Article

The Glutamate Receptor Plays a Role in Defense against *Botrytis cinerea* through Electrical Signaling in Tomato

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Abstract: Plant *glutamate-like receptor* genes (*GLRs*) are homologous to mammalian *ionotropic glutamate receptors* genes (*iGluRs*). Although *GLRs* have been implicated in plant defenses to biotic stress, the relationship between *GLR*-mediated plant immunity against fungal pathogens and electrical signals remains poorly understood. Here, we found that pretreatment with a *GLR* inhibitor, 6,7-dinitriquinoline-2,3-dione (DNQX), increased the susceptibility of tomato plants to the necrotrophic fungal pathogen *Botrytis cinerea*. Assessment of the *glr3.3*, *glr3.5* and *glr3.3/glr3.5* double-mutants upon *B. cinerea* infection showed that tomato *GLR3.3* and *GLR3.5* are essential for plant immunity against *B. cinerea*, wherein *GLR3.3* plays the main role. Analysis of the membrane potential changes induced by glutamate (Glu) or glycine (Gly) revealed that amplitude was significantly reduced by knocking out *GLR3.3* in tomato. While treatment with Glu or Gly significantly increased immunity against *B. cinerea* in wild-type plants, this effect was significantly attenuated in *glr3.3* mutants. Thus, our data demonstrate that *GLR3.3*- and *GLR3.5*-mediated plant immunity against *B. cinerea* is associated with electrical signals in tomato plants.

Keywords: glutamate receptor-like; necrotrophic pathogens; electrical signal; *Botrytis cinerea*; *Solanum lycopersicum*

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1. Introduction

Plants that grow in a natural environment are continuously threatened by a wide variety of pathogens. These biotic stressors affect crop production and often cause economic losses. As plants lack mobility, they have acquired a range of signal-transduction systems during evolution to effectively mitigate the adverse effects of environmental changes. The faster these signals maneuver, the more likely it is that plants will adapt and resist the incoming stress. The signals reported so far include calcium flow, ROS (reactive oxygen species), electrical signals, etc. [1–5], which react within seconds or minutes.

Plant *glutamate-like receptor* genes (*GLRs*) are homologous to mammalian *ionotropic glutamate receptors* genes (*iGluRs*), which trigger electrical and Ca^{2+} signal cascades [3,6]. Plant *GLRs* are hypothesized to have ligand-binding as well as ion channel activity. *GLRs* have 20 family members in *Arabidopsis* [7] and 13 members in rice [8]. There are 13 full-length *GLR* genes in tomato plants [9].

Importantly, *GLRs* are involved in the response of plants to biotic stress. For instance, *AtGLR3.3* is a vital module of plant immunity against the bacterial pathogen *P. syringae* pv. tomato DC3000 (*Pst* DC3000), the Oomycete *Hyaloperonospora arabidopsidis*, and the insect *Spodoptera littoralis* [10–12]. In addition, Wang et al. (2019) reported that *GLR3.5* functions in tomato defense against the root-knot nematode (RKN) *Meloidogyne incognita* [13]. Over-expression of radish *GluR*-complementary DNA in *Arabidopsis* plants enhances resistance to a necrotic fungal pathogen, *B. cinerea*. However, *Arabidopsis glr3.3* mutants do not

demonstrate increased vulnerability to the fungal pathogen in comparison with Col-0. Thus, the role of GLRs in plant immunity against fungal pathogens remains controversial.

Plant GLRs are involved in the generation and transmission of electrical signals. The existence of electrical signals in plants was discovered by Burden-Sanderson and Charles Darwin [14,15]. They are a rapid-response signals generated by the instantaneous change in the electric potential gradient on the plasma membrane or alveolar leaf membrane [16]. All plants can produce long-distance electrical signals, which play a role in the communication of diverse tissues and organs, leading to the integration of environmental responses [17,18]. In recent years, the function of electrical signals in GLR-mediated plant resistance to biotic stress has attracted much attention [19]. In *Arabidopsis*, insects feeding on leaves can cause changes in the surface potential, which lead to the expression of several key jasmonate (JA)-inducible regulatory genes in the adult phase of plants [3]. Root-knot nematode attack on tomato roots causes the transfer of electrical and ROS signals from roots to leaves, resulting in an accumulation of JAs in leaves [13]. Farmer et al. (2014) proposed a hypothesis that distal JA synthesis in plants upon wounding was dependent on clade 3 GLR-mediated electrical signals [20]. However, the relationship between electrical signals and GLR-induced plant immunity against fungal pathogens has not been fully substantiated.

The tomato (*Solanum lycopersicum* L.) ranks as one of the world's most significant cultivated crops in terms of economic importance [21]. Nonetheless, fungal diseases have a negative impact on tomato production, resulting in substantial economic losses [22]. In particular, *Botrytis cinerea*, a typical necrotrophic fungus, has been considered to be in second place in the top 10 fungal pathogens in molecular plant pathology [23]. It has a wide host range and can infect over 200 plant species, including a variety of cash crops [24]. *B. cinerea* can cause a significant decline in tomato yield and post-harvest fruit quality [25]. However, a tomato variety with broad resistance to *B. cinerea* has not been developed yet. Thus, understanding the molecular mechanisms of tomato resistance to *B. cinerea* is particularly important.

There are 13 full-length *GLR* genes in tomato plants. In this study, *GLR3.3* and *GLR3.5*, which play positive roles in cold tolerance [26] and defense against RKNs [13], were functionally characterized in regard to tomato plant immunity against *B. cinerea*. We found that *GLR3.3* and *GLR3.5* are important players in plant immunity against *B. cinerea* in tomato, possibly through binding to ligand glutamate (Glu) or glycine (Gly), thereby triggering electrical signals. This finding has intrinsic scientific value, with potential for developing new strategies for increasing crop resistance to *B. cinerea*.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions, and Chemical Treatments

The tomato lines (CRISPR-Csa9-edited lines of *glr3.3*, *glr3.5*, and *glr3.3/3.5*) used in this study were in the cv Ailsa Craig (AC) wild-type (WT) background, as reported previously [27]. In the case of soil-less substrate culture, tomato plants were cultivated in pots that contained a mixed substrate (vermiculite: peat, 1: 3, *v/v*) and fertilized with Hoagland's nutrient solution. In the case of hydroponic culture, tomato seeds were initially germinated in trays filled with a mixed substrate (vermiculite: perlite, 1: 1, *v/v*) and, after emergence, seedlings were transferred to plastic containers having 1.5 L capacity (four seedlings per container). The containers were filled with half-strength Enshi nutrient solution, which was continuously ventilated by aerating the air from an air pump [21]. The following were the growing conditions: 14 h light/8 h dark photoperiod; 24 °C/20 °C day/night temperature; and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (photosynthetic photon flux density). Four-week-old tomato plants were subjected to treatments.

For DNQX (6,7-dinitroquinoxaline-2,3-dione, GLRs inhibitor) pretreatment assay, the WT plants were treated by foliar spray with 1 mM DNQX or H₂O as control, 12 h prior to *B. cinerea* inoculation. Glu and Gly pretreatment assays were performed as the *glr3.3* mutants and WT plants were treated with 20 mM Glu, 20 mM Gly, or H₂O as control once per

day on both the abaxial and adaxial surfaces of leaves. Two days after pharmacological treatment, the plants were subjected to pathogen inoculation.

2.2. Pathogen Inoculation and Disease Symptom Assays

The *B. cinerea* strain BO5-10 was used in this study. The inoculation was performed using a suspension of *B. cinerea* at a density of 2×10^5 spores mL⁻¹ [28]. The whole-leaf portion of tomato plants was sprayed with the suspension. The inoculated plants were placed in a sealed box to maintain humidity of about 90%. The temperature and light were the same as in normal culture conditions.

After pathogen inoculation, *B. cinerea* actin mRNA accumulation was quantified at 1 day post-inoculation (dpi) [29]. Chlorophyll fluorescence was measured with a chlorophyll fluorometer (IMAG-MAXI; Heinz Walz) at 3 dpi. The photochemical quantum efficiency of PSII (Φ PSII) was analyzed as $F_m' - F / F_m'$ [30].

2.3. Subcellular Localization

GLR3.3 and GLR3.5 gene fragments were amplified using the primers listed in Supplemental Table S1 and cloned into a pAC402 vector with a green fluorescent protein (GFP) tag [31]. *Agrobacterium* strain GV3101, carrying plasmids, and *Agrobacterium*, carrying FLS2-mCherry as a plasma membrane localization marker, were transiently co-expressed in the transgenic *Nicotiana benthamiana* (expressed with histone 2B-mCherry for marking the nucleus localization), as previously described [32]. At 2 days after infiltration, the fluorescent signals of GFP- or mCherry-tagged proteins were assessed in leaves, using a Zeiss LSM 780 confocal microscope at previously described excitation/emission wavelengths [28].

2.4. RNA Isolation, Transcription, and qRT-PCR

Total RNA was isolated from tomato leaves using the RNAsimple Total RNA Kit (Tiangen, China) and reverse-transcribed using the ReverTra Ace qPCR RT Master Mix (Toyobo, Japan). The qRT-PCR process was carried out using the LightCycler 480 Real-Time PCR System (Roche, Switzerland). The reaction system was performed as described in the previous experiment [28]. The specific primers for the *B. cinerea* actin gene and the internal control tomato actin gene are listed in Supplemental Table S1.

2.5. Electric Potential Recording

Hydroponic tomato plants at the four-leaf stage were used for electrical potential recording. Borosilicate glass capillaries with an outside diameter of 1.5 mm and an inner diameter of 0.86 mm (Sutter Instrument Company, America) were fashioned into glass microelectrodes with a tip diameter of around 0.5 mm for intracellular cytoplasmic potential measurements. An APE-2 vertical micropipette puller (Narishige, Tokyo, Japan) was used to pull capillaries after heating. Microelectrodes were filled with 1 M KCl and inserted into the mesophyll cells of a leaf. The leaf was submerged with the basic solution (composed of 5 mM MES, 0.5 mM CaSO₄, 0.05 mM KCl, and pH 6.0) [13], which was performed as shown in Supplement Figure S1. Each electrode was connected to a differential amplifier. Two dual-channel amplifiers (FD223 and Duo773, World Precision Instruments) were used to detect the signals. The hydroponic solution was replaced by the same solution added 2 mM Glu or 2 mM Gly, using the infusion tube, when the value of measured membrane potential was stable. Amplitudes are potential differences relative to the baseline before the changes.

2.6. Statistical Analysis

The experiments were conducted via a completely randomized design, and a minimum of three replicates was used in each experiment. Unless otherwise stated, for every biological replication, two leaves from each of two plants were combined and used as an independent sample. Three separate experiments were carried out independently. We ran

an analysis of variance on the data using SAS software version 8 (SAS Institute), and then used Tukey's test to determine significance at the 5% level.

3. Results

3.1. Pretreatment with a GLR Inhibitor Increases the Susceptibility of Tomato to *B. cinerea*

To explore the role of GLRs in plant defense against *B. cinerea*, the tomato plants were sprayed with DNQX or H₂O (control), 12 h prior to inoculation with *B. cinerea*. At 3 dpi with *B. cinerea*, the inoculated leaves exhibited visible disease lesions. The *B. cinerea* infection-induced damage to leaves was assessed using chlorophyll *a* fluorescence imaging to determine the deviations in the quantum yield of photosystem II (Φ PSII) (Figure 1a,b). After inoculation with *B. cinerea*, the Φ PSII significantly decreased. Notably, the value of Φ PSII in the DNQX-treated plants was lower than that in the control. DNQX treatment also increased the abundance of *B. cinerea actin* mRNA in leaves (Figure 1c). These findings indicate that GLRs positively regulate plant immunity against *B. cinerea* in tomato plants.

Figure 1. DNQX treatment increases the susceptibility of tomato to *B. cinerea*. Four-week-old tomato plants were treated with 1 mM DNQX or H₂O, 12 h prior to inoculation with *B. cinerea*: (a) pseudocolor images of tomato leaves showing Φ PSII at 3 dpi; (b) quantitative values of Φ PSII at 3 dpi; (c) transcript abundance of *B. cinerea actin* in tomato leaves inoculated with *B. cinerea* at 1 dpi. The results in panels (b,c) are presented as mean values $\pm$ SD; $n = 4$. Statistically significant differences between treatments ($p < 0.05$, Tukey's test) are shown by different letters. The experiments described above were carried out three times, with the same findings each time.

3.2. Subcellular Localization of GLR3.3 and GLR3.5

Tomato GLR3.3 and GLR3.5 are homologous to Arabidopsis GLR3.3 and GLR3.6, respectively [9], which have been reported to participate in response to nematode attack and cold tolerance in tomato. To determine the subcellular localization of tomato GLR3.3 and GLR3.5, GLR3.3-GFP, GLR3.5-GFP, and GFP were transformed into *Agrobacterium tumefaciens*, and then transiently expressed in *N. benthamiana*. Confocal microscopy of leaf

epidermal cells showed that the control GFP signal exhibited a localization in the cells' membranes, cytosols, and nuclei (Figure 2). The fluorescent signals of GLR3.3-GFP and GLR3.5-GFP were colocalized with FLS2-mCherry at the plasma membranes of the cells. It was not observed that GLR3.3 and GLR3.5 colocalized with histone 2B-mCherry at the nuclei. The results suggest that tomato GLR3.3-GFP and GLR3.5-GFP localize at the plasma membrane of a cell.

Figure 2. Subcellular localization of GLR3.3 and GLR3.5. Tomato GLRs-GFP were transiently co-expressed with FLS2-mCherry (a plasma membrane localization maker) in *N. benthamiana* leaves. The GFP and mCherry signals were visualized using confocal microscopy 48 h following the infiltration. Bar = 25 μ m.

3.3. GLR3.3 and GLR3.5 Function in Plant Defense against *B. cinerea*

To investigate the role of GLRs in plant immunity, CRISPR/Cas 9-mediated gene-edited tomato *GLR3.3* or *GLR3.5* knockout plants and *glr3.3/3.5* double-mutants were infected with *B. cinerea*. The results showed that knockout of *GLR3.3* and *GLR3.5* resulted in an obvious decrease in Φ PSII, compared with WT control (Figure 3a,b). Accordingly, the accumulation of *B. cinerea* actin mRNA in leaves significantly increased (Figure 3c) in the *glr3.3* or *glr3.5* mutants. Meanwhile, the *glr3.3/glr3.5* double-mutants showed more attenuation in plant defense when compared with the *glr3.3* and *glr3.5* mutants. These observations suggest that GLR3.3 and GLR3.5 function redundantly to establish resistance against *B. cinerea*. Furthermore, GLR3.3 plays the main role, as *glr3.3* mutants were more susceptible to *B. cinerea* inoculation than *glr3.5* mutants. On this basis, GLR3.3 was selected for further study.

3.4. Recognition of Glu and Gly by GLRs Triggered Electrical Signaling in the Immune Response

As Glu or Gly can be recognized by GLRs to trigger membrane depolarization [33], we determined Glu- or Gly-induced electrical signaling by monitoring the effect of Glu and Gly on membrane potential in tomato leaves. Glu and Gly treatment elicited significant changes in membrane potential in a short time, with an increase in amplitude of about 17–20 mV (Figure 4). Notably, the change in membrane potential induced by Glu and Gly was significantly weakened in *glr3.3* mutants. These findings suggest that electrical signaling in tomato leaves, induced by Glu or Gly, is partly dependent on GLR3.3.

Figure 3. Effects of *GLR3.3* and *GLR3.5* editing on plant defense against *B. cinerea* inoculation in tomato: (a) pseudocolor images of tomato leaves showing Φ PSII at 3 dpi; (b) quantification of Φ PSII at 3 dpi; (c) transcript abundance of *B. cinerea actin* in tomato leaves inoculated with *B. cinerea* at 1 dpi. The results in panels (b,c) are presented as mean values $\pm$ SD; $n = 4$. Statistically significant differences between treatments ($p < 0.05$, Tukey's test) are shown by different letters. The experiments described above were carried out three times, with the same findings each time.

Figure 4. Glu or Gly affects the membrane potential of WT and *glr3.3* leaves: (a) typical membrane potential changes in tomato WT or *glr3.3* mutant leaves treated with Glu or Gly; (b) quantification of membrane potential changes of amplitude. The results in panel (b) are presented as mean values $\pm$ SD; $n = 4$. Statistically significant differences between treatments ($p < 0.05$, Tukey's test) are shown by different letters. The experiments described above were carried out three times, with the same findings each time.

The *glr3.3* mutants exhibited more sensitivity to *B. cinerea*, which was consistent with the reduction in the Glu- or Gly-induced electrical signals. On the basis of these observations, we hypothesized that the recognition of Glu and Gly by GLRs is critical

in immunity against *B. cinerea*. To verify this hypothesis, a pharmacological assay was carried out by Glu- or Gly-exogenous treatment. Before *B. cinerea* inoculation, the WT plants and the *glr3.3* mutant were pretreated with Glu, Gly, or H₂O (control). In the WT plants, the *B. cinerea* inoculation-induced reduction in Φ PSII and the increase in *B. cinerea actin* mRNA accumulation were significantly alleviated by pretreatment with exogenous Glu or Gly at 3 dpi (Figure 5). However, disease symptoms in the *glr3.3* mutant were more severe, and the beneficial effects of Glu and Gly on the resistance against *B. cinerea* significantly decreased due to the knockout of *GLR3.3*. Thus, these results indicate that Glu- and Gly-induced plant immunity against *B. cinerea* partly depends on *GLR3.3*.

Figure 5. Glu- or Gly-induced immunity against *B. cinerea* partly depends on *GLR3.3*. Four-week-old tomato plants were treated with 20 mM Glu, 20 mM Gly, or water once per day for two days before *B. cinerea* inoculation: (a) pseudocolor images of tomato leaves showing Φ PSII at 3 dpi; (b) quantification of Φ PSII at 3 dpi; (c) transcript abundance of *B. cinerea actin* in tomato leaves inoculated with *B. cinerea* at 1 dpi. The results in panels (b,c) are presented as mean values $\pm$ SD; $n = 4$. Statistically significant differences between treatments ($p < 0.05$, Tukey's test) are shown by different letters. The experiments described above were carried out three times, with the same findings each time.

4. Discussion

GLRs are cation-channel proteins that exist in bacteria, plants, and animals. In animals, GLRs are proven to be involved in neurotransmission and immune regulation, and their ligand Glu acts as a signal agent between the two systems [34–36]. In plants, GLRs play a positive role in immunity against biotic stress [10,12,13]. Notably, GLR-induced defense against RKNs and insect bites was shown to be associated with electrical signals [3]. However, the relationship between GLR-mediated fungal-disease resistance and electrical signals has not been fully substantiated. Here, we provide convincing evidence demonstrating that tomato *GLR3.3*- and *GLR3.5*-modulated plant immunity against the typical fungal pathogen *B. cinerea* is related to electrical signals.

In this study, the role of GLRs in tomato seedling immunity against *B. cinerea* was unveiled for the first time. The application of the GLR inhibitor DNQX enhanced the susceptibility of tomato plants to *B. cinerea* (Figure 1). Moreover, knocking out *GLR3.3* or *GLR3.5* significantly attenuated plant immunity, leading to increased disease symptoms in leaves (Figure 3). These findings support the notion that tomato plants require GLRs to mount an adequate defense against the necrotrophic fungal pathogen *B. cinerea*. Although overexpression of radish *GluR* inhibits the growth of *B. cinerea* [37], none of the Arabidopsis *glr* mutants were found to be more sensitive to *B. cinerea* [12], suggesting that the function

of GLRs may differ among species. In the present study, the *glr3.3/glr3.5* double-mutants showed a more sensitive phenotype compared with the single-mutant *glr3.3* and *glr3.5*. These findings suggest that GLR3.3 and GLR3.5 function redundantly. Notably, a comparison of the phenotypes between *glr3.3* and *glr3.5* mutants suggests that GLR3.3 plays the main role in defense against *B. cinerea*.

Electrical signals are a rapid response involved in GLR-mediated plant resistance to biotic stress. Here, the electrical signals induced by Glu or Gly were significantly weakened in *glr3.3* mutants. Glu or Gly treatment induced the membrane potential for a short time. However, the variation in amplitude is substantially decreased in *glr3.3* mutants (Figure 4). Similarly, in Arabidopsis, the generation and propagation of electrical signals induced by leaf damage are weakened in *glr3.3* and *glr3.6* mutants [3]. However, the weakening of wound-induced surface potential changes is manifested in the shortening of the duration, but the amplitude remained the same as WT in Arabidopsis *glr3.3* mutants after wounding the leaves. These differences in results may be due to differences in species and treatments. Consistent with our results, the knockout of *GLR3.3* inhibits Glu treatment-induced membrane depolarization in Arabidopsis roots [38]. All of these results suggest that the electrical signals induced by Glu or Gly partly depend on GLR3.3.

Exogenous treatment with Glu induces immune responses to multiple diseases, such as the rice blast fungus *Magnaporthe oryza*, the necrotrophic fungus *Alternaria alternata* in tomato fruits, and the soil-borne bacterial pathogen *Ralstonia solanacearum* [39–41]. Sun et al. (2019) reported that Glu treatment enhances the resistance of tomato fruits against *B. cinerea* [42]. In the present study, exogenous treatment with Glu or Gly induced immunity against *B. cinerea* in WT plants; however, the benefits of this treatment were significantly inhibited in the *glr3.3* mutant (Figure 5). The data presented here demonstrated that GLR3.3 was required for Glu- or Gly-induced immunity against *B. cinerea*. Thus, these findings suggest that the *B. cinerea* resistance induced by Glu or Gly is partly dependent on GLR3.3.

Based on the data provided here, we propose that GLRs are involved in defense responses to *B. cinerea* through electrical signals. Considering the phytohormone-mediated immunity, the salicylic acid signaling pathway facilitates the defense against biotrophic and hemibiotrophic pathogens, while the JA/ethylene signaling pathway functions against necrotrophic pathogens [43,44]. An antagonistic relationship between these two pathways is evident in many plant pathosystems [45,46]. However, GLR3.3 has been shown to positively mediate the resistance against the biotrophic pathogen *Pst* DC3000, while at the same time being essential to immunity against the necrotrophic pathogen *B. cinerea*. Our proposal is reasonable, as GLR3.3 can recognize Glu, which is considered to be a kind of DAMP [6,47]. DAMPs trigger early secondary signals and induce expression of JA/ethylene, an SA signaling defense gene. Furthermore, the injection of artificial current into the surface of the stem induces a significant increase in the accumulation of JAs in plants [13]. In accordance with these results, GLR3.3 may play a role in the early phase of plant resistance through Glu- or Gly-induced electrical signals, which respond in seconds or minutes, upstream of the hormone-signaling pathway. The JA signaling pathway may also be activated by electrical signals. In addition, it is well documented that AtGLR3.3 participates in plant resistance by mediating $[Ca^{2+}]_{cyt}$ variation and oxidation responses [12]. The relationship between these pathways and electrical signals in the regulation of plant resistance against *B. cinerea* warrants further investigation.

5. Conclusions

The data presented here show that GLR3.3 and GLR3.5 play a positive role in tomato plant defense against the necrotrophic pathogen *B. cinerea* through electrical signals. Our study provides new insights into the plant defense mechanism against *B. cinerea*. Nonetheless, it will be interesting to further investigate the relationship between several signaling pathways induced by GLR, which contribute to plant immunity against fungal pathogens.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app112311217/s1>, Table S1: Primers used in this study, Figure S1: Diagram of the electrode setup.

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