*Research review*

# Bitter and sweet make tomato hard to (b)eat

Author for correspondence
*Jan A.L. van Kan*
*Email: jan.vankan@wur.nl*

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**Yaohua You and Jan A.L. van Kan**

Laboratory of Phytopathology, Wageningen University, Wageningen 6708 PB, the Netherlands

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## Summary

The glycoalkaloid saponin α-tomatine is a tomato-specific secondary metabolite that accumulates to millimolar levels in vegetative tissues and has antimicrobial and antinutritional activity that kills microbial pathogens and deters herbivorous insects. We describe recent insights into the biosynthetic pathway of α-tomatine synthesis and its regulation. We discuss the mode of action of α-tomatine by physically interacting with sterols, thereby disrupting membranes, and how tomato protects itself from its toxic action. Tomato pathogenic microbes can enzymatically hydrolyze, and thereby inactivate, α-tomatine using either of three distinct types of glycosyl hydrolases. We also describe findings that extend well beyond the simple concept of plants producing toxins and pathogens inactivating them. There are reports that toxicity of α-tomatine is modulated by external pH, that α-tomatine can trigger programmed cell death in fungi, that cellular localization matters for the impact of α-tomatine on invading microbes, and that α-tomatine breakdown products generated by microbial hydrolytic enzymes can modulate plant immune responses. Finally, we address a number of outstanding questions that deserve attention in the future.

## Introduction

The conquest of South and Central America in the 16$^{th}$ and 17$^{th}$ centuries resulted in the import into Europe of several food crops indigenous to the America's, including tomato and potato. These Solanaceae were soon discovered to produce bitter-tasting glycoalkaloids in vegetative organs, of which consumption could result in serious poisoning. It was later observed that, apart from their antinutritional effect, glycoalkaloids also possess antibiotic activity, are repellent or toxic to pest insects, and may have useful medicinal applications. In the past decades, the tomato glycoalkaloid saponin α-tomatine has been extensively studied for its role in the interaction of plants with pest insects and pathogens, often with the aim of improving plant health. In this paper we present an overview of α-tomatine as a broad-spectrum toxic plant compound that protects tomato from herbivores and pathogens, and we discuss recent insights into its biosynthesis and regulation. Furthermore, we describe how microbial pathogens cope with the inhibitory activity of α-tomatine and may even exploit the hydrolytic breakdown products of α-tomatine to modulate plant immune responses.

## α-Tomatine: the major tomato saponin with antibiotic activity

The study of α-tomatine started from the exploration of fungistatic agents in tomato tissues. Over 70 yr ago, Fontaine *et al.* (1948) named the first purified compound from tomato leaves possessing antifungal properties as tomatine. It was identified as a glycosidal alkaloid, also known as steroidal glycoalkaloids (SGAs), which are a subgroup of saponins. Later studies revealed that its chemical structure is composed of a steroidal aglycone ('tomatidine') and a tetrasaccharide side branch (β-lycotetraose) containing two molecules of glucose and one molecule each of galactose and xylose. α-Tomatine is the name of the form with the tetrasaccharide, whereas the other forms lacking a terminal xylose or terminal glucose, or both terminal sugars were designated as $\beta_1$-tomatine, $\beta_2$-tomatine, and γ-tomatine, respectively (Fig. 1; Kuhn *et al.*, 1956, 1957). α-Tomatine is the major SGA, as well as the main saponin, in vegetative tissues and green fruits; its concentration can be up to several millimolar on an FW basis (Keukens *et al.*, 1995; Friedman & Levin, 1998; Kozukue *et al.*, 2004; Iijima *et al.*, 2013). The high α-tomatine levels in immature fruit decrease during

ripening by the conversion to esculeoside A (Fig. 1; Mintz-Oron *et al.*, 2008; Iijima *et al.*, 2009; Cárdenas *et al.*, 2019; Nakayasu *et al.*, 2020). The antimicrobial activity of α-tomatine was first demonstrated on the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Roddick, 1974); thereafter, antibiotic properties were reported against many tomato pathogens (including fungi, oomycetes, and bacteria), as well as pest insects (Campbell & Duffey, 1979; Sandrock & VanEtten, 1998; Kaup *et al.*, 2005; Seipke & Loria, 2008; Altesor *et al.*, 2014; Chowánski *et al.*, 2016). Because of its high concentration and broad spectrum of *in vitro* antibiotic activity, α-tomatine has long been studied as a defense compound that might confer resistance to tomato pathogens. Here, we will discuss old and recent knowledge about the relevance and mode of action of α-tomatine and illustrate its versatile biological properties, which extend well beyond the perception of a 'simple' membrane-perforating toxin.

## The biosynthetic pathway of α-tomatine and its regulation

Although α-tomatine was identified as the major SGA in tomato more than 70 yr ago, the metabolism and regulation of its synthesis are not fully understood. Initial studies on α-tomatine metabolism were driven by an interest to improve fruit quality by removing the antinutritional trait caused by α-tomatine. However, as a potent defense metabolite, insight into its metabolism can also help to increase α-tomatine levels in vegetative tissue, and thereby contribute to resistance.

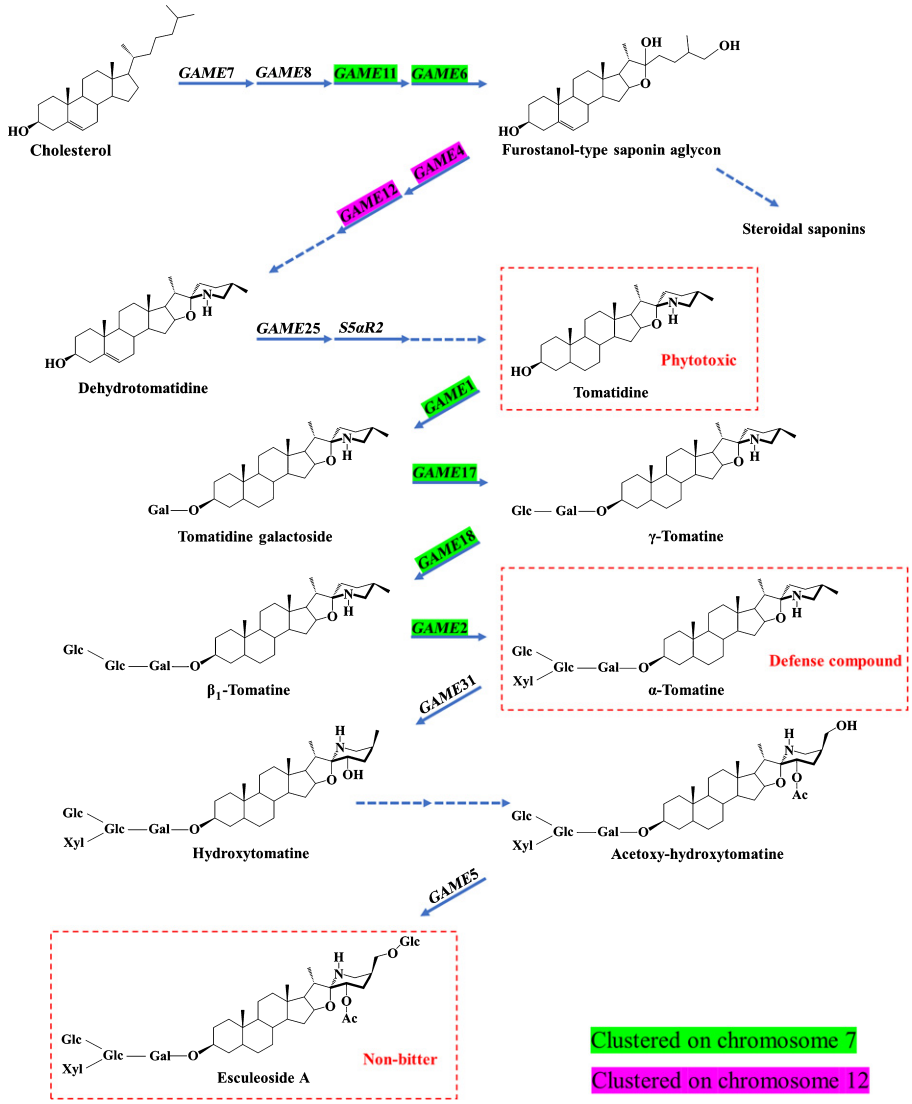


**Fig. 1** The tomato (*Solanum lycopersicum*) biosynthetic pathway of α-tomatine from cholesterol. Chemical structures and names of several biosynthetic intermediates are provided. The arrows represent catalytic conversions, with the gene name (if characterized) provided above the solid blue arrows; dashed blue arrows represent catalytic conversions for which genes are currently unknown. Genes in colored boxes are in close physical proximity in a genomic cluster on tomato chromosomes 7 and 12. The red dashed boxes highlight three key components: the phytotoxic precursor tomatidine, the defense compound α-tomatine, and the nonbitter breakdown product esculeoside A.

**Table 1** Tomato genes shown to be involved in regulation of glyoalkaloid biosynthesis.

| Gene name | Locus name | Role in regulation of α-tomatine metabolism | Target genes | References |
|---|---|---|---|---|
| *GAME9/JRE4* | Solyc01g090340 | Positive regulator | *C5-SD*, *DWF5*, *GAME4*, *GAME7*, *GAME17* | Cárdenas *et al*. (2016), Nakayasu *et al*. (2018), Thagun *et al*. (2016), Yu *et al*. (2020) |
| *MYC2* | Solyc08g076930 | Positive regulator | *C5-SD*, *GAME4*, *GAME7* | Cárdenas *et al*. (2016), Swinnen *et al*. (2020) |
| *MYC1* | Solyc08g005050 | Positive regulator | *C5-SD* | Swinnen *et al*. (2020) |
| *TAGL1* | Solyc07g055920 | Negative regulator | Unknown | Zhao *et al*. (2018) |
| *TDR4/FUL1* | Solyc06g069430 | Negative regulator | Unknown | Zhao *et al*. (2019) |
| *HY5* | Solyc08g061130 | Positive regulator | *GAME1*, *GAME4*, *GAME17* | Wang *et al*. (2018) |
| *PIF3* | Solyc01g102300 | Negative regulator | *GAME1*, *GAME4*, *GAME17* | Wang *et al*. (2018) |
| *MYB12* | Solyc06g009710 | Positive regulator | Unknown | Chen *et al*. (2019) |

SGAs, including α-tomatine, are synthesized from cholesterol, although cholesterol biosynthesis in plants itself is not fully understood. Conversion of cholesterol to α-tomatine (Fig. 1) involves multiple reactions mediated by enzymes encoded by *GLYCOALKALOID METABOLISM* (*GAME*) genes (Itkin *et al.*, 2013). The first part of the pathway requires four enzymes (GAME7, GAME8, GAME11 and GAME6) and results in the synthesis of a saponin aglycone that serves as precursor for both glycoalkaloids and steroidal saponins. The first dedicated step towards glycoalkaloid production is the oxidation of saponin aglycone by GAME4, followed by multiple additional conversions to form the aglycone alkaloid tomatidine (Itkin *et al.*, 2013). Recent research revealed that the conversion from dehydrotomatidine to tomatidine is not mediated by a single reaction, as suggested by Friedman (2002) and Itkin *et al.* (2013), but rather involves multiple steps, including oxidation, isomerization, and reduction, and requires a short-chain dehydrogenase/reductase (GAME25, also known as Sl3βHSD) and steroid 5α-reductase (SlS5αR2) (Sonawane *et al.*, 2018; Akiyama *et al.*, 2019; Lee *et al.*, 2019). As shown in Fig. 1, the synthesis of α-tomatine from its aglycon tomatidine requires four consecutive glycosylations by distinct glycosyltransferases GAME1, GAME17, GAME18 and GAME2 (Itkin *et al.*, 2011, 2013). During tomato fruit ripening, the decrease of α-tomatine content results from conversion to esculeoside A, a nonbitter steroidal glycoalkaloid. This process involves GAME31, a 2-oxoglutarate-dependent dioxygenase (also known as Sl23DOX) that catalyzes the hydroxylation of α-tomatine, and the recently identified glycosyltransferase GAME5, which produces esculeoside A (Fig. 1; Cárdenas *et al.*, 2019; Nakayasu *et al.*, 2020; Szymański *et al.*, 2020).

Interestingly, six *GAME* genes are physically clustered on tomato chromosome 7 (Fig. 1), including *GAME11* and *GAME6*, which are required in the production of the furostanol-type saponin aglycon along with the four genes encoding the glycosyltransferase that add the lycotetraose moiety to tomatidine. The potato has a similar cluster in the syntenic region; however, potato lacks orthologues to the *GAME18* and *GAME2* genes of tomato, which mediate the two final steps of tomatine biosynthesis (Cárdenas *et al.*, 2015). Also, the genes *GAME4* and *GAME12*, involved in the first two dedicated steps towards glycoalkaloid synthesis, are physically clustered in tomato chromosome 12, and this cluster is conserved in potato (Cárdenas *et al.*, 2015).

The regulation of α-tomatine metabolism involves the *GAME9* gene, a member of the APETALA2/Ethylene Response Factor family, also referred to as *JRE4* (Cárdenas *et al.*, 2016; Thagun *et al.*, 2016). More recently, additional genes involved in the (positive or negative) regulation of α-tomatine metabolism have been identified (Wang *et al.*, 2018; Zhao *et al.*, 2018; Chen *et al.*, 2019; Swinnen *et al.*, 2020; see Table 1).

## Toxicity mechanisms: membrane disruption or more than that?

Numerous studies have shown that α-tomatine is toxic to a spectrum of tomato pathogens and pests. Membrane disruption, followed by cytoplasmic leakage and cell death, was observed in cells exposed to α-tomatine (Arneson & Durbin, 1968; Campbell & Duffey, 1979; Osbourn, 1996a; Hoagland, 2009). The molecular basis for membranolytic action has been studied in depth. Membrane leakage caused by α-tomatine is dependent on the presence of sterols in the plasma membrane. A mutant of *Fusarium solani* that accumulated 20% less sterol in the membrane manifested lower sensitivity to α-tomatine (Défago & Kern, 1983). *Phytophthora* species do not synthesize sterols and, hence, were tolerant to α-tomatine; however, they gained sensitivity when grown in medium supplemented with free sterols (Steel & Drysdale, 1988). Old studies showed that α-tomatine can bind *in vitro* to different types of sterols, such as cholesterol and ergosterol, which are the major mammalian and fungal sterols, respectively, and sitosterol and stigmasterol, which are predominantly found in plant cells (Roddick, 1979). The membrane disrupting effect caused by this interaction required the intact tetrasaccharide group of α-tomatine and the presence of sterol 3β-hydroxy groups (Nepal & Stine, 2019). By contrast, membranes containing sterols lacking 3β-hydroxy groups were insensitive to disruption by α-tomatine (Roddick & Drysdale, 1984; Steel & Drysdale, 1988; Keukens *et al.*, 1995). Chemical hydrolysis products of α-tomatine lacking a single monosaccharide ($\beta_1$-

tomatine, $\beta_2$-tomatine) or multiple sugars ($\gamma$-tomatine and the aglycon tomatidine) showed > 95% reduction in their ability to disrupt membranes (Keukens *et al.*, 1995).

Despite its disrupting activity on artificial membranes, infiltration of α-tomatine into the apoplast of tomato leaves did not cause visible damage (Ökmen *et al.*, 2013). Considering the high concentration of α-tomatine in tomato tissue, tomato plants must be able to avoid self-intoxication. Indeed, tomato and potato leaves had a lower content of free sterols (*c.* 10%) and were more resistant to α-tomatine as they manifested less electrolyte leakage than plants containing higher proportions of free sterols, such as tobacco (*Nicotiana benthamiana*) and *Nicandra physalodes* (*c.* 50-%) (Steel & Drysdale, 1988). The fact that tomato cells can withstand high concentrations of α-tomatine is likely associated with substitution at 3β-hydroxyl groups, thereby forming sterol conjugates that prevent binding with α-tomatine (Steel & Drysdale, 1988). In contrast to plants, fungi predominantly accumulate ergosterol, which occurs as free sterol and in multiple esterified forms (Hartmann, 1998; Weete *et al*, 2010). The ratio between these two forms varies among fungal species, but it is unknown whether this ratio affects the sensitivity to α-tomatine (Yuan *et al.*, 2007).

Apart from the membranolytic action by sterol binding, Ito *et al.* (2007) reported that α-tomatine may induce programmed cell death (PCD) in the fungus *F. oxysporum*. Hallmarks of apoptosis, such as DNA fragmentation, depolarization of the transmembrane potential of mitochondria, and generation of reactive oxygen species (ROS), were detected in fungal cells treated with α-tomatine (Ito *et al.*, 2007). The cell death induction by α-tomatine in *F. oxysporum* was markedly reduced by the application of a specific inhibitor of PCD (Ito *et al.*, 2007), the only report thus far of PCD induction by α-tomatine in tomato pathogens. However, the PCD-inducing activity in fungi is not unexpected as α-tomatine was also reported to stimulate caspase-independent PCD in mouse colon cells and human leukemia cell lines (Chao *et al.*, 2012; Kim *et al.*, 2015). The induction of apoptosis in plant pathogens by plant defense molecules was reported in the interaction between *Arabidopsis* and the fungus *Botrytis cinerea* (Shlezinger *et al.*, 2011). The toxic effect of α-tomatine seems to be based both on membranolytic activity and the activation of the PCD machinery, although the mechanism of PCD induction remains elusive. More efforts should be made to increase our understanding of the modes of action of α-tomatine.

## How pathogens deal with α-tomatine: the role of tomatinase

One way of dealing with the toxic action of α-tomatine is the accumulation of low levels of sterols in membranes, as occurring in *Pythium* and *Phytophthora* species (Défago & Kern, 1983; Steel & Drysdale, 1988). Besides such passive tolerance, fungi can actively repair membrane damage inflicted by α-tomatine. Exposure of *Neurospora crassa* to α-tomatine triggered the recruitment of the membrane repair protein PEF1 to the lysing point at the membrane, and deletion of *pef1* increased the sensitivity of *N. crassa* to α-tomatine and other pore-forming drugs (Schumann *et al.*, 2019). Furthermore, fungi can actively export exogenous toxic compounds through ATP-binding cassette (ABC) transporters. The roles of ABC transporters in the efflux of plant secondary metabolites and synthetic fungicides are well documented (Andrade *et al.*, 2000; Schoonbeek *et al.*, 2001; Stergiopoulos & de Waard, 2002; Kretschmer *et al.*, 2009; Stefanato *et al.*, 2009). In the insect herbivore *Helicoverpa armigera*, transcript levels for ABC transporters were induced when larvae were fed α-tomatine (Bretschneider *et al.*, 2016), indicating that the adaption to α-tomatine in *H. armigera* might require ABC transporters. However, a direct role of ABC transporters in the tolerance to α-tomatine in tomato pathogens and pests remains to be characterized.

Another active way of dealing with α-tomatine is to secrete enzymes that degrade α-tomatine to reduce its toxicity (Osbourn, 1996a; Sandrock & VanEtten, 1998). Hydrolysis of α-tomatine was first reported in the fungus *Septoria lycopersici* (Arneson & Durbin, 1968). Since then, the identification and characterization of tomatinase activity has been extended to more pathogens. Although enzymes catalyzing the hydrolysis of α-tomatine are collectively referred to as tomatinase, they differ in the glycosidic cleavage sites, catalytic mechanisms, and the classification of corresponding genes (Fig. 2; Table 2). The degradation process is categorized into three main actions, based on the hydrolysis products $\beta_2$-tomatine, $\beta_1$-tomatine and tomatidine.

### Regulation of tomatinase expression

**$\beta_2$-Tomatine as the main product** Generation of $\beta_2$-tomatine by cleaving off the terminal D-glucose has been reported in the fungi *S. lycopersici*, *Verticillium albo-atrum*, and *Colletotrichum coccodes* (Arneson & Durbin, 1968; Pegg & Woodward 1986; Sandrock *et al.*, 1995; Sandrock & VanEtten, 2001). The enzyme possesses β-glucosidase activity and is named $\beta_2$-tomatinase. The cloning and sequencing of the *S. lycopersici* $\beta_2$-tomatinase gene indicated that it belongs to the Glycosyl Hydrolase (GH) Family 3 of carbohydrate-active enzymes (CAZymes) (Lombard *et al.*, 2014).

**$\beta_1$-Tomatine as the main product** Besides the terminal D-glucose, the other terminal sugar moiety, D-xylose, can be the target of enzymatic hydrolysis. Removal of the terminal D-xylose and release of $\beta_1$-tomatine was reported in *B. cinerea* (Quidde *et al.*, 1998). Genes encoding $\beta_1$-tomatinase have not been cloned, so we cannot yet attribute the activity to a GH family; however, this enzyme must possess β-xylosidase activity and is likely from the GH39 or GH43 family of CAZymes. Using the sequence of *S. lycopersici* $\beta_2$-tomatinase as a probe, Quidde *et al.* (1999) cloned a homologue (*sap1*) from *B. cinerea*. Characterization of a *B. cinerea sap1* knockout mutant revealed that this gene is not responsible for the $\beta_1$-tomatinase activity as the mutant can still hydrolyze α-tomatine (Quidde *et al.*, 1999). The characterization of genes encoding $\beta_1$-tomatinase in *B. cinerea* and other fungi awaits their cloning.

**Tomatidine as the main product** Besides hydrolytic removal of single terminal sugar moieties, several microorganisms can convert

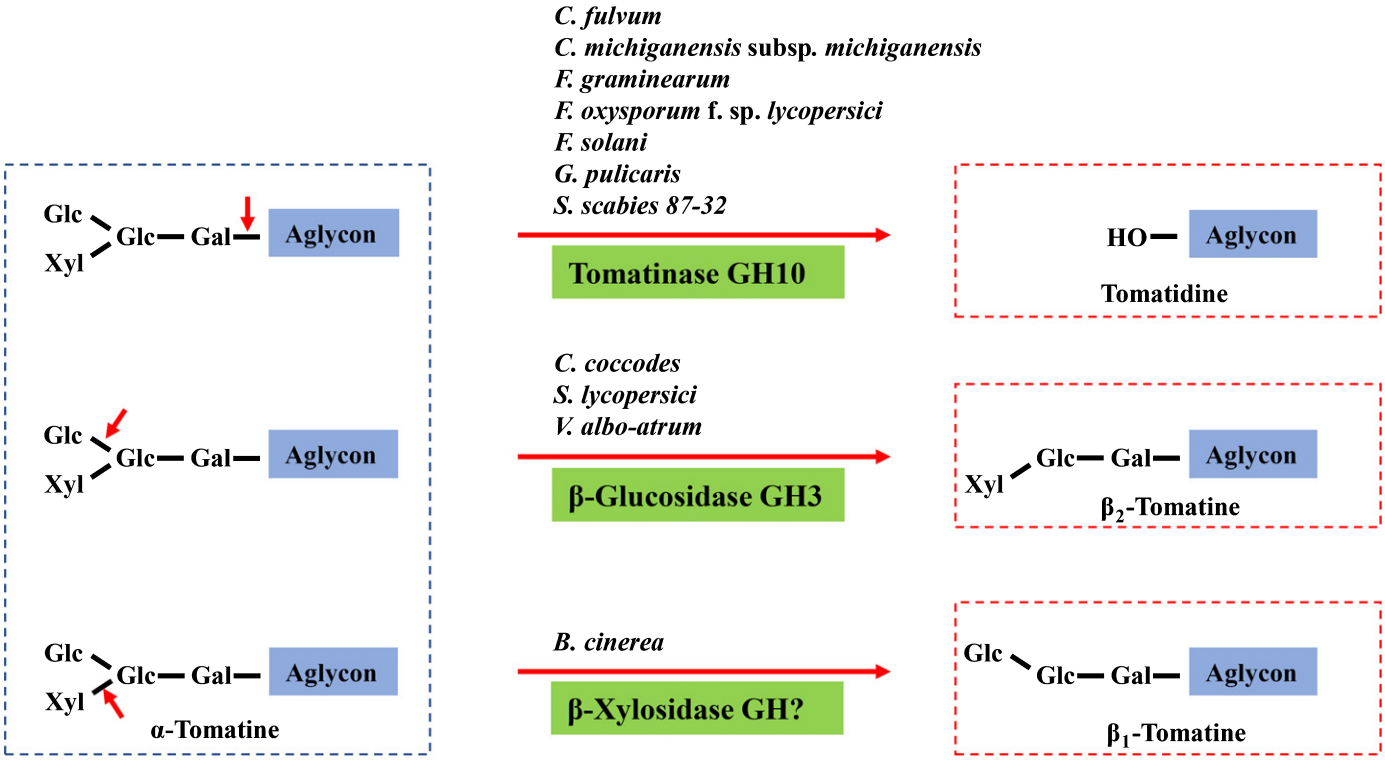


**Fig. 2** Different hydrolytic activities that detoxify α-tomatine. Chemical structures of α-tomatine are simplified. The glycosidic bonds that are cleaved are indicated by a red arrow. Carbohydrate-active enzyme activities that catalyze the reaction are indicated below the arrows, and microorganisms (*Cladosporium fulvum*, *Clavibacter michiganensis* subsp. *michiganensis*, *Fusarium graminearum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium solani*, *Gibberella pulicaris*, *Streptomyces scabies* 87-22, *Colletotrichum coccodes*, *Septoria lycopersici*, *Verticillium albo-atrum*, *Botrytis cinerea*) that were shown to possess these activities are specified above the arrows.

α-tomatine to the aglycon tomatidine (Fig. 2; Table 2): the fungi *Cladosporium fulvum* (Ökmen *et al.*, 2013), *F. oxysporum* (Roldán-Arjona *et al.*, 1999), *F. solani* (Lairini & Ruiz-Rubio, 1998), *Fusarium graminearum* (Carere *et al.*, 2017), and *Gibberella pulicaris* (Weltring *et al.*, 1998), and the bacterial pathogens *Clavibacter michiganensis* (Kaup *et al.*, 2005) and *Streptomyces scabies* (Seipke & Loria, 2008). The generation of tomatidine is through the removal of the tetrasaccharide chain (lycotetraose). Unlike the $\beta_2$-tomatinase, which belongs to the CAZy GH3 category, all tomatinase activities that detoxify α-tomatine through cleaving off the lycotetraose belong to the GH10 family (Table 2). Finally, there is one example of an organism possessing distinct, functionally redundant enzymes capable of detoxifying α-tomatine. Removing the lycotetraose group was first considered to be the mode of action of α-tomatine degradation in *F. oxysporum* (Roldán-Arjona *et al.*, 1999). However, a knockout mutant in the GH10 CAZyme gene remained able to degrade α-tomatine because it also possesses several GH3 enzymes that convert α-tomatine into $\beta_2$-tomatine instead of tomatidine. The $\beta_2$-tomatinase GH3 activity was not identified in the first place as it was masked by the presence of the GH10 tomatinase activity, which cleaves off the entire lycotetraose branch (Pareja-Jaime *et al.*, 2008).

Several aspects of the regulation of expression of tomatinase genes have been described. First, the tomatinase activity and expression of tomatinase genes can often be induced by α-tomatine (Quidde *et al.*, 1998; Roldán-Arjona *et al.*, 1999; Ökmen *et al.*, 2013). Second, induction seemed to be specific to α-tomatine treatment, as there was no induction by other saponins, such as chaconine or solanine in *B. cinerea* (Quidde *et al.*, 1998). Finally, the effect of carbon catabolite repression differed between fungi: $\beta_1$-tomatinase from *B. cinerea* was not subject to catabolite repression (Quidde *et al.*, 1998), but expression of the *F. oxysporum* GH10 tomatinase gene was repressed when glucose is present (Roldán-Arjona *et al.*, 1999).

## Degradation of α-tomatine is more than detoxification

Pathogens achieve detoxification of α-tomatine by enzymatically converting it to less-toxic products. In addition to detoxification, tomatinase activity may have additional biological repercussions. A GH3 tomatinase-deficient mutant of *S. lycopersici* caused more intense plant cell death than the wild-type in early stages of infection and induced enhanced expression of defense-related genes on tomato leaves (Martin-Hernandez *et al.*, 2000). In a different study, inoculation on *N. benthamiana* of the *S. lycopersici* GH3 tomatinase-deficient mutant, but not the wild-type, elicited intense cell death in mesophyll tissue; this resembled a hypersensitive response, and the infection was fully contained within 2 d post-inoculation (Bouarab *et al.*, 2002). These observations indicated that GH3 tomatinase not only detoxifies α-tomatine, but also mediates the suppression of plant defense responses. Further experiments showed that pre-infiltration of $\beta_2$-tomatine in *N. benthamiana* leaves enabled the *S. lycopersici* GH3 tomatinase mutant to cause expanding lesions and also compromised plant

**Table 2** Microbial glycosyl hydrolases capable of degrading α-tomatine.

| Pathogen | GH family | Accession (database) | Degradation product | Reference |
|---|---|---|---|---|
| *Colletotrichum coccodes* | Unknown | not applicable | $\beta_2$-Tomatine | Sandrock & VanEtten (2001) |
| *Septoria lycopersici* | GH3 | U35462 (NCBI) | $\beta_2$-Tomatine | Sandrock *et al*. (1995) |
| *Verticillium albo-atrum* | Unknown | Not applicable | $\beta_2$-Tomatine | Pegg & Woodward (1986) |
| *Botrytis cinerea* | GH3, GH39, GH43? | Not applicable | $\beta_1$-Tomatine | Quidde *et al*. (1998) |
| *Cladosporium fulvum* | GH10 | 188986 (JGI) | Tomatidine | Ökmen *et al*. (2013) |
| *Clavibacter michiganensis* subsp. *michiganensis* | GH10 | AAP57293 (NCBI) | Tomatidine | Kaup *et al*. (2005) |
| *Fusarium graminearum* | GH10 | EYB27127 (NCBI) | Tomatidine | Carere *et al*. (2017) |
| *Fusarium oxysporum* f. sp. *lycopersici* | GH10 | AJ012668 (NCBI) | Tomatidine | Roldán-Arjona *et al*. (1999) |
| *Fusarium solani* | Unknown | Not applicable | Tomatidine | Lairini & Ruiz-Rubio (1998) |
| *Gibberella pulicaris* | Unknown | Not applicable | Tomatidine | Weltring *et al*. (1998) |
| *Streptomyces scabies* 87-22 | GH10 | CBG74701 (NCBI) | Tomatidine | Seipke & Loria (2008) |
| *Alternaria alternata* | Unknown | Not applicable | Unknown, but not tomatidine | Oka *et al*. (2006) |
| *Corynespora cassiicola* | Unknown | Not applicable | Unknown, but not tomatidine | Oka *et al*. (2006) |

resistance to the bacterial pathogen *Pseudomonas syringae* pv *tabaci*. By contrast, infiltration of α-tomatine did not have such effects (Bouarab *et al*., 2002). Moreover, silencing of the *N. benthamiana* SGT1 gene, required for disease resistance in plants, restored the pathogenicity of *S. lycopersici* GH3 tomatinase-deficient mutant (Austin *et al*., 2002; Bouarab *et al*., 2002; Peart *et al*., 2002). These observations suggest that the capacity of tomatinase to suppress plant defense depends on the breakdown product(s) generated by tomatinase, rather than the protein itself. A dual function of tomatinase was also reported in *F. oxysporum*, which converts α-tomatine by a GH10 hydrolase to the aglycon tomatidine (Roldán-Arjona *et al*., 1999). The addition of either tomatidine or lycotetraose to suspension-cultured tomato cells can suppress the oxidative burst and hypersensitive cell death triggered by fungal elicitor (Ito *et al*., 2004). The effect of tomatidine and lycotetraose on the production of ROS was studied in more detail. *In vitro* assays revealed that tomatidine could scavenge superoxide anions as effectively as ascorbic acid, whereas lycotetraose did not possess antioxidant activity. These observations suggest that the suppression of an oxidative burst by degradation products of α-tomatine is based on different mechanisms: tomatidine can directly scavenge ROS, whereas lycotetraose might block the generation of ROS through an as yet unknown mechanism. Furthermore, treatment of tomato plant with tomatidine or lycotetraose promoted the colonization of hypocotyls by a nonpathogenic *F. oxysporum* strain lacking tomatinase activity (Ito *et al*., 2004). Besides modulating plant defense responses, tomatidine was reported to exhibit phytotoxic effects. Transgenic tomato plants in which the *GAME1* gene (Fig. 1) was silenced accumulated excessive levels of tomatidine and exhibited severe developmental defects (Itkin *et al*., 2011). This observation was substantiated by the cell-death-inducing effect on tomato leaves of exogenously applied tomatidine (Fig. 3; Ökmen *et al*., 2013). Based on these studies, it is apparent that the hydrolysis of α-tomatine during pathogen infection is not merely reducing its toxicity but is also affecting the physiology and defense responses of the plant through α-tomatine breakdown products. In some situations, the latter role appeared to be important for virulence of tomato pathogens (Bouarab *et al*., 2002; Ito *et al*., 2004).

## Subcellular localization: arsenal or battlefield?

Like other saponins, α-tomatine is thought to be localized within tomato cells and to be released upon cell damage resulting from pathogen invasion (Dow & Callow, 1978) or pest feeding. The subcellular localization can define the spatial and temporal contribution of α-tomatine to the inhibition of pathogen infection. Theoretically, if a tomato pathogen can avoid the release of α-tomatine from the host cells, it would circumvent the inhibition. Studies on the biotrophic tomato pathogen *C. fulvum* have shed light on the importance of the distribution of α-tomatine because this fungus exclusively colonizes the apoplast and causes limited damage to host cells until the final stage of infection (Stergiopoulos & de Wit, 2009). Initially, it was proposed that *C. fulvum* was less likely to encounter inhibitory concentrations of α-tomatine during infection if the glycoalkaloid predominantly localizes intracellularly. Based on this assumption, it was hypothesized that tomatinase activity might not be important for full virulence of *C. fulvum* despite high sensitivity of the fungus to α-tomatine (Dow & Callow, 1978; Kohmoto & Yoder, 1998; Melton *et al*., 1998). In order to test whether tomatinase activity contributes to virulence, Melton *et al*. (1998) expressed a GH3 tomatinase gene from *S. lycopersici* in *C. fulvum*, as this was the only characterized tomatinase gene at that time. Expression of the heterologous tomatinase resulted in enhanced virulence of *C. fulvum* (as assessed by increased sporulation) and provided evidence for a positive role of α-tomatine degradation to *C. fulvum* infection (Melton *et al*., 1998). A later study described the identification of the *C. fulvum* endogenous GH10 tomatinase gene *CfTom1* and further substantiated the role of tomatinase activity. A knockout mutant in the *CfTom1* gene displayed increased sensitivity to α-tomatine and reduced virulence on tomato (Ökmen *et al*., 2013). This study also detected the presence of α-tomatine in apoplastic fluid at 0.02 mM, which is low compared with the levels of *c.* 1 mM in total leaf extract (Ökmen *et al*., 2013). In light of these studies, there is no doubt that α-tomatine predominantly accumulates inside plant cells; however, the amounts of α-tomatine in intercellular spaces might be sufficient to exert some inhibition to invading microbes. It is unknown whether the apoplastic localization of α-tomatine

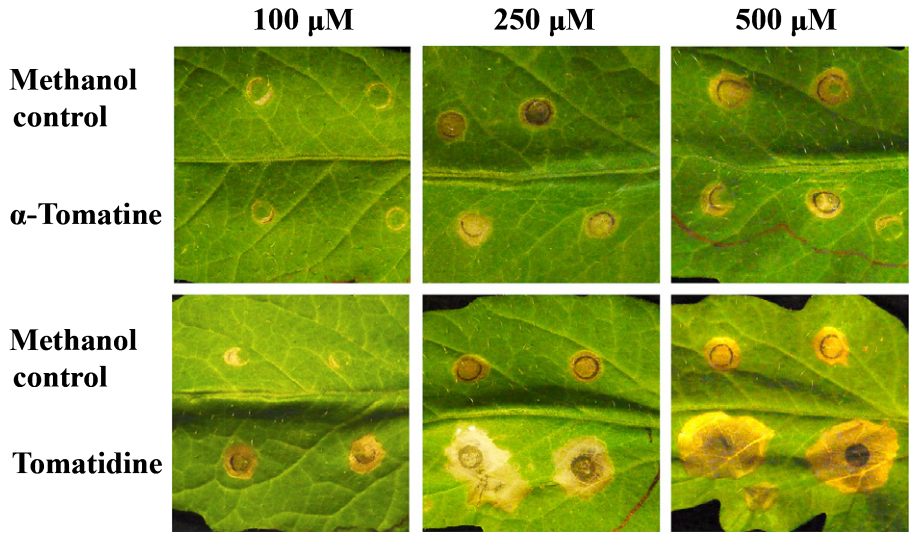


**Fig. 3** Phytotoxic effects of α-tomatine and tomatidine on tomato (*Solanum lycopersicum*) leaves. Taken from Ökmen *et al*. (2013) with permission.

involves active secretion or merely results from the leakage from cells. The impact of the intercellular distribution of α-tomatine in the defense against pathogens that employ different infection strategies, such as necrotrophic and hemibiotrophic pathogens, remains unclear.

## Effect of pH on tolerance to α-tomatine

α-Tomatine is more toxic at higher pH (Arneson & Durbin, 1968; Dow & Callow, 1978). At pH 3.0, α-tomatine concentrations almost 300 times higher were required to achieve the same inhibitory effect on fungi as at pH 8.0 (Arneson & Durbin, 1968). This effect might be partially caused by increased protonation of α-tomatine in acidic conditions, as the unprotonated α-tomatine can bind to cholesterol *in vitro* but the protonated form cannot (Arneson & Durbin, 1968). Besides influencing the toxicity of α-tomatine, ambient pH may also affect the expression of tomatinase genes. The *C. fulvum* GH10 tomatinase gene *Cftom1* was barely expressed in liquid medium containing α-tomatine at pH 4.0, whereas abundant transcript levels were detected at pH 7 (Ökmen *et al*., 2013). This observation explained why a previous study could not detect α-tomatine degradation, as the medium used to grow the mycelium was adjusted to pH 4.5 (Melton *et al*., 1998). Studies showing the impact of pH on *in vitro* assays raise questions about the role of ambient pH at infection sites in tomato–pathogen interactions, and highlight the possible impact of ambient pH manipulation by microbes during infection, such as the host tissue acidification reported for *B. cinerea* (Müller *et al*., 2018). Although the effect of pH manipulation might not occur with the specific purpose to decrease sensitivity to α-tomatine, it likely affects its toxicity and thereby could have an impact on the outcome of tomato–microbe interactions.

## Typical phytoanticipin or more than that?

The term phytoanticipin was first proposed and defined by VanEtten *et al*. (1994). Phytoanticipins are low molecular weight metabolites with antibiotic properties that are either preformed or generated from accumulated precursors when plants are challenged by pathogens. Phytoanticipins differ from the phytoalexins, which are induced upon pathogen infection. α-Tomatine has long been considered as a potent phytoanticipin because of its high accumulation in healthy tomato tissues and its toxicity against different pathogens (Osbourn, 1996b; Piasecka *et al*., 2015). A recent study on resistance to early blight (*Alternaria solani*) described a difference in metabolic profiles between a resistant wild tomato (*Solanum arcanum*) and the susceptible cultivated tomato (*Solanum lycopersicum*). This study indicated that α-tomatine can also serve as a phytoalexin in certain conditions (Shinde *et al*., 2017). A pronounced increase of α-tomatine content was detected in *S. arcanum* after *A. solani* infection, to levels 10 times higher than before infection. By contrast, the susceptible cultivated tomato had more severe symptoms, and its α-tomatine level increased by only 2.5-fold. Counter-intuitively, the expression of α-tomatine biosynthetic genes *GAME1*, *GAME17* and *GAME18*, as well as the regulator gene *GAME9*, were much higher in susceptible cultivated tomato despite the lower increase of α-tomatine levels, compared with the wild tomato upon infection. By contrast, *GAME2*, which encodes the enzyme that performs the last step of α-tomatine synthesis, was expressed at much higher levels in resistant wild tomato, highlighting an important (rate-limiting) role of *GAME2* expression in α-tomatine stimulation in response to *A. solani* invasion. To date, this is the only report showing that α-tomatine biosynthesis can be elicited by the challenge of microbes.

## The balance between α-tomatine accumulation and degradation defines the outcome on the battlefield

Although being referred to as a defense compound in many studies because of its high accumulation in tomato tissue and its toxicity against many pathogens, direct evidence of the contribution of α-tomatine to plant immunity is lacking. The importance of α-tomatine in basal defense is indirectly implied from various studies on tomato pathogens. First, tomato pathogens tend to be more

resistant to α-tomatine than organisms that are nonpathogenic on tomato (Arneson & Durbin, 1968; Steel & Drysdale, 1988). For instance, mycelia of the fungal tomato pathogens *B. cinerea*, *V. albo-atrum* and *F. solani* exhibited less electrolyte leakage than nontomato pathogens, such as *Alternaria tenuis*, *Ascochyta pisi* and *F. graminearum*, when incubated with α-tomatine (Steel & Drysdale, 1988). A comprehensive study among 23 fungal strains revealed a strong correlation between the tolerance to α-tomatine, the ability to degrade α-tomatine, and pathogenicity on tomato (Sandrock & VanEtten, 1998). A similar phenomenon was observed in pea pathogens: among 50 plant pathogenic microbes, only the taxa that were able to metabolize the pea phytoalexin pisatin could infect pea, and all isolates that were nonpathogenic on pea were unable to detoxify pisatin (Delserone *et al.*, 1999). Moreover, the pea pathogen *Nectria haematococca* can infect mature tomato fruit (low in α-tomatine) but not green fruit, which accumulates high concentrations of α-tomatine, whereas expression of the *S. lycopersici* GH3 tomatinase gene in *N. haematococca* conferred the ability to colonize green tomato fruit (Sandrock & VanEtten, 2001). These observations implicate that degrading α-tomatine is essential to achieving successful infection on tomato or determining the host range. A similar concept was described for the oat pathogen *Gaeumannomyces graminis* var. *tritici*, in which mutants that were unable to degrade the oat saponin avenacin A-1 lost their ability to infect oat (Osbourn *et al.*, 1995).

However, the mutagenesis of genes encoding tomatinase in several microbes thus far does not support the role of tomatinase as an essential determinant in pathogenicity on tomato but rather contributes quantitatively to virulence. For example, *C. fulvum* GH10 tomatinase-deficient mutants remained pathogenic on tomato despite accumulating less fungal biomass, whereas the heterologous overexpression of *S. lycopersici* GH3 tomatinase in *C. fulvum* enhanced fungal sporulation during tomato infection (Melton *et al.*, 1998). Also, in *F. oxysporum*, GH10 tomatinase-deficient mutant caused delayed disease development compared with the wild-type (Pareja-Jaime *et al.*, 2008). Moreover, the natural field isolate M3a of *B. cinerea* (from grape) was deficient in α-tomatine degrading activity and accordingly was less virulent on tomato leaves, compared with the α-tomatine-degrading strain B05.10. When infecting plant tissues lacking α-tomatine, such as bean leaves, similar lesion sizes were observed for M3a and B05.10 (Quidde *et al.*, 1998). In addition, the infection on tomato was unaffected when tomatinase was disrupted in *S. lycopersici* (Martin-Hernandez *et al.*, 2000) or in the bacterium *S. scabies* (Seipke & Loria, 2008). In these cases, it suggested that these organisms possess additional mechanisms that confer tolerance to α-tomatine. These observations are indicative of the importance of tomatinase in tomato–microbe interactions and consequently highlight the potential role of α-tomatine in tomato basal defense.

Moreover, the contribution of 'tomatinase' to plant infection might not necessarily be (exclusively) related to α-tomatine degradation. As already described, tomatinases are glycosyl hydrolases of distinct CAZyme families, which might also act on substrates other than α-tomatine and thereby play a different role in the infection. For instance, the virulence of GH3 tomatinase-deficient mutants of *S. lycopersici* was not reduced on tomato leaves; however, they failed to infect *N. benthamiana* leaves, which do not accumulate α-tomatine (Bouarab *et al.*, 2002). Similarly, a glycosyl hydrolase from *F. graminearum* possessing hydrolytic activity, an α-tomatine, acted as a virulence factor on wheat (Carere *et al.*, 2017).

In a recent study, tomato leaves overexpressing a gene encoding tomato strictosidine synthase (STR-2) accumulated more α-tomatine and exhibited enhanced resistance against *B. cinerea* and *Phytophthora infestans* (Chen *et al.*, 2019). Taken together, there are strong indications that α-tomatine participates in the basal defenses against pathogens. However, whether an increase in α-tomatine levels may increase resistance to presently notorious pathogens and the absence of α-tomatine will render tomato plants more susceptible to organisms not normally infecting tomato remain to be studied. Such studies would benefit from using α-tomatine-deficient transgenic tomato (using CRISPR) and lines accumulating a higher level of α-tomatine to provide direct evidence of the role of α-tomatine in plant immunity.

## Conclusion and perspectives

The data discussed herein provide circumstantial evidence that α-tomatine is a specialized metabolite that confers important levels of protection from herbivory and pathogen invasion. The final proof of its important function in plant defense remains to be provided by knocking out its biosynthesis or increasing its levels by selective overexpression of *GAME* genes and testing the impact of altered α-tomatine levels on the susceptibility to herbivores or pathogens. The mode of action on plant, microbial, and insect membrane and the mechanisms by which α-tomatine induces PCD in plants need to be resolved.

Enzymatic degradation of α-tomatine by three distinct types of microbial secreted CAZymes – GH3, GH10 or GH43, which, respectively, remove the terminal glucose moiety, terminal xylose moiety, or the entire lycotetraose group – provides a beautiful example of independent, convergent evolution in several pathogenic bacteria and fungi towards the detoxification of a potent antimicrobial compound. These genes probably evolved from ancestral GH genes with the appropriate catalytic site, towards specialization on a substrate that was a major obstacle for pathogen development and reproduction in a toxic environment. For detoxification of other phytoanticipins, such as avenacin and pisatin, there is generally just a single enzymatic activity reported that can inactivate these compounds. The finding of three separate detoxification activities for the same antimicrobial compound is remarkable.

It is noteworthy that enzymatic degradation products of α-tomatine, such as $\beta_2$-tomatine, tomatidine, and lycotetraose, can modulate the immune response of a plant, suggesting that the removal of sugar moieties benefits a pathogenic microbe in two ways: by reducing membrane permeating activity of α-tomatine and by lowering the plant defense machinery. The impact of antimicrobial plant metabolites on the plant immune response through different mechanisms (other than being toxic to microbes) deserves further attention. The observation that sterol glycosylation in tomato confers tolerance to the toxicity of α-tomatine raises the

question of whether microbes, and especially tomato pathogens, could protect themselves from membrane damage by glycosylating their sterols, either constitutively or in the presence of α-tomatine.

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## Author contributions

YY collected the literature and prepared the figures. YY and JALvK wrote the manuscript.

## ORCID

Jan A.L. van Kan https://orcid.org/0000-0003-3563-1550

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