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REVIEW

Could a cuticle be an active component of plant immunity?

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Abstract

The cuticle is the first physical barrier between the plant and the outer environment. The cuticle is no longer viewed as a rigid "inert sealer". Components of the cuticle were found to be responsive in their function and chemical composition to environmental signals. Cuticle creation is energy-consuming and complicated. Thus, cuticle composition and renewal dynamics are precisely regulated. Activated plant immunity is also energy "expensive". We briefly summarised our knowledge of the involvement of cuticle in plant-microbe interactions. Changes in cuticle amount and composition affect plant resistance to pathogens and treatment with cutin monomers triggers plant immunity. However, our knowledge about the effects of activated plant immunity on cuticle is scarce. We hypothesise that activated immunity influences cuticle dynamics. Our *in-silico* gene expression analysis revealed that cuticle biosynthetic genes are modulated under conditions simulating activated immunity. The analysis indicates that the cuticle is not just a rigid component of a plant reaction to the pathogen attack. Strengthening of the cuticle could prevent pathogen penetration. However, inhibition of cuticle production could save the energy needed for plant immunity. We propose questions which should be addressed in future research. Answering them would lead to a better understanding of plant defence against pathogens.

Keywords: cuticle dynamics, gene expression, microbe penetration, plant immunity, wax.

Introduction

Land plants (*Embryophyta*) evolved from green algae (*Charophyceae*) and first appeared about 450 to 500 million years ago (Niklas and Kutschera 2010, Niklas *et al.* 2017, Bhanot *et al.* 2021). Physical constraints of the environment, such as low air humidity, limited soil water content, low atmospheric CO_2 concentration, high UV radiation, or light intensity, created selective pressure on the evolution of plants functioning in the homoiohydric state (Edwards 1993). Therefore, the colonisation of the land was particularly enabled by the evolution of various morphological innovations and strategies. Here belongs also the development of a hydrophobic cuticle. Nevertheless, the cuticle is not a protective barrier only against abiotic stress. The cuticle, located on the entire plant surface, has evolved as the first site of interaction between plant and pathogen in the aerial environment (Pfeilmeier *et al.* 2016, Aragón *et al.* 2017, Lewandowska *et al.* 2020).

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Abbreviations: ACP - acyl carrier protein; CoA - coenzyme A; DAMPs - damage-associated molecular patterns; ER - endoplasmic reticulum; FAE - FA elongase; FAs - fatty acids; OX - overexpression; PAMPs - pathogen-associated molecular patterns; *Pto* DC3000 - *Pseudomonas syringae* pathovar *tomato* DC3000; ROS - reactive oxygen species; SAR - systemic acquired resistance; VLC - very long chain; VLCFA - very-long-chain fatty acids.
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The plant cuticle is a thin (typically 1 - 10 µm) extracellular hydrophobic membrane layer. It consists of a polyester cutin matrix and cuticular waxes. Cuticular waxes are composed of a complex mixture of lipidic compounds, mainly very-long-chain fatty acids (VLCFA) and their derivatives, such as aldehydes, ketones, alkanes, esters, and primary and secondary alcohols impregnating the cutin matrix. The main component of the cutin matrix is cutin, a three-dimensional polymer composed of C16 or C18 fatty acid derivates with hydroxy or epoxy substituents in the middle of the chain (Samuels *et al.* 2008, Fich *et al.* 2016, Ingram and Nawrath 2017, Bhanot *et al.* 2021).

The cuticle is formed on the surface of epidermal cells soon after the beginning of embryonic development (Szczuka and Szczuka 2003) and is then maintained throughout the whole plant life (Szczuka and Szczuka 2003, Ingram and Nawrath 2017). The cuticle was thought to be only a passive barrier deposited at an early stage and invariant for the rest of the leaf ontogeny (Bessire *et al.* 2007, Fich *et al.* 2016, Wang *et al.* 2020). However, scientists uncovered that the cuticle renews (Sachse *et al.* 2009, Kahmen *et al.* 2011, Gao *et al.* 2012, Kubásek *et al.* 2023) and changes its composition during the life of the plant (Jetter and Schäffer 2001, Neinhuis *et al.* 2001, Richardson *et al.* 2005). With the increasing age of plants, there are more alkanes with longer chains in the cuticle (Atkin and Hamilton 1982, Jenks *et al.* 1996, Jetter and Schäffer 2001). Cutin and especially waxes were found to be responsive in their function (permeability to water) and chemical composition to abiotic conditions and stresses such as drought (Karbulková *et al.* 2008, Macková *et al.* 2013, Shellakkutti *et al.* 2022), high humidity (Schreiber *et al.* 2001, Koch *et al.* 2006), abscisic acid treatment (Macková *et al.* 2013), temperature changes (Kerstiens 1996, Schreiber 2001), and mechanical wounding (L'Haridon *et al.* 2011). However, a little is known about the effect of biotic stress on cuticle composition or permeability.

Cuticle biosynthesis

Cuticle biosynthesis starts in the plastids of epidermal cells. The newly synthesised fatty acids (FAs) are transported from plastids to the endoplasmic reticulum (ER). On the ER, the synthesis diverges and proceeds separately for cuticular waxes and cutin. The synthesised wax precursors (*e.g.*, VLCFA) and cutin (C16 or C18 FA) are subsequently transported from the ER through the plasma membrane and cell wall into the cuticle, where they are incorporated into the final cuticle structure. In the text below, we summarise enzymes contributing to cuticle biosynthesis whose gene expression we monitored in our *in silico* analysis (Fig. 1).

Biosynthesis of FAs as precursors of cutin and cuticular waxes takes place exclusively in the plastid stroma of epidermal cells (Post-Beittenmiller 1996, Kunst and Samuels 2003, Ohlrogge *et al.* 2015). FA biosynthesis is catalysed by FA synthase (FAS) complex, which contributes to adding two-carbon moieties to the FA chain. The *de novo* synthesis of C16 and C18 FAs requires 7 - 8 cycles of addition of two-carbon moieties, which consume 14 - 16 NADPH molecules (Post-Beittenmiller 1996, Kunst and Samuels 2003, Samuels *et al.* 2008,

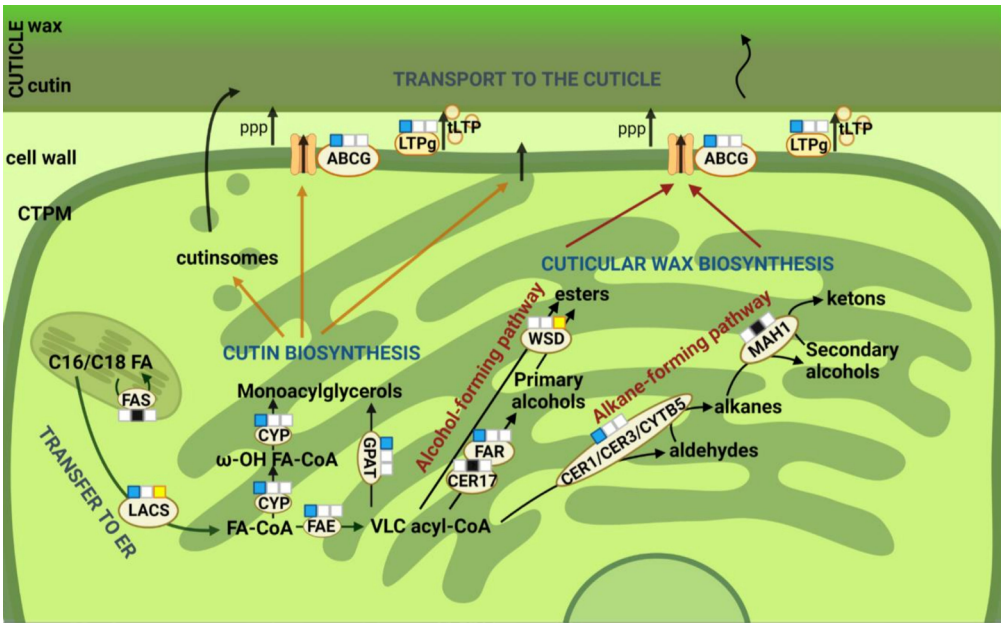

Fig. 1. Model of cuticle biosynthesis showing crucial proteins with their transcriptional levels under biotic stress. Oval beige structures with abbreviations represent proteins. The squares above the protein's abbreviations indicate the transcriptional level of their encoding genes under biotic stress. The left square is blue if the gene is inhibited, the middle square is black if there is no effect of the biotic stress on gene transcription, and the right square is yellow if the gene transcription of a certain gene is induced. The white colour indicates that this transcription status was not observed for certain genes. The transcriptional data were obtained from analysis in *Genevestigator*® (in more detail in Fig. 3) and the colour coding is the same as in Fig. 3. Fig. 1 was created in *BioRender*®.

Ohlrogge *et al.* 2015). The resulting chain of FAs then needs to be transferred from plastids to the ER. FA chains are subsequently activated on coenzyme A (CoA) acyl by long-chain acyl-coenzyme A synthases (LACSs) attached to the outer plastid membrane or the ER (Jessen *et al.* 2015, Fich *et al.* 2016, Zhao *et al.* 2021). The location of LACS orthologs may vary between species (Zhao *et al.* 2021). LACS9 is also supposed to play a role in trafficking between plastid and ER (Jessen *et al.* 2015).

At the ER, cuticle biosynthesis diverges and proceeds separately for waxes and cutin. In the biosynthesis of cuticular waxes, C16 and C18 FAs are activated into acyl-CoA and serve as precursors for very long chain (VLC) acyl-CoAs. The synthesis of VLC acyl-CoA is catalysed by enzymes comprising the FA elongase (FAE) complex elongating cyclically the acyl chain by two carbons with the consumption of NADPH (Cassagne *et al.* 1994, Samuels *et al.* 2008). The VLC acyl-CoAs are further modified *via* either the alcohol-forming pathway generating primary alcohols and wax esters, or the alkane-forming pathway creating alkanes, aldehydes, secondary alcohols, and ketones (Samuels *et al.* 2008). The VLC acyl-CoAs are converted by acyl desaturase ECERIFERUM17 (CER17) into n-6 monounsaturated FA, which is subsequently reduced to primary alcohols by fatty acyl-CoA reductase (FAR) (Rowland *et al.* 2006, Yang *et al.* 2017, Wang *et al.* 2020). Primary alcohols and VLC acyl-CoA can further be used as precursors of WSD1 (wax synthase/acyl-CoA:diacylglycerol acyltransferase1) for the formation of wax esters (Kunst and Samuels 2003, Samuels *et al.* 2008, Yeats and Rose 2013). VLC acyl-CoAs are modified to aldehydes and subsequently to alkanes by the CER1/CER3/CYTB5 complex (Bernard *et al.* 2012, Yeats and Rose 2013, Pascal *et al.* 2019, Wang *et al.* 2020). VLC alkanes can be further hydroxylated to secondary alcohols and secondary oxidised to ketones by a cytochrome P_{450} enzyme called MAH1 (midchain alkane hydroxylase1), which likely catalyses both reactions (Greer *et al.* 2007, Samuels *et al.* 2008, Wang *et al.* 2020).

For cutin biosynthesis in ER, C16 and C18 free FAs or acyl-CoAs are modified to oxygenated fatty acid-glycerol esters called monoacylglycerols (Pollard *et al.* 2008, Yeats and Rose 2013, Fich *et al.* 2016, Zhao *et al.* 2019). FAs activated by conjugation with acyl-CoA are subsequently ω-hydroxylated and hydroxylated, often with the participation of cytochrome P_{450} (CYP) enzymes (Pollard *et al.* 2008, Fich *et al.* 2016, Zhao *et al.* 2019). Finally, cutin monomers are formed by esterification, where acyl groups are transferred by glycerol-3-phosphate acyltransferase from acyl-CoA to glycerol-3-phosphate.

The transport mechanism of VLCFA and cutin (C16 or C18 FA) through the plasma membrane and cell wall into the cuticular membrane is still not fully understood. Part of the wax and cutin precursor transfer across the plasma membrane is mediated by ABC (ATP-binding cassette) transporters from the G subfamily (Yeats and Rose 2013, Fich *et al.* 2016, Li *et al.* 2016, Philippe *et al.* 2020, Stępiński *et al.* 2020, Wang *et al.* 2020, Bhanot *et al.* 2021, Xin and Herburger 2021). For cutin monomers have been also proposed a hypothetical possibility of transfer from the ER directly across the plasma membrane at points where the two membranes touch (Samuels and McFarlane 2012, Fich *et al.* 2016). The following transport step into the cuticle is the little-known transfer of hydrophobic cuticle precursors across the hydrophilic polysaccharide cell wall. This transfer may be facilitated by the coordinated action of two types of LTPs (lipid transfer proteins), LTPg and tLTPs (Edstam *et al.* 2011), but other types of LTPs are also likely involved in transport across the cell wall (Edstam *et al.* 2011, Yeats and Rose 2013, Fich *et al.* 2016, Li *et al.* 2016, Edqvist *et al.* 2018). Alternatively, cuticle precursors could be transferred *via* the cell wall using passive phase partitioning, in which cuticular precursors diffuse through the hydrophilic environment of the apoplast, accumulate outside and, due to their biophysical properties, self-assemble to form a hydrophobic cuticle (Fich *et al.* 2016).

After transferring cuticle precursors outside the cell wall, the components need to be assembled, arranged, and modified to form a functional cuticular membrane. The mechanism of these processes has not been fully elucidated. Cutin monomers need to be polymerised to form the cutin matrix of the cuticle. It has been shown that polymerisation can take place *via* an enzymatic pathway (Yeats and Rose 2013, Fich *et al.* 2016, Segado *et al.* 2020, Stępiński *et al.* 2020) or with the help of so-called cutinosomes, spherical nanostructures (40 - 200 nm) capable of self-assembly only through physicochemical processes pathway (Heredia-Guerrero *et al.* 2008, Fich *et al.* 2016, Segado *et al.* 2020, Stępiński *et al.* 2020, Bhanot *et al.* 2021, Xin and Herburger 2021). The cuticle itself has several layers. On the outermost surface of plants are epicuticular waxes, and intracuticular waxes are embedded in the cutin matrix along the cell wall.

The cutin matrix is mainly responsible for the mechanical strength of the cuticle (Riederer and Schreiber 2001, Shellakkutti *et al.* 2022), and polysaccharides incorporated into the cutin matrix may determine the elasticity and rigidity of the cuticle (Ziv *et al.* 2018). In most plants, the main transpiration barrier establishing cuticle permeability is probably the cuticular waxes (Cameron *et al.* 2006, Kosma *et al.* 2009), and cutin has a lower effect on permeability (Sieber *et al.* 2000). More recently, however, it has been shown that only intracuticular waxes establish the transpiration barrier, and epicuticular waxes have almost no effect on it (Zeisler and Schreiber 2016, Zeisler-Diehl *et al.* 2018). Even so, we cannot state that cuticle permeability depends only on intracuticular waxes, as the polarity gradient caused by the complex cuticular architecture is also important (Kamtsikakis *et al.* 2021).

Cuticle in plant-pathogen interactions

As mentioned above, the plant cuticle is the first physical barrier between the plant and the outer environment, thus protecting plants against abiotic and biotic stresses. The cuticle plays an important role in plant interactions with pathogens (Pfeilmeier *et al.* 2016, Aragón *et al.* 2017, Lewandowska *et al.* 2020).

Cuticles "help" pathogens to infect plants: Pathogens can use the cuticle for their benefit. Thus, the role of the cuticle is not only positive for plants during their interactions with pathogens. Pathogens do not try to pass through plant cuticle randomly. The cuticle's composition and the epicuticular waxes' crystallisation affect the pathogen's success in settling on the plant. The components of the cuticle (mainly VLC alkanes and aldehydes) can function as signals required for the pathogen's germination, development, and differentiation (Kolattukudy *et al.* 1995, Hansjakob *et al.* 2011, Serrano *et al.* 2014, Lewandowska *et al.* 2020). VLC aldehydes trigger the spore germination and appressorium differentiation of *Botrytis cinerea* (Ringelmann *et al.* 2009, Hansjakob *et al.* 2010, 2011), and primary VLC alcohols of avocado fruit induce the spore germination and appressorium differentiation of *Colletotrichum gleosporioides* (Podila *et al.* 1993). Cutin monomers induce appressorium germ tube formation of *Blumeria graminis* f.sp. *hordei* (Francis *et al.* 1996) and appressorium formation of *Magnaporthe grisea* (Gilbert *et al.* 1996). *Blumeria graminis* spores germinate less on the mutants in 3-ketoacyl-CoA synthase 6 (KCS6), enzymes important in FA elongation and further synthesis of epicuticular waxes in barley (Weidenbach *et al.* 2014), *A. thaliana* (Weidenbach *et al.* 2014), and wheat (Wang *et al.* 2019). Similarly, *B. graminis* f.sp. *tritici* spores germinate less on the mutants with nonfunctional enoyl-CoA reductase (ECR). ECR is responsible for the biosynthesis of wheat cuticular waxes. The same situation (lower germination of *B. graminis* spores) was shown for the mutants with impaired function in cyclin-dependent kinase 8 (CDK8) and wax inducer 1 (WIN1) with decreased wax components and cutin monomers in wheat leaves. Artificial application of wild-type cuticular wax on the surface of knock-out mutants in CDK8 and WIN1 restored *B. graminis* f. sp. *tritici* spores germination (Kong and Chang 2018). Epicuticular waxes from *Digitaria sanguinalis* (host) enhance the growth of germ tubes but do not affect the appressorium formation of *Culvularia eragrostidis*. On the other hand, the epicuticular waxes with different compositions from *Festuca arundinacea* inhibit the extension of germ tubes and the differentiation of *C. eragrostidis* appressoria (non-host) (Wang *et al.* 2008).

Cuticles help plants defend themselves against pathogens: The cuticle contributes to plant defence against pathogens. During their life, plants must interact with a broad spectrum of pathogens. Plants use passive (constantly present) and/or inducible defence strategies to defend themselves. Passive defence consists of chemical and mechanical barriers. On the other hand, inducible defence (plant immunity) requires recognition of a pathogenic attack, which is followed by triggering immune responses.

The cuticle's most widely considered and accepted role in the "battle" with pathogens is in the passive type of defence, where the hydrophobicity of the cuticle is exploited. The hydrophobic properties of the cuticle reduce adhesion forces between the plant surface and water drops (so-called lotus effect or self-cleaning process) (Barthlott and Neinhuis 1997, Watson *et al.* 2014). Such "running" drops of water rinse off spores, bacteria, or fungi, which prevents their adherence. They also remove dust and other particles that pathogens could use to survive on the plant surface. Such conditions make the life of pathogens on the plant surface challenging. Therefore, the plant cuticle and epicuticular waxes play a significant role in resistance to penetration and infection by potential pathogens (Bessire *et al.* 2007, Fich *et al.* 2016, Wang *et al.* 2020).

Some pathogens can bypass the obstacles mentioned above and enter the inner tissues through stomata, natural gaps, and wounded areas with external cuticle disruption or using their mechanical strength, such as fungal appressoria. Moreover, some pathogens can break the cuticular barrier by producing enzymes called cutinases, which hydrolyse ester bonds between cutin monomers. Other enzymes produced by pathogens are cutinolytic lipases, which cleave water-insoluble glycerides (Kolattukudy *et al.* 1995, Reis *et al.* 2005, Skamnioti and Gurr 2007, L'Haridon *et al.* 2011, Serrano *et al.* 2014, Wang *et al.* 2020, Arya and Cohen 2022). The cuticle disruption, either mechanical or enzymatic, is recognised by the plant and triggers its immune responses.

The crucial step for plant immunity is the correct and fast recognition of pathogen presence. The recognition could occur either on the plasma membrane or in the cytosol. In the cytosol, pathogen-specific molecules called effectors are recognised directly or indirectly. Pathogens actively secrete effectors into the cytosol, which inhibit plant immune responses (Jones and Dangl 2006). Regarding the cuticle, however, the plasma membrane-located recognition seems more relevant. Plasma membrane receptors called pattern-recognising receptors (PRRs) specifically recognise molecular patterns related to the pathogen attack. These could be pathogen-derived molecules (PAMPs, pathogen-associated molecular patterns) or plant-derived molecules (DAMPs, damage-associated molecular patterns) (Dodds and Rathjen 2010, Boutrot and Zipfel 2017). DAMPs are interesting in connection with the cuticle's role in plant immune responses.

The plant's perception of the degradation products of their cuticle can activate defence-related events: Based on this finding, cutin monomers and wax components were included among the DAMPs (Aragón *et al.* 2017, Ziv *et al.* 2018, Hou *et al.* 2019). However, the molecular mechanisms underlying cuticle fragments perception remain unclear and uncovering the receptor for cutin monomers would be a big step forward in this research area (Schweizer *et al.* 1996, Fauth *et al.* 1998, L'Haridon *et al.* 2011, Aragón *et al.* 2017, Ziv *et al.* 2018, Hou *et al.* 2019).

Changes in the cuticle affect plant resistance to pathogens

Different cuticular mutants have been investigated and the effect of an altered cuticle on the defence against different

pathogens has been observed. Higher susceptibility of *A. thaliana* to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) was described in *DEWAX-Ox*, *lacs2*, *att1*, *acp4*, *cer3-6*, *cer1-1* even though *CER1ox1* and *CER1ox2* mutants (Tang *et al.* 2007, Xia *et al.* 2009, Bourdenx *et al.* 2011, Aragón *et al.* 2021). Higher resistance to *Pto* DC3000 was observed in *dewax* and *myb96* mutants (Ju *et al*. 2017, Seo and Park 2010) (Table 1). For *dewax*, *lacs2*, *acp4* and *DEWAX-Ox*, the explanation of the effect on the resistance is modulated permeability of the cuticle (Tang *et al.* 2007, Xia *et al.* 2009) (Table 1). In *myb96* mutant is higher resistance very probably dependent on the induction of salicylic acid pathway (Seo and Park 2010).

Increased resistance of *A. thaliana* to biotrophic fungus *Golovinomyces orontii* was observed in *cer1-1*, *cer3-6*, and *cer3-8* mutant with reduced wax content (Table 1). On the other hand, the same mutant (*cer1-1*) exhibited higher susceptibility to the necrotrophic fungus *Sclerotinia sclerotiorum* (Bourdenx *et al.* 2011, Aragón *et al.* 2021). Similarly, *A. thaliana* plants expressing a fungal cutinase called CUTE were more susceptible to *S. sclerotiorum* (Table 1).

Most work focusing on the effects of cuticle changes on plant resistance to pathogens was done using necrotrophic fungi *Botrytis cinerea* (Table 1). Surprisingly, *A. thaliana* mutants with increased cuticle permeability (*lacs2*, *lacs2.3*, *lcr*, *bdg* or *DEWAX-Ox*) were more resistant to *B. cinerea* (Sieber *et al.* 2000, Bessire *et al.* 2007, Tang *et al.* 2007, Voisin *et al.* 2009, L'Haridon *et al.* 2011, Aragón *et al.* 2021) (Table 1). This was confirmed also by treatment with cutinase or by mechanical damage which both increased cuticle permeability and improved resistance to *B. cinerea* (L'Haridon *et al*. 2011). In all cases (genetic mutation, cutinase treatment, mechanical damage) was observed induction of plant immune responses such as reactive oxygen species (ROS) production and faster callose accumulation, which was put into the connection with observed higher transcription of genes encoding two co-receptors BAK1 (*Brassinosteroid-associated kinase 1*) and BIK1 (*Botrytis-induced kinase 1*) that play an important role in plant immunity (Liu *et al.* 2017, Aragón *et al.* 2021). Enhanced immune response is the explanation for *A. thaliana* increased resistance to *B. cinerea.* Higher susceptibility to *B. cinerea* was observed in *dewax*, *A. thaliana* mutant with lower cuticle permeability (Ju *et al.* 2017) (Table 1). CER1 lineage mutants are the most counterintuitive (Bourdenx *et al.* 2011). Although the *cer1-1*, *CER1ox1*, and *CER1ox2* mutants have opposite effects on cuticle permeability, which is increased and decreased, respectively, compared with WT, their impact on plant susceptibility to pathogens are the same. *CER1ox1*, *CER1ox2*, and *cer1-1* mutants are more susceptible to most pathogens tested compared with WT. This may indicate the importance of proper alkane formation for proper cuticle function, regardless of the amount of alkanes.

In general, higher permeability of the cuticle increased susceptibility to bacterial pathogen *Pto* DC3000 but surprisingly led to higher resistance against necrotrophic fungus *Botrytis cinerea.* This may be explained by the fact that plants can enhance the increased permeability of the cuticle to increase the efficiency of transporting antimicrobial agents to the pathogen. For example, *A. thaliana* infected with *B. cinerea* accumulates fungal cutinases, thereby increasing its cuticle permeability (Ju *et al.* 2017). Moreover, through the function of DEWAX, the plant ensures that cuticle permeability is not reduced by its production of additional waxes during the infection (Go *et al.* 2014, Ju *et al.* 2017). We propose this mechanism may be advantageous when the pathogen has already overcome the cuticle, so there is no need to improve the already "outdated armour". The increase in cuticle permeability may act as an amplifying loop, whereby increased permeability increases the number of DAMPs inducing a higher immune response. Nevertheless, the mechanism by which the cuticular membrane's composition, permeability, and structure affect the course of infection and plant immunity is still not fully understood.

Changes in the cuticle modulate jasmonic and salicylic acid signalling: The metabolism of FAs plays an important role in plant defence and is involved in the cross-talk with diverse phytohormones, including salicylic acid, a phytohormone important for plant immunity (Kachroo and Kachroo 2007, 2009). It was recently suggested that the cuticle biosynthesis pathway may be genetically connected with salicylic and jasmonic acid biosynthesis pathways. Li and coworkers studied cotton (*Gossypium hirsutum*) *GhWIN2*, a transcription factor from the *WIN* gene family that regulates the expression of cuticle biosynthesis-related genes and promotes cuticle formation. They showed that silencing of the *GhWIN2* gene increased salicylic acid content, resulting in enhanced resistance to *Verticillium dahliae* and suppressing the expression of jasmonic acid (Li *et al.* 2019). Zhao *et al*. (2021) showed that cuticle and phytohormonal biosynthesis are connected *via* the vesicle trafficking system. They studied the *A. thaliana* knock-out mutant *Atmin7.* MIN7 is an important component in the vesicular trafficking system. *Atmin7* had a thinner cuticle, reduced load of cutin, decreased content of phytohormones (salicylic acid, jasmonic acid, and abscisic acid), and increased susceptibility to the pathogen *Pto* DC3000. The artificial addition of these three phytohormones to *Atmin7* helped to restore cuticle formation and gave the resistance to *Pto* DC3000 back to WT level.

Interestingly, an intact cuticle may also play a role in the induction of systemic acquired resistance (SAR). Since the cuticle-defective mutants (*acp4* - acyl carrier protein, a part of FA biosynthesis complex, *lacs2* - long-chain fatty acid synthetase 2, *lacs9* - long-chain fatty acid synthetase 9, and *mod1* - FA biosynthetic enzyme enoyl ACP reductase) and plants with mechanically damaged cuticles failed in induction of SAR and have increased susceptibility to infection (Xia *et al.* 2009, Lim *et al.* 2020). However, these plants with negatively affected cuticle biosynthesis (*acp4*, *lacs2*, and *mod1*) do not lose the ability to generate the SAR-triggering mobile signal at the site of infection. Still, they cannot induce SAR in distal tissues. Lim *et al.* (2020) showed that cuticle-defective mutants are impaired

Table 1. Cuticular mutants with modified responses during the plant-microbe interaction (inspired by Aragón *et al*. 2017). Permeability: + = higher permeability than wt, - = lower permeability, resistance: + = higher resistance, - = lower resistance, 0 = no change. ox - overexpression, VLC - very-long-chain, TF - transcription factor, FA - fatty acid. **Pseudomonas syringae* pv. *tomato* DC3000, ***Pseudomonas syringae* pv. *maculicola*, **** Blumeria graminis* f. sp. *hordei*. [1] Bourdenx *et al*. 2011, [2] Aragón *et al*. 2021, [3] Inada and Savory 2011, [4] Lee *et al*. 2016, [5] Ju *et al*. 2017, [6] Blanc *et al*. 2018, [7] Zeier *et al*. 2004, [8] Xiao *et al*. 2004, [9] Tang *et al*. 2007, [10] Bessire *et al*. 2007, [11] Voisin *et al*. 2009, [12] L'Haridon *et al*. 2011, [13] Bessire *et al*. 2011, [14] Chassot *et al*. 2007, [15] Chassot *et al*. 2008, [16] Seo and Park 2010.

Mutant	Gene (gene locus)	Gene product	Affected component (permeability)	Pathogen	Pathogen type (kingdom)	Resistance	Ref.
cer1-1	*CER1/CER22* (AT1G02205)	wax biosynthetic gene for VLC-aldehyde decarbonylase - VLC alkane forming	wax (+)	*Golovinomyces orontii*	biotroph (fungi)	+	1,2
				Pto DC3000*	biotroph (bacteria)	–	1,2
				Sclerotinia sclerotiorum	necrotroph (fungi)	slightly –	1,2
cer1-4	*CER1/CER22* (AT1G02205)	wax biosynthetic gene for VLC-aldehyde decarbonylase - VLC alkane forming	wax (+)	*Botrytis cinerea*	necrotroph (fungi)	–	2
CER1ox1	*CER1/CER22* (AT1G02205)	wax biosynthetic gene for VLC-aldehyde decarbonylase - VLC alkane forming	wax (–)	*Pto* DC3000*	biotroph (bacteria)	–	1
				Sclerotinia sclerotiorum	necrotroph (fungi)	–	1
CER1ox2	*CER1/CER22* (AT1G02205)	wax biosynthetic gene for VLC-aldehyde decarbonylase - VLC alkane forming	wax (–)	*Pto* DC3000*	biotroph (bacteria)	–	1
				Sclerotinia sclerotiorum	necrotroph (fungi)	+	1
cer3-6	*CER3/WAX2/YRE* (AT5G57800)	wax biosynthetic gene for VLC acyl-CoA reductase	wax (+)	*Golovinomyces orontii*	biotroph (fungi)	slightly +	3
				Pto DC3000*	biotroph (bacteria)	–	4
				Alternaria brassicicola	necrotroph (fungi)	–	4
				Botrytis cinerea	necrotroph (fungi)	–	2,4
cer3-8	*CER3/WAX2/YRE* (AT5G57800)	wax biosynthetic gene for VLC acyl-CoA reductase	wax (+)	*Golovinomyces orontii*	biotroph (fungi)	slightly +	3
dewax	*DEWAX* (AT5G61590)	TF repressing cuticular wax biosynthesis	wax (–)	*Pto* DC3000*	biotroph (bacteria)	+	5
				Botrytis cinerea	necrotroph (fungi)	–	5
DEWAXox	*DEWAX* (AT5G61590)	TF repressing cuticular wax biosynthesis	wax (+)	*Pto* DC3000*	biotroph (bacteria)	–	5
				Botrytis cinerea	necrotroph (fungi)	+	5
eca2	*ECA2* (AT4G00900)	an early elicitor-response RING-H2 zinc-finger gene	wax + cutin (+)	*Phytophthora brassicae*	biotroph (fungi)	–	6
				*Psm***	biotroph (bacteria)	+	6,7
				Botrytis cinerea	necrotroph (fungi)	+	2,6
acp4	*ACP4* (AT4G25050)	plastid acyl carrier protein - FA biosynthesis	cutin (+)	*Pto* DC3000*	biotroph (bacteria)	–	8
att1	*ATT1/CYP86A2* (AT4G00360)	cytochrome P450s of the CYP86A subfamily - FA hydroxylase activity - cutin monomers	cutin (+)	*Pseudomonas syringae*	biotroph (bacteria)	–	8
				Botrytis cinerea	necrotroph (fungi)	+	9
bdg	*BDG/CED1* (AT1G64670)	α/β-hydrolase fold protein superfamily - ester-bound cutin monomers assembly	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	2,9–12
fdh	*FDH/KCS10* (AT2G26250)	β-ketoacyl-CoA synthase - FA elongation	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	11

Mutant	Gene (gene locus)	Gene product	Affected component (permeability)	Pathogen	Pathogen type (kingdom)	Resistance	Ref.
lacs2-2	*LACS2/SMA4/BRE1* (AT1G49430)	long-chain FA synthetase 2 - ω-hydroxy fatty acyl-CoA intermediates in cutin synthesis	cutin (+)	*B. graminis* f. sp. *hordei***	biotroph (fungi)	0	10
				Erysiphe pisi	biotroph (fungi)	0	10
				Golovinomyces orontii	biotroph (fungi)	0	10
				Pto DC3000*	biotroph (bacteria)	–	9
				Aleternaria brassicicola	necrotroph (fungi)	0	10
				Botrytis cinerea	necrotroph (fungi)	+	2,9–12
				Plectosphaerella cucumerina	necrotroph (fungi)	0	10
				Sclerotinia sclerotiorum	necrotroph (fungi)	+	10
lacs2-3	*LACS2* (AT1G49430)	long-chain FA synthetase 2 - ω-hydroxy fatty acyl-CoA intermediates in cutin synthesis	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	2,9–12
lcr	LCR/CYP86A8 (AT1G27340)	cytochrome P450s of the CYP86A type - FA hydroxylase activity - cutin monomers	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	2,9–12
pec1	*PEC1/ABCG32* (AT2G26910)	ATP binding cassette 32 transporters - exporting cutin precursors	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	13
CUTE	-	fungal cutinase-expressing	cutin (+)	*B. graminis* f. sp. *hordei****	biotroph (fungi)	0	10
				Erysiphe cichoracearum	biotroph (fungi)	0	10
				Hyaloperonospora parasitica	biotroph (oomycota)	0	10
				Phytophthora brassicae	biotroph (oomycota)	0	10
				Alternaria brassicicola	necrotroph (fungi)	–	14
				Botrytis cinerea	necrotroph (fungi)	+	14
				Plectosphaerella cucumerina	necrotroph (fungi)	–	14
				Sclerotinia sclerotiorum	necrotroph (fungi)	–	14
WT	-	cutinase application	cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	12
WT	-	mechanical damage	wax + cutin (+)	*Botrytis cinerea*	necrotroph (fungi)	+	12,15

in long-distance accumulation of salicylic acid, a crucial molecule in SAR, due to increased transpiration and reduced water potential. It indicates an active role of the plant cuticle in SAR-related molecular signalling.

Does activated plant immunity affect the cuticle?

Although the involvement of the cuticle in plant defence against pathogens is undisputed, many issues remain unresolved. This leads us to several questions – the answers to which would bring novel and important insights into plant-pathogen interactions.

The changes in cuticle composition/structure/rigidity can induce immune responses (Fig. 2). However, less is known about the effects of activated immunity on cuticle dynamics, composition, and quantity. The question is whether plants actively modify their cuticle structure, permeability, and properties in response to the presence of the pathogen. If yes, what is the purpose of this strategy? A more permeable cuticle could lead to higher resistance or higher susceptibility to the pathogens (Table 1). Is a strengthening of a physical barrier or its weakening for volatiles and other chemical compounds necessary for plant defence signalling? And is this actively driven process within plant immunity?

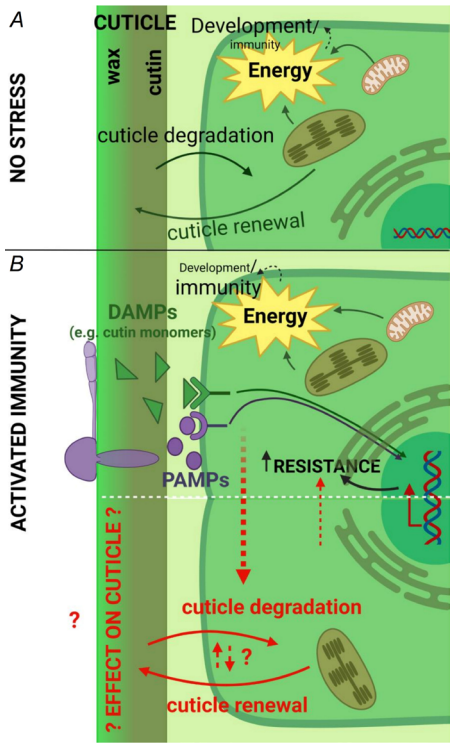

Fig. 2. Schematic overview of missing knowledge of cuticle dynamics under biotic stress. *A* - Plant cells under normal ("no stress") conditions when energy is mainly used for life-sustaining, plant growth, and development. The cuticle dynamics was measured for such plant cell stage, and cuticle renewal/degradation was demonstrated. *B* - Plant cells with activated immunity (under biotic stress), here triggered by fungal infection. The energy is prioritised for immunity rather than for development. PAMPs and DAMPs are recognised by receptors and trigger immune responses, resulting in increased resistance against pathogens. Among DAMPs belong to cutin monomers. Below part of (*B*) are highlighted gaps in our knowledge about the connection between plant immunity and cuticle dynamics. The cuticle dynamics under activated immunity have not been studied yet. DAMPs - damage-associated molecular patterns; PAMPs - pathogen-associated molecular patterns. Fig. 2 was created in *BioRender*®.

Until now, the study of cuticle involvement in plant immunity was mainly performed using mutants affected in cuticle formation. But to get the answers to the above questions, we need to first trigger the plant immunity and look at how it affects the cuticle dynamics and composition (Fig. 2). Different approaches to monitor the effect of activated immunity exist. Various mutants with permanently triggered immunity are available, especially for *A. thaliana*. However, *A. thaliana* is not the best model for studies focused on the cuticle. Other plants with thicker cuticles with less trichomes, such as *Prunus laurocerasus*, *Hedera helix*, *Ficus elastica*, *Clusia rosea*, *Hordeum vulgare*, or *Zea mays*, could be a better choice. The recent progress in the gene editing of non-model plants provides more possibilities for such research. A reasonable approach for non-model plants would be the treatment with chemicals. Many molecules, such as PAMPs or DAMPs, trigger plant immunity. It would be interesting to look at what changes they trigger in the composition, permeability, or dynamics of the recovering cuticle and the expression of cuticular genes in distinct plant species.

To get the first brief insight into whether activated immunity has the potential to affect the cuticle, we used available transcriptomic data from *Genevestigator*® (Zimmermann *et al.* 2004) and analysed the gene expression of known genes involved in cuticle biosynthesis in response to conditions mimicking activated plant immunity (treatment with PAMPs) and after presence of pathogens or pests (Fig. 3). For further analysis, we selected genes that responded to more than six stimuli with a $\log_2$ ratio greater than 1, or less than -1. Most of the cuticle biosynthesis genes appeared to be inhibited in response to the presence of pathogens or under activated immunity (Fig. 3). The *ABCG* and *LTPG* genes involved in the transport of components of the future cuticle across the cuticular membrane appear to be the most inhibited. Does inhibition of these genes lead to decreasing cuticle thickness? Does it modulate cuticle permeability? Does inhibition of these genes affect plant resistance to pathogens? Opposite to *ABCG* and *LPTG*, gene expression of *WSD6* and *WSD7* is induced (Fig. 3). These genes are involved in ester synthesis. This result is interesting because *A. thaliana* generally has a small number of esters in its cuticle (Bernard and Joubès 2013). Such transcriptional change indicates that the chemical composition of the cuticle is affected by activated plant immunity. We also compared the difference in response when the plant is host (H) or non-host (NH) for the pathogen, but no clear difference was observed.

The comparison of different biotic stimuli and elicitors shows that cuticle-related genes are most responsive

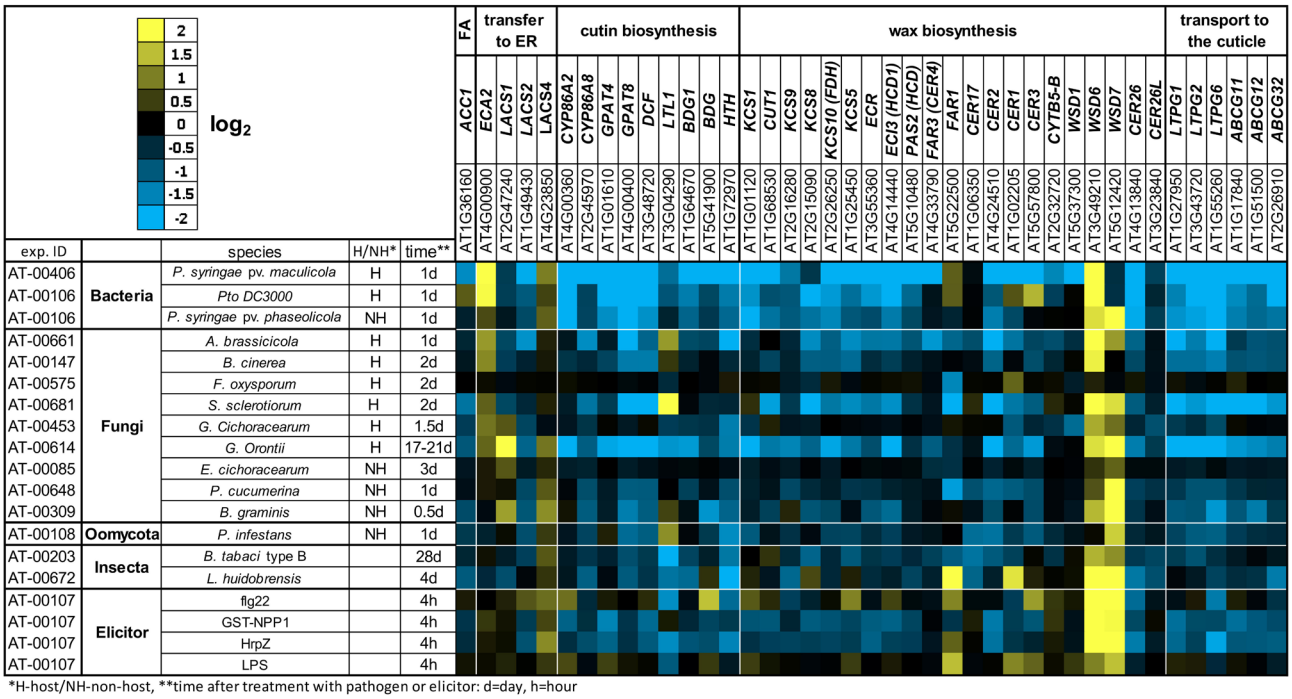

Fig. 3. Transcriptional analysis of the genes involved in cuticle biosynthesis under biotic stress conditions. The analysis was performed using *Genevestigator*® and only *Arabidopsis thaliana* Col-0 samples were used. All plants used in the analysis were 3 - 4 - 5 weeks old, and the leaves were adult. The colours represent the gene transcription level under biotic stress conditions. The legend for the gene transcription intensity is in the top-left corner of the figure. Blue colour represents inhibition, yellow colour represents increased gene transcription, and black colour indicates no effect on gene transcription. The terms in the top row classify genes according to their function during cuticle synthesis: FA - fatty acid synthesis, ER - endoplasmic reticulum.

after treatment with bacteria, when the transcription was altered in the largest number of monitored genes (Fig. 3). The question is if the effect of biotic stress on cuticle-related genes transcription is direct or pleiotropic. And if it directly affects plant immunity, could it relate to the classical growth-defence trade-off hypothesis? This hypothesis states that under activated immunity, the energy that is normally used for growth and development is used for defence. Both plant immunity and cuticle biosynthesis are an energy-consuming events, thus, the emerging question is: does plant-mediated inhibition of cuticle-connected genes prevent wasting energy on cuticle formation?

We are convinced that the answers to the raised questions and progress in the field of cuticle-immunity connection would provide necessary and interesting insights into plant-microbe interactions.

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