Suppression Subtractive Hybridization Technique in Wheat for the Identification of Disease Resistance Differentially Expressed Genes

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Abstract – A resistant variety with high yielding potential is key for increasing crop production to fulfill the food requirement of the ever increasing world populations. Consequently, the aim of plant breeders is to develop high yielding varieties or cultivars that are resistant or tolerant to specific diseases or insects. To develop a resistant variety, it is enormously indispensable to incorporate or introgress the specific resistant genes of that particular disease into the recipient. Suppression subtractive hybridization (SSH) is a powerful technique for the identification of disease specific differentially expressed genes that are expressed in a resistant or susceptible variety. This paper presents a brief review on the SSH technique with examples focusing on the identification of the wheat disease specific differentially expressed genes and their defense mechanisms against fungal pathogens in global wheat cultivars. This review is helpful for wheat researchers for the updated information on the SSH technique for the identification of differentially expressed genes in the global wheat cultivars and varieties. Eventually, the identified genes could be used to develop the disease resistance variety through marker-assisted backcrossing programme or conventional breeding.

Keywords: Differentially expressed genes, expressed sequence tags (ESTs), resistance variety, Suppression subtractive hybridization (SSH), Triticum aestivum

Introduction

Wheat is among the top cereal crops and it ranks first in global crop production (Cao et al., 2011). More than half of the total world population depends on wheat to meet their daily food demand. Wheat is affected by several fungal, bacterial and viral diseases. Among these diseases, leaf rust and leaf blight e.g. Bipolaris leaf blight (Bplb) are the major and common fungal diseases distributed worldwide. The aim of plant breeders is to develop high yielding varieties or cultivars that are resistant or tolerant to specific diseases or insects. To develop a resistant variety, it is necessary to incorporate or introgress the resistant gene of that particular disease into the recipient.

The suppression subtractive hybridization (SSH) method is valuable for identifying differentially regulated genes important for cellular growth and differentiation. It is a widely used method for separating DNA molecules that distinguish two closely related DNA samples. Two of the main SSH applications are cDNA subtraction and genomic DNA subtraction. SSH is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. It is based primarily on a suppression polymerase chain reaction (PCR) technique and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the populations being compared. This dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs or genomic DNA fragments, and simplifies the analysis of the subtracted library. The SSH technique is applicable to many comparative and functional genetic
studies for the identification of disease and developmental, tissue-specific or other differentially expressed genes as well as for the recovery of genomic DNA fragments distinguishing the samples under comparison. The SSH technique may result in over 1000-fold enrichment for rare sequences in a single round of subtractive hybridization (Lukyanov et al., 2007).

Differentially expressed genes are those whose expressions are limited to a particular tissue or stage or whose expression patterns changed after a stimulus is received by the organism (Habib, 2011). They are of interest because they often have important functional roles in specific stage or tissue such as attack of wheat plant by leaf blight and leaf rust pathogens at the vegetative stage in which they are expressed (Jones & Harrower, 1998).

The level of expression of all genes of an organism in different types of cells, tissues, developmental stages, or disease processes constitutes essential information for understanding the function of different genes, and to unravel the complex network of biological processes acting in every biological system (Eickhoff et al., 2000). Several challenges pertaining to gene expression studies take account of low abundance, tissue-specific or stage-specific gene expression patterns. By means of a normal gene isolation method such as cDNA library screening, these genes are the most likely overlooked during the isolation process. Therefore, these genes are the most likely to be missing in the database. A number of approaches can be used to increase the opportunity of isolating tissue-specific, stage-specific and low abundance genes by collecting the sample at a particular period of time e.g. leaf blight attack at the vegetative stage and smut disease at the heading stage, manipulating the growth condition e.g. to make the plant more resistant by applying a silicon fertilizer or make the plant more susceptible by using a nitrogen fertilizer to express the particular genes, and pulling specific tissues to increase the mRNA level for the low abundance gene. Studies of such genes are promising as they include key regulatory factors responsible for differentiated phenotypes, developmental progression, or cell growth regulation (Martin & Pardee, 2000).

In SSH two populations of samples, one treated and one controlled, are generally used to compare the expression of genes. The mRNA which is obtained from total RNA, are used as a starting material. First, both mRNA populations are converted into cDNA: the cDNA that contains specific (differentially expressed) transcripts referred as the tester, and the reference cDNA as the driver. The tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA. The process is shown in Figure 1.

**Comparison of SSH with other methods**

Several molecular methods exist for isolating low-abundance genes and for identifying differentially expressed genes among biological samples, namely differential display reverse transcription polymerase chain reaction (DDRT-PCR) (Liang & Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), microarrays (Shena et al., 1995), and subtraction and normalization method called suppression subtractive hybridization (SSH) (Diatchenko et al., 1996). Each of the methods has specific advantages and each also has inherent disadvantages.

The differential display (DDRT-PCR) method developed by Liang and Pardee (1992) uses a combination of arbitrary PCR primers and oligo (dT)-anchored cDNA primers to amplify cDNA sequences by reverse transcription of mRNA followed by PCR. This method is simpler, quicker and more sensitive and is widely used for the identification of differentially expressed genes when the RNA sample is limited such as in pre-implantation embryos. Although this method requires a relatively small amount of RNA sample, false positive results can generate a large number of false sequences that do not represent differentially expressed genes. The cDNA-AFLP method is sensitive for gene discovery in plants and more reliable than the DDRT-PCR method. The serial analysis of gene expression (SAGE) method allows a rapid, detailed analysis of thousands of transcript (Song, 2003), but much time is required to set up a gene expression profile for a eukaryote (Velculescu et al., 1995). The microarray technique has been widely used for the isolation of differentially expressed genes in model plants such as *Arabidopsis thaliana* and maize (Brosche et al., 2002) but these
techniques are expensive and their use is commonly limited to the most standardized models. Libraries constructed through SSH, followed by colony blot hybridization or cDNA amplified fragment length polymorphism (AFLP), are time consuming because positive results have to be confirmed by an independent technique (Massart & Jijakli, 2006). The combination of SSH and negative subtraction chain (NSC) ensures that target sequences are dramatically and efficiently enriched but may not have enough sensitivity (Li et al., 2005) to direct isolate differentially expressed genes from highly complex eukaryote genomes (Lisitsyn et al., 1993).

In the SSH method subtraction and normalization are performed simultaneously (Mahalingam et al., 2003) thus equalizing the abundance of target cDNAs in the subtracted population (Diatchenko et al., 1996). The subtraction method overcomes the problems of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics. The SSH method eliminates any intermediate step(s) for the physical separation of single and double stranded cDNAs in one round of subtractive hybridization.

![Diagram of SSH process]

**Figure 1**: Brief overview of the suppression subtractive hybridization (SSH)

**Powdery Mildew Resistance Genes Identification by SSH**

Powdery mildew of wheat is caused by *Blumertia graminis* (DC.) E.O. Speer f. sp. *Tritici* Em. Marchal (*Bgt*) =*Erysiphe graminis* DC. *Ex Meraf.* sp. *Tritici* Em.Marchal. It is the most devastating fungal disease of common wheat worldwide (Alam et al., 2011). Due to this disease, global yield loss is accounted for 13 to 34% (Griffey et al., 1993; Leath & Bowen, 1989). A marker-assisted selection is a common molecular procedure for the identification of mildew resistance gene of wheat. The use
of resistant cultivars is the more effective and environmentally safe approach to avoid the yield loss due to powdery mildew. The resistance to powdery mildew in wheat is mainly defined as two types: monogenic (vertical) or race specific resistance, and adult plant resistance (APR) (Alam et al., 2011). The adult plant resistance is also known as “slow mildewing” (Shaner, 1973) and “partial resistance” (Hautea et al., 1987). Approximately more than 60 genes/alleles for resistance to powdery mildew have been identified and located on 18 different chromosomes in bread wheat. The 29 resistance genes/alleles have been tagged with common molecular markers such as RFLPs, RAPDs, AFLPs, STS and SSRs (Alam et al., 2011). However, identification of powdery mildew resistance genes using SSH is very limited. In a comparison of SSH technique with conventional cDNA library construction using a powdery mildew resistance wheat cultivar (BaiNong 321/Mardler BC5F4), Luo et al. (2002a) concluded that the subtracted cDNA library is a more advantageous technique in gene expression profiling of disease resistance than the simple library construction procedure in rich and specific DRR (disease-resistance-related) genes. They found 760 ESTs from the subtracted cDNA library and the redundancy of disease resistance genes such as those related to photosynthesis and ribosome are less than those in conventional cDNA library. About 54.1% function-known ESTs in the SSH cDNA library were identified to be DRR genes by screening. There were 247 clones of the SSH cDNA library that had positive signal in the repeated hybridizations with the pathogen uninfected probe. The identified DRR genes are distributed in the whole procedure of powdery mildew resistance, but they are mainly focused on the SAR (systemic of acquired resistance). Luo et al. (2002b) also conducted another study using the Bainong 3217 × Mardler BC₅F₄ wheat line for the construction of a cDNA library with an inoculation of powdery mildew pathogen (Erysiphegraminis DC) at the initial stage of plant by the SSH technique. Totally 199 differentially expressed ESTs with related diseases were obtained out of 760 ESTs based on BLASTn and BLASTx comparison sequencing analysis. The obtained powdery mildew resistance genes in wheat were predominant in terms of kinds (antioxidation substances and cell protection mechanism) and quantity. The average size was 83 to 276 bp and 91 ESTs were known functional homology in nature (34.0% of total ESTs), and their functions were mainly involved in energy metabolisms and protein synthesis (Table 1).

A study was conducted by Ma et al. (2015) using two wheat lines, L693 and L1095 (Liu et al., 2014), which were resistant and susceptible to powdery mildew to understand physiological changes during the host–pathogen interaction as well as gene expression, photosynthetic and chlorophyll fluorescence parameters, chlorophyll content, and antioxidant activity, specific to the resistance response to powdery mildew. Wheat (Triticum aestivum L.) photosynthesis- and energy-related pathways are directly affected by powdery mildew. From their investigation, 58 and 26 ESTs were identified from forward and reverse SSH cDNA libraries constructed from Blumeriagraminis (DC.) Speerf. sp. Triticci Em. Marchal infected L693 and L1095 leaves respectively, and their average length with detailed information are given in Table 1. A total of 9% of the ESTs in the forward library was high homology, further classified into carbon metabolism, photosynthesis pathway, nitrogen metabolism, protein modification pathway, and transport of metabolic products. In contrast, 50% in the reverse cDNA library showed high homology and further classified into photosynthesis, cell wall catabolism, nitrogen metabolism, and carbon metabolism related functions.

A wheat line N0308 with resistance to powdery mildew was used for the construction of a cDNA library by the SSH technique (Alam et al., 2014). The leaves were inoculated by Bgrtrace E09 at the two-leaf stage. In response to Bgr infection in wheat line N0308, the differentially expressed genes were identified, and a total of 175 positive clones from the library were sequenced, and 90 expressed sequence tags (ESTs) were subjected to clustering, BLAST alignment, functional annotation, and classification into different categories. Among these, 7 ESTs were differentially expressed and associated with the resistance reaction of powdery mildew by using semi-quantitative reverse transcription-polymerase chain reaction based on BLAST search analysis, whereas 5 genes (sulfatase, pathogenesis-related protein 17, betacarboxylic anhydrase 2, thiorodoxin h-like protein, and coronatine-insensitive) transcripts were induced at the early stage at 72 h after Bgr infection. Two genes violaxanthin de-epoxidase and gag-pol-polyprotein were at the highest level at 12 and 18 hours post-
infection respectively, which suggested that these genes are induced at an early stage of infection and are transcriptionally activated for the host defense response (Table 1).

Another related study was conducted by Michel et al. (2006) using susceptible wheat (*Triticum aestivum* L.) cultivar Fidel and inoculated with a Swiss field isolate of the powdery mildew fungus *Blumeria graminis* f.sp. *tritici* for the construction of a cDNA library by the SSH technique. After the differential sequencing analysis, 158 uni-gene sequences were obtained and among these 71 (45%) exhibited > 90% sequence identity to ESTs present on the Affymetrix wheat gene, and their average size were 80 bp (Table 1).

<table>
<thead>
<tr>
<th>Cultivar/ Variety</th>
<th>Name of the disease with pathogen</th>
<th>Identified genes</th>
<th>Forward library of SSH</th>
<th>Reverse library of SSH</th>
<th>Post inoculation time</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L693, L1095</td>
<td>Powdery mildew, (<em>Blumeria graminis</em> f.sp. <em>tritici</em>)</td>
<td>ESTs, Singletons, Contigs, UniESTs</td>
<td>L693 as the tester and L1095 as the driver</td>
<td>L693 as the driver and L1095 as the tester</td>
<td>24, 48 and 72 hours</td>
<td>Ma et al., 2015</td>
</tr>
<tr>
<td>N0308</td>
<td>Powdery mildew, (<em>Blumeria graminis</em> f.sp. <em>tritici</em> Bgt race E09)</td>
<td>ESTs</td>
<td>7</td>
<td>200-100</td>
<td>-</td>
<td>24, 48 and 72 hours</td>
</tr>
<tr>
<td>“Bainong 3217 × Mardler” BC₅aturity populations</td>
<td>Powdery mildew, (<em>Erysiphe graminis</em> DC)</td>
<td>ESTs</td>
<td>91</td>
<td>83-276</td>
<td>Functional homologus in nature</td>
<td>24, 48 and 72 hours</td>
</tr>
<tr>
<td>Fidel</td>
<td>Powdery mildew, (<em>Blumeria graminis</em> f.sp. <em>tritici</em>)</td>
<td>ESTs</td>
<td>71</td>
<td>80</td>
<td>Affymetrix wheat gene in nature</td>
<td>-</td>
</tr>
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</table>

**Table 1**: Differentially expressed genes in wheat cultivars against powdery mildew disease

**Fusarium graminearum resistance genes identification by SSH**

Fusarium head blight (FHB) is a serious disease of wheat caused by *Fusarium graminearum*. Resistance to FHB in wheat is mainly defined by Type I (resistance to initial infection) and Type II (resistance to spread of the disease within a spike) (Mesterhazy, 1995) identified in several wheat genotypes. Multiple quantitative trait loci (QTL) mapping has been used in the identification of regions of the wheat genomes referred to as *Fhb1* in resistant cultivar Sumai 3, and its derivatives (Bai et al., 1999; Waldron et al., 1999; Anderson et al., 2001; Buerstmayr et al., 2002; Zhou et al., 2002; Liu et al., 2006). A study was conducted by Bernardo et al. (2007) for the identification of the expression patterns of transcriptomes from wheat spikes of FHB resistant cultivar Ning 780, and susceptible cultivar Clark were used after 72 hours of inoculation with *Fusarium graminearum*. From this investigation, 44 significantly and differentially expressed genes between cv. Ning7840 and cv. Clark, and maximum differentially expressed genes were found in susceptible libraries compared to resistance libraries. The resistant cultivar Ning7840 related to the susceptible cultivar Clark during the early fungal stressed on the basis of up-regulation defence related genes. Three up-regulated unknown function genes in cv. Ning7840 were investigated which played a significant role for enhancing the FHB resistance. Muhovski et al. (2012) used *Fusarium graminearum* infected spikes of a resistant
Belgian winter wheat (Triticum aestivum L.) variety Centenaire and susceptible genotype Robigus for the construction of a SSH cDNA library, exhibiting Type II resistance to FHB. From this investigation, 43 differentially expressed transcripts were identified and these were classified in different categories according to their predicted functions, including proteins involved in defence response, signaling, transport of molecules, metabolism and proteins with unknown functions. An ontime-course gene expression analysis on 12 selected genes was carried out between the FHB resistant genotype Centenaire and the susceptible genotype Robigus in order to validate the SSH screening. The selected transcripts were differentially expressed between the resistant and the susceptible genotypes at three-time points (24, 48 and 72 h) after the inoculation with the pathogen showing real-time quantitative polymerase chain reaction. The transcripts’ accumulation rates were higher in the FHB resistant as compared to the susceptible one.

Another study was conducted by Kong et al. (2005) involving F. graminearum and Chinese wheat cultivar Ning7840 (resistance to FHB) using the SSH for identifying the differentially expressed genes. From this study, 24 differentially expressed genes were identified between this fungus and wheat interaction, out of which 8 showed homology with genes from the pathogen, and 16 with wheat genes, including those encoding cytochrome P450, actin depolymerizing factors, chitinase, histone H4, pyruvate decarboxylase, and S-adenosylmethionine decarboxylase.

A related study was carried by Golkari et al. (2009) for the investigation of QTL-specific differential gene expression between the resistant Chinese cultivar Sumai-3 and two susceptible near isogenic lines (NILS) inoculated with Fusarium graminearum by the SSH technique. From this investigation, 25 wheat unigenes were found to express differentially, responsible of F. graminearum infection. Three pathogenesis-related (PR) encoding gene proteins, viz., β-1, 3-glucanase (PR-2), wheatins (PR-4) and thaumatin-like proteins (PR-5) were identified, which showed a significant regulation in genotype Sumai-3 3BS region. Simultaneously, susceptible genotypes NILS have less of these three genes, and the gene activity indicated the importance of both the 2AL and 3BS regions in the activation of effective defense responses for the infection of F. graminearum. Significant up-regulation of phenylalanine ammonia-lyase was also detected only in the resistant Sumai-3, and all expressed differentially genes were quantitative in nature in both the resistant cultivar (Sumai-3) and susceptible lines (NILS).

The resistant Chinese cultivar Sumai-3 was also used for the construction of another subtracted library by Al Taweel et al. (2014) to clone and identify the differentially expressed genes between FHB-inoculated and water-inoculated wheat spikes. After the EST sequencing based on the BLAST homology search programme (BLASTn and BLASTx), 31 ESTs were obtained in differentially expressed genes and among these, 7 genes were selected based on their putative involvement in pathogen-defence response and/or high signal expression in differential screening (Table 2). Another gene expression was studied by Goswami et al. (2006) for preparing a subtraction library using the susceptible wheat cultivar Norm, inoculated with a highly aggressive F. graminearum strain (NRRL 31084) or mock-inoculated (less aggressive strain NRRL 28303) with water for identification and functional analysis of novel genes potentially involved in the pathogenicity, and also for the identification of fungal genes differentially expressed in planta during interactions between wheat and a highly or less aggressive strain. They found four differentially expressed genes (Abc2, Lyp1, Rrr1 and Zbc1) potentially involved in the pathogenesis and development of this fungus in wheat cultivar Norm (Table 2). Based on BLASTx comparisons, Abc2 and Zbc1 appeared to encode protein and Lyp1, Rrr1 appeared putative regulatory in nature. Different differentially expressed gene details are given in Table 2 with respective wheat cultivars.
Table 2: Differentially expressed genes in wheat cultivars against Fusarium head blight (FHB) disease

<table>
<thead>
<tr>
<th>Cultivars/ Varieties</th>
<th>Name of the disease with pathogen</th>
<th>Identified genes</th>
<th>Post inoculation Time</th>
<th>References</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Using method for library construction</td>
<td>No.</td>
<td>Nature</td>
</tr>
<tr>
<td>Clark and Ning 780</td>
<td>(Forward Library of SSH)</td>
<td>44</td>
<td>Homology</td>
<td>72 hours</td>
</tr>
<tr>
<td></td>
<td>Clark as a tester and Ning 780 as a driver</td>
<td></td>
<td></td>
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<tr>
<td>Robigus and Centenaire</td>
<td>Robigus as a tester and Centenaire as a driver</td>
<td>43</td>
<td>Homology</td>
<td>24, 48, and 72 hours</td>
</tr>
<tr>
<td>Ning 7840</td>
<td>Forward and Reverse subtracted cDNAs</td>
<td>24</td>
<td>8 genes homology</td>
<td>2, 6, 12, 24, 36, 72, and 96 hours</td>
</tr>
<tr>
<td>Sumai-3 and its isogenic lines (NILS)</td>
<td>Fusarium head blight (Fusarium graminearum)</td>
<td>Isogenic line of Sumai as a tester and Sumai as a driver</td>
<td>25</td>
<td>Quantitative in nature with up-regulation</td>
</tr>
<tr>
<td>Sumai-3</td>
<td>Quantitative real time reverse transcriptage PCR</td>
<td>31</td>
<td>Up-regulated putative defense genes</td>
<td>6, 12, 24, 36, 48, 72, and 144 hours</td>
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<tr>
<td>Norm</td>
<td>(Reverse Library of SSH)</td>
<td>4</td>
<td>Putative and protein regulator in nature</td>
<td>48 hours</td>
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<td></td>
<td>NRRL 28303- inoculated wheat heads as a tester and NRRL 31084- inoculated wheat as a driver.</td>
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</table>

Leaf rust of wheat and SSH

*Pucciniatriticina* is the most common and widely distributed pathogen among the three wheat rusts. Globally, leaf rust damages are greater than stem and stripe rust (Goswami et al., 2005; Huerta-Espino et al., 2011). More than 70 leaf rust resistance genes are identified which can be pyramiding to develop a resistant variety of wheat through marker-assisted selection programmes (MAS) (Kolmer, 1996). The use of SSH method for the identification of differentially expressed genes in the case of leaf rust disease is very few. A study was conducted by Venkatappa et al. (2003) to understand the molecular basis of wheat-rust interactions by using SSH to construct a cDNA library. From this study, 26 differentially expressed genes were identified, and non-ribosomal protein genes were induced in rust-infected leaves. Among these genes, 21 of the induced genes were from the rust fungus, indicating that the majority of the induced genes were rust *PIGs* that were likely to play a role in parasitism. Some of the induced genes were homologous to known *PIGs* or virulence genes in other fungi, suggesting similarities in parasitism among different fungi. Eight clones correspond to novel PIGs that have not been reported in any organism. Another finding was observed by Li et al. (2008) using leaf rust resistance wheat cultivar TcLr41 as a driver and a susceptible host Thatcher as a tester to identify the differentially expressed genes by the SSH method. From their investigation, 240 differential expression sequence tags were obtained and 137 qualified ESTs showed high homology (Table 3) with the function known genes or ESTs. After the analyses, these genes with their functions were further classified into glutathione-S-transferase, mitogen-activated protein kinase, W1R1 protein, serine/threonine protein kinase, lipid transfer protein, thaumatin-like protein, GTP-binding protein, chitinase, and calreticulin for the involvement in the process of the incompatible interaction between wheat TcLr41 and the *Pucciniatriticina* race.
Stripe rust of wheat and SSH

Stripe rust is one of the most economically destructive diseases in common wheat (Triticum aestivum L.) worldwide caused by Puccinia striiformis f. sp. tritici (Pst). The identification of stripe-rust resistance genes is comparatively more difficult than other rust pathogens due to the frequent emergence of new virulent races (Dadrezaei et al., 2013). Although more than 40 stripe-rust resistance genes have been identified using different molecular marker breedings, approaches derived from wheat and wheat related species such as Triticum spelta album (Yr5), Secalis cereale (Yr9), Triticum dicoccoides (Yr15, Yr35) (Cao et al., 2008; Peng et al., 2000) Triticum tauschii (Yr28) (Marais et al., 2005) and Aegilops species (Yr37, Yr38 and Yr40) (Peng et al., 2000; Singh et al., 2000; Marais et al., 2006) identification of differentially expressed genes for stripe rust in wheat using SSH technique is very rare. A study was conducted by Zeng et al. (2010) using wheat germplasm NR1121 infected by Chinese Pstrace CYR32 as a tester and susceptible Mingxian169 and as a driver (control) for the identification of differentially expressed genes in response to Pst infection in wheat by the SSH technique (Table 3). A total of 550 positive clones from the library was identified and subjected to sequencing. The 162 ESTs were grouped into different cluster patterns based on BLAST alignment and functional annotation and classified into different categories. Among these, eight genes were detected by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time polymerase chain reaction. The expression of six gene (acycylcoenzyme A synthetase, glutathione S transferase, Lipid transfer protein, ubiquitin protein ligase 2, cytochrome P450, and serine/threonine protein kinase SNT7) transcripts were induced and up-regulated to their highest levels at 24 hours after Pst infection, while those of two genes (serine Hydroxy-methyl transferase and S-adenosylmethionine decarboxylase) were significantly expressed at 48 hours (hpi). These genes were transcriptionally activated for the host defence response and highly induced at an early stage of infection.

Another SSH cDNA library was constructed from Pst infected seedling leaves of resistant wheat cultivar Shaanmai 139 (Zhang et al., 2011). They obtained a total of 84 expressed sequence tags with high quality and alignment of the sequences identified in 56 Unigenes. After the analysis by BLASTx search, 46 Unigenes showed homologous and encoding proteins of known functions such as signal transmission, energy and metabolism, transcription regulation, phenylpropanoid pathway, and defence response (Table 3). The wheat cultivar Suwon11 was inoculated with stripe rust (Puccinia striiformis f. sp. tritici) strains CYR23 and CYR31 for the construction of SSH cDNA library to analyze the expression patterns of wheat HSP70 (Heat Shock Proteins) gene in wheat plants exposed under abiotic stress (Duan et al., 2011). From this study, the differentially expressed gene designated as TaHSC70 was identified during an incompatible and compatible interaction with wheat-stripe rust and was up-regulated by the MeJA treatment. The identified gene TaHSC70 played a role of stress-related responses, predicted to encode a protein of 690 amino acids, and also demonstrated in a homolog of chloroplast HSP70s by the phylogenetic analysis (Table 3).

The Chinese winter wheat cultivar Xingzi 9104 (XZ) (XY is susceptible to CYR32 at the seedling stage, but is resistant at the adult-plant stage) inoculated with high aggressive Pst race CYR32, was used for the construction of SSH cDNA library to identify differentially expressed genes involved in wheat-Pst interactions on adult plants (Huang et al., 2013). From this study, a large number of genes were found involved in defence and signal transduction based on putative functions. Twelve genes specifically expressed in inoculated adult plants of XZ were isolated and identified, and these genes are valuable candidates for functional analyses of stripe rust APR in wheat cultivar XZ.

Wheat common bunt and SSH

A common bunt is caused by Tilletia caries (DC) Tul., T. foetida (Wallr) Liro., and T. controversa, respectively, and can still cause yield and quality losses of wheat (Zeng et al., 2010). Very few molecular markers have been used for the identification of common bunt resistance genes in wheat. More than 15 specific common bunt resistance genes named Bt genes have been identified using different molecular markers which can be used to breed resistant cultivars (Ciucu, 2011). The use of SSH technique for the identification of differentially expressed genes is very rare. A related study was conducted by Zhen-Xiang et al. (2005), using susceptible wheat line Neepaw and resistant line...
BW553 for the identification of differentially expressed genes against wheat common bunt disease (pathogen: *Tilletia tritici*). They identified 193 differentially genes (168 up-graded and 25 downgraded genes) (Table 3) and sequenced them. Among these, more than 70% sequences had significant homology to genes of known functions, and these homology sequences have been categorized into three based on their cellular functions, viz., 59% cellular metabolism and development, 24% in abiotic/biotic stress responses, as well as 3% involved in transcription and signal transduction responses. Furthermore, two putative resistance genes and a transcription factor were identified among the up-regulated sequences.

**Bipolaris sorokiniana** of wheat and SSH

*Bipolaris sorokiniana* is a dominant pathogen causing leaf blight or blotch on wheat and yield losses up to 15% in Bangladesh (Alam et al., 1998), and average yield losses range from 15.5 to 19.6% in the world wheat production (Dubin & Van Ginkel 1991; Chowdhury et al., 2013). To date only eight resistant genes have been identified in wheat against *Bipolaris sorokiniana* pathogen by using SSR and microsatellites marker-assisted breeding. However, only one research was conducted using the SSH technique for the identification of differentially expressed genes in bread wheat. A resistant wheat (*Triticum aestivum*) genotype, BH1146 and one of the F2 lines (line 47) of the cross between BH 1146 and *Bipolaris sorokiniana* susceptible genotype Sonalika were selected for the preparation of cDNA library using the SSH technique (Chowdhury et al., 2013). From this study, 1000 clones were obtained and among these, 480 clones after the colony PCR was subjected to sequencing. The sequences were analysed by the BLAST comparison analysis for homology search against the gene bank non-redundant database. Out of 480 clones, 36% of the clones (172 clones) were high quality clones having > 200 base pair size. They further used 172 clones for sequence analysis and found that all the 172 clones matched with ESTs of different plant species in the NCBI database. High redundancy for putative puroindoline b-protein, putative NADH dehydrogenase and hypothetical protein OSJ 021-069 were observed in the BLAST search. Finally 48 differentially expressed genes were identified (Table 3) and these were further classified into 7 categories based on their putative functions, viz., plant defense related genes, genes which have role in signal transduction, genes associated with transcription and translation, energy related genes, genes involved in metabolism and unknown functional. Furthermore, out of 48 differentially expressed genes, 20 gene sequences matched with *Arabdiopsis* spp, 5 matched with *Brassica* spp. and 3 matched with *Triticum aestivum*. In addition to defense-related genes analysis, out of 48 differentially expressed clones, 12 clones (25%) were classified as plant defense responsive genes based on their putative functions observed in NCBI blast search, while 21% genes were having metabolism related functions, 17% genes were related to transcription/translation and 10% genes had energy related functions and cellular transport each. Only 4% genes were related to signal transduction while 13% were unknown genes. For the clones of SSH, 135 matched with putative puroindoline b proteins from *Triticum aestivum* which are encoded by puroindoline gene (*pin B* gene). This gene contains tryptophan rich hydrophobic domain, which is responsible for grain hardness and also has antimicrobial properties having lipid binding sites.

**Table 3**: Differentially expressed genes in wheat cultivars against rust, common bunt and Bipolaris leaf blight of wheat

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<thead>
<tr>
<th>Cultivars/ Varieties</th>
<th>Name of the disease with pathogen</th>
<th>SSH library</th>
<th>Identified genes</th>
<th>Post inoculation time</th>
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<td>Thatcher and TcLr41</td>
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<td>ESTs 137 High homology in nature</td>
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<td>NR1121 and Mingxian 169</td>
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Advantages of SSH
The molecular mechanisms of host pathogen interaction through SSH is useful. The SSH is used to enrich the rare cDNA transcripts by more than 1000 fold (Honnareddy et al., 2014). The construction of cDNA library by the SSH technique includes more genes related to disease resistance and defense reaction comparing to an ordinary cDNA library by any other molecular approaches (Luo et al., 2002c). The SSH is the powerful method for studying the function of genes involved in a particular disease a well as the development of plants and/or pathogens with the differential expression of tissue-specific proteins etc (Diatchenko et al., 1999). It is also used for identification of differentially expressed genes between hybrids and their parents which are responsible for the heterosis (Muhovski et al., 2012).

Limitation of SSH
The SSH is laborious multi-step technique (Alice et al., 2013). For getting stable hybridization results, experiments must be repeated several times and a large amount of mRNA and radioactive isotope are needed (Yao et al., 2005). It is totally dependent on different enzymes and reagents, so the purity of enzymes and the use of reagent are highly significant for getting the actual findings. The construction of cDNA libraries is one of the key factors of this technique for obtaining the differentially expressed sequences and is an essential need for the identification of genes involved in contrasting situations (Yao et al., 2005). The identification of expressed genes requires two or more than two sample at a time, and hence the handling of these samples are more complicated compared to other methods.

Direction for further research
This review presents a good number of differentially expressed genes identified by wheat researchers using the SSH technique against different fungal diseases of wheat. The updated information will be helpful to further design the experiments to develop durable disease resistant varieties against fungal pathogen through marker-assisted backcrossing (MABC) or gene transformation through genetic engineering. These could be planned against specific pathogen, or combination of pathogens using gene pyramiding.

Conclusion
The use of a resistant cultivar of any crop is the sustainable and environmentally friendly approach to enhance the production of any crop. The SSH is an easy and powerful approach to identify resistant genes of any resistance cultivar. Ultimately, these resistant genes may be used to develop a new resistant variety or cultivar by gene pyramiding using different molecular approaches. This technique
is also helpful to identify pathogen-host interaction mechanism. Through this paper, researchers will be enormously benefited to find out the different resistant wheat cultivars in the world, and in brief they can obtain clear information about the differential expressed genes and their functional activities.

Acknowledgement
The author, Md. Akhtar-Uz-Zaman, would like to express his gratitude to the Bangladesh Agricultural Research Institute for giving him deputation to conduct research with the Institute of Tropical Agriculture, Universiti Putra Malaysia.

References


