

# Effect of *Fusarium culmorum* infection on survivability of a T-DNA tagged mutant of *Arabidopsis thaliana* harboring a mutation in the peptide transporter gene At5g46050

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## Abstract

Previously, we have reported a T-DNA tagged mutant (TAG\_009) of *Arabidopsis thaliana* exhibiting a significant sensitivity to biotic stresses. We have also cloned and analyzed the tagged gene At5g46050. Based on bioinformatic and molecular characterization, we proposed that At5g46050 is involved in the transport of peptides participating in plant defense against biotic stresses. To provide further evidence for supporting our proposal, this time we exposed this mutant to *Fusarium culmorum*, a potential fungal pathogen. Besides TAG\_009 line, in our investigations we included two SALK insertion mutants (SALK\_003119 and SALK\_145209), two wild-type ecotypes (WT\_C24 and WT\_Col-0) and an additional T-DNA tagged mutant (TAG\_197-6) of *A. thaliana*. We have found that the highest degree of leaf damage was exhibited by TAG\_009 line (damage score 4.37), whereas the lowest was observed in WT\_Col-0 ecotype (damage score 3.43). The highest rate of mortality after eight weeks of inoculation with *F. culmorum* was also observed in TAG\_009 line (85.24%), while the lowest was in WT\_Col-0 line (37.22%). We have also found that plants of SALK\_145209 line, despite being infected with *Fusarium*, produced the highest number of leaves (average 14.17 leaves per plant), whereas the lowest number of leaves was produced by plants of TAG\_197-6 line (average 9.5 leaves per plant). Statistical analyses showed that the differences between the T-DNA tagged line TAG\_009 and WT\_Col-0 were significant, whereas in comparison with wild-type control plants WT\_C24, they were insignificant. Based on these results, we can conclude that the gene we have tagged by using T-DNA-mediated *in vivo* gene fusion is indeed involved in the plant defense against *Fusarium* infection.

**Key words:** *Fusarium culmorum*, *Arabidopsis thaliana*, T-DNA tagging, disease resistance

## Introduction

Sustainable crop production is a very significant factor in the context of declining natural resources and growing population, particularly in the developing countries. Recent reports presented by the United Nations (UN), the Food and Agricultural Organization (FAO), and the Third World Academy of Sciences (TWAS) indicate that within the next three decades, the world's population will have grown in such a manner that to avoid food shortage its production will have to be increased by at least 60% (BBC News, 2004; Myers and Kent, 2001). Unfortunately, besides the natural calamities like drought, soil salinity, low temperature, flood etc, the biotic stress factors have severe impacts on global food production. It is estimated that every year approximately one-third of the global plant harvest is lost due to many plant pathogenic diseases and insect injuries. Besides

viral and bacterial pathogens, fungal plant pathogens are the major players that contribute to the severe loss of harvest. Despite these biotic or abiotic stress factors, the world will have to solve this problem by creating an improved and sustainable crop production system using new knowledge and techniques that have been developed recently in the area of plant molecular biology.

Plants exhibiting hardiness to severe biotic and abiotic stresses harbor several adaptive and defense mechanisms that are developed from complex metabolic, hormonal and signaling networks (e.g., jasmonic acid JA, ethylene ET, abscisic acid ABA, reactive oxygen species ROS etc.) responsive to these stress conditions. Apart from these networking safeguards, there are other key players involved in plants defense signaling pathways. They are highly conserved mitogen-activated protein kinase (MAPK) cascades and groups of responsive trans-

cription factors that regulate diverse molecular and biochemical processes in plant cells, such as growth inhibition and stress responses (Kunkel and Brooks, 2002; Mittler, 2002; Blokhina et al., 2003; Dolferus et al., 2003; Wang et al., 2003a; Nürnberger et al., 2004; Shinozaki and Yamaguchi-Shinozaki et al., 2007). The morphology and distribution of visible abiotic and biotic stress symptoms provide a primary diagnostic indication helping to verify the stress types and the plant responses. In addition, it is possible to differentiate the stress symptoms from natural senescence symptoms (Vollenweider and Günthardt-Georg, 2005).

Previously, in order to identify and isolate the genes that might be involved in plants tolerance to biotic stresses, we employed T-DNA-mediated gene tagging in the model plant *Arabidopsis thaliana* (Karim et al., 2005 and 2006). *A. thaliana* has become one of the most widely used plants in biological research during the past decades. Its relatively small genome (~125 Mbp) with a low number of repetitive sequences and a short life cycle are some of the reasons as to why this plant has been adopted as a model system for molecular and genetic studies (Meyerowitz, 1987; Meinke et al., 1998). In addition, the value of *Arabidopsis* as a model plant increased significantly at the end of the year 2000 when it became the first plant to have its genome sequenced (The Arabidopsis Genome Initiative 2000). T-DNA tagging, on the other hand, has become a powerful approach for identifying new genes and determining gene functions. By inserting a DNA fragment with a known sequence into the plant genome, a knockout mutation in a particular gene can be generated (Alonso et al., 2003; Mandal et al., 1995; Topping et al., 1995; Feldmann, 1991). Knockout of the plant gene may consequently result in plants with a recognizable mutant phenotype. Insertion of the T-DNA does not need to occur in the exon of a functional gene to result in a mutant phenotype. Several researchers have shown that insertions can also occur in the introns and in 5' or 3' non-coding regions of the gene leading to a mutant phenotype of the plant (Azpiroz-Leehan and Feldmann, 1997). Furthermore, the insertional mutagen not only creates a mutation, but also "tags" the affected gene. This enables researchers to identify, isolate and study the gene in question.

This paper is a continuation of our previous research towards cloning and characterization of genes involved in the hardiness of plants to biotic stresses

carried out by using T-DNA-mediated gene tagging and *in vivo* gene fusion in *Arabidopsis thaliana* (Karim et al., 2005 and 2006). We have isolated a T-DNA tagged mutant (line TAG\_009) that exhibits an increased sensitivity to mechanical injuries and infection with bacterial pathogens *Erwinia carotovora* and *Pseudomonas syringae*. We have also cloned the T-DNA tagged gene, At5g46050 (AtPTR3), from *A. thaliana*. Based on the results obtained while characterizing the function of this gene, we have postulated that At5g46050 encodes a protein which is involved in transporting peptides required for the developing of the plant hardiness to biotic stresses (Karim et al., 2006). Here we report further evidence supporting our previous postulate regarding the function of the T-DNA tagged gene At5g46050. In order to do so, we exposed the plants of the tagged line TAG\_009 to another severe fungal plant pathogen *Fusarium culmorum*. Besides TAG\_009, two other insertion mutants (SALK\_003119 and SALK\_145209), two wild-type ecotypes (WT\_C24 and WT\_Col-0) and another T-DNA tagged mutant of *A. thaliana* TAG\_197-6 were included in these investigations and used as the control material.

## Materials and methods

### *Plant material*

All plant materials used in our study consisted of *Arabidopsis thaliana*. Mutant lines TAG\_009 and TAG\_197-6 were generated by T-DNA mediated gene tagging and *in vivo* gene fusion in ecotype C24 as described previously (Mandal et al., 1995). Cloning and characterization of the genes tagged in the TAG\_009 line (Karim et al., 2005 and 2007) and TAG\_197-6 line (Svensson et al., 2004 and 2005) have also been reported. Two insertion mutants, SALK\_003119 and SALK\_145209 (Alonso et al., 2003) were kindly supplied by the Arabidopsis Stock Center at the SALK Institute, USA (<http://www.signal.salk.edu>). SALK\_003119 harbors an insertion mutation almost identical to that in the T-DNA tagged line TAG\_009 (gene At5g46050), whereas SALK\_145209 contains a mutation identical to that in the T-DNA tagged line TAG\_197-6 (gene At4g20010). Wild-type WT\_Col-0 and WT\_C24 ecotypes were obtained from the Swedish University of Agricultural Sciences, Uppsala.

### *Preparation of plants*

*Arabidopsis thaliana* seeds were first surface sterilized for 5 minutes with 70% ethanol and then rinsed

three times with sterilized water. Under axenic conditions, seeds were then plated on Petri dishes containing 1% sterile agar medium (0.2 g of agar diluted in 20 ml of sterile water). Each plate contained approximately 20 seeds. In order to enhance the germination rate (Hensel et al., 1993; Scholl et al., 1997), the plates were kept in a growth chamber for 7 days at 7°C by maintaining 12-h day (light intensity 130  $\mu\text{mol}/\text{m}^2/\text{s}$ ).

#### **Preparation of *Fusarium culmorum***

*Fusarium culmorum* (W.G.Sm.) Sacc. strain IPO348-01 was kindly supplied by the Plant Protection Institute, Wageningen, The Netherlands. The virulence of this strain has been previously studied in wheat (Wojciechowski et al., 1997) and barley (Chelkowski et al., 2000). Incubation of the fungus was carried out on Petri dishes containing 19 g of PDA (Potato Dextrose Agar, Sigma Chemical Company) per liter. Petri plates were kept in the microbiological growth chamber (Heraeus B 6060) maintaining complete darkness for 7 days at 22°C.

#### **Leaf damage**

Germinating seedlings (2–4 leaf seedlings) were transferred onto disks ( $\varnothing$  2 mm) of PDA culture that contained rapidly growing *Fusarium culmorum*, strain IPO 348-01, and incubated for 7 days at temperature 22°C/20°C (day/night) using a 12 h day (light intensity 130  $\mu\text{mol}/\text{m}^2/\text{s}$ ) with a relative humidity of 100%. Control seedlings were treated with sterile water and allowed to grow under identical growth conditions. The experiment was carried out in a completely randomized design with three replications. Each replication consisted of 40 plants/investigated line. The level of seedling damage caused by *Fusarium culmorum* infection was evaluated on the basis of a five degree scale (Chelkowski and Manka, 1983) as follows: 1, very weak infection; 2, weak infection; 3, medium infection; 4, high infection and 5, very high infection.

#### **Mortality of plants**

Germination of *A. thaliana* seeds and cultivation of IPO 348-01 strain of *F. culmorum* were performed in the same way as described above (experiment with leaf infection). After seven days of germination, the seedlings were transferred onto soil pots. The soil was first sterilized by autoclaving. Young roots were then inoculated with disks ( $\varnothing$  2 mm) of PDA culture of *F. culmorum* (W.G.Sm.) Sacc. isolate IPO348-01. To enhance

infection, high humidity was maintained by covering all pots with plastic foils. Plants were kept in the growth chamber under following conditions: temperature 15 to 17°C, photoperiod 16 h/day (130  $\mu\text{mol}/\text{m}^2/\text{s}$ ), relative humidity 100%. Pots were watered regularly every third day. Mortality of each line was estimated every seventh post-inoculation day based on the difference between the number of inoculated plants and the number of plants that could survive and grow further after eight weeks of infection. The number of leaves per plant was also determined after eight weeks of inoculation. The flowering time was defined as the time lapse between the sowing of the seeds and the appearance of the first flower buds.

#### **Experimental design**

For the estimation of the survival rates of the inoculated plants, we made seven experimental combinations. The experiments were carried out in a completely randomized design with three replications. The data obtained from these observations were calculated statistically. By using the software STATISTICA 7.0 – StatSoft (STATISTICA System Reference, StatSoft 2002), we have performed one factor analysis of variance, ANOVA (Snedecor and Cochran, 1989) to estimate significance of the differences observed between the investigated lines. In some cases, conclusions were made on the basis of least significant differences (LSD) between these lines.

## **Results**

#### **Leaf damage**

As mentioned in Materials and methods section, the level of leaf damage caused by *Fusarium culmorum* was estimated seven days after inoculation based on a 5-degree evaluation scale. A variance analysis was performed to estimate the significance of the differences seen in the investigated lines. The results are shown in Table 1. As can be seen the examined lines differ statistically at a significance level  $\alpha = 0.05$ . The data collected in Table 2 indicate that among the mutant plants, the highest degree of leaf damage (score 4.37) was observed in the plants of TAG\_009 line, whereas the lowest (score 3.43) was seen in WT\_Col-0 line. The level of damage in wild-type plants of ecotype WT\_Col-0 differs significantly from that observed in the remaining investigated lines (except for plants of SALK\_145209 line, score 3.80).

The difference in the degree of damage between the wild-type ecotypes WT\_C24 (score 3.93) and WT\_Col-0 (score 3.43) was also significant as the Least Significant Difference (LSD) at  $\alpha = 0.01$  for this analysis was 0.48 (Table 2). However, the difference between SALK\_145209 and SALK\_003119 resulted in LSD 0.40 (less than the wild-type) and remained statistically insignificant (Table 2).

**Table 1.** Analysis of variance for leaf damage score of *Arabidopsis thaliana*. Data obtained were calculated statistically by using StatSoft and variance analysis was done by using ANOVA

Source of variation	Degrees of freedom	Square sum	Mean squares	F emp
Lines	5	1.6369	0.3274	4.564*
Error	12	0.8607	0.0717	
Total	17	2.4977	–	

\* indicates significant at  $\alpha = 0.05$

**Table 2.** Effects of *Fusarium culmorum* infection on leaf damage in wild type (WT) *Arabidopsis thaliana* and its mutants (TAG/SALK). The level of damage was estimated after 21 days after inoculation, based on a five-degree scale (1-5). One represents a very low level, whereas five represents a very high level of leaf damage

Lines	Disease score
TAG-009	4.37
SALK_003119	4.20
TAG-197-6	4.10
WT_C24	3.93
SALK_145209	3.80
WT_Col-0	3.43
Mean	3.97
LSD	0.48

### Survival of plants

The rate of mortality of the transgenic and the WT control plants was estimated on the basis of differences between the number of inoculated plants and the number of plants that could survive infection and grow further as a normal flowering plant. The results obtained from the variance analysis (Table 3) revealed that the difference in surviving capacity between plants was highly significant (at  $\alpha = 0.01$ ). The result presented in Table 4 concerns mortality of plants after eight week of *Fusarium* infection. However, we measured mortality every

seventh post-inoculation day. We observed that the difference in surviving capacity of WT\_Col-0 (37.22% mortality) and the T-DNA-tagged mutant TAG\_009 (85.24% mortality) was statistically significant as well as between WT\_C24 and its mutant TAG\_009 (LSD 38.57%). Plants that survived *Fusarium* inoculation for a period of eight weeks could grow further and complete all the developmental cycles (Meinke et al., 1998, www.arabidopsis.org).

**Table 3.** Analysis of variance for mortality of *A. thaliana* lines. Data obtained were calculated statistically by using StatSoft and variance analysis was done by using ANOVA

Source of variation	Degrees of freedom	Square sum	Mean squares	F emp
Lines	5	6025.71	1205.14	7.4503**
Error	12	1941.08	161.76	
Total	17	7966.80	–	

\*\* indicates significant at  $\alpha = 0.01$

**Table 4.** Mortality of wild type *A. thaliana* (WT) and its mutants (TAG/SALK) after exposition to *Fusarium culmorum*. Mortality was estimated in percentages after eight weeks of inoculation on the basis of differences between the number of inoculated plants and the number of plants that could survive this infection and grow further to a normal flowering plant

Lines	Mortality (%)
TAG_009	85.24
SALK_145209	83.33
SALK_003119	66.67
TAG_197-6	50.00
WT_C24	46.67
WT_Col-0	37.22
Mean	61.52
LSD	22.63

Table 4 indicates that the lowest rate of mortality was found in plants of WT\_Col-0 (37.22%), whereas the highest in plants of lines TAG\_009 and SALK\_145209, 85.24% and 83.33% mortality, respectively.

However, the difference in the mortality rate between TAG\_009 and the insertion mutants SALK\_003119 and SALK\_145209 remained insignificant. Difference in the lethality in these mutants varied from 85.24 to 66.67% (Table 4) and was less than the calculated LSD (22.63).

### Development of leaves and flowers

Plants of all of the investigated ecotypes could develop leaf rosettes after two weeks of germination regardless of the inoculation with *F. culmorum*. However, eight weeks after inoculation, the average number of leaves per plant varied significantly among the genotypes. One-factor analysis of variance, ANOVA (STATISTICA System Reference, StatSoft 2002) conducted for leaf formation revealed that the difference in the number of leaves developed after eight weeks *Fusarium* infection was highly significant (at  $\alpha = 0.01$ ) – Table 5. Table 6 indicates that after eight weeks *Fusarium* infection, the highest number of leaves per plant (14.17) was found for SALK\_145209 insertion line, whereas the lowest for TAG\_197-6 line (9.5 leaves per plant). Number of leaves/plant in wild type control plants, WT\_Col-0 and WT\_C24, was 9.67 and 12.7, respectively (Table 6).

**Table 5.** Analysis of leaf number variance in *Arabidopsis thaliana*. Data obtained were calculated statistically by using StatSoft and variance analysis was done by using ANOVA

Source of variation	Degrees of freedom	Square sum	Mean squares	F emp
Lines	5	52.923	10.585	6.411**
Error	12	19.816	1.651	
Total	17	72.74	–	

\*\* indicates significant at  $\alpha = 0.01$

**Table 6.** Effects of *Fusarium culmorum* infection on the leaf production in wild type *Arabidopsis thaliana* (WT) and its mutants (TAG/SALK). Average number of leaves per plant was estimated eight weeks after inoculation

Lines	Number of leaves
SALK_145209	14.17
SALK_003119	13.08
TAG_009	12.46
WT_C24	12.17
WT_Col-0	9.67
TAG_197-6	9.50
Mean	11.84
LSD	2.29

The difference in the number of leaves developed during eight weeks *Fusarium* infection was significant

for WT\_Col-0 and both SALK mutants as well as for WT\_C24 and mutant TAG\_197-6 (Table 6). However, the difference in leaf production between TAG\_009 and WT\_C24 was only 0.29 (which is lower than the calculated LSD 2.29) and therefore, remained insignificant (Table 6). Besides wild type controls (WT\_Col-0 and WT\_C24) only one mutant line, TAG\_197-6, could grow further and produce flowers and fertile seeds (data not shown). Plants of WT\_C24 flowered earlier, approximately after 30 days from sowing, whereas the plants of line TAG\_197-6 flowered late approximately after 55 days from sowing.

### Discussion

This paper is a part of our T-DNA-mediated gene tagging project towards isolation and characterization of *Arabidopsis thaliana* genes that might be involved in the tolerance of plants to biotic stresses. Previously, we have reported regarding the identification of the T-DNA tagged mutant TAG\_009 and isolation of the tagged gene AtPTR3 (Karim et al., 2005 and 2007). Based on our investigations with a bacterial pathogen *Erwinia carotovora* we have postulated that the gene we have isolated is involved in the tolerance of plants to bacterial infection and/or mechanical wounding. In this paper, by exposing the mutant line TAG\_009 to another potential fungal pathogen *Fusarium culmorum*, we provide further evidence that supports our previous postulates. In our recent investigations, we have introduced both the T-DNA tagged and the SALK insertion mutants. It should be mentioned here that SALK\_003119 and SALK\_145209 mutants were generated from WT\_Col-0 background (Alonso et al., 2003), whereas TAG\_009 and TAG\_197-6 mutants were generated from WT\_C24 background (Mandal et al., 1995). Therefore, the data obtained in the tagged mutant TAG\_009 have been compared with those obtained in wild type WT\_C24 controls, whereas the data resulting from the SALK mutants have been compared with those seen in wild type WT\_Col-0 controls. The results presented in Table 2 clearly show that when exposed to *Fusarium* infection, the level of leaf damage in plants of line TAG\_009 was insignificantly higher (score 4.37) than that observed in the plants of wild type control WT\_C24 (scores 3.93). However, the difference in leaf damage between SALK\_003119 (score 4.20) and the wild type WT\_Col-0

(score 3.43) remained significant. Surprisingly, the difference in leaf damage between the WT\_C24 (score 3.93) and WT\_Col-0 (score 3.43) was also significant. This can be explained by the fact that, as described above, these are two different ecotypes. WT\_C24 contains comparatively less trichomes on the surface of the leaves in comparison with the leaves of WT\_Col-0. It has been postulated that trichomes are involved in the protection of plants against pathogen infection (Meyerowitz, 1987) and this is why WT\_Col-0 ecotype is less susceptible to pathogen infection than WT\_C24 ecotype. In addition, lack of trichomes may also be a reason that explains the insignificant difference in leaf damage between WT\_C24 and TAG\_009.

In our experiments, we have determined the resistance or susceptibility of the investigated lines to *Fusarium* infection based on the differences between the number of inoculated plants and the number of plants that survived this infection, grew further and bloom. The results presented in Table 4 demonstrate that the difference in mortality caused by *Fusarium* infection between the plants of line TAG\_009 (85.24%) and the wild type WT\_C24 controls (46.67%) was significant. Similar significant difference in mortality rate was also obtained between the plants of SALK\_003119 (66.67% mortality) and WT\_Col-0 controls (37.22% mortality). However, the difference in the mortality rate between the wild type control plants, WT\_Col-0 and WT\_C24 was insignificant (Tables 3 and 4). Similar experiments concerning susceptibility of *A. thaliana* to several plant pathogenic bacteria or fungi have been previously reported by many researchers. Studies conducted by several researchers revealed that the survival rate of the *esa1* mutant when exposed to necrotrophic pathogens (either *Alternaria brassicicola*, *Botrytis cinerea* or *Plectosphaerella cucumerina*) was much lower than that observed in the wild type control plants (Tierens et al., 2002; Kishimoto et al., 2006). Van Hemelrijck et al. (2006) reported that the *esa1* mutant was also susceptible to cereal pathogens such as *Fusarium culmorum* and *Fusarium graminearum*. These researchers observed that only after nine days of inoculation, the mutant *esa1* was much more susceptible to *Fusarium* infection in comparison with the wild type WT\_Col-0 plants. Some reports have also proved that wild type *A. thaliana*, ecotype Columbia (WT\_Col-0) is less susceptible to the cereal pathogens *F. graminearum* and *F. culmorum* as com-

pared to the *esa1* mutant (Urban et al., 2002; Van Hemelrijck et al., 2006). In our studies, the mortality rates observed in the wild type controls was significantly lower than those observed in the respective mutants lines (Table 4).

Our investigations concern not only the mortality rates of the investigated lines when exposed to *Fusarium* infection but also their capacity to recover from the fungal infection and to grow further to produce flowers and seeds. By comparing the results obtained for SALK\_003119 and WT\_Col-0 lines, we found that the difference in number of leaves between these two lines was statistically significant. In these lines, the number of leaves per plant was 13.08 and 9.67, respectively (Table 6). But when comparing the results obtained for TAG\_009 and WT\_C24, we could not find any statistically significant difference (Table 6). These data do not correlate with our expectation that “the higher the mortality the lower the number of leaves per plant”. Our explanation to these results is that the mortality of *A. thaliana* ecotype C24 caused by *Fusarium* infection and its capacity to develop rosette leaves after recovery from this infection are two separate features and not necessarily correlated. We should remember that both lines WT\_C24 and TAG\_009 are derived from C24 background and contain less trichomes on the leaf surface than the Col-0 ecotype. Trichomes may also play a significant role in developing rosette leaves in C24 ecotype. Moreover, we observed that the plants of the mutant line TAG\_197-6 exhibited a late flowering phenotype in comparison with the WT\_C24 controls. The flowering time occurred approximately after 55 and 30 days, respectively. These results are almost identical to those we have reported previously (Svensson et al., 2004 and 2005).

In conclusion, the T-DNA tagged mutant TAG\_009 that we isolated and characterized previously also exhibits a higher degree of susceptibility towards *Fusarium culmorum* infection. The degree of TAG\_009 susceptibility to this fungus remains in agreement with the study we conducted with bacterial infection, particularly with *E. carotovora* (Karim et al., 2005 and 2006). This confirms our previous postulate that the mutant we have identified is less resistant to pathogenic infection (either bacterial or fungal) in comparison with the wild type control plants. Secondly, it supports our previous postulate concerning the involvement of the tagged gene in plants defence against biotic stresses.

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