REGULATION OF THE JUB1 STRESS-RELATED TRANSCRIPTION FACTOR IN *ARABIDOPSIS THALIANA* IN RESPONSE TO BIOTROPHIC FUNGUS *OIDIUM NEOLYCOPERSICI*

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Abstract: Biotic stresses influence fitness of the plants and may decrease their productivity. Especially biotrophic fungi govern complex regulation of host metabolism to fulfill their nutritional needs, while the plant tries to avoid this fungal activity. The defense system of the plant may involve transcription of stress-dependent genes, activation of signaling pathways or production of antimicrobial compounds. The responsible signal molecule in biotrophic pathogen-host interaction was found to be mostly the salicylic acid (SA). We studied here, whether the salicylic acid (SA) is required for the induction of a stress-related transcription factor JUB1 by the biotrophic fungus of tomato *Oidium neolycopersici* or not. To prove the regulation of JUB1, we isolated the upstream region of the gene and transcriptionally fused to *gus* reporter gene. This promoter::reporter fusion was then transferred into wild type and two salicylic acid insensitive mutants of *Arabidopsis thaliana* (*nim1-1* and *nahG*). The transgenic plants were infected by powdery mildew (PM) *O. neolycopersici*. We found an obvious increase of reporter gene expression in all three lines mostly at the area, where the pathogen was contacted to the plants. Based on the result, the stress-dependent JUB1 transcription factor is probably not influenced by the SA mutations of *nim1-1* and *nahG*. However, the induction appeared along with hydrogen-peroxide development, which suggests that this gene regulation in response to powdery mildew is probably regulated via H_2O_2 and not by SA signaling.

Keywords: biotic stress, H_2O_2 signaling, *Oidium neolycopersici*, salicylic acid, transcription factor

Introduction

Pathogens may cause extreme decrease in crop yield, which may have serious economic consequences. The *Oidium neolycopersici* powdery mildew (PM) is one of the major diseases of tomato (*Solanum lycopersicum*). Additionally it has a broad host range (over 60 species) in 13 plant families, mostly in *Solanaceae* and *Curcubitaceae*. High humidity promotes pathogen proliferation, therefore the *O. neolycopersici* causes disease mostly in greenhouse grown tomatoes. In spite of the PM does not infect the fruit, a marked decrease is observed in fruit size and quality on the infected plants (Jones et al., 2001), which results in significant shortfall for growers. Although plant-pathogen interactions have been under intense scientific research, many details of the interaction are still unclear. Studies searching the genetic and molecular backgrounds of these interactions may provide solution for enhancement of resistance in cultivated plants.

The biotrophic pathogens subsist on living plants only, since the host plant metabolism satisfies their nutritional needs. Therefore, these organisms are able to modulate plant metabolic pathways to support their growth and reproduction. Especially in the case of fungi, this manipulation places at the direct contact site between fungus and the plant. Fungi, during their infection process, develop a specific feeding structure – named haustorium – inside the plant cell lumen without puncturing the cytoplasm membrane. This particular organ is through which the nutrient exchange and signal transduction take place. The metabolic system of host plant may be modified by fungal effector molecules, which trigger increased expression of certain genes. However, the plants try to avoid the parasitic activity of the attacker since this may decrease their fitness and survival rate. Therefore, after recognition of infection a signal transduction process is activated, mediating by the plant defense responses against the pathogen (Blumwald et al., 1998).

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In biotrophic-host interaction usually the salicylic acid (SA) is the specific signal, which induces the components of immunity (Glazebrook, 2005). This defense mechanism may involve the activation of resistance *(R)* genes, which recognize effector molecules along with induction of immune response, or may include the production of antimicrobial compounds such as the phytoalexins. Earlier studies demonstrated that the transcription factors regulate the expression of defense genes in immune response, such as the WRKYs, MYBs and NACs does (Ambawat et al., 2013; Nuruzzaman et al., 2013; Pandey and Somssich, 2009). The NAC (NAM/ATAF/CUC) transcription factors compose a large gene family and are specific to plants (Riechmann et al., 2000). The members of this family are found to regulate both abiotic and biotic stress responses, especially the JUNGBRUNNEN 1 encoding *JUB1/ANAC042* gene, which reacted to necrotrophic fungus *Alternaria brassicicola* infection. The *jub1* knock-out mutants represented high susceptibility to this fungus and failed to accumulate camalexin, which is a specific *Arabidopsis* phytoalexin (Saga et al., 2012). Camalexin has protective function against fungal and bacterial pathogens, and *JUB1* was found to participate in biosynthesis of this molecule (Saga et al., 2012). Additionally, this gene was found to negatively regulate senescence, while overexpressorion delayed bolt development, and repressed intracellular H_2O_2 levels along with increase in abiotic stress tolerance. During abiotic stress the JUB1 was found to interact with 5'- RRYGCCGT-3' consensus core sequence of *DREB2A* promoter. DREB2A (DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2) acts as regulator in drought and high-temperature stress (Lim et al., 2006; Liu et al., 1998). Shahnejat-Bushehri et al. (2012) have found that the JUB1 associates with thermomemory genes, such as Heat Shock Proteins (HSP) and Heat Shock Factors (HSF). The heat-stress tolerance is probably regulated via activation of these genes, since *JUB1* overexpression resulted in up-regulation of *HSPs* and *HSFs* (Wu et al., 2012). Although the *JUB1* was found to be slightly influenced by the mutation of isochorismate synthase (*ics1*) gene in response to *Golovinomyces orontii* (Chandran et al., 2009), however, the SA induction–deficient (*sid2-2*) did not alter the induction of *JUB1* in response to Flg22 elicitor compared to the wild type. Our aim was to study the regulation of this *Arabidopsis NAC* gene in response to *O. neolycopersici*, whether its PM-mediated induction is dependent on SA or not.

Materials and methods

Cloning process using Gateway® Technology

For analyzing the regulation of the *JUB1* gene in response to powdery mildew infection, the upstream region of the gene was isolated using PCR with the following primers:

(P15'-TTACAGCGAGGGAGATAATGA-3', P2 5'-TCGATCTCTTTAGAACACCAATCA-3').

The primers were designed based on the sequence of *JUB1* gene (AT2G43000) of The Arabidopsis Information Resource (TAIR) database. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used for the PCR reaction. This enzyme provides blunt end for the amplified fragment, which is required for the process of Gateway Cloning Technology (Invitrogen). The PCR product was loaded on the agarose (1% TAE) gel, excised and isolated back using the Wizard® SV Gel and PCR Clean-Up System of Promega. The fragment was cloned into the pENTR D-Topo vector (Invitrogen), and then subcloned into pGWB633 binary vector (Nakamura et al., 2010) mediated by the LR clonase enzyme of Invitrogen. The recombinant positive binary vector contained the promoter of *JUB1* (*pJUB1*), which was transcriptionally fused to the *gus* reporter gene. The cloning success was confirmed by PCR and sequencing. Additionally, the pGWB633 vector contains the *bar* glufosinate-ammonium

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resistance gene in the T-DNA region as a selection marker of positive transformants.

For control experiments a no-promoter (*pØ::GUS*) fusion was also created in pGWB633, which was also confirmed by PCR and sequencing.

Plant preparation

The GV3101::pMP90 *Agrobacterium tumefaciens* strain was transformed with the recombinant pGWB633 binary vectors containing the *pJUB1::GUS* and *pØ::GUS* fusion. The surviving colonies were checked by PCR, and the positive bacteria were used for transformation of *Arabidopsis thaliana* lines. The seeds of the lines were obtained from Arabidopsis Biological Resource Center (ABRC): three lines were selected for the experiment; wild type (WT), *nim1-1* mutant and *nahG* transgenic line. All the lines originated from Wassilewskija ecotype. The *nim1-1* is defective in SA-mediated signal transduction, while the *nahG* eliminates all the innate SA by constitutively expressing the salicylate-hydroxylase gene (Delaney et al., 1995; Gaffney et al., 1993). If the *pJUB1::GUS* fusion is regulated in response to PM in these lines, the SA is neither the responsible signal for regulation of *gus*, nor of *JUB1*. The *Arabidopsis* seeds were sowed on water-saturated soil and kept on 4ºC for two days. After germination the plants were cultivated in growth chamber for four weeks with the following conditions: 22-24ºC, cool white light, long day illumination, 60% RH. The first bolts were cut back to induce multiple bolt development. The six-week old *Arabidopsis* lines were transformed with the GV3101::pMP90-pGWB633 (*pJUB1::GUS*) and with the GV3101::pMP90-pGWB633 (*pØ::GUS*) after the description of Clough and Bent (1998). After transformation the plants, T0s were transferred back to the growth chamber and cultivated for further weeks till the siliques were ripen. The seeds of T0 plants were harvested and sowed into soil as described above. Two weeks after germination the T1 seedlings were sprayed with 10 mg/l glufosinate-ammonium containing herbicide (Finale). The spray was repeated three times, when the positive transformants were clearly distinguishable from non-transgenic plants. The survived plants were selected and transplanted. The plants were grown to T3 generation to select transformants containing one copy of the transgene in homozygote form. Three transgenic lines for each genetic background (WT, *nim1-1*, *nahG*) were selected for further experiments. The selected T3 plants were used for testing the basal expression of the *gus* and its induction in response to PM infection.

Four-week old transgenic plants were mock-inoculated and inoculated with *O. neolycopersici* following the description of Huibers et al. (2013). The plants were grown in growth chamber (22-24ºC, cool white light, long day illumination, 60% RH) for further two weeks. Fourteen days after inoculation (dai) the PM colonies were visible on the leaves and these leaves were harvested to stain them histochemically.

Histochemical staining of transgenic plants

Histochemical staining was performed after the descriptions of Jefferson et al. (1987), with the modification in the GUS-Buffer: 100 mM phosphate buffer (pH 7.0), 10 mM EDTA, 1% Triton X-100, 0.3% H_2O_2, 0,5 mg/ml X-Gluc/5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt. The leaves were incubated in the GUS-buffer overnight at 37ºC, and on the next day the chlorophyll was removed using 70% ethanol wash. The fungal filaments were stained with cotton blue solution. The leaves were observed using stereo and light microscopes.

DAB-staining of hydrogen-peroxide

Wild type non-transgenic plants were inoculated with *O. neolycopersici* as described above. The accumulated H_2O_2 in response to PM was stained using the method

of Thordal-Christensen et al. (1997). After 8 hour incubation the leaves were dipped into 70% ethanol to remove chlorophyll. The fungal filaments were stained with cotton blue solution. The leaves were observed using stereo and light microscopes.

Results

Histochemical staining of non-infected transgenic plants with all three types of genetic background (WT-*pJUB1::GUS*, *nim1-1-pJUB1::GUS* and *nahG-pJUB1::GUS*) expressed the β-glucuronidase on a basal level, which expression is not tissue specific. The expression of the reporter gene was detected in the root caps (the meristematic zone was free of *gus* expression (Figure 1F, G)), in axillary buds, in the junction of root and hypocotyl, in style tissue of the carpel and at the margins of leaves (Figure 1). The *gus* expression was lacking from the no-promoter (*pØ::GUS*) control plants (Figure 1H).

After the infection of these transgenic plants we observed the colonies covered leaves at 14 dai. The *nim1-1* and *nahG* lines were infected in a higher rate compared to the wild type, since the SA deficiency increase susceptibility to PM (Delaney et al., 1995; Gaffney et al., 1993). The histochemical staining demonstrated that *gus* expression was induced significantly in the infected plants compared to the mock-infected ones. This phenomenon was detected in all three types of transgenic plants, especially in the plants with *nim1-1* and *nahG* genetic background. During microscopic observation of the infected leaves we detected the induction mostly at that area, where the pathogen was in direct contact with the host (Figure 2). The uninfected area displayed basal expression of the *gus*. The no-promoter (*pØ::GUS*) control plants did not display *gus* expression after the infection.

Earlier studies demonstrated the hydrogen-peroxide accumulates in response to biotrophic pathogen invasion (Wang et al., 2009). Therefore, we tested the location of the increased H_2O_2 in wild type non-transgenic plants in response to *O. neolycopersici*. We found that the hydrogen-peroxide accumulated at the site of infection. Microscopic observation demonstrated that the increased H_2O_2 was detected in the epidermal pavement cells and only around the haustorium, in the extrahaustorial matrix (EHM) (Figure 3). Since the *JUB1* promoter was induced in SA signal defective plants, it is probably not regulated by SA. Inversely, the hydrogen-peroxide may be an inducer in response to pathogenic infection, since this molecule was detected at the site of infection.

Figure 1. Expression of pJUB1::GUS *in transgenic* Arabidopsis thaliana *plants without powdery mildew (PM) fungus infection. A) 4-week old seedling leaf margin, B) Axillary bud, C) Junction of hypocotyl and root, D) Stigma and style of the carpel with pollen grains, E) 5-day old seedling cotyledon, F) Root tip, G) Root cap, H) No-promoter* (pØ::GUS) *control plant. Scale bars indicate 100 µm.*

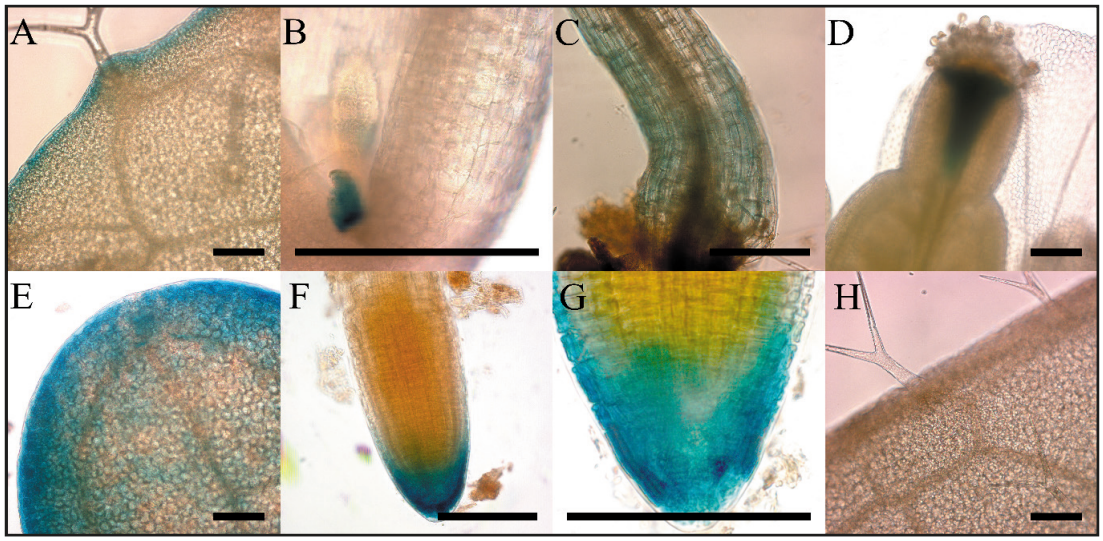

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Figure 2. Stereomicroscopic observation of PM-induced gus *expression in* pJUB1::GUS *transgenic* Arabidopsis thaliana *plants compared to the mock-infected plants. Genetic background of transgenic plants: WT – wild type;* nim1-1 *– non-induced immunity;* nahG *– contains the gene which encodes salicylate hydroxylase. Scale bars indicate 1 mm.*

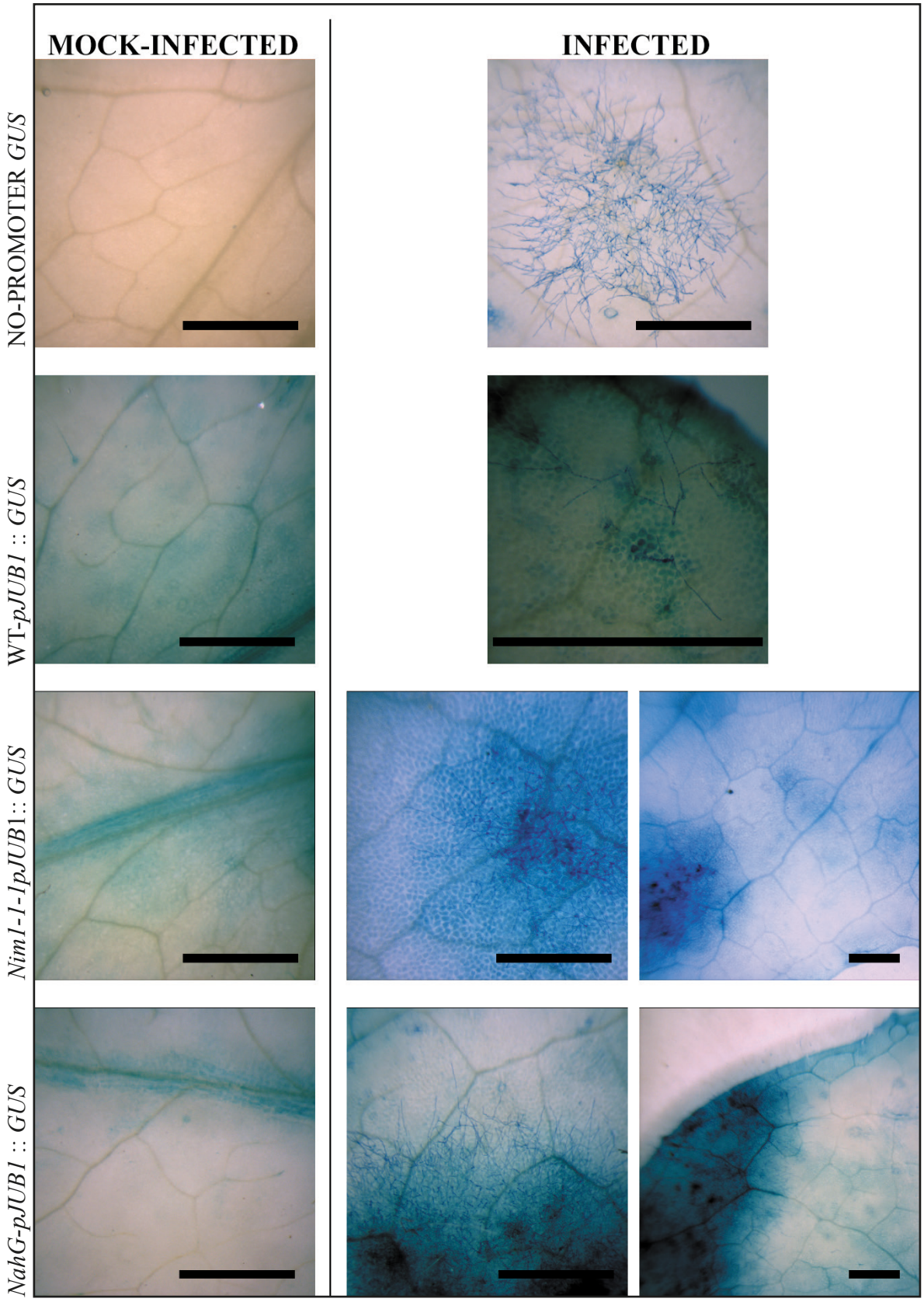
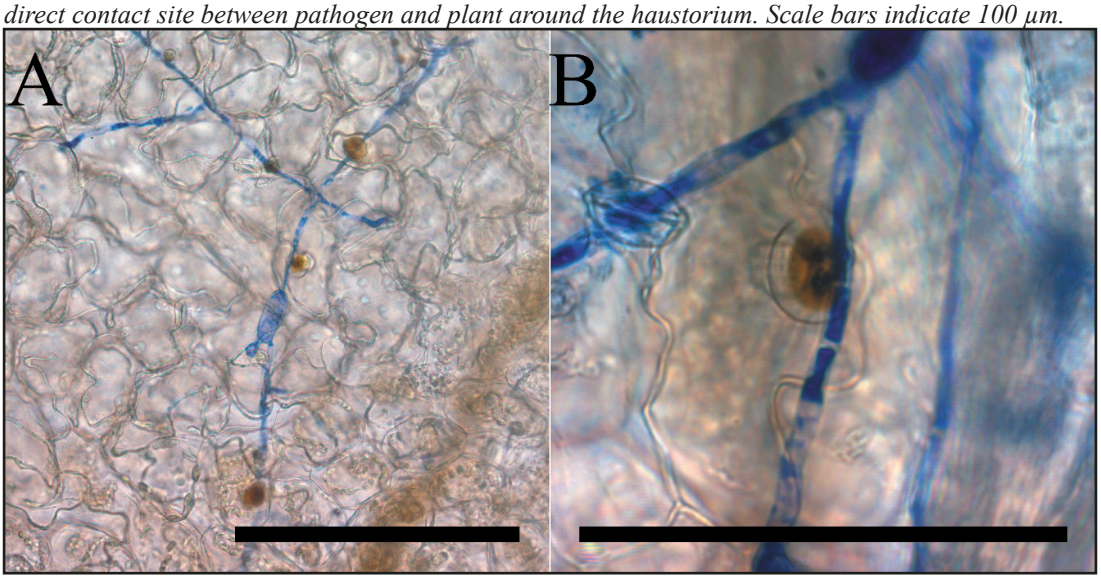


Figure 3. Accumulated hydrogen-peroxide in response to Oidium neolycopersici *infection in* Arabidopsis thaliana*. A) H_2O_2 oxidized DAB (brown stain) only at the area where the pathogen induced the hydrogen-peroxide accumulation. B) The increased level of H_2O_2 was detected specifically at the direct contact site between pathogen and plant around the haustorium. Scale bars indicate 100 µm.*



Discussion

The goal of this study was to observe the regulation of an *Arabidopsis* stress-related transcription factor in response to a biotrophic fungus *O. neolycopersici*. In preliminary experiments we found that the *pJUB1* induces basal expression in various tissues such as in the root cap, in axillary buds, in the junction of root and hypocotyl, in style tissue of the carpel and at the margins of cotyledons and young leaves. This basal expression was detected in all three types of transgenic plants (WT-*pJUB1::GUS*, *nim1-1-pJUB1::GUS* and *nahG-pJUB1::GUS*), meaning that SA is probably not required for this basal regulation.

On the effect of the infection with *O. neolycopersici*, a significant induction of *gus* expression was observed in the infected leaves compared to the PM-free ones, which suggests that this gene probably plays a role in the defense reaction against the pathogen. The induction was observed in all three types of transgenic plants, meaning that neither the SA, nor the nim1-1-mediated SA signal transduction is required for induction by the biotrophic *O. neolycopersici*. Although, the *G. orontii* is also a biotrophic fungus, it was found that the SA biosynthesis mutant *ics1* slightly modulated the induction of *JUB1* in response to *G. orontii* (Chandran et al., 2009). However, the *JUB1* reacted to PM in this mutant, but in a bit lower rate (7-fold) compared to the induction in the wild type (8-fold) (Chandran et al., 2009). Saga et al. (2012) demonstrated that *JUB1* expression was significantly lower in *ein2-1* (ethylene-insensitive) mutant in response to Flg22 in the primary root apex compared to the wild type. This suggests that *JUB1* may be induced by the elicitor via ethylene signaling. Additionally the *JUB1* responded intensively to *Sclerotinia sclerotiorum* to regulate camalexin against the pathogen, but the coi1-2 (coronatine-insensitive) mutation did not influence the up-regulation (Stotz et al., 2011).

Microscopic observation of the infected leaves showed that the induced expression was mostly around the PM colonies, in the haustorium containing plant cells. Recent study has demonstrated that plant cells around the haustoria express a different gene set compared to the uninfected area of the leaf. Interestingly, among these specifically induced genes the most are not previously associated with defense responses. Many of them manage

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endoreduplication process in mesophyll cells, which is activated by the fungus. The multiplication of chromosomes results in overstrain of overall host metabolism, which benefit the fungus survival and reproduction. Interestingly among the specifically induced genes the *pathogenesis-related 1* (*PR-1*) was identified, which participate in SA-mediated defense response (Chandran et al., 2010). However, ortholog of *JUB1* in grape was found to co-express with the *Vitis PR-1* in response to *Erysiphe necator* (Fung et al., 2008).

Earlier results showed that biotrophic fungus infection induce hydrogen-peroxide accumulation at the site of infection (Wang et al., 2009). Observation of infected wild-type non transgenic plants lead to recognize, the increased H_2O_2 is constricted around the developed haustorium, in the extrahaustorial matrix. Based on the phenomenon the H_2O_2 may be the inducer signal of *JUB1* expression, since this molecule also has signaling function during biotic stress (Slesak et al., 2007). The *JUB1* was found to be H_2O_2 inducible along with back-regulation of innate H_2O_2 content (Shahnejat-Bushehri et al., 2012; Wu et al., 2012), which suggests that *JUB1* probably responds to *O. neolycopersici* via H_2O_2 homeostasis. Wu et al. (2012) also represented that *JUB1* functions as a regulator of plant longevity. The overexpression of *JUB1* resulted in delayed senescence and increased cytokinin level, which suggests that this gene is up-regulated at the site of infection to retard aging of the leaf. Additionally, a recent study demonstrated that the highest cytokinin level in the primary root apex was detected in the root cap (Antoniadi et al., 2015). Adding our results to this, the JUB1 may participate in cytokinin biosynthesis. Therefore, during infection, the up-regulation of *JUB1* by the biotrophic fungi may corporate in the well-known 'green island' symptom (Thomas and Ougham, 2014), putatively regulating susceptibility and not defense. Although *JUB1* gene was found to play a role in biosynthesis of camalexin, and it acts as defense gene against necrotrophs (Saga et al., 2012), its activation may benefit the pathogen in the host-biotroph interaction.

Conclusion

The goal of this study was to observe the regulation of the stress-related *JUB1* gene during biotic stress. We have found that *JUB1* is inducible by biotrophic fungus *O. neolycopersici*, and the up-regulation can be detected at the area of the infection site. The expression of *JUB1* is probably dependent on hydrogen-peroxide and not SA homeostasis during the infection. *JUB1* possibly play a role in cytokinin biosynthesis, therefore incorporates in the 'green-island' effect.

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