نقش ژن شبه استریکتوزیدین سینتاز-٦ در مقاومت گیاه آرابیدوپسیس تالیانا در برابر *Alternaria brassicicola*

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(تاریخ دریافت: ۱۳۹۴/۱۱/۷؛ تاریخ پذیرش: ۱۳۹۵/۱۱/۲۰)

چکیده

استریکتوزیدین سینتاز یک آنزیم کلیدی است که در بیوسنتز الکالوئیدهای مختلف گیاهی نقش دارد. دومین همولوگ استریکتوزیدین سینتاز در موجودات مختلف از جمله گیاه مدل آرابیدوبسیس تالیانا یافت شده است. با انجام مطالعات بیوانفورماتیک و بیان در یکی از ژن های شبه استریکتوزیدین سینتاز (*SSL6*) در گیاه آرابیدوبسیس مشاهده شد که ژن به طور معنی داری به تیمار با سیگنال های مولکولی، تیمار با آلترناریا و ویروس CMV واکنش و افزایش بیان داشته است. به منظور مطالعه بیشتر نقش ژن *SSL6* در سیستم دفاعی گیاهان، از ژنتیک معکوس استفاده و موتانت T-DNA ناک اوت ژن مذکور آنالیز شد. گیاهان با قارچ *Alternaria brassicicola* که دارای کنش متقابل ناسازگاری با آرابیدوبسیس می‌باشد تلقیح شدند. کمیت سنجی قارچ با استفاده از شمارش اسپور و تکنیک Real-Time PCR انجام شد. علاوه بر این، قطر لکه های حاصل از واکنش ناسازگاری در ژنوتیپ های موتانت و وحشی نیز اندازه گیری شد. نتایج نشان داد که موتانت *ssl6* به طور معنی داری از ژنوتیپ وحشی Col-0 به بیماری حساس تر است. اندازه گیری میزان کلروفیل های a و b حاکی از میزان بالای کلروفیل در موتانت در مقایسه با وحشی بوده است. این مطالعه اولین در نوع خود در خصوص کارکرد ژن *SSL6* در گیاهان می باشد.

کلیدواژه: واکنش ناسازگاری، موتانت T-DNA، ژنتیک معکوس، Real-Time PCR

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The role of a Strictosidine synthase-like gene-6 in *Arabidopsis thaliana* defense against *Alternaria brassicicola*

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(Received: 27.1.2016; Accepted: 8.2.2017)

Abstract

Strictosidine synthase is a key enzyme in the biosynthesis of plant alkaloids. The homologous of the strictosidine synthase domain has been found in different organisms such as *Arabidopsis thaliana*. Bioinformatics and expression studies on one of the strictosidine synthase-like gene (*SSL6*) in *Arabidopsis* plants showed that gene responded to the signaling molecules, fungal and viral pathogens. The functional role of *SSL6* has been further studied using an *Arabidopsis* knockout mutant with a T-DNA inserted into exon 1. The plants were inoculated with *Alternaria brassicicola*, which had incompatible interaction with *A. thaliana*. The fungal biomass was quantified by quantitative real-time PCR and spore count techniques. The assays indicated that T-DNA mediated *ssl6* mutant was more susceptible than Col-0 genotype. The chlorophyll content and the size of necrotic lesions were significantly smaller in the *ssl6* mutant compared with the Col-0. This is the first report with reference to the functional analysis of *SSL6* suggesting that might be *SSL6* plays an important role, among other factors, in plant defense against *A. brassicicola*.

Keywords: Real-Time PCR, incompatibility, T-DNA mutant, Reverse genetic

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Introduction

Plants are exposed to a wide range of microbial pathogens and herbivores. They responded by the evolving sophisticated mechanisms in order to efficiently defend themselves against these threats (Chisholm *et al*., 2006). Appropriate adjustments of defensive reactions are important for plants to be healthy (Walters, 2015).

The plant immune system recognizes pathogen-associated molecular patterns (PAMPs), the molecules that are produced by pathogens through receptor proteins termed as pattern recognition receptors (PRRs). The PAMPs are conserved structures (patterns) such as fungal chitin or bacterial flagellin that recognitions of them by PRRS would activate the first tier of the plant immune system named as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Nürnberger and Kemmerling, 2009). Pathogens could overcome PTI by using secreted proteins so called effectors that target different cellular components involved in mediating PTI (Van der Hoorn and Kamoun, 2008; Gassmann and Bhattacharjee 2012). As a consequence, compatible interaction establishes and colonization of the host takes place. This is expected to either delay or block the induction/activation of all 'downstream' executors including phytoalexins and phytoanticipins. Additionally, resistance proteins can recognize the presence of effectors and build up the second layer of defense termed as effector-triggered immunity (ETI; Jones and Dangl, 2006; Dodds and Rathjen, 2010). The latest interaction that occurs via PTI or ETI is termed incompatible causes suppression of pathogen growth and reproduction (Steinbrenner et al., 2016). Pathogens can also secret toxins to manipulate host hormonal signaling pathway and suppress ETI (Friesen *et al*., 2008).

Insects and plants can rely on similar molecules in their inherent defenses. The first report in this regard is on fruit flies (*Drosophila melanogaster*). After being picked with a bacteria-soaked needle, *Drosophila* larva and adult *Drosophila* produce large quantities of a potent antifungal peptide homologous to a family of brassicaseae antifungal peptides which is involved in the defense against microorganisms. (Fehlbaum *et al*., 1994). Another example in this context is a new gene family similar to the plant strictosidine synthase enzyme (Fabbri *et al*., 2000). Strictosidine synthase is a key enzyme in the biosynthesis of plant alkaloids (Ziegler and Facchini, 2008). In *Arabidopsis*, strictosidine synthase-like genes (*SSL*) constitute a multi-gene family that may act as antifeedant and antibacterial materials. *AtSSL4 - AtSSL7 Arabidopsis* sequences appear to be more related to hemomucin than strictosidine synthase (Fabbri *et al*., 2000). That's why this gene family is also known as hemomucin like (*HML*; Kibble *et al.* 2009). Hemomucin is a *Drosophyla* cell surface mucin that may be involved in inducing antibacterial effectors after binding to a lectin (Helix pomatia A hemagglutinin; Theopold *et al.* 1996). *AtSSL4 - AtSSL7* RNAi lines show a reduction in secologanin consumption which is a strictosidine precursor in other species. This fact suggests a role for this class of protein in the secologanin metabolism. *Arabidopsis* SSL proteins possibly lack strictosidine synthase activity (Kibble *et al.* 2009).

Gene expression studies have also shown *SSL6* (At3g51440) responses to the treatment of signaling molecules, fungal and viral pathogens. The gene was over-expressed after salicylic acid, UV-B and salt treatment. *SSL6* gene expression profiles revealed its possible role in the functions associated with the salicylic acid pathway and reactions against *Cucumber mosaic virus* (CMV) as well as *Alternaria brassicicola (Schwein.) Wiltshire* (Sohani *et al*., 2009; Kibble *et al*., 2009).

In the present research, the biological role of *SSL6* in *Arabidopsis* defense mechanisms against an incompatible necrotrophic pathogen, *A. brassicicola*, has been further studied. *Arabidopsis* wild type ecotype Col-0 as a resistant control genotype and T-DNA-mediated *ssl6* mutant were challenged with the *A. brassicicola* and their susceptibility to this fungus has been monitored.

Materials and methods

Plant maintenance

Seeds of Columbia-0 (Col-0) ecotype of *Arabidopsis* were obtained from CSIRO, Adelaide, Australia. The seeds were sown in two different ways. They were directly sown on the surface of *Arabidopsis* soil mix (perlite and peat moss in 1:1 ratio plus the following amount of fertilizers for 500 litters soil: iron sulphate 500 g and 200 g hydrated lime) using a yellow tip pipette. Pots were covered with a glad wrap till seedling reached the four-leaf

stage. In the second method, surface sterilized seeds were sown onto petri's plates (10 * 10 cm) containing MS medium (Sigma, St. Louis, USA) plus 0.1 % sucrose and 0.8% agar. Plates were stacked in the growth room at 22-25ºC. The seedlings were transplanted to *Arabidopsis* soil mix after they reached the four-leave stage. The pots were covered with the glad wrap for 48 hours to plants be adapted to the normal conditions and grown in growth chambers with 16-hr dark/8-hr light photoperiod before being inoculated with any pathogens. The first generation of knockout lines was grown under long day (16 hr light). To multiply seeds or to make back-crosses, seedlings were grown at 23-25ºC and under florescent light (90-100 µE/sec/m2) at the level of rosette leaves.

Seed sterilisation

About 30 seeds were placed in an Eppendorf microtube. Seeds were soaked in 1 ml 70% ethanol for 2 minutes. Bleach solution (NaClO, 13% active chlorine, the same volume of dH_2O and 1 µl 20% Triton-X (Sigma, St. Louis, USA) was added to the seeds and the tube was placed on a rotator for 5 minutes. Seeds were spun down at about 600 rpm for 20 seconds and the supernatant was removed. Sterilized seeds were rinsed four times with sterile water each 5 minutes. A cut off yellow tip was used to pipette up the seeds and aliquot them out evenly on the surface of media.

The Analysis of knockout lines by direct PCR

To find a T-DNA knockout line in *SSL6* gene, the open reading frame was submitted to "T-DNA–Express" Gene Mapping tool of Salk Institute Genomic Analysis Laboratory (SIGnAL; http://signal.salk.edu/cgi-bin/tdnaexpress), a web-based accessible graphical interface, which provided both text and DNA searches of the insertion sequence database (Alonso *et al.*, 2003).

To confirm the presence of T-DNA insert in the *SSL6*, a line with accession number SALK_046142 was analyzed using direct PCR with T-DNA left border (LB) and gene specific (GS) primers. The two following specific left border primers for SALK knockout lines (LB1 5'-TGG TTC ACG TAG TGG GCC ATC G-3' and LB2 5'-GCG TGG ACC GCT TGC TGC AAC T-3') in combination with forward- or reverse-gene specific primers (oriented towards the insertion) were used to do the first and second PCR reactions, respectively. Isolated border fragments were sequenced by Bioneer (Daejeon, South Korea) and the insertion point was identified doing BLAST search against The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org) database.

Genetic characterisation of mutants

In order to genetically analyse the mutants, to estimate the number of possible unlinked insertion(s) sites, and to resolve the tagging status, the seeds were plated on selection medium containing ½ MS medium, 0.3% sucrose and 50 µg/ml kanamycin. Putative *ssl6* SALK line (SALK_046142) was first plated on the same medium lacking antibiotics. The two-week-old seedlings were transferred to the soil and were grown under long day photoperiod (16 hrs) to bulk the seed. Seeds from each individual were directly sown on the soil surface using yellow tips. Ten days later, seedlings were sprayed with 120 µg/L glufosinate ammonium (active ingredient of Basta herbicide; Hoechst, Germany) and 2 more times subsequently over the next 2 weeks. At least 50-60 seedlings were scored for each individual.

Quantitative Real Time-PCR analysis of transcripts

Transcription levels of the *SSL6* and the quantity of *A. brassicicola* biomass (Kariola *et al.*, 2004) in leaf tissues were monitored using Quantitative Real Time-PCR (RT-qPCR). Stock solutions of the PCR product from each pair of primers were purified and quantified by HPLC. One µl of 10X diluted cDNA derived from samples were amplified in each PCR reaction, which was comprised of 10 µl of SYBR Green (Bioneer, Daejeon, South Korea) RT-PCR reagent, 3 µl of each forward and reverse primers (4µM concentration) and 3 µl water. The amplification was performed in a CFX cycler (Bio-Rad, Hercules, USA). The light cycler was programmed as follows: Initial activation step for 15 min at 95ºC followed by 45 cycles of 20s at 95ºC, 30s at 55ºC, 30s at 72ºC, and 15s at 80ºC. To carry out melting curve analysis at the end of the amplification, the temperature was increased from 70ºC to 99ºC. To obtain the abundance of each individual mRNA, fluorescence data were acquired at 72ºC and 80ºC. The melting curve was used to obtain optimal temperature for data acquisition.

Independent samples of 20 µl PCR reactions were combined and purified using High-performance liquid chromatography (HPLC). The PCR products were injected into the HPLC system (Agilent Eclipse, USA) equipped with a UV-visible detector, a column (ODS C_{18}; 2.1-mm X 15-cm 3.5–micron reverse phase column; Agilent Technology, Palo Alto, USA). The mobile phase was a mixture of solvent A [95: 5 (v/v) water : acetic acid] and solvent B [90 : 10 (v/v) acetonitrile : water], all HPLC grade. Samples and columns held at 25°C, the eluent flow was at 1.0 ml min^{-1}, and the run time was 40 min. The products were first dried and then dissolved in water in a final concentration of 20 ng/µl. Sequencing was done by Bineer (Daejeon, South Korea) in both directions to confirm the size and identity of the products. Finally, a stock solution containing 10^9 copies of the PCR product per micro litter was prepared. A dilution series from original solutions was prepared and was used as the standard. In each QPCR experiment three replicates of each of seven standard concentrations together with a two "no template" control ("no SYBER" and "no DNA") were included.

To select the seqences of the gene specific primers, primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used. Three pairs of primers for amplification of Arabidopsis internal genes were designed. For Actin gene forward 5'- GAG TTC TTC ACG CGA TAC CTC CA-3' and reverse 5'-GAC CAC CTT TAT TAA CCC CAT TTA CCA-3' primers; for Cyclophilin forward 5'-TGG CGA ACG CTG GTC CTA ATA CA-3' and reverse 5'- CAA AAA CTC CTC TGC CCC AAT CAA-3' primers; and for GAPDH 5'-TGG TTGA TCT CGT TGT GCA GGT CTC-3' and 5'-GTC AGC CAA GTC AAC AAC TCT CTG-3' were used as forward and reverse, respectively. The RT-PCR normalisation factor was calculated according to Vandesompele *et al.* (2002). To obtain a normalisation factor for each experiment, geometric means of internal genes were calculated, and the best performing ones were chosen.

Real-time PCR reaction was performed using forward (5'-CGG ATC TCT TGG TTC TGG CA-3') and reverse (5'-AAT GAC GCT CGA ACA GGC AT-3') *Alternaria* 5.8S rRNA gene specific primers. The *SSL6* forward and reverse primers were as follow respectively: 5'-TAT GGC GGC TAA GTA CGG CTAT-3' and 5'-GGT GGT TAT GTG AGA GAG CGC-3'. The Starting quantity (absolute quantification) of the fungal DNA from different samples was calculated and the results were analyzed using SAS software version 9.1.

Southern blotting of T-DNA

An enzyme was chosen in order to cut at least 1000 bp of DNA which were flanking the T-DNA borders and allowing the generation of the fragments containing sufficient flanking genomic DNA. *Arabidopsis* genomic DNA (~5 µg, as estimated on the gel), 4U/µg *Hind*III restriction enzymes, 10X buffer E and the final concentration of 2.5 mM spermidin were mixed to set up a digestion reaction with a total volume of at least 50 µl. The volume of digests, after incubation overnight at 37ºC, was adjusted to about 20 µl using speed-vac. Three µl DNase-free RNase (10 ug/µl) was added to the sample just before being loaded on gel. Digested genomic DNA from primary transformants, untransformed plants, uncut genomic DNA and a molecular weight marker were run on a 0.8% agarose gel at 60 V/cm for about 2 hrs. Gel photographs were taken with a ruler laid beside it and with a minimum exposure to UV light. The gel was placed in a dish and covered with a 0.25M HCl solution for exactly 10 minutes with gentle agitation. HCl solution was removed and gel was rinsed with dH_2O three times each for five minutes. The gel was covered with the denaturation buffer (1.5M NaCl , 0.5M NaOH) for 30 minutes with gentle agitation and was rinsed in dH_2O three times. The gel was covered with neutralisation buffer (1.5M NaCl, 0.5M Tris-HCl, pH adjusted to 7.5) with gentle agitation and the solution was removed 30 minutes later. DNA was transferred to the nitrocellulose membrane using 20X SSC as the transfer buffer. DNA was immobilized on the damp membrane by cross-linking with UV using GS Gene Linker UV Chamber (Bio-Rad, Hercules, USA) at 150 mJ. The filter was wrapped in plastic wrap and stored dry between the sheets of Watman paper. Hybridisation of the membrane was done using the probes of 500 bp length from left borders primers including pROK2 5'-TTCCAGTTTGGAACAAG AG -3' and 5'-TTAGAGTCCCGCAATTATAC -3' as a forward and reverse primers, respectively. The digestion was done using *sal1* enzyme, which do not cut the T-DNA fragment.

Another approach to estimate the number of inserts was to use the segregation ratio of resistant to

sensitive seedlings after spraying with the Basta herbicide. The T_3 seeds from the provider were bulk-propagated and 50 seeds from each individual in T_4 generation were sown on separate pots and sprayed with Basta.

Growth of fungus Alternaria brassicicola and plant inoculation

Necrotroph fungus *A. brassicicola* were cultured on the potato sucrose agar (PSA) medium (200g potato, 10g sucrose, 15g agar per litter; Yamaguchi and Mutsunobu, 2010). The potatoes were boiled in distilled water until cooked and strained through a double layer of gauze to collect the broth. The pH was adjusted to 6.5. Fungi were grown on 22ºC for 1-2 week. The harvesting of spores was done by flooding plate culture with sterile water and rubbing with a sterile spatula (Broekaert *et al.*, 1990). Spores were collected with a pipette and spore concentrations (5×10^5 spores per ml) were determined using a hemocytometer (van Wees *et al.*, 2003).

To ensure the growth of fungi in the leaves, two methods of inoculation were used. In the first method, one or two drops of spore suspension with a total volume of 5 microlitres were placed on each leaf (van Wees *et al.*, 2003). In the second method, small lesions were created on the leaf surface using micropipette tips. Then, a drop containing 5 microlitres inoculum was placed on the top surface of each lesion (Kariola *et al.*, 2005). Inoculated plants were kept inside an air tight plastic box at 100% RH at 22ºC on a 10-h-light/14-h-dark cycle.

Analysis of the symptoms following fungi inoculation

According to Thomma *et al.* (1999a), different methods were employed to monitor the susceptibility of mutants to *A. brassicicola*. The newly *in planta* formed spores were counted six dpi. The batches of five leaves containing 20 lesions were collected and placed in 5 ml of 0.1% Tween 20 in a test tube. Tubes were vigorously shacked and the leaves were removed from the suspension and checked under the microscope for remaining spores. The suspension containing the spores was centrifuged at 5000g for 15 min and the spores were resuspended in 200 µl of 0.1% Tween 20. A haemocytometer was used to count the spores (van Wees *et al.*, 2003).

In order to monitor the susceptibility of *Arabidopsis* genotypes to the *A. brassicicola*, the size of necrotic lesions was measured using the Image J software (Savchenko *et al.*, 2010). The average size lesion from each genotype was measured 3, 5, and 7 dpi.

Chlorophyll measurement

Leaves from six inoculated plants (0.05 g) were harvested and grinded in 1mL methanol. The resulting mixture was centrifuged for 10 minutes at 6000 rpm and the supernatant was kept in the dark for 30 minutes. The chlorophyll content was measured at 663 and 645 nm wavelength (Arnon, 1967).

Data analysis

Data were analyzed based on a completely randomized design (CRD), and Tukey's significant difference test at two levels of α (α = 0.05 and α = 0.01) was performed.

Results and Discussion

Analysis of T-DNA-mediated SSL6 mutant

To use a T-DNA *SSL6* insertion line for phenotypic analysis several properties need to be examined beforehand, included confirmation of the presence of a T-DNA insert within the *SSL6* gene, the homozygosity of the insert, the estimation of the number of un-linked T-DNA inserts and the expression levels of *SSL6* locus.

Characterisation of genomic DNA flanking T-DNA

As a result of sequencing, the T-DNA insert was localised in the first exon of *SSL6*. The exact LB-genomic DNA junction (▼) and the site of the LB-specific primers used for border isolation were depicted in Figure 1. Additional 4-5 base pairs called "filler DNA" were also detected at the junction of vector and genomic DNA, which belonged to neither vector nor genomic DNA.

Selection of homozygous T-DNA insert in the specific SSL knockout loci

To select homozygous T-DNA plants, a direct PCR method was used. Since the ORF of the gene was interrupted as a result of T-DNA insertion, forward and reverse gene-specific primers was not able to amplify any products and due to limited extension time in PCR cycles, the primers could not extend beyond the insert. Accordingly, LB-specific primers in combination with a GS primer

SALK_046142

Figure 1: Sequence of genomic DNA flanking T-DNA in *ssl6* knockout lines. The results of DNA sequencing showed that insert is located in exon 1. The junction of the genomic DNA-TDNA at left border (▼) and filler DNA (bold letters) are presented in this figure.

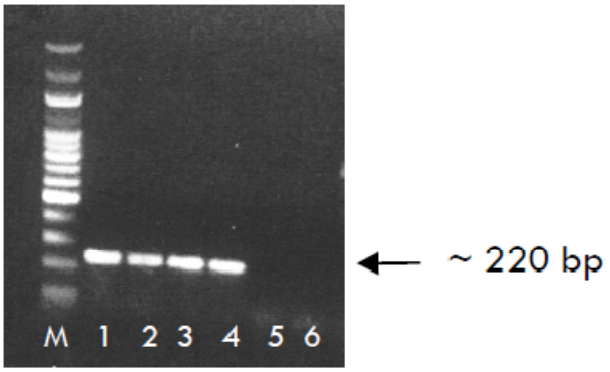

Figure 2: Homozygosity analysis of SALK_046142 (*ssl6*) T-DNA mutant by means of polymerase chain reaction. PCR were performed using *SSL6* reverse gene specific and T-DNA left border primers in four replications (1-4); forward and reverse *SSL6* gene specific primers (5); T-DNA left border primer (6), in an individual genotype. M: marker.

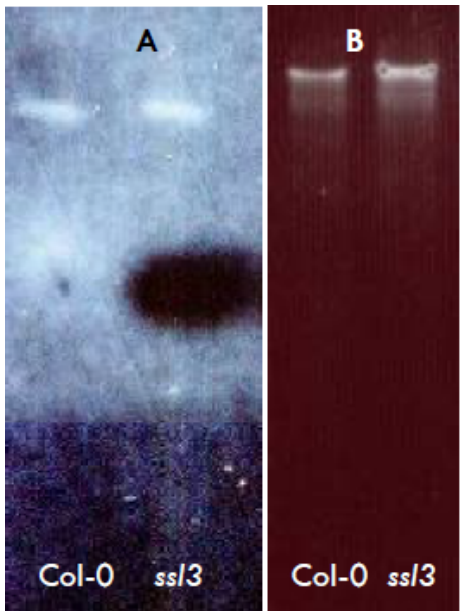

Figure 3: Southern blot hybridisation of *ssl6* knock-out line. (A) Hybridisation of the membrane with left border probe suggested the presence of single T-DNA insert; (B) The gel image of corresponding digested genomic DNA.

were able to amplify the T-DNA-genomic border. A positive control (Col-0) and negative control, containing only one left border primer, were included in the reactions. In some cases, a single LB primer could amplify the TDNA (Figure 2).

Estimation of the number of unlinked T-DNA insertions

Southern blot showed that *ssl6* contained a single T-DNA insert (Figure 3).

The chi-squared test proved that some of the *ssl6* genotype segregation ratio was in agreement with 3:1 for survived and dead individuals; respectively i.e. there is one T-DNA insert in *ssl6* (Figure 4).

According to the SALK institute, some of the lines showed silencing of NTPII (kanamycin resistance) after several generations of growth; selection is more reliable using sprays of Basta (http://signal.salk.edu/tdna_FAQs.html).

Figure 4: Genetic analysis of segregating populations of T-DNA-mediated *ssl6* mutant. Two-week old plants were sprayed with Basta and the number of resistance:susceptible individuals were counted to estimate the number of insert(s).

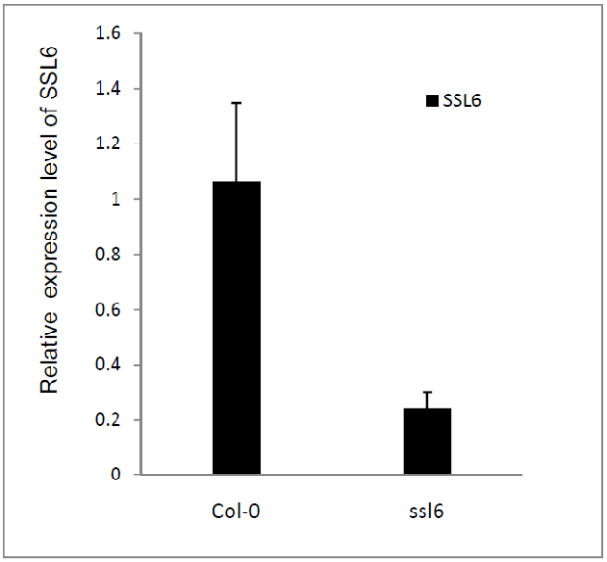

Figure 5: Suppression of *SSL6* expression level in the knockout line. According to the quantitative real-Time PCR analysis, the expression of *SSL6* in the mutated line decreased five times compared with that of Col-0.

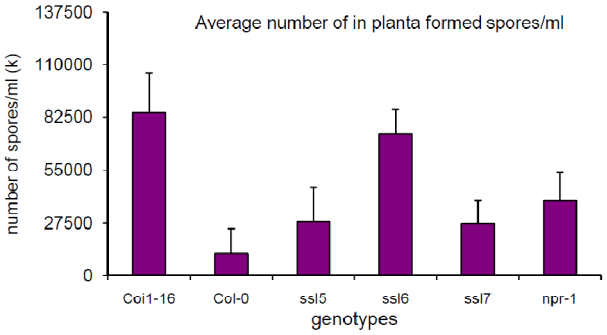

Figure 6: Analysis of *Alternaria brassicicola in planta* spores formed on various *Arabidopsis* genotypes. Mean of the genotypes were compared using each pair student's t-test. According to this analysis *ssl6*, *coi1*-16 and *npr1* showed a significantly higher number of spores compared to wild type Col-0.

Expression of the SSL6 genes in the related knock-out line

A quantitative real time PCR (q-PCR) was performed in order to exam the expression level of *SSL6* upon insertion of the T-DNA into the exon 1. As a result of q-PCR, SALK_046142 mutant exhibited low expression levels of *SSL6* compared with that of in the wild type Col-0 (Figure 5).

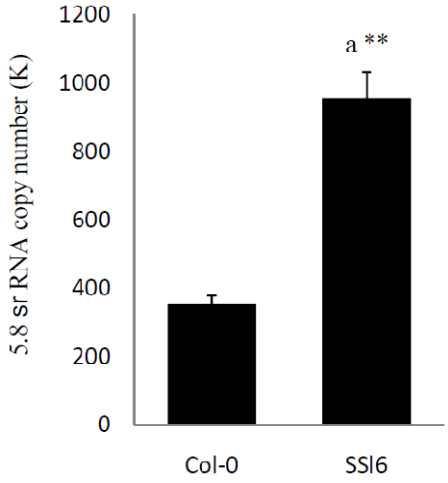

Figure 7. The quantity of *A. brassicicola* fungal biomass in terms of rRNA copy numbers, three days after *A. brassicicola* wounding-inoculating in *Arabidopsis* leaves.

Alternaria brassicicola assay

According to the spore count assay, the average numbers of *in planta* spores for *ssl6* mutant were significantly higher than the numbers in the Col-0 genotype. The experiment was repeated four times with the same results (Figure 6).

In the current experiment, *ssl5* and *ssl7* T-DNA mutants with accession numbers SALK_045029 (At3g51430) and SALK_041885 (At3g51450), respectively have been included in similar pathogenicity assay as well. The result indicated that the latest mutants were susceptible as wild type Col-0 (Figure 6).

The number of *in planta* spores, counted six dpi, is usually considered a measure for the susceptibility or resistance of a genotype to *A. brassicicola* (van Wees *et al.*, 2003). The *in planta* spores were easily distinguishable from the ones grown *in vitro* by their larger size and more hyaline appearance.

The quantity of *A. brassicicola* fungal biomass in *ssl6* and Col-0 genotypes was studied through q-PCR approach. The Average initial copies of the fungal gene (Starting quantity) on the third day post wounding-inoculation were 351k copies for Col-0 and 956k copies for *ssl6* genotypes (Figure 7). The results indicated that the difference in the fungal biomass between the two genotypes was at

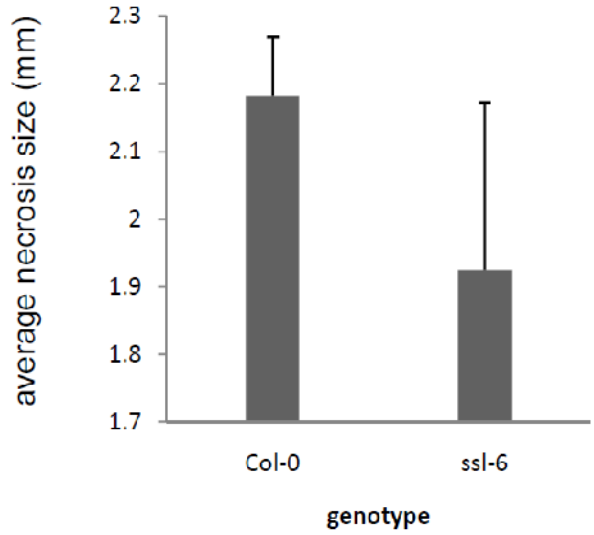

Figure 8. The diameter of necrotic spots on the leaves of Arabidopsis, seven days after A. brassicicola inoculation using wounding method.

0.01 level of significance.

In fact, nucleic acid-based techniques are faster, more specific, and more reliable than the immunoassay assessment for tracking, differentiation and quantification of the microbial pathogens. Real-time PCR techniques can be employed to measure the fungal biomass before manifestation of the visible symptoms of the disease (Narayanasamy *et al.*, 2008).

In a wounding inoculation method, the size of the lesions was measured seven dpi. The results suggested that the average diameter of *ssl6* necrotic spots was significantly less than the size of lesion in Col-0 (α=0.05) (Figures 8 and 9). However, at 7 dpi, no significant differences in the average diameter of the necrotic lesions were observed between the two genotypes using non-wounding method.

The inoculation of *A. thaliana* Col-0 ecotype by the fungus *A. brassicicola* could cause hypersensi-

Figure 9. Necrotic spots on the leaves of *Arabidopsis*, seven days after *A. brassicicola* inoculation in *Arabidopsis* leaves with the wounding method.

tivity responses, leading to the small necrosis lesions which were limited to the infected sites. Measuring the diameter of the necrotic lesions is a scale to assess the sensitivity and the resistance of the plants against the pathogens (van Wees *et al.*, 2003). In some trials due to the incompatibility of the fungus *A. brassicicola* with *Arabidopsis* plants, no occurrence of the necrosis has been observed.

The diameter of the lesions suggested that *SSL6* silencing was associated in some degree with the failure in development of hypersensitive response and restriction of fungal growth. The smaller lesions might be the result of limited incompatible interactions in *ssl6*. Considering the increased expression of *SSL6* under salicylic acid treatment, the increased resistance of the knockout mutant to cell death is explicable. Metabolite profiles of *SSL1-4* RNAi lines have shown significant reduction in the amount of salicylic acid beta-glucoside (SAG) (Kibble *et al.* 2009). Salicylic acid beta-glucoside is a storage form of a defense signal against pathogens that releases free salicylic acid. Excess salicylic acid induces localized cell death after oxidative burst and Ca^{2+} influx in plants (Kawano *et al.*, 2004).

In comparison to Col-0, smaller necrotic spots in *ssl6* genotype on the seventh dpi and higher chlorophyll content on the fifth dpi may be affected by the lower levels of ROS and salicylic acid glucoside in *ssl6*. Lower amounts of peroxidase in *ssl6* genotype could confirm this hypothesis as well. Salicylic acid glucoside induces slow and prolonged superoxide production and gradual release of salicylic acid is probably peroxidase-dependent (Kawano *et al.*, 2004). Salicylic acid may also indirectly and through the reactive oxygen species affect resistance against *A. brassicicola* (Thomma *et al.*, 1999b).

Camalexin or a camalexin-related metabolite and jasmonic acid are pathogen inducible effectors and mediators of *Arabidopsis* resistance against *A. brassicicola* and are controlled by separate pathsways (Thomma *et al.*, 1999b). Considering the increase of *SSL6* expression in the treatments/inoculation with *A. brassicicola*, it was assumed that this gene might play a role in camalexin (3-thiazol-2'yl-indole) production (Sohani *et al.*, 2009).

In the present research, *Arabidopsis* wild type Col-0 was used as a control. A known susceptible mutant to *A. brassicicola* with a defective MJ

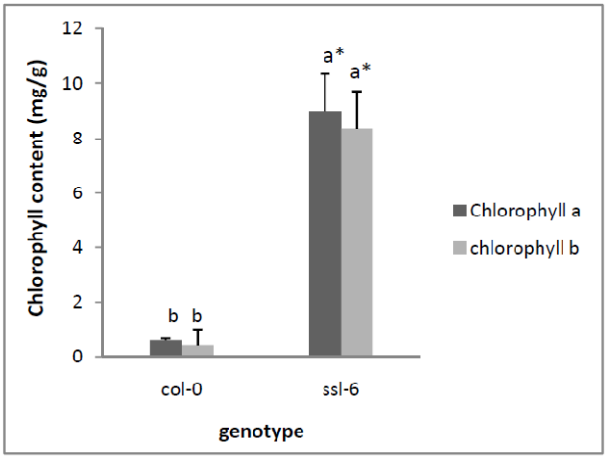

Figure 10. The content of chlorophyll a and b, on the fifth day after wounding-inoculation of *A. brassicicola* in *Arabidopsis* leaves.

pathway (*coi1-16,* kindly provided by Professor John G. Turner, UK) and a mutant with a blocked SA pathway (*npr*-1, kindly provided by Dr. Xinnian Dong, NC, USA) and a mutant with defective ethylene pathway (*ein2-1*) were also included in the experiment as a positive control. Pair-wise comparison using student t-test showed that *coi1-16, ein2-1* and *npr-1* were significantly more susceptible than Col-0 regarding the size of the lesion (Figure 6). Enhanced tissue colonization by the pathogen was observed in *coi1-16* compared to wild type (Thomma and Broekaert, 1998) while *npr-1* and *ein2-1* was slightly different from Col-0. This is an indication that SA- and ethylene-dependent pathways are only marginally effective against *Alternaria*.

Chlorophyll a and b content of ssl6 following A. brassicicola inoculation

The chlorophyll a and b content of the *ssl6* and wild-type Col-0 genotypes was measured at the fifth day post wounding-inoculation. The content of chlorophyll a and b in the *ssl6* genotype was significantly higher than Col-0 genotype (Figure 10).

The induction of plant defense response following pathogen attack is cost-intensive, which increased demand for assimilates in the plant (Swarbrick *et al.*, 2006). The withdrawal of carbohydrate metabolism for its own use by the pathogen will further increase the demand for assimilates. Moreover, pathogen infection often leads to the development of chlorotic and necrotic areas and to a

decrease in photosynthetic assimilate production (Mukherjee *et al.*, 2010).

Down-regulation of quantum efficiency of Photosystem II in compatible interactions with biotrophic as well as necrotrophic pathogens has been reported (e.g. Bonfig *et al.*, 2006). A decrease in photosynthesis has also been reported in incompatible interactions (Swarbrick *et al.*, 2006). The results led to the proposal that plants switch off photosynthesis and other assimilatory metabolism to initiate respiration and other processes required for defense (Berge *et al*, 2007). The same phenomenon most likely not occurs in *ssl6* since defense reaction was failed and higher quantity of fungal biomass detected. The higher quantity of chlorophyll a and b in *ssl6* compared to Col-0 might be as a result of failure in the development of hypersensitive response and incompatible interaction in *ssl6* genotype. Incompatible interaction took place in wild type Col-0 as it was observed by significantly a lesser amount of *A. brassicicola* biomass and bigger lesion diameter.

A comparison of the interaction of Arabidopsis with a virulent and an avirulent strain of *P. syringae* demonstrated that the major difference was the speed rather than the quality of photosynthesis. A decrease in photosynthesis was detectable earlier with the avirulent strain than with the virulent strain (Tao *et al.*, 2003).

Pathogen effects on the plant photosynthesis are observable through the expansion of the chlorotic and necrotic tissues, loss of the leaf, reduction of the chlorophyll and chloroplasts and also the stomata closure. Depending on the pathogens and growth conditions, photosynthesis can be increased decreased or it can remain unchanged (Orcutt *et al.*, 2000).

As the chlorotic leaf area increases, the chlorophyll content is reduced (Mukherjee *et al.*, 2010). Due to the photoactive nature of the chlorophylls, the chloroplast is one of the sources of reactive oxygen species (ROS) generation. Accumulation of ROS is essential in response to several plant pathogens (Karpinski *et al.*, 2003). This may lead to the release of the chlorophyll in tylakoid membranes. In such circumstances, the chlorophylls must be rapidly degraded to avoid the photodynamic cellular damage (Takamiya *et al.*, 2000).

Uncoupling, or inhibition, of the photosystem machinery in the chloroplast and photorespiration associated with chloroplast and peroxisome function can lead to the formation of high levels of ROS that can dramatically affect cellular homeostasis. Its Interference with the chlorophyll degradation pathway also results in over-accumulation of ROS and an increase in susceptibility to some necrotrophic pathogens (Kariola *et al.*, 2005).

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