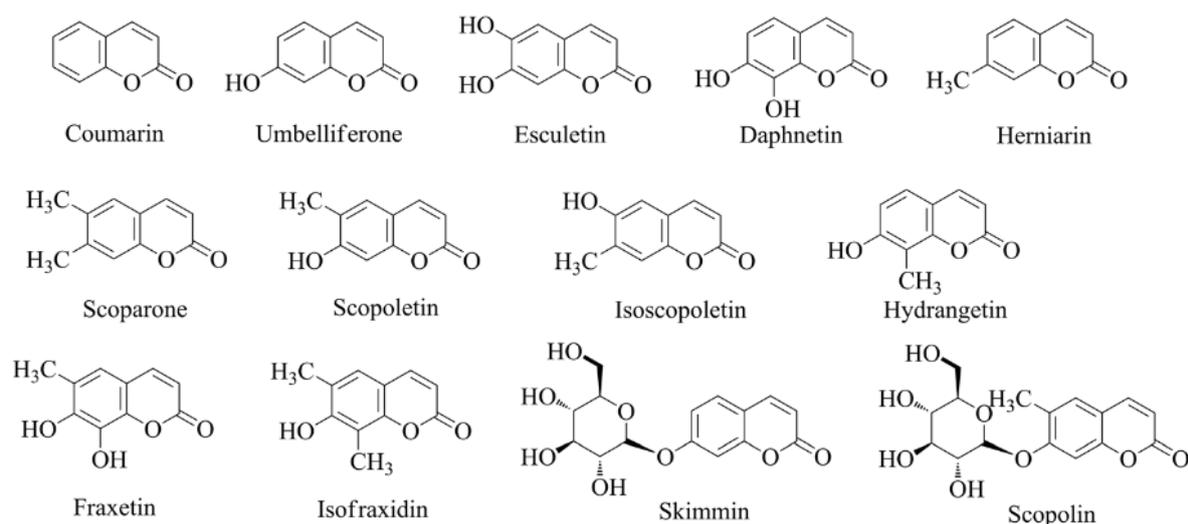


Figure 1 Structures of common simple coumarins. Includes several glucosylated coumarins, such as scopolin and skimmin (Chu et al., 2017).

Coumarins have been found to have multiple functions in plants. One of the most well-researched functions is their role in defence against pathogens, and their involvement in the induced system resistance (ISR), a broad resistance response that is induced by beneficial microbes and increases plant disease resistance against pathogens (Pieterse et al., 2014, Zamioudis et al., 2014). This antimicrobial effect and resistance-improving effect is of great interest when searching for methods for protecting crops from infections, and several studies have already shown that artificially applied coumarins and coumarin derivatives are effective

at protecting crops (Chen et al., 2016). In addition to their antimicrobial effects, coumarins also play a large role in mobilising iron for plant uptake if there is an iron shortage (Robe et al., 2021). Understanding this function is relevant for adapting crops to allow them to grow in iron-depleted soil, and may help better utilise these soils for agriculture. Coumarin secretion has also been linked to having a role in the plant response to phosphate shortages (Ziegler et al., 2016). Lastly, another role of coumarins and coumarin derivatives that has been researched for a relatively long time is their role in dealing with radical oxygen species (ROS) during oxidative stress in plants (Matos et al., 2017). In recent years, this topic in particular has attracted attention in medical research, as coumarins have also been found to exhibit these antioxidant functions when applied to animal cells (Borges Bubols et al., 2013). While the roles of coumarins in microbial interactions, iron mobilisation and ROS mitigation in plants have so far mostly been studied separately, these three topics combined make coumarins one of the most intensely studied groups of plant secondary metabolites of recent years, while large-scale practical application in for example agriculture is yet to occur.

Phenylpropanoid pathway and coumarin synthesis

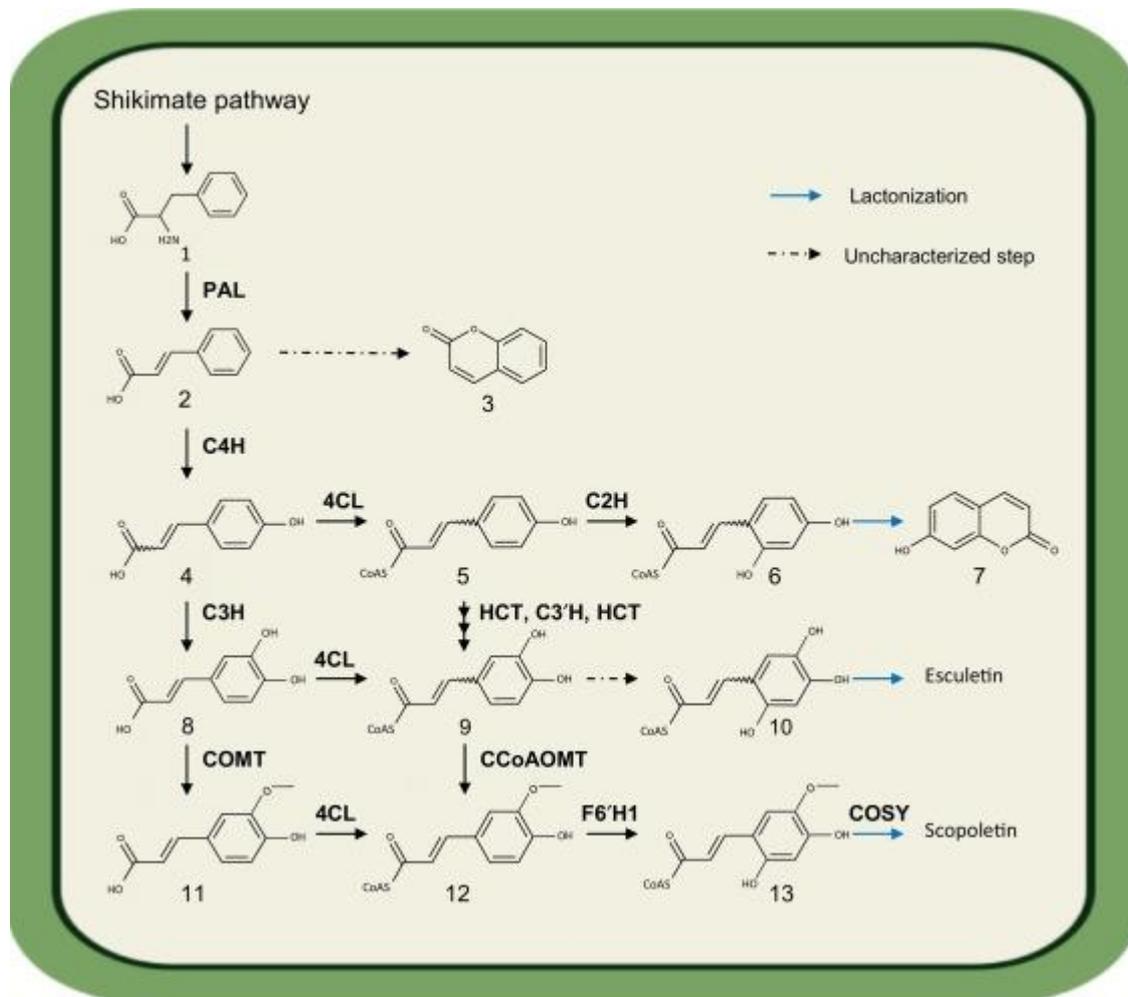
Coumarins are considered members of the phenylpropanoid family of compounds. Synthesis occurs via the phenylpropanoid Biosynthesis pathway, which is also responsible for many other secondary metabolites, including lignin and flavonoids (Biała & Jasiński, 2018). Understanding the coumarin biosynthesis pathway is important for identifying how certain regulatory mechanisms influence coumarin production.

The phenylpropanoid pathway starts with the production of phenylalanine, an aromatic amino acid, via the shikimate pathway (Vogt, 2010). Contrary to animals, plants are capable of synthesising phenylalanine by themselves by different mechanisms (Vogt, 2010, Yoo et al., 2013). As much as 20 to 30 percent of all fixated carbon in plants goes to the synthesis of phenylalanine, of which most feeds into the phenylpropanoid pathway. However, the vast majority of the phenylalanine in the phenylpropanoid pathway is eventually used for the production of lignins (Bonawitz & Chapple, 2010), a class of polymers primarily used for strengthening cell walls and creating wood-like structures. A smaller part of the phenylalanine is used for the biosynthesis of other secondary metabolites, including coumarins.

The branching point for the synthesis of different types of simple coumarins is cinnamate (fig. 2), an intermediate compound of the phenylpropanoid pathway that is produced directly from phenylalanine by the enzyme phenylalanine ammonia-lyase (PAL) (Sui et al., 2019). The compound coumarin, after which the coumarin family is named, is synthesised from cinnamate via a currently unknown pathway (Robe et al., 2021). Of the coumarins umbelliferone, esculetin and scopoletin, the synthesis pathway is known. Cinnamate is converted into three other types of hydroxycinnamates: p-coumaric acid, caffeate and ferrulate (Shimizu, 2014). These hydroxycinnamates are converted via intermediates into the coumarins umbelliferone, esculetin and scopoletin, respectively (fig. 2).

After the synthesis of the coumarin umbelliferone, a part of the synthesised umbelliferone is used in other pathways to produce furanocoumarins and pyranocoumarins (Shimizu, 2014). This is done via the addition of prenyl groups (Karamat et al., 2014). In addition, scopoletin has been found to be converted into the coumarin fraxetin (Rajniak et al., 2018). Subsequently, fraxetin is then converted into the coumarin sideretin. The final steps of simple coumarin production, trans-cis isomeration and lactonisation, have been confirmed to be performed by

a single enzyme, coumarin synthase (COSY), for the production of scopoletin (fig. 2) (Vanholme et al., 2019). It is possible that the isomerisation and lactonisation steps for the other simple coumarins are also catalysed by a shared enzyme.



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Figure 2: Coumarin biosynthesis via the phenylpropanoid pathway. Arrows indicate characterised enzymatic steps, broken lines indicate uncharacterized steps. Numbers denote intermediate compounds. 1: L-phenylalanine, 2: cinnamic acid, 3: coumarin, 4: p-coumaric acid, 5: p-coumaroyl-CoA, 6: 2-hydroxy-p-coumaroyl-CoA, 7: umbelliferone, 8: caffeate, 9: caffeoyl-CoA, 10: 6-hydroxycaffeoyl-CoA, 11: ferulic acid, 12: feruloyl-CoA, 13: 6-hydroxyferuloyl-CoA (Robe et al., 2021).

The main purpose of this literature review is to provide an extensive overview of the three main functions of coumarins in plants, as well as the molecular pathways underlying these functions. The first part will delve into the function that coumarins have in managing the plant microbiome. The second part will discuss the functions and mechanisms of coumarins in response to low iron availability. Finally, the role coumarins have in reducing the damage of oxidative stress in plants will also be outlined.

Chapter 1: Coumarins in plant-microbe interactions

One of the most well-researched aspects of plant coumarins is their role in managing the plant microbiome. In this chapter, several coumarins will be discussed in the context of their response to the microbiome. This will include the induction and regulation of biosynthesis, storage and accumulation. Finally, the effect the coumarins have on the microbiome itself for pathogenic and beneficial microbes will also be discussed. The coumarin scopoletin has been found to play a major role in plant defence and interactions with the microbiome (Stringlis et al., 2019), and the majority of research into coumarins in a plant-microbe interactions context focusses on this coumarin. Several other coumarins will also be mentioned if their role is known within that topic.

Triggering of coumarin production by MAMPs

Many different microbe-associated molecular patterns (MAMPs) have been identified as having a role in activating defence mechanisms. The general response of the plant to these MAMPs includes a switch of focus from growth to defence. Examples of MAMPs that induce defence responses include flagellin, lipopolysaccharide (LPS), and peptidoglycan (Kunkel & Harper, 2018). MAMPs have also been linked to increased coumarin concentrations. For example, the elicitor and MAMP flg22 has been found to induce the production of scopoletin (Schenke et al., 2011). The flg22 MAMP is recognised by the receptor flagellin-sensing 2 (FLS2) (Yi et al., 2014). The defence response triggered by flg22 is one of the most extensively researched mechanisms of microbial-induced coumarin production. Recently, a pathogen-induced glycosyltransferase UGT73C7 has been discovered that creates a response to infection by *Pseudomonas syringae* by redirecting the phenylpropanoid pathway towards coumarin production (Huang et al., 2021). UGT73C7 itself glycosylates p-coumaric acid and ferulic acid, and causes their increased synthesis, increasing the coumarin output of the phenylpropanoid pathway. This is an important example of the phenylpropanoid pathway being redirected towards coumarin production.

Glucosylation of coumarins

Under non-stress conditions, coumarins like scopoletin are generally stored in a glucosylated form, which for scopoletin is scopolin. Glucosylated forms of metabolites are often less active, and this is also the case for scopolin (Goy et al., 1993). Glucosyltransferases couple glucose derived from UDP-glucose to scopoletin, creating the glycoside scopolin (Fraissinet-Tachet et al., 1998). For scopoletin and esculatin, glucosyltransferases TOGT1 and TOGT2 have been shown to facilitate this conversion (Fraissinet-Tachet et al., 1998). While these glucosyltransferases are located in the cytosol, scopolin accumulates in the vacuole during low stress conditions (Werner & Matile, 1985). Under high stress conditions, including physical stress such as lesions and cell damage, scopolin is released from the vacuole. It is then in the cytosol converted back into active scopoletin by β -glucosidases that are present in the cytosol (Morant et al., 2008). One such β -glucosidase is BGLU42, which has been found to be essential for the conversion of scopolin into scopoletin (Stringlis et al., 2018). In addition, several other β -glucosidases, including BGLU21, BGLU22 and BGLU23 in *Arabidopsis*, have been found to be able to perform this conversion (Ahn et al., 2010).

Most β -glucosidases are compartmentalised; they are not present in the cytosol under normal circumstances to prevent too much scopoletin from accumulating in the cytosol and avoid

toxicity (Morant et al., 2008). The location where the β -glucosidases are compartmentalised outside of stress conditions varies between monocotyledons and dicotyledons. In monocotyledons, storage takes place in the plastids (often chloroplasts), and in dicotyledons, they are usually stored either in the apoplast or located inside protein bodies inside the cytoplasm (Morant et al., 2008). Only during stress conditions are both the β -glucosidases and coumarin glucosides such as scopolin present in the cytoplasm together, allowing the formation of the bioactive form in great numbers. For BGLU42 specifically, it is not known whether it is also compartmentalised.

β -glucosidases that hydrolyse scopolin are not exclusive to plants; at least one microbial β -glucosidase that can do this has also been identified (Deflandre & Rigali, 2022). The pathogen *Streptomyces scabiei* mass produces the phytotoxin thaxtomin A when colonising root and tuber crops, which is a compound that inhibits the biosynthesis of cellulose. The synthesis of thaxtomin A in the bacterium is inhibited by scopoletin. β -Glucosidase BglC has been identified in *S. scabiei*, and has been found to hydrolyse scopolin to scopoletin (Deflandre & Rigali, 2022). While it may seem counterproductive for the bacterium to initiate the production of antimicrobial compounds, this activity is performed inside the bacterial cytosol, allowing the amount produced to be controlled. How the scopolin enters the bacteria remains unknown. The suspected function of this mechanism is to prevent overproduction of thaxtomin A in this particular virulence strategy. This mechanism is an interesting recently discovered example of pathogens influencing coumarin availability as a regulation mechanism for their own virulence, and not necessarily focussing on the mitigation of coumarin damage.

Glycosylated coumarins like scopolin can be considered phytoanticipins, metabolites that are stored in a non-toxic form with the goal of using them in the future to provide an immediate response to acute danger, such as in the case of scopoletin invasion by herbivores or pathogens (Morant et al., 2008). Phytoanticipins are often contrasted against phytoalexins, which are compounds that are synthesised directly in response to microbial stress and are generally not present during low stress conditions. Scopoletin fits this definition, as it is produced from scopolin at the time of infection, as well as being *de novo* synthesised (Huang et al., 2021). Thus, the stored glycosylated forms of coumarins could be considered phytoanticipins, while the active forms could be considered as phytoalexins. Despite scopolin usually being regarded as an inactive form of scopoletin and not being as toxic to microbes, some evidence exists that scopolin does play a direct antimicrobial role in some interactions *in vitro* (Li & Wu, 2016). This effect was concluded to be directly due to scopolin, and not due to scopoletin. However, no follow-up studies on this topic have been performed yet that confirm or disprove these findings.

Molecular pathways of inducing coumarin accumulation during pathogen-induced stress

The MYB15 transcription factor has been confirmed to be necessary for *de novo* scopoletin production (Chezem et al., 2017). An increase in MYB15 presence has been shown to lead to increases in feruloyl-CoA 6'-hydroxylase 1 (F6'H1) enzyme synthesis. F6'H1 is an enzyme in the production pathway of scopoletin, and is a part of the phenylpropanoid pathway (fig. 1). Experiments in Chinese wild grape have shown that the *MYB15* promoter region is induced during the immunity response that is triggered by flg22 by itself, indicating that this elicitor and potentially others trigger the expression of *MYB15* (Luo et al., 2019). Thus, MYB15 and the

F6'H1 production that it induces appear to have an important role in increasing scopoletin production, and *MYB15* is upregulated in response to at least one MAMP.

MYB72 is a different transcription factor that is believed to be one of the major factors in inducing the ISR. *MYB72* production is induced by colonisation by certain microbes that also activate ISR (Van der Ent et al., 2008) (fig. 3). The transcription factor has been found to be essential for proper induction of the ISR. *MYB72* transcription factor has also been linked to scopoletin production (Zamioudis et al., 2014). β -glucosidase *BGLU42* was shown to be essential for the ISR-inducing effect of *MYB72*, and *BGLU42* is upregulated during the ISR. This upregulation of *BGLU42* is regulated by *MYB72* (Zamioudis et al., 2014). Since *BGLU42* can convert scopolin into scopoletin, it is likely that at least a part of the ISR response induced by *MYB72* is the result of increased scopoletin availability.

So far, it has not yet been identified how much *BGLU42* is present under non-stress conditions, and if so where this enzyme is stored. As mentioned earlier, many β -glucosidases related to plant defence have been found to be present during non-stress conditions in bodies such as the apoplast and plastids (Morant et al., 2008). If this would also turn out to be the case for *BGLU42*, *MYB72* may have a role in activating the transport of these enzymes into the cytosol, where they can convert scopolin into scopoletin. Although it is known that *BGLU42* is upregulated during the ISR (Zamioudis et al., 2014), it may be the case that in case of an infection, there is a combined effect of increased *BGLU42* production and increased localisation. This could also have a role in plant priming and the long-lasting effects of the ISR, as a high amount of *BGLU42* that is already present can cause a faster response to infection than *de novo* synthesis.

Hormonal regulation of coumarin production

There is strong evidence for the involvement of several hormones with the accumulation of coumarins during pathogen infection. The hormones jasmonate (JA) and salicylic acid (SA) have been found to be involved in scopoletin production via *MYB15* (Chezem et al., 2017). SA concentrations are increased in response to flg22 exposure (Yi & Kwon, 2014), and these increased concentrations have been linked to increases in *MYB15* induction (Luo et al., 2019). In addition, SA has been discovered to elicit accumulation of the coumarins umbelliferone and hernianin in leaves (Pastřiová et al., 2004). This indicates that SA has an important role in the production of multiple different coumarins. In addition to SA, *MYB15* was also observed to have increased transcription when JA was added (Balfagón et al., 2019). JA has been found to be necessary for scopoletin biosynthesis in tobacco plants, and is likely essential for its accumulation (Sun et al., 2014). Lastly, ethylene biosynthesis and signalling is also essential for scopoletin accumulation together with JA (Sun et al., 2017).

The production of scopolin from scopoletin has been found to be induced by synthetic auxin in tobacco plants (Hino et al., 1982). MAMPs have previously been found to cause reduced auxin sensitivity in plants (Navarro et al., 2006). This is achieved with the reduction of auxin receptor production by inducing a microRNA that negatively regulates receptor mRNA. The repression of auxin signalling, combined with the increase of JA and SA concentrations, is one of the key factors driving the plant focus shift from growth to defence in the case of an infection. The effect of auxin on scopoletin (and potentially also other coumarins) reflects that, as a focus on growth means in most cases mean defence is a lower priority. In the case of infection, the hormonal changes will cause scopolin to no longer be produced, which increases the amount

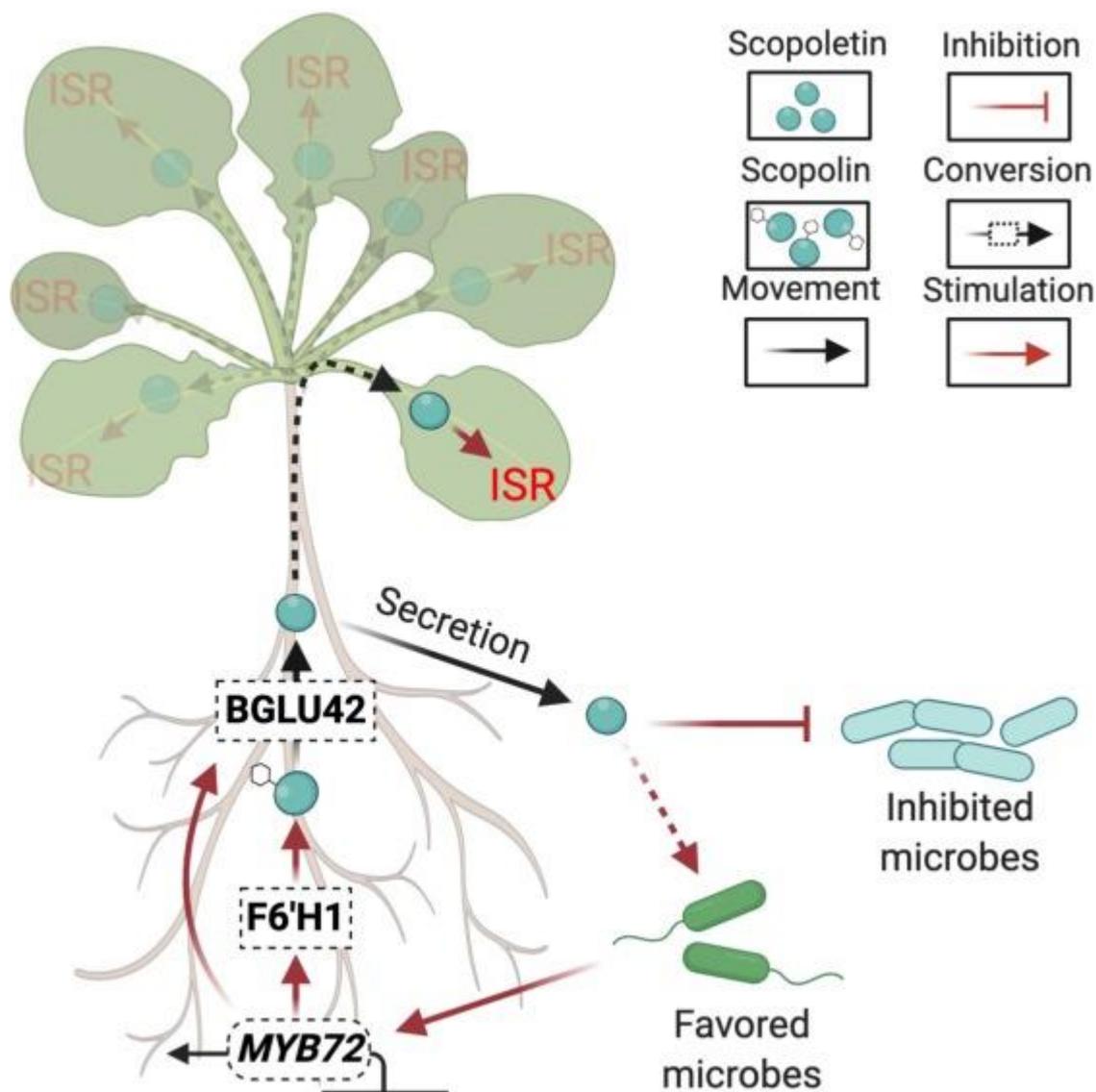
of free scopoletin. The interplay for all these hormones, SA, JA, ethylene and auxin, is a major topic of research in the plant-microbe interactions field, but so far it remains elusive how they interact with each other and the phenylpropanoid pathway in relation to coumarin biosynthesis.

Coumarin toxicity to pathogens

Many plant pathogens, including bacteria, fungi and oomycetes, have been shown to be highly sensitive to scopoletin, esculetin and other coumarins, often resulting in reduced reproduction, activity or growth of these pathogens (Valle et al., 1997, Sun et al., 2014). The toxicity of coumarins to microbes appears to originate from biochemical traits that differ between different coumarins. For example in the case of some coumarins, it is suspected that a methoxy group at the C7 position, and a hydroxy group at the adjacent C6 or C8 position play a major role in toxicity for bacteria (Kayser & Kolodziej, 1999). However, not all coumarins have these groups. Scopoletin (6-hydroxy-7-methoxycoumarin) is an example of a coumarin that has these groups. In some cases such as the coumarins tomentin and umckalin, the hydroxy group of C6 is replaced with a methoxy group, and this type of coumarin appears to be specifically toxic to certain specific bacteria including *Streptococcus pneumoniae* and *Haemophilus influenzae*. However, these coumarins showed reduced toxicity to other types of bacteria. Esculetin and umbelliferone both have a specific 7-hydroxy group instead of a methoxy group, and they showed a more broad effectiveness to the tested bacteria but showed less toxicity to the specific bacteria to which the C6 methoxy coumarins were effective. Scopoletin showed a similar effect to esculetin, but with a slightly higher toxicity to these specific bacteria as well, appearing to have one of the broadest ranges of toxicity against bacteria. The compound coumarin itself also has this broad range, while it has no special side groups, it was nonetheless effective against all tested bacterial strains (Kayser & Kolodziej, 1999). This may in part be caused by the lack of side chains, allowing for easier diffusion across pathogen membranes and cell walls (Kayser & Kolodziej, 1999, Johnson et al., 1989). The difference in toxicity against different groups of bacteria that different coumarins show suggest that having a large variety of different coumarins is important for plants to combat a broad range of pathogens, as not all coumarins are as effective against specific pathogens, with some having broader effects than others.

Beneficial microbes

Not all plant-microbial interactions involve pathogens. In many cases, microbes can provide beneficial effects to the plant, and one of these effects is an increase in plant resistance, which is believed to occur via ISR, or via the direct competition with soil-borne pathogens (Van Wees et al., 2008). Coumarins have been found to play a role in shaping the microbiome by selecting for beneficial, mutualistic microbes (Stringlis et al., 2018). Arabidopsis beneficial bacterium *Pseudomonas simiae* WCS417 for example, has been found to be substantially more tolerant to scopoletin compared to a number of soil-borne fungal pathogens (Stringlis et al., 2018) (fig. 3). In this case, the beneficial microbes have also been found to induce the excretion of coumarins by triggering the plant immune response, thus increasing the plant resistance and harming pathogens.



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Figure 3: Scopoletin and its effect on beneficial microbes and the ISR. Beneficial microbes in the root microbiome induce *MYB72* expression, which results in both more coumarin production via an increase of *F6'H1* activity, and an increase in coumarin conversion from their glucosylated form (in this case scopolin) into their active form (scopoletin). Scopoletin is secreted, which harms non-favoured microbes but not favoured microbes, thus selecting which microbes survive near the roots. In addition, scopoletin also activates the ISR response (Stassen et al., 2021).

How these microbes develop an increased tolerance to coumarins while pathogens fail to do so is still a matter of study, but recent research into bacterial transcriptome changes in response to coumarins have shown a role for genes that are involved in bacterial motility (Yu et al., 2021). Most of these genes have also been found to be required for root colonisation by *P. simiae* WCS417 (Cole et al., 2017). Several bacterial flagellar biosynthesis genes are downregulated in the beneficial bacterium during coumarin exposure, while generally being upregulated in other bacteria during colonisation (Yu et al., 2021). This suggests that coumarins act on the flagellum of the microbes, and that reducing the presence of these proteins is one of the methods by which *P. simiae* WCS417 avoids being targeted by coumarins. Due to the flagella and motility in general being important for colonisation, it is possible that this downregulation only occurs after initial colonisation of the roots. Recently, it has also been shown that resistance against coumarins like scopoletin can evolve within 6

months in the microbiome of *Arabidopsis* in the case of mutualistic microbes (Li et al., 2021). This proves that coumarin resistance can occur relatively easily in certain mutualistic bacteria if the right conditions are met. In addition to interfering with the flagellum, it has also been found that the compound coumarin itself has the ability to interfere with bacterial quorum sensing *in vitro* (Gutiérrez-Barranquero et al., 2015). Further research *in vivo* is needed to determine whether or not this ability plays a major role during plant infection by pathogens.

Application of coumarins as antimicrobial agents

The antimicrobial properties of coumarins and their abundance in nature make them interesting targets for societal situations in which antimicrobial compounds are needed. A large amount of research has been performed for the effectiveness of coumarins and coumarin derivatives against human pathogens. Due to their antimicrobial effects, coumarins and coumarin derivatives are actively considered as new antibiotics and antifungals for medical use (Kayser & Kolodziej, 1999). Many of these compounds are promising, and in particular coumarin antifungals have proven to be highly effective against human pathogens *in vitro* (Geweely, 2009, Prusty & Kumar, 2020). In addition to medical use, coumarins are also being considered as antibacterial agents in agriculture. The artificial application of the compound coumarin has proven effective against pathogen *Ralstonia solanacearum* in tobacco plants (Chen et al., 2016). In addition, coumarin-derivative seed coatings are also proposed to combat soil-borne pathogens, and have so far been shown to be effective (Brooker et al., 2008). These broad opportunities for application of coumarins for their microbial traits

Chapter 2: Coumarin roles in improving iron uptake

Iron is an essential nutrient for plant growth. Although iron is typically an abundant element in soils, not all forms of iron can be directly taken up by plants, limiting their ability to acquire this resource (Guerinot & Yi, 1994). Most iron that is absorbed by plant roots is in the form of Fe^{2+} ions. However, very little soil iron is available as Fe^{2+} . Most soil iron is in the form of iron hydroxides and iron oxides, which are insoluble (Lindsay & Schwab, 1982). This insolubility makes it difficult for plants to absorb them from the soil via the root system. Iron mobilisation is required, which involves the dissolution of iron-containing minerals to create Fe^{3+} , and reduce Fe^{3+} to Fe^{2+} , which is taken up by the roots. Compared to Fe^{3+} , Fe^{2+} is more soluble even at neutral or alkaline pH, making it more suitable for uptake (Kaplan & Ward, 2013). In soils with low pH values, the reduction of Fe^{3+} to Fe^{2+} is relatively easy, since Fe^{3+} is soluble under these circumstances. However, in alkaline soils with higher pH values, this solubility of Fe^{3+} is greatly reduced (Guerinot & Yi, 1994). This complicates iron uptake by plant roots in alkaline soils because it makes plants unable to reduce Fe^{3+} to Fe^{2+} , as well as making mineral dissolution more difficult. In alkaline soils, mechanisms are therefore required that increase iron mobilisation from the soil to avoid a plant iron deficiency.

One technique that is employed by plants to solve the solubility problem is causing a local acidification of the soil surrounding the roots (Lindsay & Schwab, 1982) (fig. 4). This response consists of using proton pumps such as *AHA2* to release a great number of protons from the roots, lowering the pH of the surrounding soil. The acidification has two functions: firstly, it causes iron-containing minerals such as the widely available ferric oxide (Fe_2O_3) to dissolve, producing Fe^{3+} ions (Lindsay & Schwab, 1982). Secondly, the Fe^{3+} is now more soluble due to the lower pH, allowing the membrane-bound plant enzyme ferric reduction oxidase 2 (*FRO2*) to reduce the now available Fe^{3+} to Fe^{2+} , which can then easily be absorbed by the roots via iron transporter *IRT1* (Jeong et al., 2017, Martín-Barranco et al., 2020). *FRO2* has previously been identified to be the rate-limiting step in iron acquisition via this mechanism (Connolly et al., 2003). This system may be sufficient for combating iron deficiency in neutral and mildly alkaline soils, but when the pH of the soil is too high, which is often the case in alkaline soils, the activity of *FRO2* is strongly reduced, limiting the amount of Fe^{2+} that can be produced (Martín-Barranco et al., 2020). In addition, the acidification caused by the proton pumps is not strong enough to provide a large enough pH shift for Fe^{3+} to become soluble. This is suspected to be one of the main reasons why iron deficiency in plants is more common in strongly alkaline soils.

Coumarin involvement via Fe^{2+} reduction

Thus, to reduce iron deficiency in alkaline soils, additional mechanisms are required. Studies have found that significant amounts of coumarins are excreted in the case of iron deficiency in plants (Clemens & Weber, 2016). Coumarins appear to be important in iron uptake under high pH conditions, in which case *FRO2* activity is low (Schmid et al., 2014). Coumarin biosynthesis mutant *f6'h1* has shown that coumarin production is essential for proper iron uptake in high soil pH conditions (Schmid et al., 2014). While this proves that coumarins play a role in iron uptake, the molecular function they have in this process is still relatively unclear, for which multiple methods have so far been suggested. One such method would be direct reduction of Fe^{3+} . The coumarins sideretin, fraxetin and esculetin have been found to have the

ability to reduce Fe^{3+} to Fe^{2+} (Rajniak et al., 2018). This method is generally referred to in iron uptake research as strategy I (Baune et al., 2020). This ability is similar to that of the FRO2 enzyme, which leads to the suggestion that coumarins may take over this function when FRO2 activity is reduced under alkaline conditions. However, they do not appear to have identical functions in iron uptake, as coumarin application is not able to rescue the iron deficiency phenotype of *fro2* mutant plants (Fourcroy et al., 2016). In addition, experiments have shown that in *f6'h1* coumarin biosynthesis mutants, the same increase in ferric reductase activity occurs during iron deficiency as in wild type Arabidopsis, indicating that coumarins do not impact this process (Schmid et al., 2014). These last two discoveries interestingly suggest that increasing the available amount of Fe^{2+} by reducing Fe^{3+} may not be the most important function of coumarins in iron uptake.

Iron mobilisation via chelation

If the iron-mobilising function of coumarins is not their own reducing ability and increasing Fe^{2+} availability alone, other coumarin effects must be partially responsible for the iron uptake increase. In vitro study has proven that coumarins can chelate Fe^{3+} (Mladenka et al., 2010). Chelation is the forming of a ring structure of a compound surrounding a metal, binding the metal via a dipolar bond. This chelation could for example be useful for a plant to control the localisation and uptake of iron. Generally, complexation of plant compounds with iron is referred to as strategy II for iron uptake, in contrast with strategy I, which involves direct reduction (Baune et al., 2020). How this chelation would relate to an improved iron uptake is currently a matter of speculation. Recently, coumarins such as fraxetin and esculetin that contain a catechol part, which consists of a benzene ring with two adjacent hydroxy groups (fig. 1), have been identified to be able to be taken up by plant roots (Robe et al., 2020). Therefore, it may theoretically be possible for the coumarins to be secreted, then chelate the Fe^{3+} , after which the coumarins are reabsorbed by the plant with the iron attached (fig. 4). However, such a mechanism is currently not known, and it may not be possible at all for a coumarin-iron complex to be taken up in such a way.

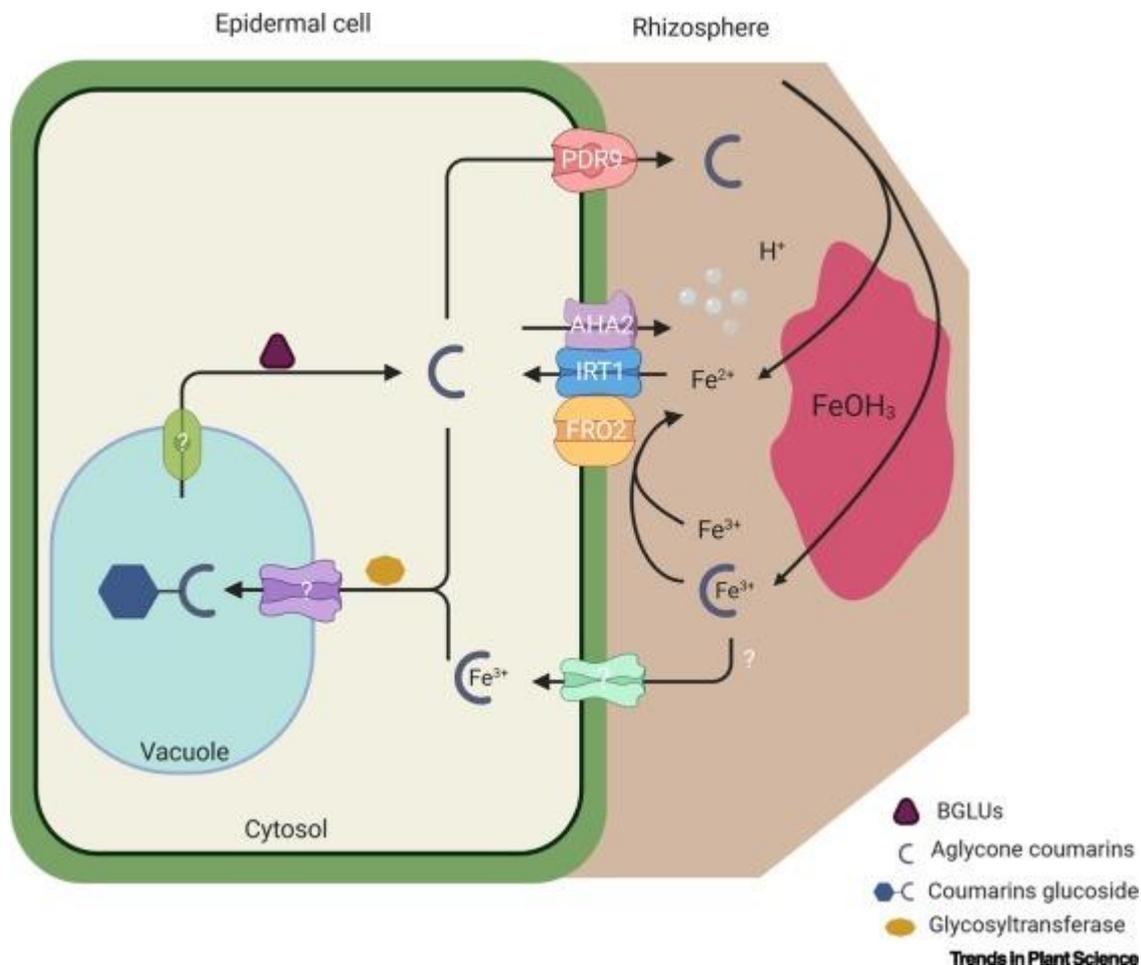


Figure 4: Different mechanisms of iron uptake. Without coumarin involvement, FeOH₃ is dissolved by acidifying the surrounding soil using the AHA2 proton pump. Fe³⁺ is then reduced to Fe²⁺ by FRO2, and Fe²⁺ is taken up via the IRT1 transporter. Alternatively, with coumarin involvement, coumarins are secreted via PDR9, after which they dissolve iron from FeOH₃. How iron transport into the roots is achieved in this case is uncertain, one way may be reduction from Fe³⁺ to Fe²⁺, while another option may be providing direct transport of Fe³⁺ into the cells via a currently unknown mechanism (Robe et al., 2021).

The role of coumarin oxidation products

Recently, a third method by which coumarins could assist in iron uptake has been proposed. It has been found that the oxidation products of coumarin may be of importance in mobilising iron (Baune et al., 2020). Because coumarins have the ability to reduce iron, this means that the redox counterpart, the coumarin itself, is oxidised. Oxidised coumarins are generally more reactive than their unoxidised counterparts. The different oxidation processes of coumarins include hydroxylation, quinone formation and dimerization (Maier et al., 2018, Baune et al., 2020), and many different varieties of these compounds are formed. These products tend to be more reactive than the original coumarins, including having better chelating properties (Baune et al., 2020). It was found that these coumarin oxidation products contribute to mobilisation of iron from iron-containing minerals *in vitro*, indicating that they could be at least partly responsible for the iron mobilising effect of coumarins in the soil (Baune et al., 2020). Therefore, the purpose of coumarins being able to reduce iron may not only be producing a supply of Fe²⁺, but also creating the oxidised form of the coumarin itself, which can have iron-mobilising properties.

Differences between coumarin effects

Research suggests that different coumarins have different functions and optimums in an iron-mobilising context. For example, scopoletin was previously thought to be unable to mobilise Fe^{3+} (Schmid et al., 2014). However, in contrast with earlier findings, recently it was found that scopoletin in fact causes the highest mobilisation of iron of tested coumarins under high pH (8.5) conditions with the iron-containing mineral lepidocrocite as substrate (Baune et al., 2020). Interestingly, different coumarins have shown to have different effectiveness at iron mobilising at different pH levels. Fraxetin, for example, was shown to be the most effective coumarin in mobilising iron at pH 4.5, but the least effective at pH 8.5, at which scopoletin was most effective (Baune et al., 2020). In the early study finding that scopoletin was ineffective in mobilising iron, pure iron hydroxide was used, while in the study that did find it was effective, different minerals were used (Schmid et al., 2014, Baune et al., 2020). This suggests that coumarin effectiveness in mobilising iron is not only dependent on pH, but also on the mineral form that iron assumes in the soil. In addition, these discoveries could also indicate that the different oxidation products of coumarins have different roles, as the oxidation products of scopoletin differ significantly from those of fraxetin and esculetin. This is mostly due to fraxetin and esculetin having a catechol part, while scopoletin does not, leading to different oxidation products (Baune et al., 2020). This may partly explain the differences in the iron uptake effectiveness.

Research has also shown that not all coumarins are secreted in the same place (Robe et al., 2020). While all coumarins have been found to be excreted via the MYB72-induced PDR9 transporter, different root tissues appear to excrete different types of coumarins (Fourcroy et al., 2016). Scopoletin, for example, has been shown to be mostly excreted by the root hairs (Robe et al., 2020). Fraxetin and esculetin, on the other hand, are mainly excreted by epidermal cells. The fact that different coumarins are secreted in different places further supports the observations that different coumarins have different roles in iron uptake.

Signalling under low iron conditions

Upstream regulator of IRT1 (URI) is a transcription factor that is currently suspected to be the primary recognition mechanism for low iron (Kim et al., 2019). During an iron shortage, phosphorylated URI accumulates, and likely forms dimers with IVc bHLH transcription factors, and becomes active. Downstream, its activity leads to the upregulation of for example *F6'H1* (involved in coumarin biosynthesis) and *BGLU42*, which both have been found to be important for the iron deficiency response in alkaline soils (Zamioudis et al., 2014, Palmer et al., 2013). *MYB72* is a direct target of URI, which likely accounts for a large part of the coumarin production-inducing effect of URI (Kim et al., 2019). However, the induction of *MYB72* is not just a consequence of direct binding of URI, as other transcription factors have also been identified to act on not just *MYB72* but also on *IRT1* and *FRO2*, which are also important for iron uptake (Robe et al., 2021). These genes are induced by FER-like iron deficiency-induced transcription factor (FIT), of which the production is induced by IRONMAN (IMA) transcription factors (Gautam et al., 2021). IMA transcription factor production is directly induced by URI. In addition, IMA activity appears to be dependent on pH, with there being higher activity under high pH conditions, which is also when iron mobilisation is more difficult (Gautam et al., 2021). Thus, IMA might be a factor that increases coumarin production based on high soil pH, likely due to increased chances of iron deficiency under those conditions.

The wide array of transcription factors so far found within this mechanism allow for strict regulation of *MYB72* induction. This serves two purposes: firstly, it would allow an increase in uptake under iron deficient conditions, and secondly, regulation of the uptake mechanism prevents iron concentrations from becoming dangerously high to prevent iron toxicity (Gao et al., 2020). The phosphorylation of URI appears to be somewhere at the beginning of the detection system, but how iron itself interacts with the regulatory mechanism, and whether or not iron directly interacts with this mechanism at all, remains unknown.

In conclusion, the exact molecular mechanism by which coumarins assist in iron uptake remains elusive. However, many different effects that coumarins and oxidised coumarin products have on iron and iron-containing minerals have been identified. It is likely that a combination of most of these effects is active to increase iron uptake under high pH conditions. At the same time, due to different coumarin activity at different pH levels and mineral substrates, it appears likely that different coumarins each have somewhat of a niche role in improving iron uptake under a particular pH and substrate conditions (Baune et al., 2020).

Chapter 3: Coumarins as ROS scavengers

Oxidative stress occurs in many different situations in plants, most commonly in response to environmental stresses. Oxidative stress is a consequence of the creation of ROS, which are highly reactive and have the potential to greatly damage cellular components (Chaki et al., 2020). A large number of mechanisms are in place to mitigate the damages of oxidative stress, partly consisting of ROS scavenging enzymes. However, there are also a variety of compounds such as flavonoids that can scavenge ROS (Chapman et al., 2019). A number of coumarins have also been found to have this ability, indicating yet another function of coumarins in a plant stress response (Payá et al., 1992a).

The function of coumarins in ROS scavenging appears to vary among different coumarins and different types of oxidative stress reactions. For example, some but not all coumarins have been found to be able to combat peroxidation of lipids, showing their ability to prevent cellular damage from oxygen radicals (Symeonidis et al., 2009). In addition, most coumarins have been discovered to have the potential to scavenge hydroxyl radicals, one of the most common ROS in plants (Payá et al., 1992b). Several coumarins, including esculetin, scopoletin, daphnetin and fraxetin, were found to also be effective at scavenging superoxide anions. Scopoletin specifically has been found to oxidise rapidly in the case of ROS produced in the case of an infection, reducing the amount of harmful ROS (Simon et al., 2014). In contrast to these effects, glucosylated coumarins typically show no scavenging activity (Payá et al., 1992b).

Fraxetin has been found to be able to scavenge hypochlorous acid (Payá et al., 1992b). A synthetic coumarin, 5,7-Dihydroxy-4-methylcoumarin, was found to perform this action even more effectively. Hypochlorous acid is not typically a radical that occurs in plants, but it is sometimes used in cultivation for its antimicrobial activity (Eryilmaz & Palabiyik, 2013). The fact that a plant coumarin is still able to scavenge it indicates that while different coumarins may scavenge different groups of radicals, the chemical ROS scavenging effect is not specific to only the ROS that occur during plant oxidative stress. This wide variety of ROS against which coumarins can be effective, not just plant ROS but also for example human ROS, is one of the main reasons for the interest in coumarins as antioxidants from medical research.

Antioxidant effects on the molecular level

In a medical context, antioxidant capabilities have been heavily studied (Matos et al., 2017). Coumarins have been linked to the antioxidant effects that some Asian traditional medicinal plants have (Thuong et al., 2009). However, different coumarins have very different ROS mitigation effectiveness when tested *in vitro*. Scopoletin, aesculetin, fraxetin, umbelliferone and daphnetin have been implicated as the coumarins from medicinal plants that have the strongest antioxidant properties. These coumarins all have in common that they possess a catechol group, which may be of particular importance in their radical scavenging activity (Thuong et al., 2009). This antioxidant activity of catechols has also been observed outside of coumarins (Cao et al., 2018). In addition, the α -pyrone ring shared among all coumarins makes them more hydrophobic than many other ROS scavengers, which improves their ability to neutralise lipid peroxy radicals (Thuong et al., 2009), which have their reactive part in the hydrophobic lipid tail. This may partly explain the large role coumarins have previously been found to have in mitigating the peroxidation of lipids (Symeonidis et al., 2009). Specific

coumarin chemical properties such as hydrophobicity could contrast coumarins from other ROS scavengers that are more hydrophilic in nature, for example phenolic acid (Aboul-Enein et al., 2007, Thuong et al., 2009). Because glucosylated coumarins have so far not been found to show no scavenging activity (Payá et al., 1992b), it is likely that coumarins need to be in their active, non-glucosylated forms to have their ROS-scavenging effect. These forms of coumarins are typically not present during low stress conditions. A large part of the increase of coumarin availability needed to combat ROS may occur as a secondary effect of the coumarins that are already produced during stress conditions, for example microbial stress or low iron stress (Clemens & Weber, 2016, Huang et al., 2021). As cellular damage also promotes release of glucosylated coumarins from the vacuole which are then converted into non-glucosylated forms, this may also be an important activation mechanism, as physical cell damage is typically associated with high oxidative stress (Morant et al., 2008). Cell damage is of course not only limited to microbial or iron deficiency stress, but can also be the consequence of many other types of physical stresses. Due to these overlaps in situations in which both the ROS scavenging effects and other coumarin effects are required, it is entirely possible that the activation mechanism of coumarins for combatting ROS at least partly overlaps with mechanisms for coumarin accumulation that have already been identified, but have not directly been implicated as having a role in ROS mitigation.

Not only coumarins themselves have been found to have an antioxidant effect. Coumarin analogue NFA is produced by the fungus *Aspergillus fumigatus*, an endophytic symbiont of rice plants (Qin et al., 2019). This symbiont is known to reduce rice yields and quality (Liang et al., 2015), but in the case of drought, it actually leads to increased drought tolerance compared to non-infected plants (Qin et al., 2019). One of the major causes of this has been found to be NFA secreted by the fungus, which was discovered to be able to reduce oxidative stress during drought in rice plants. A coumarin analogue having this effect during drought could be an indication that coumarins themselves can also play a role in reducing oxidative stress in this situation. This would further support the hypothesis that coumarins have a ROS mitigation role during a large variety of different stress conditions. Further research is needed into the effect of native plant coumarins under different types of stress than microbial stress and low iron stress, in particular abiotic stresses that involve high oxidative stress conditions.

Practical applications of antioxidant coumarins

The antioxidant functions of coumarins can be useful in a variety of ways. For example, high antioxidant contents in crops are often sought after for their health benefits. In addition to health benefits, knowledge about the antioxidant functions of coumarins may also contribute to preventing post-harvest deterioration of crops. For example, cassava, which is an important source of carbohydrates for people residing in the tropics, has a very limited shelf life of only one to three days. This short shelf life is mainly caused by deterioration that is started by an oxidative burst in response to root injury during harvest (Reilly et al., 2003). Scopoletin presence has been found to be greatly increased during this oxidative burst, and this coumarin was discovered to scavenge free radicals during this event, reducing the damage that the oxidative burst does. A stronger induction of scopoletin and potentially other coumarins such as esculetin likely leads to reduced deterioration, corresponding with longer shelf life of the cassava after harvest (Reilly et al., 2003). Selecting for this trait in crops therefore has the potential to make transport and sale easier, as well as reducing food wastage due to deterioration.

Discussion

In conclusion, the functions of coumarins are diverse, as are the mechanisms by which they operate. Coumarin research is also highly diverse, as many groups with different areas of expertise delve into coumarin research. This diversity of research angles is very useful, as it helps to create a more complete picture of all the plant functions of coumarins, as well as their biologically relevant chemical properties and the different regulatory mechanisms. Currently, as more and more becomes known about the regulation of coumarin activity, the question is raised how much of the coumarin pathways is shared between responses to different types of stresses that require coumarin action. This requires looking beyond any single type of stress when studying coumarin regulation, and will need comprehensive study also involving factors that have already been identified to be associated with coumarin activity, albeit for a different function. An example of factors that have already been found to be involved in multiple processes are MYB72 and BGLU42, which are involved in coumarin activity under both iron deficiency and microbial stress (Palmer et al., 2013, Van der Ent et al., 2008). More regulatory mechanisms may be shared between the two processes.

Recently, the similarities between the response to iron deficiency and microbial stress was further investigated, and as expected, plants suffering from an iron deficiency showed increased resistance against pathogens (Trapet et al., 2021). However, contrary to expectations, this effect was found to be independent of MYB72, suggesting a different shared mechanism that is triggered by iron deficiency and has an antimicrobial effect. However, overexpression of *BGLU42* in non-iron deficient plants caused a similar resistance effect to the iron starvation (Trapet et al., 2021). As the function of BGLU42 is activation of coumarins via deglycosylation, this does heavily imply coumarin involvement, albeit independent from MYB72 (Zamioudis et al., 2014). In addition, in a different study *Arabidopsis f6'h1* mutants failed to show the iron deficiency-induced resistance effect that the wildtype showed (Perkowska et al., 2021). Results from the same study suggested a stronger iron deficiency-induced resistance effect when tested with pathogens that employ iron-chelating siderophores to obtain iron from host plants during the infection process. This may imply that coumarins produced during the low-iron response decreases the amount of iron that the pathogen can acquire, potentially due to chelating most of the available iron with coumarins instead of siderophores.

Hormone interactions

Due to coumarin accumulation signalling mechanisms sharing components under different stress conditions, the hormonal regulation of coumarin production that has been described for coumarin production under microbial stress may also be applicable under other stress conditions. SA, JA and ethylene have been found to be involved in inducing coumarin accumulation, while auxin has an opposite effect, instead inducing the conversion of coumarins into glucosylated forms (Sun et al., 2014, Luo et al., 2019). This last effect is possibly applicable to coumarins in all three discussed functions, as auxin is often involved in shifting plant focus from defence and stress mitigation to growth (Naseem et al., 2015). In most cases when large amounts of growth occur, stress is generally low and demand for coumarins will also be low.

JA has previously been found to suppress several genes involved in the iron deficiency response (Maurer et al., 2011). Ethylene, on the other hand, likely positively regulates the iron deficiency response (Romera et al., 2011). SA is also required for this response (Shen et al., 2016). However, none of those hormones have been directly connected to coumarin production under low iron stress conditions. Despite the lack of research into this, all of the hormone effects appear to match their involvement in microbe-induced coumarin accumulation, with the exception of JA, whose function is opposite. The effect of JA in microbial stress conditions has been linked to the induction of the *MYB15* transcription factor gene, which induces the production of F6'H1 coumarin biosynthesis enzyme (Shen et al., 2016, Chezem et al., 2017). However, *MYB15* has so far not been proven to be induced during the iron deficiency response. The fact that *MYB15* may not be strongly involved in the low iron response could explain the discrepancy between the positive role of JA in the microbial stress response and the negative role of JA in the low iron stress response. For example, it could be that in the case of microbial stress, induction occurs via both *MYB15* and *MYB72*, while in the case of low iron stress there is a stronger dependence on *MYB72* alone. In conclusion, the exact crosstalk of hormones during low iron-induced coumarin accumulation remains elusive, and more research is needed to determine the exact roles of the hormones in this process.

ROS scavenging as a secondary effect

The role that coumarins have in ROS scavenging has so far mostly been researched separately from their plant functions in microbial interactions and iron deficiency. Mostly, this is because coumarin ROS scavenging activities mostly have been investigated for medical purposes. However, since coumarins are typically already present in the case of high stress conditions, this ROS scavenging role also makes sense in plants. While there are other mechanisms in plants that also have the function of ROS scavenging, coumarins have a strong potential to supplement these mechanisms in high stress conditions. Scopoletin for example has been found to have a dual role in both inhibiting pathogens and mitigating ROS damage in the case of infection (Simon et al., 2014). In addition, there is strong evidence that different coumarins have different effectiveness in combating certain ROS (Payá et al., 1992b).

Opportunities for future research

The large amount of research that has been performed into coumarins greatly improves our understanding of how they operate in plants. Despite this vast amount of available research, considerable gaps in knowledge about coumarin functioning still exist. For example, a lot of research has been performed into discovering why certain beneficial microbes develop coumarin tolerance, while others do not. Despite many hypotheses existing, it is still not fully understood how this tolerance develops. Another gap in knowledge concerns the method by which coumarins help in iron mobilisation. This process has been thoroughly investigated, but it is still unknown by which molecular mechanism the mobilisation actually takes place. More research into how coumarins interact with iron, minerals and plant roots on a molecular level is needed to shed light on how they contribute to iron mobilisation and uptake.

While the research in the iron mobilisation mechanism requires more study on the molecular level of coumarin activity, the opposite is true for the research into the ROS scavenging effects of coumarins. Studies in this field have mostly focussed on the molecular effects of the coumarins themselves due to their relevance for medicine, and rarely on the ROS scavenging role in plants and the regulation involved in that process. This has provided valuable information on how these coumarins can scavenge ROS, but there is very little information on

the actual scavenging role they have in plants. Specific studies that explore this aspect could give important new insights in how ROS-induced stress is managed in plants, in addition to the currently known mechanisms.

In conclusion, the number of studies focussing on coumarins are numerous. Many practical applications have already been proposed for coumarin research, and early studies into influencing coumarin presence in agricultural settings are promising. Applied coumarin research could lead to improved microbial resistance and reduced iron deficiency in crops. While certain key questions still remain before this becomes reality, future research will likely result in those questions being answered, and pave the way for new crops that are better adapted for the environment they are in.

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