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# Research Article
# Changes in Microsatellite Motifs in Response to Abiotic Stresses: a Case Study Using Wheat and Rice RNA-sequencing Data

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## Abstract

**Background and Objective:** Microsatellites or simple sequence repeat (SSR) markers play an important role in plant breeding projects. While a large number of SSRs in plants has been recently identified, only a few SSRs have been randomly validated in the lab. Therefore, to cope with the large numbers of SSRs, it is suggested that a targeted selection scheme may be more efficient in identifying functional biomarkers than the random selection of SSRs. The aim of this study was to develop a new method for identifying functional SSR markers in plant breeding. **Methodology:** For this study, *in silico* analyses of available wheat RNA-seq data under heat SSR stress were conducted and unique SSR patterns were obtained. In addition, alterations of SSRs under other stress conditions were confirmed through RNA-seq data of rice subjected to salt, drought and cold. The Audic and Claverie, R of Stekel and Falciani Fisher and General Chi-squared tests were all applied for comparisons of data. **Results:** The results of the study revealed that GC/GC and GCC/GGC repeats were significantly more common under stress conditions compared with controls for both wheat and rice samples. Interestingly, genes containing these motifs have been found to participate in abiotic stress responses and to include various heat-shock proteins (HSPs) and DREB/CBF (DRE-binding protein/C-repeat binding factor) proteins. **Conclusion:** The overall findings of this study suggest the possibility of using genes with altered SSRs as functional markers. The applied workflow and the results presented here are expected to help establish a new paradigm for future studies involving genetic diversity, breeding, molecular biology and association studies of plants grown under adverse environmental conditions.

Key words: Abiotic stress, genetic diversity, heat shock protein, microsatellites, rice, RNA-seq SSR, wheat

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Plant breeding for tolerance to abiotic stresses can be accelerated and made more efficient using marker assisted selection (MAS) based on simple sequence repeat (SSR) markers. Abiotic stresses such as drought, salinity and high and low temperatures are regarded as the stress agents responsible for considerable decreases in the growth and productivity of economically important crops[1-3]. Thus improved cultivars for these highly diverse agro-ecological environments are important for increasing food production. Acquired plant tolerance to abiotic stresses can be achieved both through genetic engineering strategies and through conventional plant breeding combined with the use of molecular markers in MAS[2,4,5]. Among molecular markers, SSRs are a suitable choice because they are co-dominant, more polymorphic and stable and also much easier to assay compared with other markers[4,6].

SRRs are tandem repeats of one to six nucleotides in nucleic acid sequences that are found on both non-coding and coding regions of genomes. The EST-SSR is a type of SSR marker developed from expressed sequence tag (EST) libraries[7-10]. The polymorphism derived from EST-SSR is associated with the coding regions of the genome. While preparation and sequencing of traditional EST libraries is a difficult and costly process, next generation sequencing (NGS) technologies make possible the efficient identification of a large number of sequences at a fraction of the cost and effort required for traditional approaches. High-throughput RNA sequencing (RNA-seq) is one of these NGS techniques and it is rapidly emerging as a major quantitative transcriptome profiling approach[11].

Despite the discovery of large amounts of SSRs in recent NGS studies, only about 1% of reported SSRs have been tested, nearly half of which were characterized as polymorph markers[10]. The challenge, therefore, is to manage and validate the large number of SSRs in order to obtain the best results at the least expense. In an effort to meet this challenge, liorizzo *et al.*[12] suggested computational detection of polymorphic SSRs prior to actual laboratory verifications. Zalapa *et al.*[10] have also proposed ways to reduce the cost of screening the ever-growing number of SSRs. In recent study, Alisoltani *et al.*[13] used parallel consideration of SSRs and differentially expressed (DE) genes under cold stress to develop informative markers in almond trees. The most recent findings indicate, however, that only a small fraction of SSRs have yet been randomly validated in more than one plant species.

Here it is suggested that, in addition to these approaches, the targeted selection of sequences with different SSR patterns under stress has the potential to yield many benefits that are unavailable using the random application of SSRs. Since SSRs on coding regions can cause changes in gene function and bring about phenotypic variations[8,14], they can be used to identify candidate functional genes and thus to increase the efficiency of MAS. The novel contribution of this study involves the comparative analysis of the SSR motifs among various stress conditions using RNA-seq data. Sequences with altered SSRs can be annotated further and applied to experimental validations. These insights can then be translated into functional markers in breeding programs. Furthermore, the cumulative increase in the RNA-seq datasets of various stresses suggests an opportunity to integrate and utilize these sources for the discovery of more rigorous SSR markers.

In the current study, a workflow was developed for detection and comparison of RNA-seq SSRs under abiotic stresses in order to identify functional and informative markers. Using available RNA-seq data, the differential alteration of SSRs was investigated under various stress conditions. Sequences with altered SSRs were then characterized as stress responsive genes, which may have potential uses in genetic diversity and association studies.

## MATERIALS AND METHODS

**Designed workflow:** In the present study, a simple workflow was developed for RNA-seq SSR comparison under abiotic stresses. The workflow was tested using publicly available RNA-seq data of wheat (*Triticum aestivum* L.) obtained under normal and heat stress conditions. Additional RNA-seq data from rice (*Oryza sativa* cv. Nipponbare) under salinity, drought and cold stresses were also analyzed to confirm the results. The workflow of the study is presented in Fig. 1. It involves primarily the detection of the SSRs followed by differential analysis of SSR motifs under a variety of environmental conditions.

**Data collection:** RNA-seq data of wheat (*Triticum aestivum* L., 454 platform) and rice (*Oryza sativa* cv. Nipponbare, Illumina platform) were obtained from the National Center for Biotechnology Information (NCBI). The details of sequence read archive (SRA) datasets used for SSR analysis are presented in Table 1. These datasets included two libraries of wheat (untreated and heat stress libraries) and twelve libraries of rice (three libraries each for untreated, drought, salinity and cold stress conditions).

Table 1: SRA data used for SSR analysis together with total reads and reads containing SSRs

| Accession | Plant | Description | Total No. Reads | Bases | Total No. reads with SSRs | Sequence length (nt) |
|---|---|---|---|---|---|---|
| SRR534593 | Wheat | Untreated (pollination stage) | 110,233 | 37.3 M[1] | 1581 | 26-536 |
| SRR542343 | | Heat Stress (pollination stage) | 121,684 | 39.6 M | 1877 | 26-536 |
| SRR074152 | Rice | Untreated- Leaves (2 weeks) | 2,813,770 | 73.2 M | 4141 | 26 |
| SRR074153 | | Untreated-Leaves (2 weeks) | 4,100,071 | 106.6 M | 5945 | 26 |
| SRR074154 | | Untreated-Roots (2 weeks) | 5,132,699 | 133.5 M | 6625 | 26 |
| SRR074141 | | Drought Stress- Leaves (2 weeks) | 3,435,848 | 89.3 M | 4831 | 26 |
| SRR074142 | | Drought Stress- Leaves (2 weeks) | 4,231,853 | 110 M | 6163 | 26 |
| SRR074143 | | Drought Stress- Roots (2 weeks) | 5,162,129 | 134.2 M | 6358 | 26 |
| SRR074138 | | Cold Stress- Leaves (2 weeks) | 2,846,609 | 74 M | 4278 | 26 |
| SRR074139 | | Cold Stress- Leaves (2 weeks) | 4,275,874 | 111.2 M | 6044 | 26 |
| SRR074140 | | Cold Stress-Roots (2 weeks) | 4,977,823 | 129.4 M | 6421 | 26 |
| SRR074148 | | Salt Stress- Leaves (2 weeks) | 3,365,676 | 87.5 M | 5055 | 26 |
| SRR074149 | | Salt Stress- Leaves (2 weeks) | 4,563,398 | 118.6 M | 6867 | 26 |
| SRR074150 | | Salt Stress- Roots (2 weeks) | 3,893,781 | 101.2 M | 6405 | 26 |

1: Mega base, 2: Nucleotide

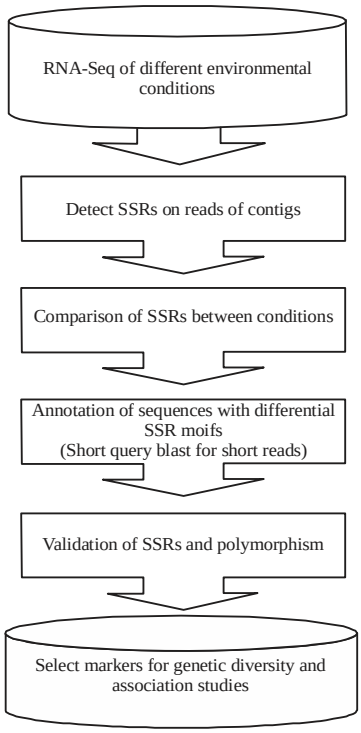


Fig. 1: Workflow of plant RNA-seq SSR analysis

**Simple sequence repeats analysis:** The sequences of each normal and treated sample mentioned above were analyzed for SSR motifs using SSR locator [15]. All reads were searched for SSR motifs ranging from 2-6 nucleotides. Mononucleotide repeats were excluded from the transcript analysis because of the abundance of poly-T repeats, which resulted mainly from poly-A tails. SSR loci with motifs of 2-6 nucleotides that featured a minimum of 5, 5, 4, 4 and 4 repeats for di-, tri-, tetra-, penta and hexa-nucleotides, respectively, were thus considered for this study.

**Comparison of SSR motifs under different stresses:** For each dataset, differential representation of SSR motifs between normal and stressed samples was analyzed based on SSR counts using a web tool, IDEG6[16]. In the case of rice leaf samples, summation of two replications with untreated samples was compared to the integrated results of treated samples.

**Annotation and functional analysis of sequences containing SSRs:** Among altered SSRs, sequences containing GC/GC and GCC/GCC motifs (which were the most significant altered motifs) were extracted from the heat stress library of wheat. These sequences were annotated using BLASTX as described on the AmiGo website[17]. The AgriGo web tool was also used to determine the biological process of the sequences[18].

**Statistical analysis:** The Audic and Claverie test as well as the Fisher exact test were conducted for comparison of two conditions, while R of Stekel and Falciani and General Chi-squared tests were applied for comparisons of three or more conditions. Bonferroni's correction were applied in case of multiple comparisons. The significance level was set at % 0.05 ($p \leq 0.05$) for all statistical tests and the significance threshold at 0.05[16].

## RESULTS

**Differential representation of RNA-Seq SSRs under heat, salinity, drought and cold stresses:** The total number of RNA-seq reads of wheat and rice data is shown in Table 1. A large number of SSRs located on RNA-seq reads was identified in both untreated and heat stress treated libraries of wheat (Table 1). In the case of rice data, four conditions, including untreated, drought, salinity and cold treated leaves and roots were analyzed for SSR motifs.

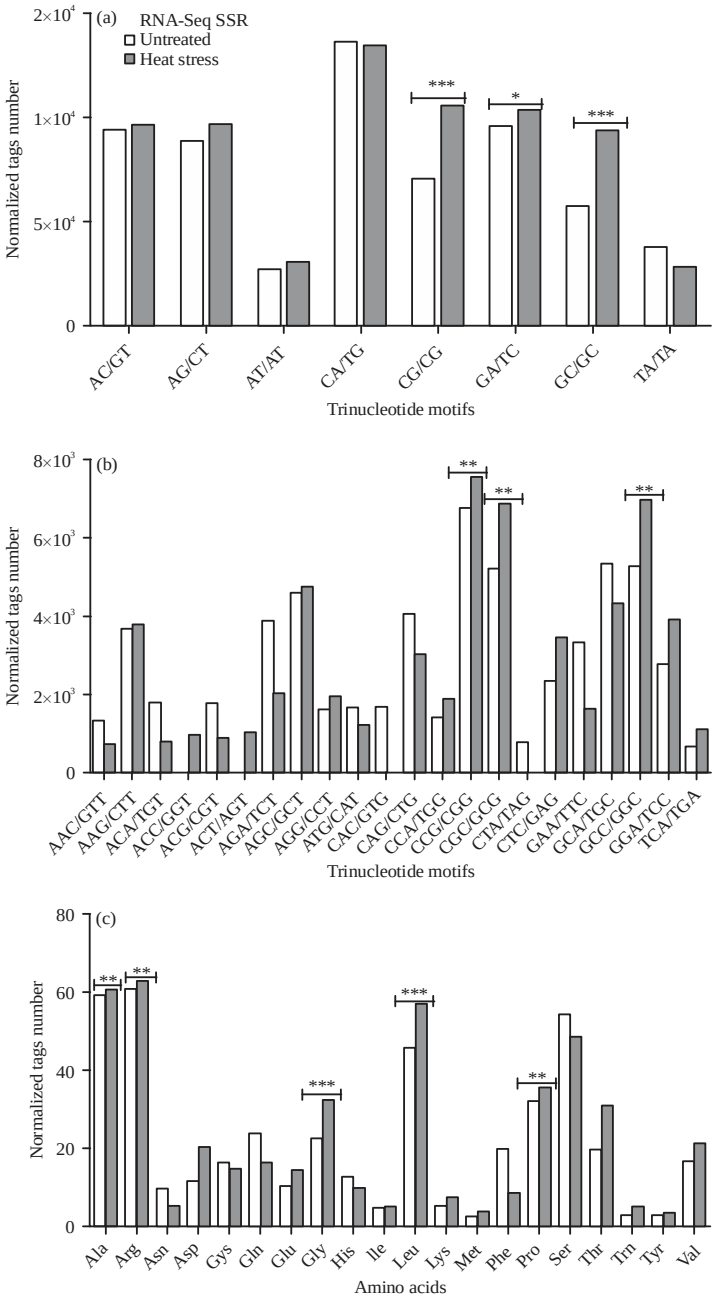


Fig. 2(a-c): Distribution of di, trinucleotides SSR motifs and amino acids contents of RNA-seq data sets of wheat under heat and normal conditions. Panels A and B represent dinucleotides and trinucleotides on sequences of RNA-seq libraries, respectively; "c" shows the types of amino acids encoded by sequences of RNA-seq libraries. Data has been normalized based on Bonferroni Correction and are presented as mean ratio ±SD. All of presented motifs show significant differences ($p<0.05$) between treated and untreated samples. The*, ** and *** symbols indicate significant differences at 0.05, 0.01 and 0.001 levels, respectively

Significant alterations ($p<0.05$) in SSR motifs were observed in both wheat and rice grown under different stress conditions. Owing to the higher frequency of di- and tri-nucleotides (82 and 97% in wheat and rice libraries, respectively) compared with other types of SSRs, the patterns of these two types of motifs were further analyzed in the context of normal and stressed libraries (Fig. 2 and 3).

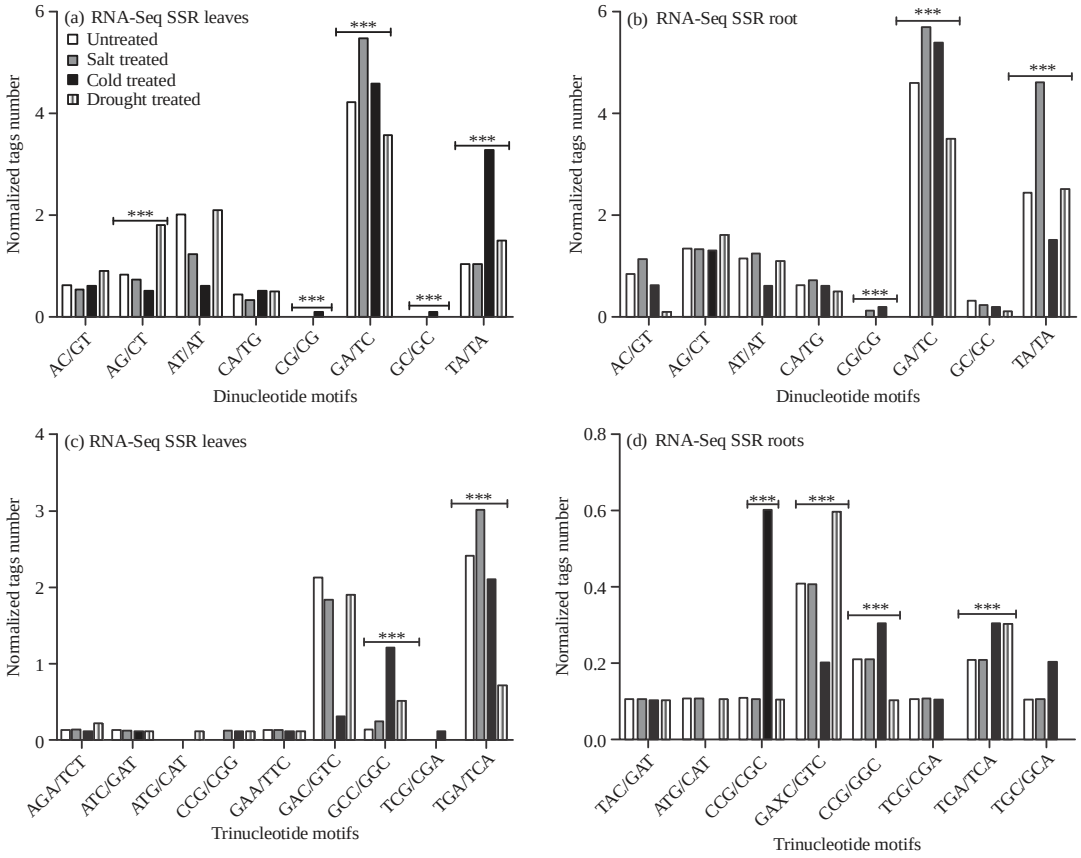


Fig. 3(a-d): Distribution of di and trinucleotides on RNA-seq data of rice under drought, salt, cold and normal conditions. Panels A and B show types of dinucleotides and trinucleotides on sequences of leaf RNA-seq libraries, respectively; C and D represent normalized based on Bonferroni Correction and are presented as mean ratio ±SD. All of presented motifs show significant differences ($p<0.05$) between treated and untreated samples. ***Symbol indicates significant differences at 0.001 levels

Motifs displayed both consistent and mixed altered patterns under different stresses (Fig. 2 and 3). Three dinucleotide motifs, including GC/GC, CG/CG and GA/TC, were identified as being over-represented under stress conditions in both wheat and rice samples (Fig. 2a, 3a and 3b). The TA/TA motif was identified as the most abundant SSR in leaves under cold and drought stresses (Fig. 3a), while in rice this motif was present mainly in salt stressed root tissue (Fig. 3b). In the case of trinucleotide motifs, GCC/GGC and CCG/CGG were observed as motifs with significant alterations ($p<0.05$) under heat stress (Fig. 2b). Interestingly, the GCC/GCC motif was found to increase under stresses in rice also (Fig. 3c and 3d). Another noteworthy alteration in rice was recorded for TGA/TCA and GAC/GTC under drought, salt and cold stresses (Fig. 3c and 3d).

In addition, RNA-seq harboring SSRs caused significant differences ($p<0.05$) in types of dinucleotides and trinucleotides on sequences of root RNA-seq libraries, respectively. Data has been amino acid content under abiotic stresses. Within different amino acids corresponding to triplet motifs, Ala and Arg were significantly over-represented in RNA-seq data of wheat under heat stress, in which they together account for 27% of total amino acids (Fig. 2c). By contrast, Asn, Gln, Phe and Ser decreased significantly under heat shock in wheat (Fig. 2c).

**Sequences with specific altered SSRs contribute to stress response processes:** In an effort to shed light on the role of transcripts that harbor specific altered SSR motifs, sequences containing GC/GC and GCC/GGC were extracted from the heat-treated library of wheat. All of the significant GO terms for sequences containing GC/GC and GCC/GGC are presented in Fig. 4 and 5, respectively. The results show that genes with these motifs participate in various biological processes, such as protein metabolism, response to abiotic stimulus, cell recognition and regulation of metabolic processes (Fig. 4 and 5).

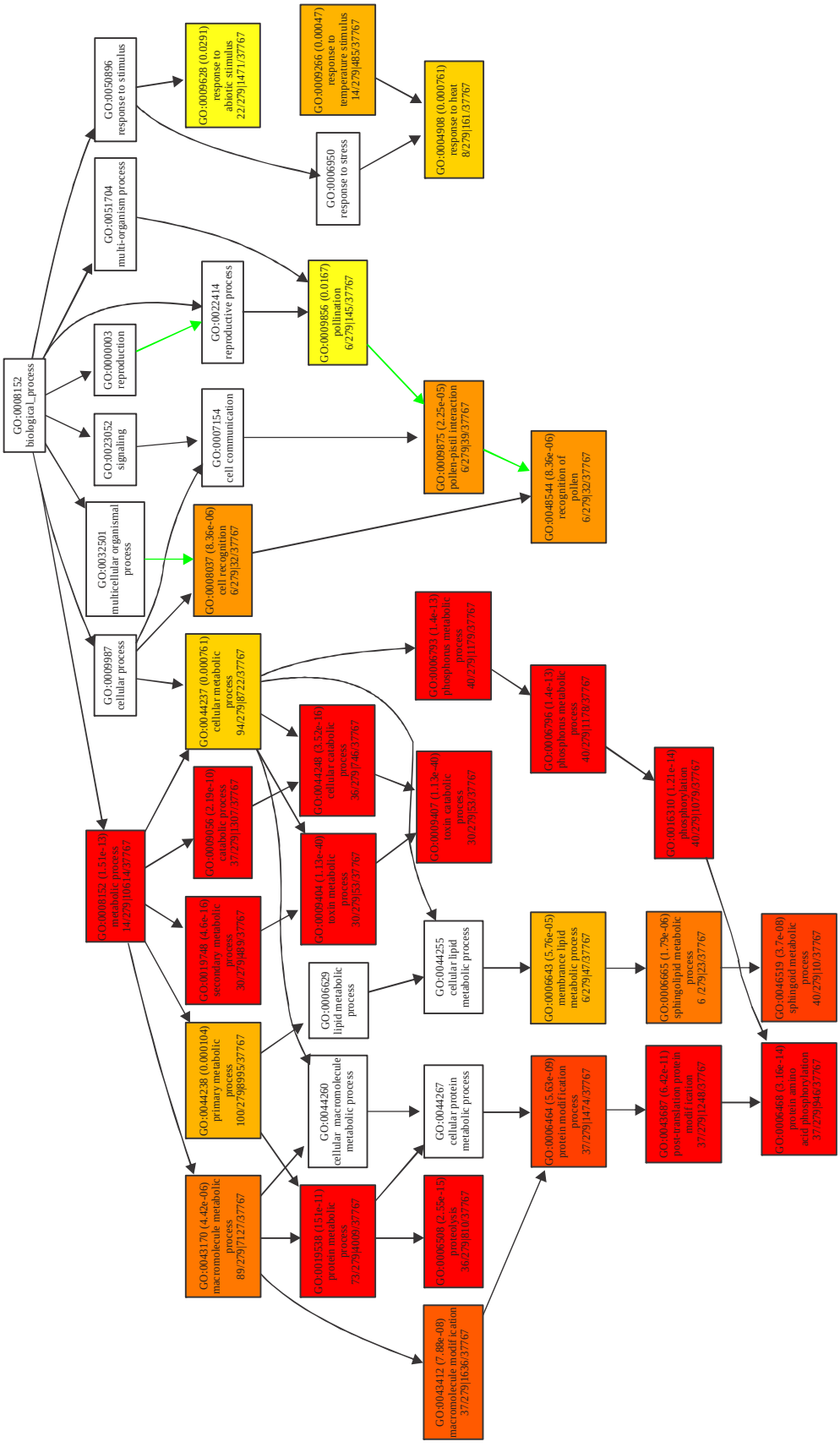


Fig. 4: Biological processes of sequences harboring GC/GC

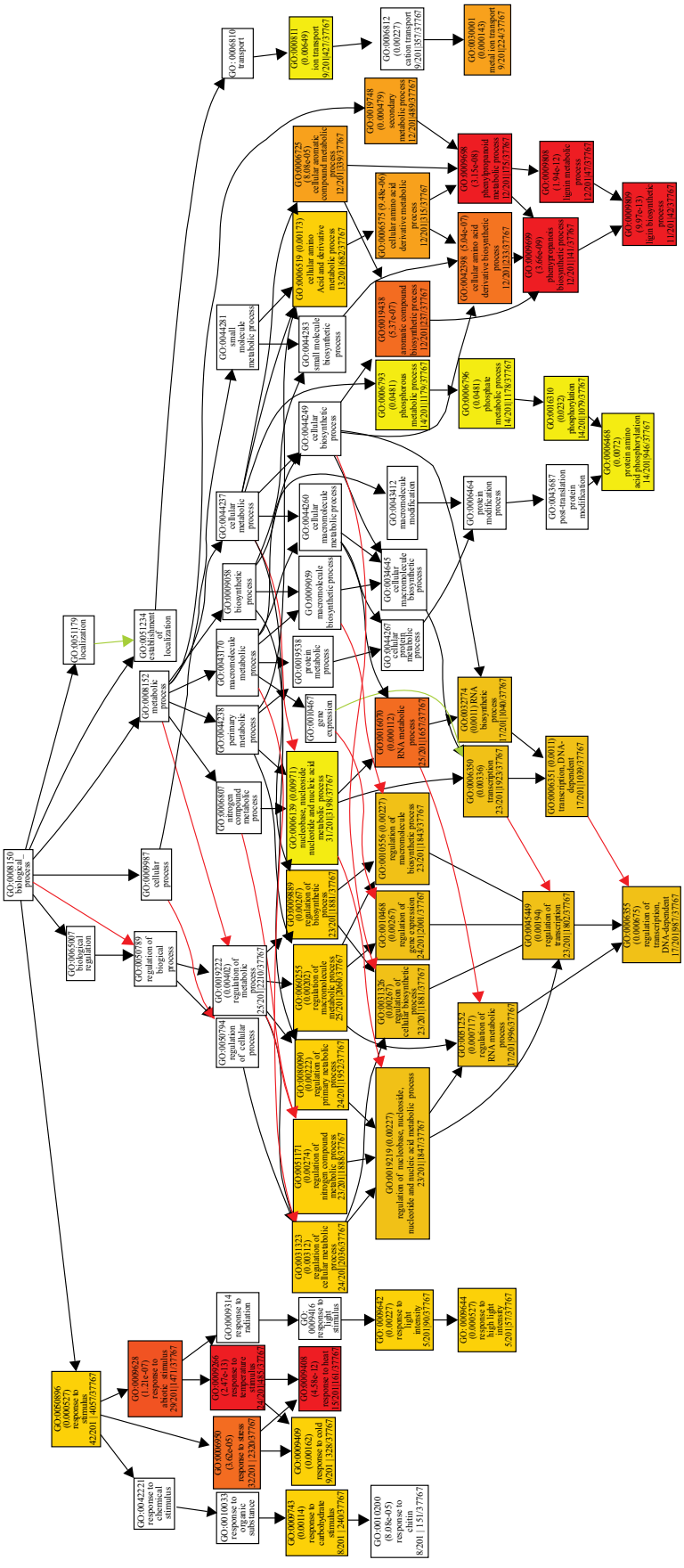


Fig. 5: Biological processes of sequences harboring GCC/GGC

Table 2: Sequences containing GC/GC and GCC/GGC motifs in heat treated library of wheat

| RNA-seq ID | Length of sequence | SSR | Start SSR | End SSR | Annotation* | Biological process |
|---|---|---|---|---|---|---|
| SRR542343.sra.41087 | 481 | (GCC)5 | 277 | 291 | HSP | Response to heat and abiotic stresses |
| SRR542343.sra.12225 | 465 | (GGC)5 | 45 | 59 | GRP2 | Cold acclimation |
| SRR542343.sra.34313 | 460 | (GCC)5 | 16 | 30 | UCC | Heat acclimation |
| SRR542343.sra.46784 | 469 | (GCC)5 | 117 | 131 | HSFA | Response to heat and hypoxia |
| SRR542343.sra.45029 | 507 | (GC)5 | 34 | 43 | DREB/CBF | Response to osmotic stresses |
| SRR542343.sra.63358 | 488 | (GC)6 | 111 | 122 | HSP | Response to heat and abiotic stresses |
| SRR542343.sra.54346 | 242 | (CG)5 | 228 | 237 | PAL | Defense response |

*Gene symbol of top hits

Among the various terms, responses to stresses, in particular heat stress, were over-represented in the ontology analysis of the sequences (Fig. 4 and 5). Some of these detected genes/proteins and their related processes are listed in Table 2, such as HSPs, DREB/CBF, heat stress transcription factor (HSFA) and uclacyanin (UCC) proteins.

## DISCUSSION

In this study, considerable changes in SSRs were recorded in wheat and rice transcription under abiotic stress conditions. The main objective of this report was to develop a simple workflow for more effective and targeted selection of SSRs. It has shown that SSR markers are one of the most informative and versatile biomarkers for numerous research approaches[4,6,8-10,19]. RNA-seq is a newly-developed means to profile mRNAs using deep-sequencing technologies[11] that has been widely been applied to the discovery of SSR markers and to genetic diversity assessments[19-25]. The main challenge faced in these studies was the selection and validation of suitable SSRs among a huge number of detected SSRs.

The recent growth in the number of RNA-seq studies provides a source of data for the development of functional SSR markers. Analysis of the wheat and rice RNA-seq data showed significant alterations ($p<0.05$) of SSRs in responses to heat, drought, salt and cold stresses. Among the various motifs analyzed, GC/CG and GCC/GGC accumulated in stress treated samples but not in untreated ones. Changes in the expression of genes containing SSRs might explain the observed alterations in them under stress conditions in the current study. The SSRs also may undergo quantitative and qualitative variation as a consequence of mutations that add or subtract repeat units[26]. In other words, the in uence of SSRs on gene regulation, transcription and protein function might be mediated through changes in the number of repeats or the types of motifs[26]. Whatever the mechanism, the sequences or genes with specific altered SSRs could confer stress tolerance and therefore have considerable potential for use in marker-assisted breeding programs.

Surprisingly, many of the annotated sequences in this study (those containing GC/GC and GCC/GGC) were related to a variety of processes, in particular abiotic stress responses. SSR repeats were found to be specifically enriched in regulatory genes that encode for transcription factors, DNA-RNA binding proteins and chromatin modifiers[27,28]. In the case of coding regions, Kim *et al.*[29] recently demonstrated the over-representation of microsatellite instability in euchromatic and intronic regions compared with heterochromatic and intergenic regions. Within the detected genes harboring altered SSRs in this study, HSPs and DREB/CBF are known to be induced in response to osmotic stresses and these genes have also been reported to play important roles in improving stress tolerance in plants[30,31]. It is also noteworthy that HSPs have been used in genetic diversity analyses of some organisms [32,33].

## CONCLUSION

In this study, SSR motifs demonstrated significant alterations under stress conditions compared with normal conditions. In particular, GC/GC and GCC/GGC were over-represented under abiotic stresses in both wheat and rice. Interestingly, sequences harboring these altered SSRs play important roles, whether directly or indirectly, in responses to heat and other abiotic stresses. Thus at least some of these markers may prove to be informative for use in MAS projects. In general, SSR analysis under various stress conditions can be used, in conjunction with ontology analysis of sequences with specific altered SSRs, to assess associations between microsatellites and the biological roles of known genes.

## SIGNIFICANCE STATEMENTS

This study discovers differential SSRs between normal and stress treated tissues of wheat and rice based on RNA-seq SSR analysis. Two SSR motifs GC/GC and GCC/GGC were significantly higher under stress conditions compared to controls for both wheat and rice samples. Interestingly, genes containing these motifs have been found to participate in

abiotic stress responses and to include various heat-shock proteins (HSPs) and DREB/CBF (DRE-binding protein/C-repeat binding factor) proteins. The major highlight of this study is obtaining functional biomarkers and introduction of a novel idea for SSR analysis and SSR marker discovery. Using RNA-seq SSR, biomarkers with potential use in genetic diversity and association studies may be discovered. The applied workflow in this study would create a new paradigm for future biomarker development in plants for breeding purposes.

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