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Identification, validation of Comp121307 C0 Seq4 gene against leaf rust resistance gene in ILT598, ILT756 and WL711 derived from *Aegilops geniculata*

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Abstract
The current investigation was conducted during the 2017-18 sample collection under six-time intervals 0, 12, 24, 48, 72 and 96hr. All six different time interval samples were collected from the Genetics area, school of agricultural biotechnology (SOAB) PAU Ludhiana (state university). To find out the candidate gene from a raw sequence provided by bioinformatics lab SOAB as well based on the candidate gene to check to fold expression in IL T598, IL756 and WL711 as well visual fungal biomass investigation. Result directly shown *comp_121307_c0_seq4* is the candidate gene which reveals the maximum fold expression at 24hr among all six-time interval was 5.40 - ILT598, 1.22- ILT756 and WL711 simultaneously less fungal mass was observed at 24hr in ILT598, more in IL756 and more than all two in WL711. Fungal biomasses in challenged leaves of ILT598 were less compare to ILT756 and WL711 which directly correlated with host-pathogen interaction (Cheng *et al.* 2012).

Keywords: Wheat, stripe rust, leaf rust, differential expression, qRT PCR, fluorescence microscopy

Introduction
Wheat is a crop of the tropical and subtropical regions mainly grown for carbohydrates and protein. This crop is worldwide demanded and its demand in India at number second after rice. One of the major problems in wheat production is rust disease leaf rust (Knott *et al.* 1981) [6] and stripe rust caused by *Puccinia triticina Erik* and *Puccinia striformis* respectively. Loss due to leaf and stripe rust is at the level of grain development due to major leaf area cover with rust spot ultimately less amount of photosynthates accumulate. Leaf and stripe rust spread through the air from spore from one to another plant resulting in loss of yield. The disease management by chemical treatment was reported but the rate of spread cannot be controlled whereas developing genetically resistant variety is the best way to control the leaf rust and stripe rust disease. There is two-stage of resistance development seen in wheat plant one at seedling and other at the adult stage. The same is gene is responsible for resistance at seedling and at adult resistance only pathways of resistance are different. V kuraparthy (2007a) [7] is a scientist of Biological science introduced *Lr57* and *the Yr40* gene responsible for the leaf as well as stripe rust resistance in *Ae. geniculata* derived introgression line (Chen *et al.* 1994) ILT598 and ILT756 respectively through linkage map (chromosomal location 5DS). Its confined position was a unique location T5DL.5DS-5M (g) S(0.95) for leaf rust in the F2 population of the introgression line derived from *Ae.geniculata*. This study was undertaken to validate the novel gene *comp121304_c0_seq4* in ILT598, ILT756, and WL711 against six-time interval 0hr, 12hr, 24hr, 48hr, 72hr and 96hr. The leaf sample was isolated from the infected leaf of wheat under the controlled condition of temperature and humidity. All six-time interval of the infected leaf was analyzed for fold change using expression analysis (Cong *et al.* 2010) [8] the study designed for getting the confirmation of a number of the transcript at the critical stage of host-pathogen interaction and also grab the information of critical stage of resistance (in terms of time interval).

Material and method
Leaf rust and stripe rust-resistant Wheat-*Ae.geniculata* introgression lines (IL) T598 (TA5601), ILT756 (TA5602) and susceptible cultivar WL711 NN=Non-Necrotic). F_4 population derived from an ILT598*WL711 and ILT756 respectively.

Methods
Validation of candidate gene contributing towards leaf rust

Developing the seedlings, treatment and sample isolation

Seedlings of IL (BC_2F_4 generation) T598, IL T756, and a parental cultivar WL711 were raised and the first leaf of 7D old seedlings had infected with a Yr race 110S119 + talc powder. The inoculated seedling was incubated in a dark chamber maintained at 20°±1 °C at 100% RH for 16h (Nayar *et al.* 1997a, 2007b) [13].

Leaf samples were collected at the different time intervals of 0 hours, 6 hours, 12 hours, 24 hours, 48 hours 72 hours and 96 hours in three biological replicates followed by 14th days rust appearance.

Total RNA extraction and c DNA conversion

Using the Trizol manual (Wang *et al.* 2011) [9] method and RNA quantification done with 1.2% denaturing gel-based running gel unit as well as Nano Drop® ND-1000 and ND-8000 8-Sample Spectrophotometers instrument with 2.0 O.D value. Prime Script™ first strand cDNA Synthesis Kit based cDNA Synthesis. cDNA quantification using the Nano Drop instrument as above said with 1.8O.D value for qRT-PCR running.

Transcriptome analysis and identification of candidate gene

The previous study on transcriptome analysis by Yadav *et al.* 2016 was differential gene expression (Guo *et al.* 2018) during infection at different time intervals 0hr, 12hr, 24hr, 48hr, 72hr, and 96hr. Pathogen induced differential gene expression was characterized in susceptible cultivar WL711 and introgression line T598 having Lr57 and Yr40 resistance gene. They isolated RNA at six abovementioned time points with leaf rust pathotype 77-5 inoculated. They investigated a total of 3020 transcripts that were differentially expressed with 1458 and 2692 numbers of the transcript in susceptible cultivar WL711 and introgression line T598. They found at 12hr highest number of differentially expressed. They use Blast2GO bioinformatics tool for classifying the R genes into the Biological process, the cellular process and their molecular function. Overall investigation revealed that comparative analysis of the differentially expressed transcripts, few were expressed only IL T598. Which deduced the specific R genes in IL T598. The RNA sequence data and differentially expressed genes (DEGs) were generated in Yadav *et al.* 2016 experiment. Developing an ideal assay for QRT PCR includes a selection of primers that will result in an mRNA specific amplification product. It should not amplify the degraded genomic DNA. Using SYBR green I. one of the PCR primers needs to stretch the intron such as 5/6 base of the 3 prime ends of one primer hybridize to the single exon of the gene. While remaining is hybridized to the adjacent exon. Taq Man probe or molecular beacon hybridization probe spread the junction of two consecutive exons to increase the surface area for SYBR green binding (Devid 2002). Results in again Cq value obtained and relative quantification accomplished.

Primer designing from RNA sequence data

RNA Sequence data of IL T598 and WL711 was available in Bioinformatics lab SOAB PAU Ludhiana. This data was generated after inoculating both the wheat lines with leaf rust race 77-5, leaf samples were collected six different time intervals of 0hr, 12hr, 24hr, 48hr, 72hr and 96hr after inoculation. Raw reads of RNA seq data were trimmed by the Trimmomatic tool, mapped by RSEM mapping tools and final reads were assembled with Trinity assembly. Using edgeR differentially expressed genes (DEGs) were identified. Based on the up and down-regulation of gene expression, resistant (R) genes were fetched out. Genes IDs with genes expressed at six different time intervals were noted and six candidate genes were selected based on FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value. URGI BLAST of all these six candidates was done and all Tophits with the range of 98 to 100% were selected. To reconfirm, gene IDs of these six candidate genes were matched with the annotation file present in our bioinformatics lab. One of the genes with selected gene ID *comp_121307307_c0_seq4* was annotated NBS-LRR and high confidence gene.

Real-time quantitative RT-PCR

Expression analysis (An *et al.* 2011) [9] using real-time quantitative PCR conducted with cDNA samples of IL T598, IL T756 and cultivar WL711 for six different time intervals. The PCR amplification efficiency was determined for each primer combination by the slope of the standard curve obtained by plotting the fluorescence versus concentration of the individual cDNA of all samples.

Real-time quantitative PCR- assay

Done all the QRT PCR reactions with LightCycler96 well plate qRT PCR (Roche). A total of 10 microliters of PCR reaction was performed (Table 3.) at the School of Agricultural Biotechnology in wheat genomics lab. QRT-PCR reaction per performed with cDNA and two types of primer one was constitutive primer (Tubulin primer) and the second was target primer (*comp_121307_c0_seq4*) within the single white PCR Plate of 96-well, segmented, semi-skirted. Simultaneously a negative-template control (NTC) negative control was performed in the same 96 PCR well plate. The PCR profile was used as initial denaturation at 94 °C/4 min, denaturation at 94 °C/1min, annealing at 56 °C/1min extension 72 °C/1min, and final elongation 94 °C/7min.

Table 1: Different components used in the qRT-PCR reaction

S. No	Component	Volume (µl /well)
1	SYBR-Green	5:0
2	Forward primer	1:0
3	Reverse primer	1:0
4	cDNA	1.5
5	Nuclease-free water	1.5

Result and Discussion

Background

A leaf rust (LR) and stripe rust/yellow rust (YR) resistant accession of a non-progenitor species *Aegilops geniculata* (2n=28, UUMM) was crossed with a YR susceptible cultivar WL711 (2n=42). F_1 plants generated were backcrossed with WL711 resulting in the development of alien disomic substitution line DS5M (5D) (Jiang *et al.* 1994). Further, this substitution line was crossed with Chinese spring (*ph*^{i}) stock carrying inhibitor for *Ph1* locus to allow crossing over between chromosome 5U of *Ae. geniculata* and 5D of wheat.F_1sobtained were backcrossed twice with bread wheat cultivar WL711 to generate BC_2stable introgression line (IL) (Chen *et al.* 1994). One of these ILs, ILT756 was completely resistant to LR and moderately resistant to YR. Linked *Lr57-Yr40* genes were mapped in this IL (Kurapathy *et al.* 2008). Another IL (developed from the same process) named ILT598 is completely resistant to YR. The present research was undertaken to study the genetics of LR resistance in ILT598 and its molecular characterization.

The sequence of comp_121307_c0_seq4 was isolated based on the fluctuation of the FPKM value shown in table 4. Primer was designed based on the above-mentioned methods and sequence shown here.

Table 2: Mean FPKM value of comp121307_c0_seq4 of all 6 different time interval

S. No	Time interval	ILT598	WL711
1	0hr	0.176667	0.38
2	12hr	0.845	0.09333
3	24hr	0.58	0.25667
4	48hr	0.055	0.66333
5	72hr	0.375	0.30667
6	96hr	2.09	1.23

From picture 1. it depicted that ILT598 were showed total resistance, ILT756 showed and WL711 showed total susceptible for both leaf and stripe rust.

WL711 IL 756 ILT598

Fig 1: Stripe rust reaction on the CV. WL711, ILT756 AND IL T598

Table 3: Primer information for RT-PCR

S. No	Primer	Gene sequence	Tm value (^{0}C)
1	Alpha tubulin F	5'AGGAGGATGCAGCCAACAAC3'	58
	Alpha-tubulin R	5'AGGGCCAGAGCCAGTTCCA3'	
2	Lr57_cds_RT_F	5'AGATTCCTGAGCCTTGTTACTTCGG3'	58
	Lr57_cds_RT_R	5'TAAGCTGTCgGGAAGATTGCCTAC3'	

A comparison was made relative *comp_121307_c0_seq4* gene expression among six different time interval cDNA (synthesize from RNA of leaf rust pathotype 77-5 inoculated sample isolated at six different time intervals) through qRT-PCR analysis (Nayyar *et al.* 2017). The result reveals that 24hr was the time at which maximum host-pathogen interaction in ILT598, 12hr for ILT756 and negligible in WL711. The jumping of data (Fig 3.) from 0 -12-24hr emphasized the increase in expression of *comp_121307_c0_seq4* was showed at 5.40, 24hr for ILT598, 2.10, 12hr for ILT756 and no any expression for WL711 at any time interval (Table 4.). The result expresses a peak expression of leaf rust resistance gene (R-gene) from ILT598 was compared to ILT756 indicating a different R gene controlling resistance against leaf rust. *Comp_121307_c0_seq4* was found to be a sequence of NBS-LRR family which also proven its novelty in the category of R gene (fig 2,3.).

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>comp121307-c0-seq4-T598
TTCAGATATTCTTAGAGAAGAAGGTGGTCAGGATCAAAATGTTGTAAAAGGATCAATGACCTGATCACAAACACAAAGGTATCATCGCTTCAAAATCTGCAGCCTCTGAGATGGCATGTACAT
CAAAATAGCAGACAATAGAAACATTCAGCAAACTGCAATACCCACACGATCGAAAGCTGACACGGTCGACGGGTTCTTGTGCTACGACCCCGACACCATCCGCCTCATGCTAAGGAGAGAGCA
ACCGCGTCTCATCATCGGCGTCCACGATGACAACGAAAGGATCTGGATGATAGAGGTGGACACGAGGAAGAAGGCGCCGCGATCCTTCGCCCGATATGCCAGCCAGAGCAACAAGGACGGCAC
ACCAATGACTGGTCACTTTAACCTGATTGGCTTCTTGCCTAGTGAGGTCTCCAAGTACCTGCCTACCTGCTGAACCAGCACGTATTACATCAAGCAACATTATTTAAGCGACTAGATTCTGTA
TTAGTCCATATCCACACGTCTTACAATACAAGTACTTTGCCCCCTCAAACCACCCGAAGTGTTTATCATGAAAAGAACTTATCACGGATATATTTATGCTAATACATACTACATGCAATAATT
ATCATACTTGCTTCTTCTATGATGTCACCGCCGGATCATCACATGGCATTCTCCATGGACGGCCCGCGACATGTCAGAGCCCCACCACAAGTCCCAGCACAAGGCCGAAATAAAAGGTCAGTG
GAACAAGCTCTTGCTTACTGCTTCCGACGTCGCCATGAATGATGGAAGAATTATTTCCTGAACAACTTCTGCTGAGAGGAGGCCCACAAAGCCCACTGTTGCCGATGTATATGTTGTCCGAAA
TGAGGGTGTCAAGTTGCGGGCCAGAAGGTATCCTTCCGGACAAATTGTTGTAGGACAGGTTAAGGGCGGACAAATACGTCAGGCTTGACAAGCTCCATGGGATTTCACCAGAGAGCTCGTTCT
CAGAGAGGTCGAGAGATTCCAGTGACCGCATGGCCCCGATCATGTTTGGGATCTGTCCACTCAATCTGTTCGACGACAGGTTCAAATTCATGAGCTCGGCAAGGGAAGTGATGTCTGTAGGGA
TTTCACCGGTCAAGGAGTTGTCTGATAGATCGATGCTCACAACATAAACAATTGCTCCACCATATAAAAGTTGATGTCCTTTTGTTACTACTGGCAATATTTCTCCCAGGTGACCAGCTCCCA
TCTCGCCAATGTGCGCGGATGCATTATATGTGTCATACATACCCATGAATTCCTTTTGTGTCCCTTTCATAAATGTCAGGTTTGATAGATGCCAAGGTACCGCACCAGAGAAATTATTGGCTG
ATAGATCCAAGTATTGAAGATTCCTGAGCCTTGTTACTTCGGCCGGAATATTATCACAAAATGCATTGTGGCACAGTAGTACAAATCTTAATTTCCCCAGATCTCCTATCCATGTAGGCAATC
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Fig 2: Primer sequence as highlighted in *Comp_121307_c0_seq4* sequence derived from ILT598

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	probable leucine-rich repeat receptor-like protein kinase At2g33170 isoform X2 [Aegilops tauschii subsp. tauschii]	163	219	98%	2e-47	93.83%	XP_020194588.1
☑	LRR receptor-like serine/threonine-protein kinase GSO2 [Triticum urartu]	164	205	98%	5e-45	92.59%	EMS45946.1
☑	unnamed protein product [Triticum turgidum subsp. durum]	164	220	98%	5e-45	92.59%	VAI12485.1
☑	probable LRR receptor-like serine/threonine-protein kinase At4g36180 isoform X1 [Aegilops tauschii subsp. tauschii]	161	216	98%	4e-44	93.83%	XP_020194587.1

Fig 3: *Comp_121307_c0_seq4* sequence derived from ILT598 showed NBS-LRR gene family in online BlastX search

Table 4: Fold expression change ($2^{-\Delta\Delta Ct}$) of *comp_121307_c0_seq4* gene in leaf rust inoculated sample taken at six-time intervals

ILS/Time interval	ILT598	ILT756	WL711
0HR	1	1	1
12HR	1.153	2.109	0.332
24HR	5.404	1.220	0.029
48HR	1.652	0.732	0.335
72HR	0.059	0.011	0.000
96HR	0.007	0.000	0.000

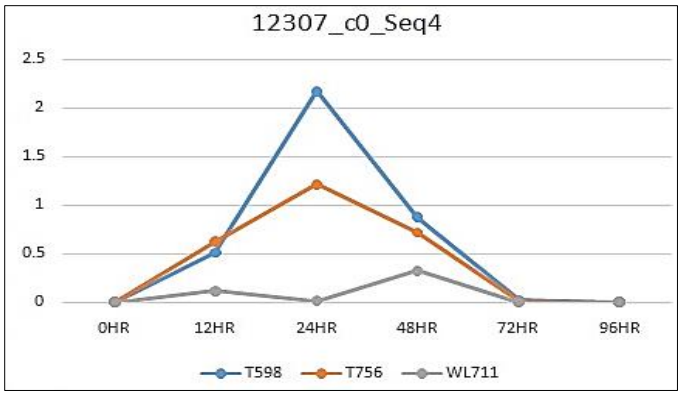

Fig 3: Fold expression change ($2^{-\Delta\Delta Ct}$) of *comp_121307_c0_seq4* gene

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