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Assessing functional role of three water deficit stress-induced genes in nonhost disease resistance using virus-induced gene silencing in *Nicotiana benthamiana*

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Nonhost disease resistance is the most common form of disease resistance exhibited by all plants and to date this phenomenon is not yet completely understood. Understanding the mechanisms behind nonhost resistance may facilitate engineering crop plants with durable resistance. Our previous studies identified putative roles for three genes *flavonol-3-O-glucosyl transferase* (*F3OGT*), an *alcohol dehydrogenase* (*ADH*) and *trans caffeoyl coA-3-O methyl transferase* (*CcoAOMT*) in water deficit stress tolerance. Preliminary information from our earlier study also suggested that Arabidopsis null mutants for these genes exhibited altered levels of tolerance to bacterial pathogens. In this manuscript we document more evidences to show the relevance of these genes in nonhost resistance using *Nicotiana benthamiana*. By using virus-induced gene silencing (VIGS), we independently downregulated these three genes and analyzed the response of gene silenced plants to bacterial pathogens. Our results showed that *F3OGT*, a gene implicated in anthocyanin biosynthesis, silenced plants compromised resistance against a nonhost pathogen. Based on this and previous results, we propose that anthocyanin might play a role in regulating plant defense against bacterial pathogens. Response of *ADH* or *CcoAOMT* gene silenced plants to bacterial nonhost pathogens was similar to wild-type. However, *CcoAOMT* gene downregulated plants were slightly more susceptibility to a host pathogen.

Key words: anthocyanin, drought tolerance, F3OGT, hypersensitive response, lignin, multi-stress tolerance, nonhost resistance, *Pseudomonas syringae*, VIGS

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The phenomenon that an entire plant species is resistant to all genetic variants of a non-adapted pathogen species is referred to as nonhost resistance.[1,2] In contrast to *R*-gene mediated resistance, nonhost resistance is expressed by every plant towards the majority of potential pathogens. Based on defense responses exhibited by plants, nonhost resistance is divided into two types, namely, type-1 (no visible symptoms) and type-2 (that induces hypersensitive cell death).[2] For example, *Nicotiana benthamiana* plants defend *P. syringae* pv. glycinea and *Pseudomonas syringae* pv. tomato T1 by type-1 and type-2 nonhost resistance mechanisms, respectively. Plants achieve this broad spectrum of resistance though complex set of mechanisms and deciphering this will help to identify potential candidate genes for engineering plants for durable disease resistance.[2] Although some information are available, exact mechanisms leading to nonhost resistance in plants is not yet completely understood.

Some inducible defense responses are common for both abiotic stress tolerance and disease resistance.[1,3] For example, *activated disease resistance 1* (*ADR1*) gene that has previously been shown to impart broad spectrum disease resistance, is recently implicated in drought tolerance in Arabidopsis.[4] Proline, flavonoids, lignin and few other compounds are induced under various stresses in plants and some of these are shown to play a role in both biotic and water deficit stress responses.[5-10] Earlier, we have documented the response of Arabidopsis mutants of *flavonol-3-O-*

glucosyl transferase (*F3OGT*), an *alcohol dehydrogenase* (*ADH*) and *trans caffeoyl coA-3-O methyl transferase* (*CcoAOMT*) genes to abiotic stresses, host- and non-host-bacterial pathogens.[11]

F3OGT, *ADH* and *CcoAOMT* gene transcripts are known to be induced under water deficit stress in *N. benthamiana* and tobacco.[11,12] Further, virus-induced gene silencing (VIGS) and RNAi of these three genes in tobacco and also Arabidopsis T-DNA insertion mutants for these three genes demonstrated their involvement in water deficit stress tolerance. Under non-stress conditions, independent knockdown or knockout of these three genes in *N. benthamiana* (by VIGS), *N. tabacum* (by RNAi) and Arabidopsis (mutant) showed normal phenotypes similar to that of their respective wild-types.[11-13]

In this study, we further analyzed the role of these genes specifically in nonhost resistance. VIGS has been shown to be an effective method for reverse genetic studies in many species and to silence gene family members.[14] Hence, in the present study we silenced *F3OGT*, *ADH* and *CcoAOMT* genes in *N. benthamiana* by tobacco rattle virus (TRV) based-VIGS and analyzed the response of silenced plants to nonhost pathogens. Experimental methodology of VIGS and pathogen infection assays used here are described in our earlier manuscripts.[11,12,14,15]

F3OGT Gene Silenced Plants Compromised Type-1 Nonhost Resistance and Showed Enhanced Spread of Disease-Induced Cell Death

The enzyme UDP glucose F3OGT, a class of the major sub family UDP glucosyl transferase (UGT) genes, is shown to be involved in anthocyanin biosynthesis.[7,16] In Arabidopsis, UGTs are encoded by a large multigenic family of 120 members. Few studies showed their actual function in planta, particularly during plant-pathogen interactions.[7,17] For example, flax plants overproducing a UGT from *Solanum sogarandinum* (*SsGT1*) showed higher resistance to Fusarium infection.[18] However, relevance of *F3OGT* gene in nonhost resistance is not yet studied.

In the present study we have silenced one of UGT member, *F3OGT* gene, in *N. benthamiana* by VIGS. In our earlier study we showed that more than 70% reduction in endogenous transcripts of this gene can be achieved using VIGS.[12] The gene silenced plants harbored marginal growth of a nonhost pathogen *P. syringae* pv. glycinea (**Fig. 1B**) but not *P. syringae* pv. tomato T1 (**Fig. 1A**) indicating a compromise of type-1 nonhost resistance. In addition, Arabidopsis *f3ogt* mutants have also showed susceptibility to a nonhost pathogen *P. syringae* pv. tabaci.[11] Our earlier study showed that silencing *F3OGT* gene in *N. benthamiana* lead to reduction in anthocyanin content.[12] Apart from *F3OGT* gene itself, anthocyanins and other flavonoids are also known to be induced upon pathogen infection and such anthocyanins impart disease resistance.[19-22] Also, present study provides clues that these compounds may be involved in imparting nonhost resistance to bacterial pathogens. Nevertheless, both in the present study (**Fig. 1C**) and in our earlier study,[11] the *F3OGT* gene silenced plants have shown similar bacterial growth as that of wild-type when inoculated with a host pathogen.

F3OGT gene silenced *N. benthamiana* plants showed disease-induced cell death at the same time as that of wild-type plants when inoculated with host pathogen *P. syringae* pv. tabaci. However, the cell death spread was faster in the gene silenced plants compared to wild-type. Similarly, Arabidopsis *f3ogt* mutants also showed enhanced disease symptoms compared to wild-type, although bacterial growth between mutants and wild-type were same.[11] Role of anthocyanins in scavenging oxygen radicals and inhibiting lipid peroxidation has been well documented.[23] Hence, it is possible that enhanced spread of cell death to adjacent cells in *F3OGT* gene silenced *N. benthamiana* plants may be due to lack of oxidative stress control that are otherwise achieved in wild-type plants by adequate induction of anthocyanins. In concurrence with this result, our earlier studies also showed the role of *F3OGT* gene in regulating free radicals during water deficit stress.[11-13] Hence, it is likely that F3OGT may play a similar role (i.e., controlling oxidative stress and lipid peroxidation) during disease-induced cell death. These data indicate that *F3OGT* gene is a potential candidate to investigate the role of anthocyanin in pathogen-induced cell death. Taken together, our data and other literature information emphasize the importance of UGTs in plant-pathogen interactions and water deficit stress.[7,17,18,24,25] Conclusively, we propose that *F3OGT* plays role in both water deficit stress tolerance and non-host pathogen-induced defense via regulating anthocyanins and other flavonoid compounds.

Silencing of *ADH* Gene Delays Hypersensitive Response (HR) Produced during Nonhost Pathogen Infection

The *ADH* gene used in this study shares more than 80% nucleotide identity with *ADH1* gene from mango, grapes and several tobacco species. *ADH1* is known to be induced during low oxygen stress.[26-28] Both *ADH1* and *ADH2* genes are shown to be involved in flooding tolerance in rice seedlings.[26] Further, the expression of these genes are also responsive to wounding in maize and lettuce seedlings.[27] Our earlier report also showed that silencing of *ADH* gene in *N. benthamiana* and *N. tabacum* led to increased susceptibility of these plants to water deficit stress.[11,12] Consistent with this observation the *adh* Arabidopsis mutant plants also showed susceptibility to water deficit and salinity stress.[11] In the present study, we found that occurrence of HR against *P. syringae* pv. tomato T1 was delayed by 24 h in the *ADH* gene silenced *N. benthamiana* plants compared to wild-type (**Fig. 2**). Currently, there is only little evidence to link between ADH and pathogen stress. For example, ADH enzyme activity is known to be induced during different pathogen derived elicitor treatment in wheat and during hypersensitive cell death due to *tobacco mosaic virus* infection in tobacco.[28,29] However, exact mechanism of this gene action in pathogen induced cell death remains unclear.

Nevertheless, both the *N. benthamiana* *ADH* gene silenced plants (**Fig. 1A and B**) and Arabidopsis *adh* mutants did not harbor nonhost pathogen growth.[11] Also, response of gene silenced *N. benthamiana* (**Fig. 1C**) and Arabidopsis mutant plants to virulent host pathogen was similar to

wild-type.[11] At this point more research is required to explain the reason for delay in HR without leading to more nonhost pathogen growth. Together our studies indicated that *ADH* gene plays a role in regulating HR during type-2 nonhost resistance in addition to imparting water deficit stress- and salinity-tolerance.[11-13]

Response of *CcoAOMT* Gene Silenced *N. benthamiana* Plants to Bacterial Pathogen Infection

CcoAOMT is one of gene involved in lignin biosynthesis.[30] In our earlier study we showed the response of *ccoaomt* Arabidopsis mutant plants to both host and nonhost pathogens.[11] The multiplication of host pathogen in the mutant plants were similar to what was observed in wild-type plants but interestingly the mutant plants showed more disease symptoms.[11] However, in the present study, *CcoAOMT* gene silenced *N. benthamiana* plants harbored more host pathogen (*P. syringae* pv. tabaci) when compared to non-silenced control plants (**Fig. 1C**). One of the reasons for Arabidopsis *ccoaomt* mutants not being hyper-susceptible is probably due to redundant function of gene family members. VIGS can silence all gene family members. Nevertheless, our earlier and the present studies clearly showed that the silenced plants do not compromise nonhost resistance (**Fig. 1A and B**).

Several studies demonstrated that manipulating the expression of single genes within the monolignol biosynthetic pathway can affect interaction of engineered plants with biotic environment.[8,9,11] *CCoAOMT* promoters have been shown to respond to biotic and abiotic stress in poplar.[31] Furthermore, de novo formation of lignin has been shown to be induced at sites of pathogen infection. At these sites, lignin deposition has been shown to play a role in plant defense by strengthening the cell wall to prevent spread of invading pathogens.[30,31] However, different research groups came to contradictory conclusions about lignin content and its relation to disease resistance.[9,32] Reasons for these contradictory results are probably due to differences in levels of lignin downregulation and alteration in composition of two major lignin monomers, namely guaiacyl (G) and syringyl (S) units (S:G). Particular lignin content and alteration in ratio of S:G lignin ratio is depend on which gene in lignin biosynthesis is silenced.[30] Hence, a detailed analysis of not only total lignin downregulation but also composition

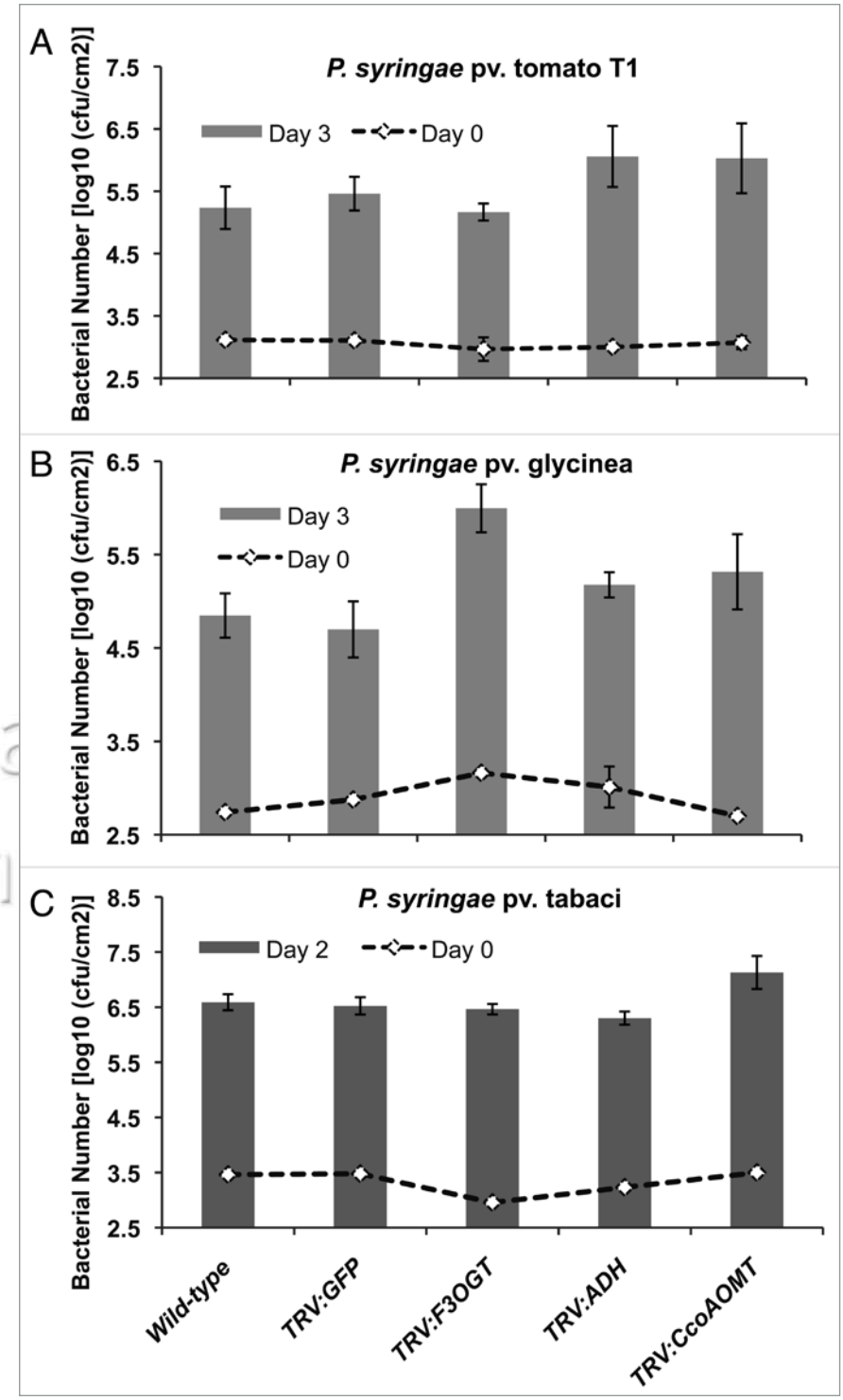

Figure 1. Growth of nonhost and host pathogens in *F3OGT, ADH* and *CcoAOMT* gene silenced *N. benthamiana* plants. Nonhost pathogens *Pseudomonas syringae* pv. tomato T1 (A) and *P. syringae* pv. glycinea (B); host pathogen *P. syringae* pv. tabaci (C) were inoculated on to the silenced plant leaves. Growth of each pathogen was quantified at the indicated time period. TRV:*GFP* represent vector control. Error bar represents standard deviation.

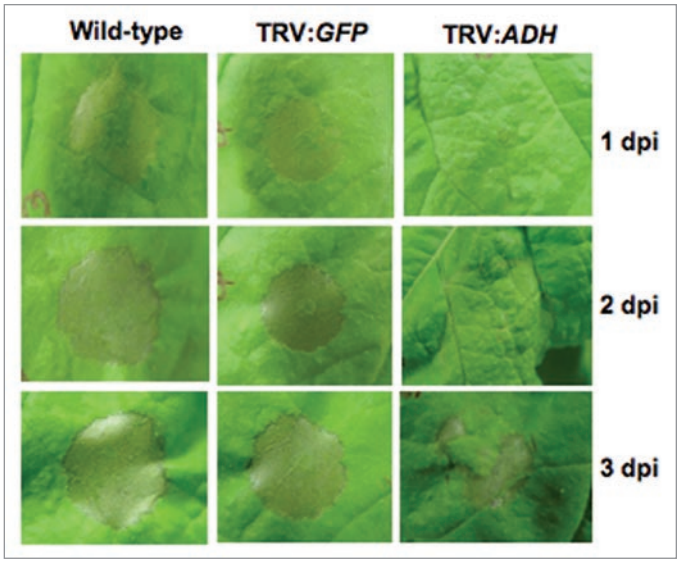

Figure 2. Hypersensitive response (HR) of *ADH* gene silenced *N. benthamiana* plant against type-2 nonhost pathogen. The gene silenced *N. benthamiana* plant leaves were inoculated with nonhost pathogen *P. syringae* pv. tomato T1. The differences in HR symptoms were photographed at 1, 2 & 3 days post inoculation (dpi).

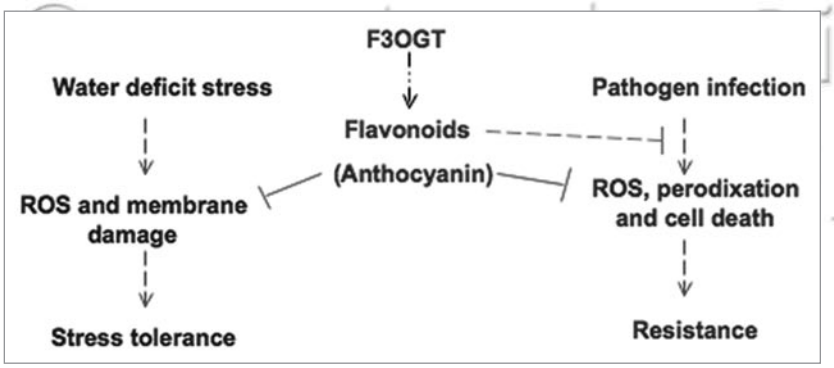

Figure 3. Hypothetical model showing a possible role for *F3OGT* gene in water deficit stress tolerance and pathogen resistance in *N. benthamiana*. Both water deficit stress and pathogen infection can induce anthocyanin accumulation through *F3OGT* gene regulation. This gene can also influence total flavonoid pool in the cell. Free radical scavenging property of anthocyanin can reduce reactive oxygen species (ROS), reduce lipid peroxidation and thus protect cell membrane during pathogen induced cell death and water deficit stress. Apart from anthocyanin, other molecules of general flavonoid pool can also be involved in protecting cell during pathogen infection. This model indicates that anthocyanin plays a role both in biotic and abiotic stress tolerance.

of lignin in the *CcoAOMT* gene silenced plants is needed to better conclude the role of this gene in pathogen defense.

Common Mechanisms Related to Abiotic Stress Tolerance and Nonhost Disease Resistance in Plants

General stress responses in plants namely activation of reactive oxygen species scavenging system, proline accumulation, anthocyanin production, lignin deposition, and alterations in certain other metabolites are known to be common under both biotic and abiotic stresses.[4,5,8,10,23] Especially, nonhost resistance and water deficit stress tolerance are controlled by multitude of complex mechanisms and share several basal plant defense strategies. Earlier we have demonstrated that *F3OGT* gene through higher anthocyanin accumulation protects membrane integrity and cell viability during water deficit stress in *N. benthamiana*.[11-13] Further, this gene is also important in preventing total chlorophyll and pheophytin degradation during water deficit stress.[12] Maintenance of chlorophyll pigments are important for photosynthesis during stress. Conclusively, our studies and others clearly demonstrated the role of this gene in water deficit stress tolerance. In the present study, we have showed that *F3OGT* is also involved in nonhost resistance of plants probably by regulating anthocyanin levels in the cell. Taken together, using results from the present and other studies,[3,5,22] we propose a model showing the involvement of *F3OGT* gene and flavonoids (specifically anthocyanin) during water deficit and pathogen stress (**Fig. 3**).

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