Understanding the drought mechanism and designing strategies to mitigate drought stress in chickpea (*Cicer arietinum* L.)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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December, 2020

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Sapna Badhan

01 December 2020

Acknowledgements

The current year has been both challenging and equally rewarding having taught me mental resilience within my quest. My quest has led to the journey of being able to present my thesis exploring the drought mechanism in chickpea allowing further development into the drought resilient chickpea in future years. It is both a delight and proud moment that I take this opportunity to thank those who have guided me throughout my journey.

It's with a great pleasure to pay gratitude to my supervisor **Associate Professor Nitin Mantri** for his supervision throughout my candidature. You always supported me and welcomed me whenever I approached you abating any doubt or request for material for my work. Professor Nitin, your encouragement and expertise encouraged me to soar to new heights with both my research and investigations. This journey would not have been complete without your insight for this study.

I would also like to thank **Professor Andrew Stephan Ball** for all his direction and optimistic support throughout my candidature. Furthermore, the positivity exuded when reviewing my progress and results encouraged me to continue within my darkest hours. A big thanks to Dr Pravas Kole and Dr Vijay Bhaskarla for assisting with sequencing experiments. Thank you to all the members of the Plant Biotechnology lab for their support and friendship during this PhD.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship. Huge thanks to RMIT University and administrative staff for their help. I would also like to say thanks to all technical staff for providing research support especially Michael Senior and Kavitha Somasundaram for their help with the plants.

A special thanks to my parents **Mr Karam Chand Badhan** and **Mrs Kaushalya Badhan** for their love and support. Your hard work has always given me the strength and courage to take the road less travelled upon. Thank you to my siblings Hemlata, Sangita and Lokesh for their unconditional love and care. I am grateful to my In-laws for their support.

Last but most significantly, a huge thanks to my husband **Bhuwan Panwar,** my pillar of strength for his affection and encouragement throughout this period. Special thanks to my gorgeous daughter **Kyra** for all the cuddles and smiles, which inspired me all the times. Love you all more than I can express.

Dedication

I dedicate this thesis

to

my parents,

husband

and daughter

who supported me during this period and encouraged me throughout my PhD journey.

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List of Abbreviations

4CL	4-coumarate ligase
ABC	ABC transporter family
Cas9	CRISPR associated protein 9
CDPK	Calcium-dependent protein kinase
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DMCs	Differentially methylated cytosines
DMRs	Differentially methylated regions
DNMTs	DNA methyltransferases
DSB	Double-strand break
FB	Flowering bud
FOF	Fully opened flower
GH	Glycosyl hydrolase
GMO	Genetically modified organisms
GO	Gene ontologies
gRNA	guide RNA
GS	Genomic selection
HDR	Homology-directed repair

ICE	Inference of CRISPR Edits
LOX	Lipoxygenase
MSAP	Methylation-sensitive amplified polymorphism
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
PAM	Protospacer adjacent motif
PEG	Polyethylene glycol
PEPC	Phosphoenolpyruvate carboxylase
POF	Partially open flower
QTL	Quantitative trait locus
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RRBS	Reduced representation bisulphite sequencing
RT-PCR	Reverse transcriptase polymerase chain reaction
RVE7	Reveille 7
SAM	Shoot apical meristem
sgRNA	Synthetic guide RNA
SNPs	Single nucleotide polymorphism
SSH	Subtractive suppressive hybridization

SuperSAGE	Super Serial Analysis of Gene Expression
TALEN	Transcription activator-like effector nuclease
TFs	Transcription factors
trcrRNA	tracer RNA
YP	Young pod

Abstract

Chickpea (*Cicer arietinum* L.) is a valued pulse crop that is consumed globally and is becoming increasingly important as its agricultural productivity is predicted to drastically affected by climate change in coming years. Chickpea is also important as a nitrogen-fixing leguminous crop cultivated in rotation with cereal crops such as wheat and barley. This rotation strategy replenishes soil nitrogen whilst providing a break from pests and diseases. Abiotic stresses such as drought, salinity and heat are key factors limiting chickpea productivity worldwide. Drought is the major cause of the global decline in chickpea production. In plants, extreme drought conditions adversely affect growth, physiology and reproduction leading to reduced yield. To date, research has provided only limited insight into different genetic pathways related to drought tolerance/response. The aims of this thesis were first, to conduct RNA sequencing of leaf tissues from drought-tolerant and sensitive chickpea genotypes and identify candidate genes and pathways associated with drought tolerance/sensitivity. Secondly, to identify DNA methylation patterns that potentially regulate drought tolerance/sensitivity of selected chickpea genotypes. Thirdly, to perform gene knockout of selected drought tolerance associated genes using modern technique CRISPR/Cas9 DNA free editing to understand their role in drought tolerance. The first chapter of the thesis comprises the literature review discussing the challenges in chickpea production and examines the different mechanisms currently known to confer drought tolerance. The experimental chapter 2 describes the identification of drought tolerance associated genes by RNA sequencing of the leaf tissue from two contrasting chickpea genotypes under drought stress. In Chapter 2, to identify the genes/pathways that are differentially expressed under drought stress in the leaves of drought-tolerant and sensitive genotypes, leaf tissue from the shoot apical meristem developmental stage of drought-tolerant (ICC8261) and drought-sensitive (ICC283) genotypes were analysed using RNA sequencing.

It was found that in the tolerant genotype, genes associated with ethylene response, MYB-related protein, xyloglucan endotransglycosylase, alkane hydroxylase MAH-like, BON-1-related, peroxidase 3-related, a cysteine-rich and transmembrane domain, vignain and mitochondrial uncoupling were explicitly upregulated, while in the sensitive genotype, the same genes were observed to be downregulated. The results confirm that the sensitive genotype was more affected by the drought stress compared to the tolerant genotype.

The crosstalk between the different hormones and transcriptional factors involved in the resistance and vulnerability to drought in both genotypes makes them perfect candidates for future studies. As a genetic donor, the tolerant genotype ICC8261 can be used to further enhance the drought-tolerant characteristics of the chickpea germplasm. This study will help to understand the putative role of candidate genes in response to drought stress in chickpea. Future research can use more systematic experimental approaches to define the individual role of these identified genes, their roles, pathways involved and application in a field study for promising drought-resilient chickpea crops.

The work conducted in Chapter 3 aimed to study the correlation between DNA methylation and gene expression under drought stress in the consensus genes identified in Chapter 2. The purpose of this study was to obtain a clear picture of the drought mechanism involved in two contrasting genotypes of chickpeas using both, RNA sequencing and RRBS (Reduced representation bisulphite sequencing). To conduct RRBS under drought stress, leaf tissues from the shoot apical meristem of the drought-sensitive and drought-tolerant genotypes plants employed in Chapter 2 were used again. Compared to the tolerant genotype, the findings showed a larger number of differentially methylated regions (DMRs) and bases in the sensitive genotype. It was reported that in the sensitive genotype, hypermethylation in the upstream/promoter region can be associated with the downregulation of gene expression. In comparison, in the tolerant genotype, hypomethylation was interrelated to the upregulation of

gene expression in the upstream/promoter region. In both genotypes, gene ontology (GO) was enriched with GO terms related to cellular metabolic processes, biosynthetic processes, oxidation-reduction processes, gene expression control, transcription regulation, DNA prototype, oxidation-reduction processes, signal transduction, lipid metabolic processes and intracellular signal transduction. This is the first study to examine DEG-associated differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in these two significant chickpea genotypes. An indication of the association of DNA methylation with gene expression under drought stress is the differential methylated regions and genes associated with the DMRs in contrasting genotypes. For both genotypes, the results of the study produced insightful datasets that will contribute to future studies aimed at unmasking the function of these strong epigenetic regulatory mechanisms involved in drought tolerance and sensitivity.

The final aim of the study was to use CRISPR/Cas9 DNA-free editing in chickpea protoplast for knockout of the *4CL* and *RVE7* genes. This is the first research using CRISPR/Cas9 DNA-free gene editing of candidate genes linked to drought in chickpea protoplast. In several plant-based applications, the genome editing method CRISPR/Cas9 has already proven its high proficiency, adaptability and simplicity. This, along with many of the available genome and transcriptome databases, represents a great opportunity for plant breeding and research in coming years. It is possible to facilitate the delivery of pre-assembled ribonucleoprotein complexes (RNPs) consisting of the Cas9 enzyme and synthetically engineered sgRNA using conventional transformation methods or using modern nanoparticles to attain targeted mutagenesis in plants. There is minimal research focused on CRISPR/Cas9 gene editing in legumes; to date, no studies have employed in chickpea. The two genes selected for CRISPR editing were related to drought tolerance. *4CL* (4-coumarate ligase) is a central enzyme in the lignin biosynthesis pathway for phenylpropanoid metabolism. This is known to

manage the aggregation of lignin in other plants under stress conditions. *RVE7* is a transcription factor for MYB that is part of the circadian regulatory rhythm in plants. A novel approach to achieving selective mutagenesis in chickpeas is the knockout of these selected genes in the chickpea protoplast using DNA free CRISPR/Cas9 editing. The results showed high efficiency of editing in the *RVE7* gene *in vivo*. For the *4CL* gene, the *in vitro* digestion worked well compared to the *in vivo* knockout. The results obtained in this study show the potential for using CRISPR/Cas9 in chickpea, leading to improved stress tolerance and enhanced productivity. Further validation of the role of these genes in drought stress is proposed. The study provides a comprehensive dataset as well as CRISPR/Cas9 editing method which can be exploited to improve chickpea productivity through a targeted breeding program.

Chapter 1

Literature Review

Literature Review

This literature review is focused on studies conducted on the genetic and epigenetic response of chickpea under drought stress, addressing recent advances in chickpea genomics research and the future of DNA-free edited chickpea varieties using CRISPR/Cas9 gene editing. The initial part highlights the importance of chickpea in comparison with other crops, assessing the demand for chickpea and the distribution of chickpea production globally. The following section addresses the effect of drought stress on chickpea production and assesses knowledge of the regulation of different mechanisms activated by the stress. Genomic approaches for understanding the tolerance mechanisms are also presented. Additionally, the role of epigenetic response such as DNA methylation to drought stress is discussed. Finally, the role of modern CRISPR/Cas9 DNA gene editing is discussed along with approaches that can be used to edit the chickpea genome that will lead to enhanced food security in the future. Evaluation of the recent DNA-free genome editing studies conducted using CRISPR/Cas9 is also explored in the section along with challenges and upcoming opportunities for the use of the CRISPR/Cas9 system in crop breeding for drought stress. The main aim of this review is to review information regarding the scope of current genomics techniques related to chickpea growth and production. The information presented here will assist in the development of strategies for the development of drought-resilient chickpea varieties. Conclusions and limitations of previous studies directed the foundation of this thesis and research conducted in all three experimental chapters.

1.1 Introduction

1.1.1 Chickpea and its importance

Chickpea (*Cicer arietinum* L.) is a pulse crop cultivated around the world and is ideally suited to cold, dry climates. It belongs to the Fabaceae family, and in some parts of the world, it is a well-known winter legume crop. It is a diploid with 2n=2x=16 chromosomes

(Ladizinskyand Adler, 1976) and a genome size of ~738 Mbp and 28,269 genes (Arumuganathan and Earle, 1991; Varshney et al., 2013a). It is a self-pollinating ancient crop that originated from south-eastern Turkey and adjacent Syria (Singh, 1997). Chickpea is also known as Garbanzo bean, Bengal gram, Egyptian pea, Chana or Hamaz, nohud, lablabi, shimbra, katjang, Cece in diverse areas of the world. The production of chickpea ranks third after beans with an average annual output of over 11.5 million tonnes, with most of the production India focused. In recent years, the area dedicated to chickpea has grown; it is currently estimated to be 14.56 million hectares (Merga and Haji, 2019). In the semi-arid regions of South Asia and sub-Saharan Africa, the cultivation of chickpea is performed using residual soil moisture. India is the prime producer of chickpea, contributing 60% of global production with 7,818,984 tonnes per year; Australia is the second-largest chickpea producer with an annual production of 874,593 tonnes (https://www.atlasbig.com/en-au/countries-by-chickpea-production, Accessed 31-03-2020). Other chickpea producing countries include Canada, Turkey, Mexico, Pakistan and the United States (Merga and Haji, 2019). Figure 1 presents the regional production share of chickpeas worldwide from 1994 to 2018 with 84.9% in Asia, 4.7 % for Africa, followed by 4.4% for America and Oceania and lastly, 1.5 % for Europe.

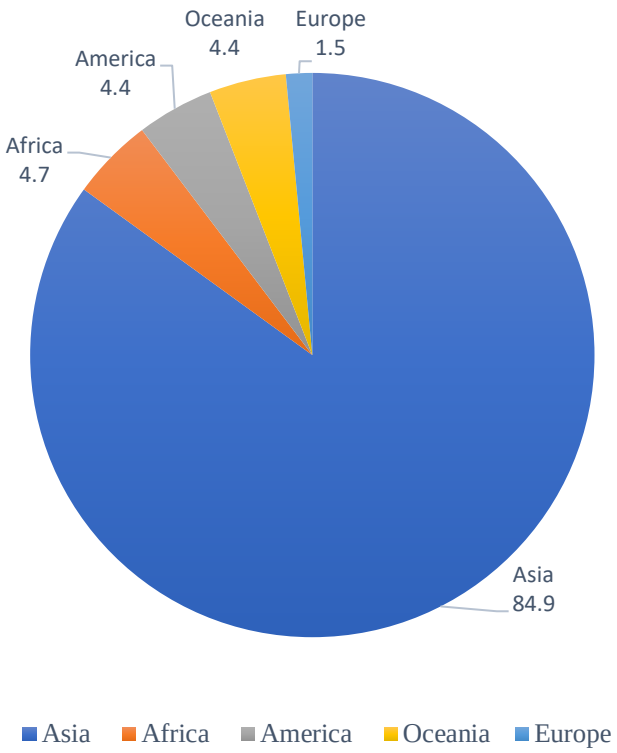

Figure 1: The regional production of chickpeas worldwide from 1994 to 2018 (FAOSTAT, http://www.fao.org/faostat/en/#search/Chick%20peas).

The two main types of chickpeas: the *Desi* chickpea is brown, with small angular seeds. The *Desi* chickpea is mainly purchased by India and Bangladesh. The *Kabuli* chickpea is white to cream in colour with large seeds; buyers for this type of chickpea include nations from the Mediterranean region and Central Asia (Varshney et al., 2013a).

The importance of chickpea being a leguminous crop is in crop rotation because as it assists in enhancing soil fertility by nitrogen fixation (Idris et al., 1989). For wheat and barley in crop rotation, chickpea provides disease break and weed control (Gunes et al., 2007). Nutritionally, chickpeas are high in proteins, amino acids and dietary fibre as well as additional nutrients (Iqbal et al., 2006; Jukanti et al., 2012) (Table 1). Chickpeas are well known as an excellent source of both carbohydrates and proteins, which together make up about 80 percent of the total weight of dry seeds. When compared to wheat and rice, the protein percentage of chickpea is around 20-25%.

Table 1: Shows the nutritional value of chickpea in a serving size in 100 g compared to other legumes. (Source: Adapted from Pulse Australia, http://www.pulseaus.com.au/using-pulses/health-benefits).

Nutrition per 100 g raw							
Nutrition	**Chickpea**	**Faba Bean**	**Green Lentil**	**Red Lentil**	**Field Pea**	**Lupin**	**Mungbean**
Energy	968 kJ	1680 kJ	1550 kJ	886 kJ	1840 kJ	1840 kJ	1800 kJ
Protein	13 g	25 g	14 g	27 g	18 g	32 g	26 g
Fat	3.8 g	1.3 g	0.4 g	2.5 g	0.8 g	5 g	2 g
Carbohydrate	41 g	57 g	44 g	58 g	40 g	NA	72 g
Fibre	17 g	8 g	8 g	10 g	19 g	15 g	12 g

Over recent decades, food security has become an increasingly important subject, given the forecasted population increase. About 7.8 billion people are currently residing on our planet, and it is predicted that by 2050, it will rise to over 9 billion growing by nearly 50 per cent. In developing countries, there has been a focus on the improvement in the production of highly nutritive food crops, which can combat food security and malnutrition (Malunga et al., 2014). Increasingly there is a need to grow more food for more individuals with fewer resources and crops like chickpea provides a lead in achieving this farming race (Merga and Haji, 2019). Chickpea plays a leading role in world food security by resolving the protein shortage in the daily food ration of the Indian and African Sub-Saharan populations. However, there are significant constraints to the production of chickpea worldwide despite the fact cultivars

adapted to a wide range of latitudes and with the disease, tolerance continues to be established in the breeding programmes (Siddique et al., 2000).

1.1.2 Drought stress and its implications

There are diverse types of abiotic stresses experienced by plants, commonly faced are drought, cold, heat and salinity which effect chickpea growth worldwide. However, between all these stresses, drought represents the chief constraint in chickpea growth and can result in up to 50% production losses worldwide (Sabaghpour et al., 2006). More than 90% of chickpea growing areas in Australia are arable land in the northern grain region that consists of some parts of central Queensland and southern Queensland and also covers the northern part of New South Wales. However, it is becoming more popular in southern Australia, particularly in Victorian and Western Australia regions (Pulse Australia, 2013) (http://www.pulseaus.com.au/growing-pulses/bmp/chickpea, Accessed 31-03-20) because they are well adapted to the shorter seasoned and medium rainfall environments. Also, in South Australia recent reports shows that chickpea is grown more as it shows good adaptation to soil type, weed issues and season length limitations compared to other legumes (https://pir.sa.gov.au/search?query=chickpea&collection=PIRSA-web, Accessed 10-02-21).

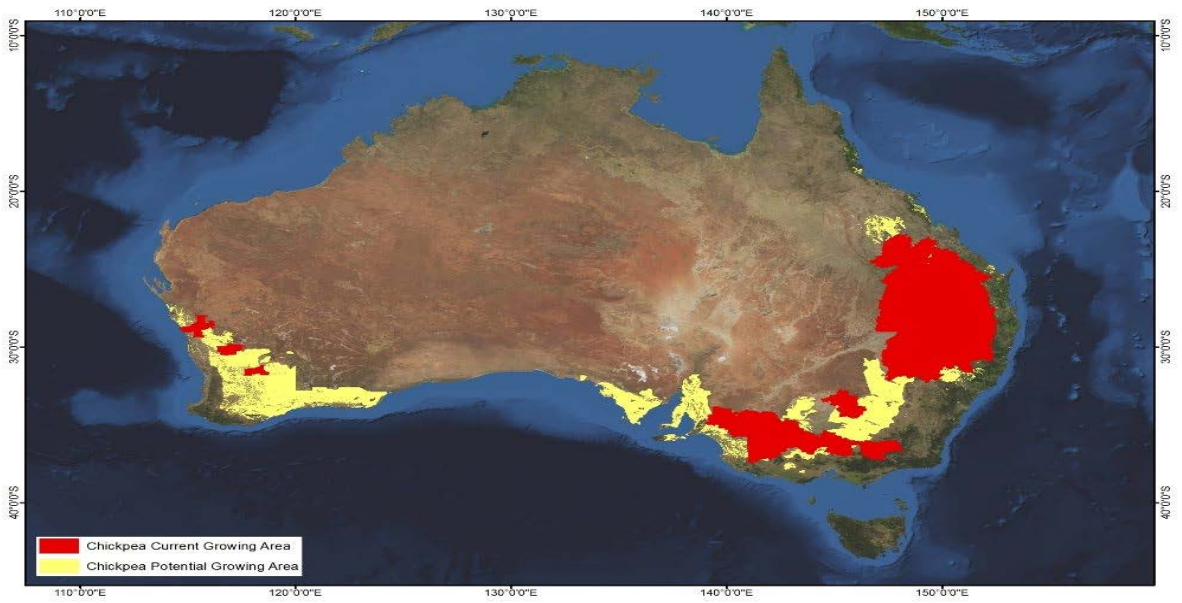

Figure 2: Current and potential growing regions for chickpea in Australia. Areas highlighted in red are current growing areas and yellow are potential growth areas. (Source: https://www.agrifutures.com.au/farm-diversity/chickpea/ Accessed 31-03-20).

1.1.3 The effects of drought stress on chickpea

In rainfed areas, the production of chickpea as affected by terminal drought stress above all other abiotic stresses. Drought stress disturbs many physiological and biochemical processes in chickpea, ranging from photosynthesis, accessibility of CO_2, cell growth, respiration, stomatal conductance and other cell metabolic processes (Rahbarian et al., 2011). The reduction in photosynthesis and stomatal conductance that occur during drought stress leads to the biosynthesis of abscisic acid (ABA). Pang et al. showed that water deficit increased ABA during chickpea podding, which can impair pod setting, cause pod abscission, and eventually major yield losses (Pang et al., 2017). Subsequently, the plant faces oxidative stress, caused by reductive oxidative species (ROS): H_2O_2, O_2^-, O^-, and HO^- (Farooq et al., 2017). As a result, the plant defence system in the form of antioxidants and scavengers is activated (Waqas et al., 2019).

In one study on the role of osmotic adjustment (OA) in drought tolerance of chickpea, eight cultivars differing in OA capacity were investigated. The results indicated that water

potential, osmotic potential, and relative water content of the crop decreased gradually with soil moisture stress (Moinuddin and Khanna-Chopra, 2004b). However, Turner et al. suggested that OA varies in chickpea and phenotypic expression of OA is not stable within varieties, varying from year to year in each genotype. This may be a consequence of variation in rates of accumulation and consumption of solutes rather than an intentional accumulation of solutes in response to water stress (Turner et al., 2006). Another study showed that drought imposition affected the vegetative growth of pea plants and it was noted that yield was affected more by limiting a significant pod number (Mafakheri et al., 2010). Additionally, a significant decrease in chlorophyll *a*, chlorophyll *b* and total chlorophyll content was noted in the vegetative stage, with higher proline accumulation in the drought tolerant variety compared to the drought sensitive plant (Mafakheri et al., 2010). Drought-tolerant chickpea cultivars have been reported to have greater relative water content, ascorbic acid and proline concentrations, but compared to drought-sensitive chickpea genotype cultivars, a lower rate of water loss and membrane permeability was reported (Gunes et al., 2008). Rahbarian et al. reported that the water use efficiency and CO_2 assimilation rate were higher in the drought tolerant genotype compared with the sensitive genotypes in all stages of growth which were investigated under drought stress (Rahbarian et al., 2011). The results from another study by Gunes et al. suggests that the mineral nutrient uptake in chickpea cultivars grown under stress from drought may contribute to drought tolerance as contrasting results were obtained in drought tolerant and susceptible cultivars (Gunes et al., 2006). In 2013, Pouresmael et al. demonstrated in chickpea that under drought stress, yield and stomatal conductance showed maximum reduction in contrasting chickpea genotypes. Furthermore, in the susceptible genotype, ILC3279, the rate of reduction reached 95% for yield components and stomatal conductance. The study suggests that relative water content, photochemical efficiency of photosystem II, and stomatal conductance are among the major physiological traits which contributes towards drought tolerance (Pouresmael

et al., 2013). Another analysis by Mansourifar et al. showed that the effects of drought stress on yield and its components, the impact of cultivars on grain yield and protein yield under drought stress is relatively significant. They noted that with amplified level of drought stress yield and protein content decreased under drought conditions (Mansourifar et al., 2011). A summary of the effects of drought stress on chickpea plants is shown in Figure 3.

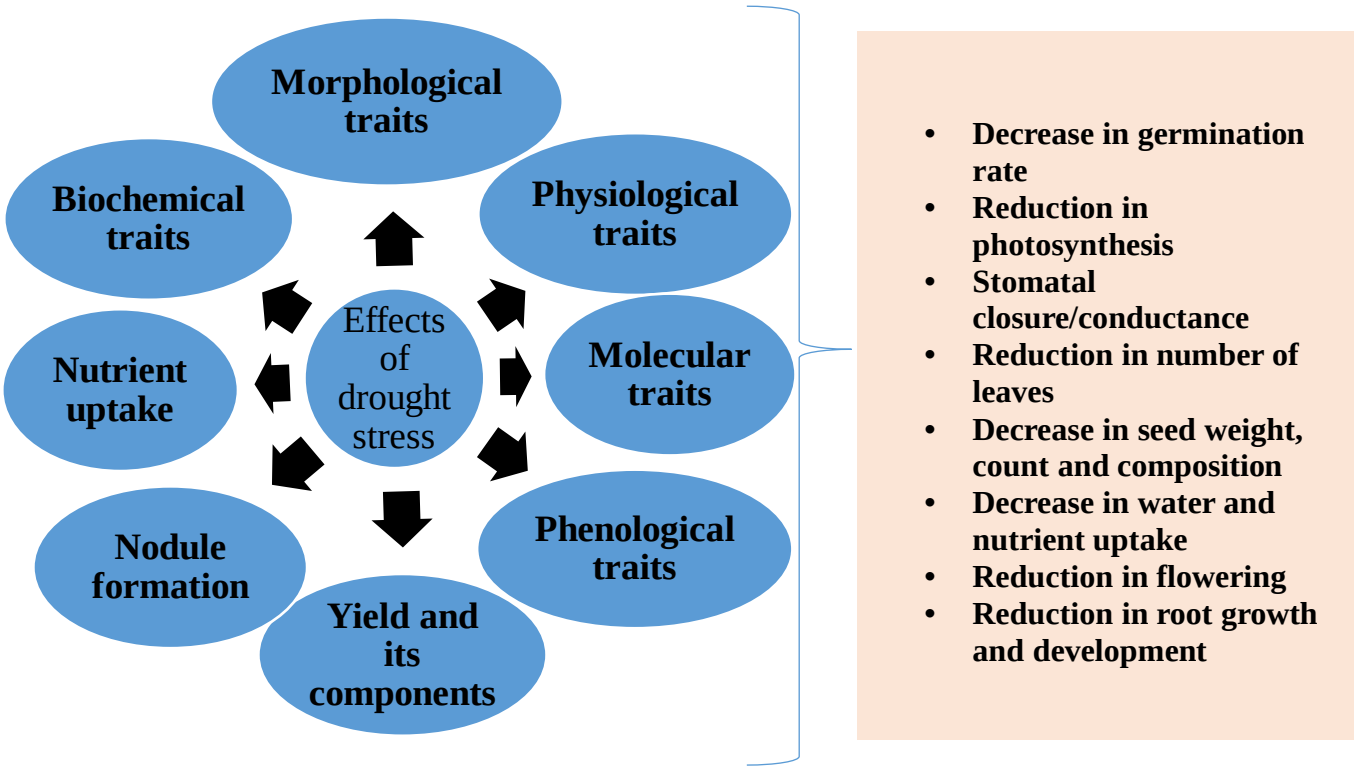

Figure 3: A summary of the overall effects of drought stress on chickpea. Adapted from: (Maqbool et al., 2017; Nadeem et al., 2019).

1.1.4 Strategies to cope with drought stress

Plants respond in distinct ways to retort and adapt to stress conditions by initiating various biochemical, morphological and physiological responses including drought avoidance, drought escape, drought tolerance and recovery (Farooq et al., 2017; Farooq et al., 2009).

Figure 4 shows the impact of drought stress on plants and potential responses. A brief of all these mechanisms employed by plants is discussed below.

1.1.4.1 Drought avoidance

Plants can avoid the drought stress by initiating water control resulting in a reduction in water loss from plants, due to stomatal closure during transpiration. They can also maintain uptake of water through a more extensive, deeper root system, as observed in most drought-tolerant plants (Kavar et al., 2008). Variations in root characteristics, such as biomass, density, depth and length, are the essential characteristics of drought prevention that lead to crop survival under drought conditions (Turner et al., 2006). The development of deeper root systems is regarded as an important trait for drought avoidance enabling increased water uptake by extracting water from substantial soil depths (Kashiwagi et al., 2006). In chickpeas, both root density and maximum root depth were correlated with the positive effect on the seed yield under terminal drought (Gaur et al., 2008). The two accessions ICC4958 and ICC8261 are well known for their root systems. In a study of the different mini germplasm collection at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), the greatest genetic difference in terms of root length density (RLD) (heritability, h2= 0.51 and 0.54) was observed 35 days after sowing, accompanied by a ratio of plant dry weight to root length density of h2 of 0.37 and 0.47 for the first and second seasons, respectively. An outstanding mini germplasm ICC8261 was identified for having a deeper root system under drought conditions (Kashiwagi et al., 2005). However, accession ICC4958, previously characterised as exhibiting chickpea drought avoidance, has been confirmed as having one of the most prolific and deep root systems, although many higher accessions have also been recognized (Saxena et al., 1993; Serraj et al., 2004).

1.1.4.2 Drought escape

Escape from drought stress is a primary adaptation which is achieved by shortening the life cycle or growth season by rapid growth, which allows plants to reproduce before they get exposed to dry conditions and complete their life cycle before drought onset (Nadeem et al., 2019). A field study of six legumes suggested that legumes can escape drought stress by reducing their growth cycle to avoid stress by retaining high tissue water potential, reducing water loss and/or improving water absorption by leaves and roots (Siddique et al., 1993). Interestingly, to complete the reproduction cycle drought escape leads to early flowering and early maturity (Silim and Saxena, 1993). In terminal stress conditions, it was observed that drought escape by early crop duration such as early flowering can contribute to the drought yield (Bidinger et al., 1987; Kashiwagi et al., 2013). The strategy of short-term drought escape has been used in the chickpea breeding process to bring yield stability in chickpea (Kashiwagi et al., 2013).

1.1.4.3 Drought tolerance

Drought tolerance is characterised as the ability to grow, flower and provide economic yield under suboptimal water conditions. It is a highly complex mechanism in which plants have higher yield under water deficit (Varshney et al., 2014). Osmotic adjustment, osmoprotection, antioxidation, cell wall hardening, ROS detoxification and a scavenging defence system are the most important factors responsible for drought tolerance. Variation in osmotic adjustment between chickpea cultivars was observed in response to soil drought, and chickpea seed yield was associated with the degree of osmotic adjustment when grown in the field under a line-source irrigation system (Moinuddin and Khanna-Chopra, 2004a). A study in 35 chickpea genotypes showed that drought-tolerant genotypes had higher relative water content, pigment and pigment concentrations and bigger K^+ accumulation compared to Na^+ (Talebi et al., 2013). The waxy bloom on leaf surfaces is considered as a desirable trait for

drought tolerance as it aids with the maintenance of high tissue water potential (Richards, 1996). Breeding strategies in chickpea are mainly focussed on the drought tolerance trait and higher yield. Additionally, phytohormones also play an important role in combating drought tolerance as well as having a major role in regulating plant development and growth. The role and importance of phytohormones in drought tolerance is further discussed in Chapter 2 of this thesis.

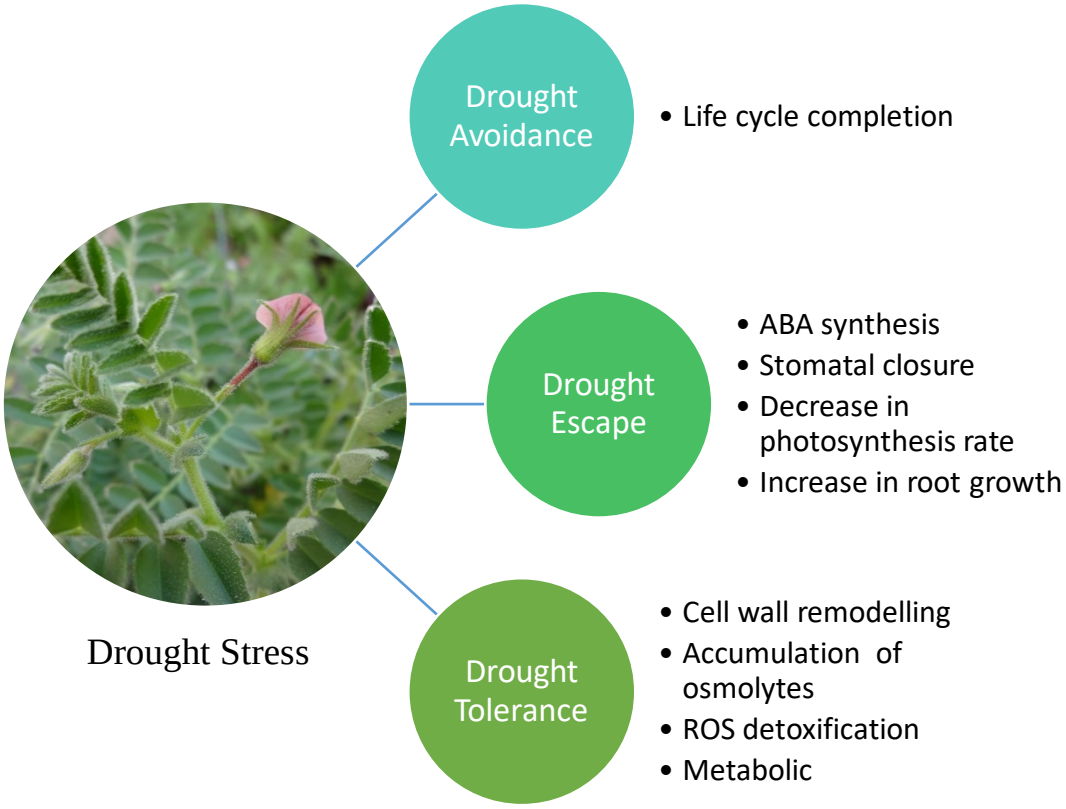

Figure 4: The different plant response to dehydration. The three main drought resistance strategies employed by plants to cope with water shortage periods are drought avoidance, drought escape and drought tolerance. Modified from (Osmolovskaya et al., 2018).

1.2. Role of omics in improving drought tolerance in chickpea

1.2.1 Genetic response

Advances in omics-based tools such as gene-based technology has allowed new strategies to be applied which can help in understanding the mechanisms behind the tolerance/sensitivity to abiotic stresses and provide new approaches to the development of

novel crop varieties. For example, Le et al. investigated the possibility of incorporating genome-wide association study (GWAS) results into a genomic selection (GS) model for exploring drought tolerance in chickpea. Compared to using all single nucleotide polymorphism (SNPs), the GS model improved the prediction accuracy of three yield and yield-related characteristics by more than double the prediction accuracy utilising subsets of SNPs substantially correlated with the characteristics under investigation (Li et al., 2018). Furthermore, omnics-based technologies namely, transcriptomics, genomics, proteomics and metabolomics can be used to detect candidate genes, thereby assisting in quantitative trait locus (QTL) analysis (Nadeem et al., 2019). In improving characteristics such as longer generation cycles and complex processes involving large numbers of small effect QTLs in chickpeas, genomic selection may play a major role. In addition, it suggested that yield prediction accuracies in rainfed environments were much lower than in irrigated environments (Roorkiwal et al., 2016). A schematic illustration of the 'omics' approach for drought tolerance in chickpea are presented in Figure 5.

For drought tolerance research, numerous genomic tools have been used in the past, including subtractive suppressive hybridization (SSH), Super Serial Gene Expression Analysis (SuperSAGE), cDNA libraries, DNA microarray, cDNA microarray, and next-generation sequencing (Jha et al., 2014). The first draft sequence of the desi type chickpea genome was created using next-generation sequencing in 2013 (Varshney et al., 2013b). Prior to the arrival of RNA sequencing, to elucidate the preliminary function of differentially expressed genes, microarray-based technologies and expressed sequenced tags (ESTs) were chiefly used (Mantri et al., 2007; Varshney et al., 2009). The differential expression of genes coding for functional and regulatory proteins was elucidated using cDNA microarray in leaf, root and flower tissues of tolerant and susceptible chickpea genotypes under abiotic stress conditions (Mantri et al., 2007). Many tissue-specific and stress-responsive genes activated under stress conditions were

identified by gene expression analysis performed using RNA-Seq. In chickpea root and shoot tissue subjected to desiccation, cold and salinity, RNA-Seq analysis was performed (Garg et al., 2014) this gave a detailed view of the complex transcriptional response of chickpea tissues to various conditions of abiotic stress. In addition, differential expression analysis allowed 1,640 chickpea transcripts to be identified that showed a response to at least one of the stress conditions imposed (Garg et al., 2014). RNA-Seq study of the chickpea roots tissue of drought and salinity tolerant genotype identified a total of 4954 and 5545 genes particularly regulated in drought and salinity tolerant genotype (Garg et al., 2016a). Drought-induced leaf senescence allows the accumulation of nutrients that can be used by the rest of the plant (Munné-Bosch and Alegre, 2004; Guo and Gan, 2014). The complex phenomenon of leaf senescence, stomatal conductance, stomatal closure and regulatory mechanism of leaves under drought stress still needs to be investigated in detail in chickpea (Blackman et al., 2009; Maqbool et al., 2017). A comparative study in chickpea showed that 92 genes were differentially expressed under drought stress and these genes were involved in diverse biological progresses. The highest number of up-regulated genes were linked to drought tolerance, and the down-regulated genes were associated with photosynthesis (Gao et al., 2008). Studies have shown that the crop growth stage also contributes to drought adaptation. While experiments with different chickpea tissues were performed, these were restricted to one stage of development or single genotypes. In addition, most of the studies conducted so far focussed on the different traits of root, flower and seed in chickpea under drought stress. However, very limited studies have focused on the mechanism of leaf stomatal conductance, density and stomatal closure as contributors to drought stress tolerance (Garg et al., 2016b; Rani et al., 2020). The identification of drought-responsive genes, transcription factors and key metabolic pathways by RNA sequencing can aid in the development of new chickpea varieties through genomics-assisted breeding programs. Scientists are now focussing on the development of new chickpea cultivars with

improved drought tolerance. A stage specific regulation of genes related to transcription factors, kinase activity, detoxification, ROS signalling and transporter activity was recently revealed in two contrasting chickpea genotypes under drought stress using RNA sequencing (Bhaskarla et al., 2020). The insufficient insight into different genetic pathways involved in drought tolerance has been provided by the studies conducted so far. To enhance our understanding, further studies on the drought mechanism and underlying mediators of drought tolerance in chickpea is required.

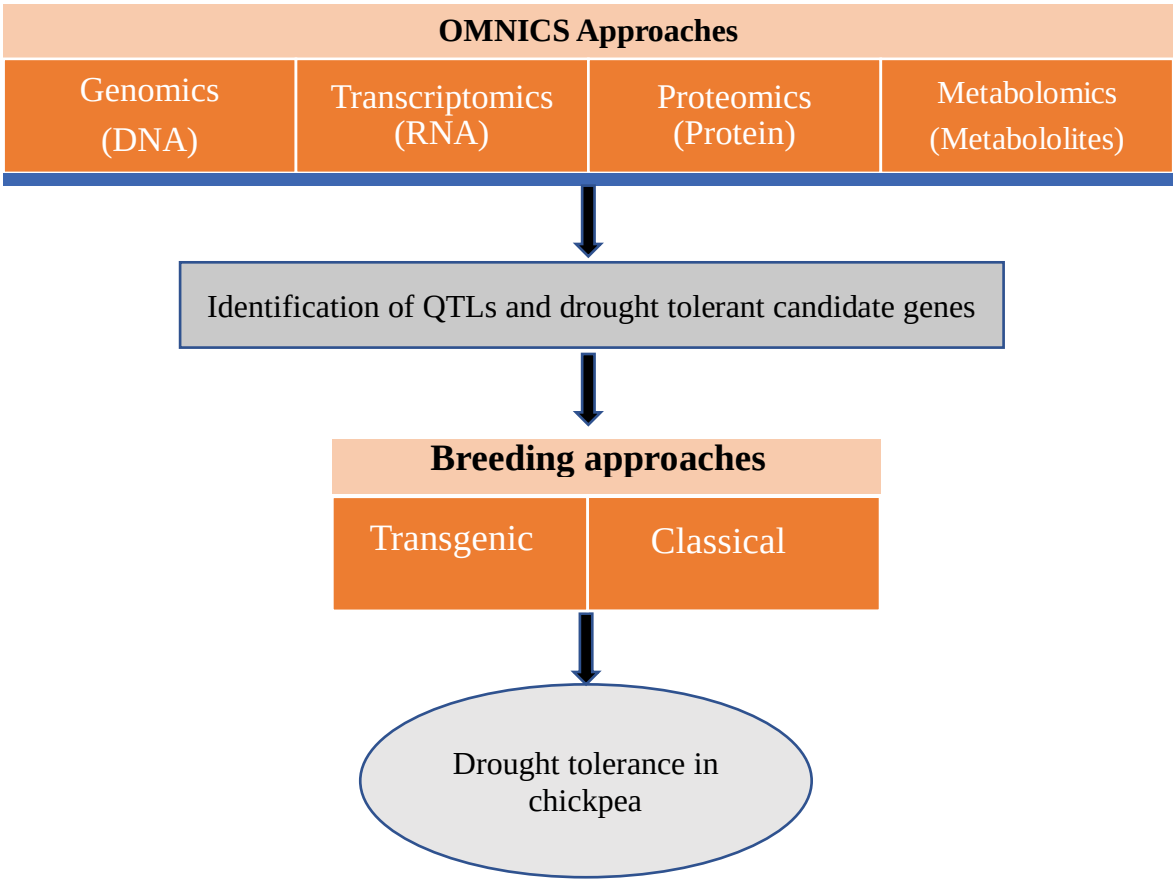

Figure 5: A schematic illustration of the 'omics' approach for drought tolerance in chickpea. Modified from: (Nadeem et al., 2019).

1.2.2 Epigenetic response

Epigenetic modifications are an organism's way of altering the expression of specific genes in response to environmental stress (Chinnusamy and Zhu, 2009). Figure 6 represents the overview of how epigenetic responses regulate gene expression under stress conditions. The most prevalent type of epigenetic modification is DNA methylation and histone modifications (Tariq and Paszkowski, 2004), DNA methylation is the addition of methyl group on the cytosine of genes and often occurs at fifth carbon atom of a cytosine ring. This pattern is particularly common in plants compared to other eukaryotes (Kumar et al., 2013; Tariq and Paszkowski, 2004). However, DNA methyltransferases (DNMTs) catalyse this conversion of cytosine bases to 5-methylcytosine (Aufsatz et al., 2004). These modified cytosine residues typically lie next to a guanine base (CpG methylation) and result in two diagonally positioned methylated cytosines on opposite DNA strands positioned diagonally towards each other. Different approaches used to study methylation are methylation-sensitive amplified polymorphism (MSAP) (Dong et al., 2006; Shan et al., 2013), methylated DNA immunoprecipitation sequencing (Wang et al., 2016) and bisulphite sequencing (Garg et al., 2015).

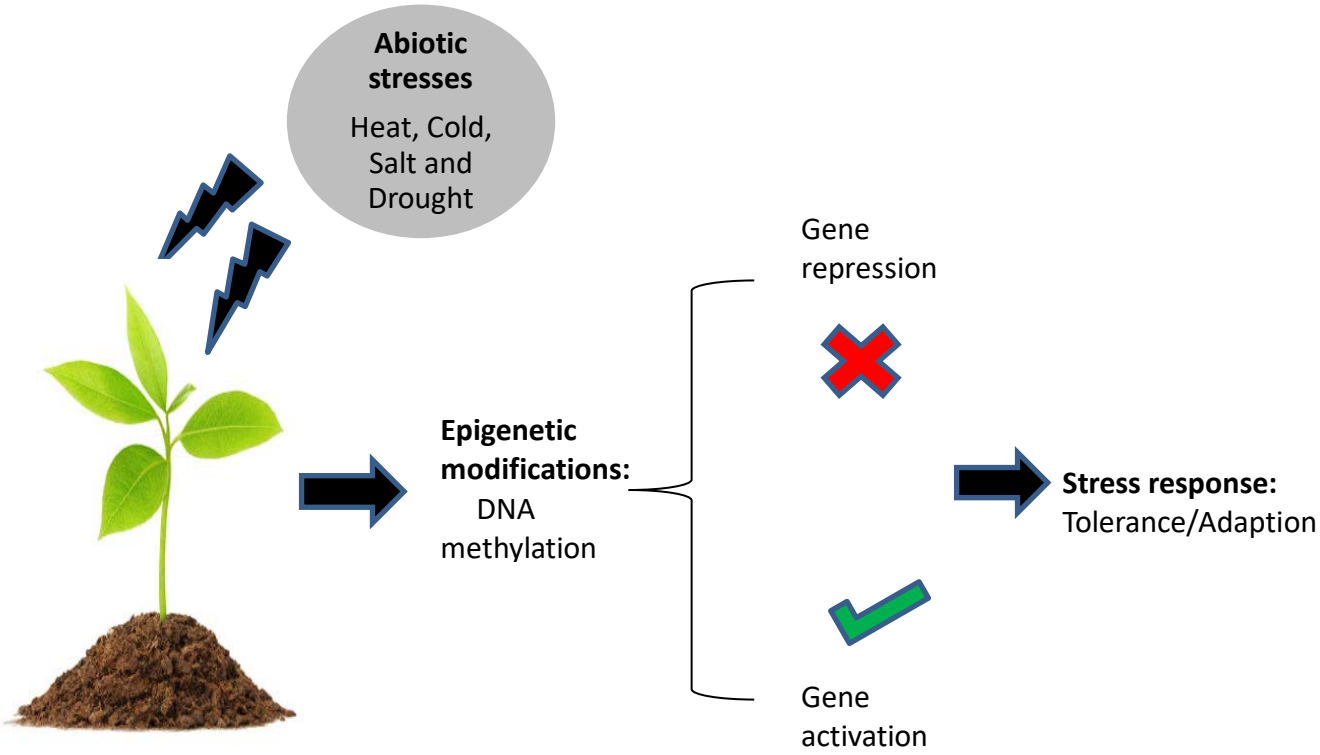

Figure 6: An overview of how plants are affected by different abiotic stresses and which leads to epigenetic modifications such as DNA methylation. This regulates the expression of genes under stress conditions, finally helping plants to adapt and tolerate stress.

1.2.3 Role of DNA methylation in abiotic stress adaptation/tolerance

A study in root tips of pea suggested that water stress induces cytosine hypermethylation in the pea genome and activities related to methylation and demethylation in control and stressed plants were consigned to specific DNA sequences (Labra et al., 2002). In 2011, Wang et al. showed that the induction of genetic changes in the rice genome can help in adaptation and response to drought tolerance (Wang et al., 2011). A study in rice germplasm also suggested that specific epigenetic changes may play an important role in identifying and regulating salt stress in the expression network of a cascade of genes involved in tolerance mechanism (Karan et al., 2012). In response to drought and salinity stress, opposing DNA methylation patterns were associated with gene expression in rice cultivars. The results from the study provided insight into the interplay between DNA methylation and gene expression in rice cultivars (Garg et al., 2015). A study in rice concluded that differentially methylated

regions (DMR)-associated changes in DNA methylation genotype were gene-specific (Wang et al., 2016). In legumes, a study in soybean examined DNA methylome in following organs: roots, stems, leaves, and seed cotyledons using deep bisulphite sequencing and 216 DMRs were identified in respective organs and hypomethylation was correlated with upregulation of flanking gene expression (Song et al., 2013). DNA methylation can play a crucial role in activation/deactivation of certain transcription factors involved in salinity stress tolerance was suggested in soybean earlier (Song et al., 2012). Moreover, a comparative epigenetic analysis in soybean and common bean by Kim et al provided reference methylomes for future legume crops epigenetic investigations (Kim et al., 2015). However, recently DNA methylation study conducted in two chickpea genotypes; cold tolerant and cold susceptible, suggested that DNA methylation levels were higher in cold susceptible genotypes compared to demethylation in tolerant genotypes (Rakei et al., 2016). In another study, Bhatia et al. identified a positive correlation in promoter hypermethylation and increased transcript abundance in flower development via DMR identification. Studies related to DMR identification under drought stress will provide the basis for how methylation-dependent gene expression pattern occurs under stress conditions and their role in the discovery of novel gene regulatory mechanisms in chickpea (Bhatia et al., 2018).

Recent advances in gene editing technology has allowed information from genetic and epigenetic studies to be used to edit genes of interest in any crop using CRISPR/Cas9 gene editing. However, the debate on commercialisation of CRISPR/Cas9 edited crops remains a major concern for the scientists.

1.3. CRISPR/Cas9 gene editing

The latest gene editing method employing CRISPR/Cas9 is based on the natural immune system that bacteria use to protect themselves against intruding infectious viruses. In 1987, the

presence of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) was first reported in *Escherichia coli* during investigation of the gene responsible for isozyme conversion of alkaline phosphatase (Ishino et al., 1987). Mojica investigated multiple copies of repeated sequences which were roughly palindromic in bacterial species which were not resembling the family of any microbes (Mojica et al., 1995). The term CRISPR was coined by Jansen and his group and later accepted by all peers (Jansen et al., 2002). When the bacteria were attacked by viruses, they detect the viral DNA and produce two types of short RNA sequences (Barrangou et al., 2007). The sequence of the invading virus matches one of these short RNA sequences. Further, these two RNA sequences build a complex with a nuclease enzyme named Cas9. The Cas9 (also known as Csn1) protein is a Type II CRISPR associated, RNA-guided DNA endonuclease enzyme (Makarova et al., 2011; Sapranauskas et al., 2011). Moreover, in the invading viral genome, the matching sequence known as guide RNA finds its target sequence. Subsequently, the Cas9 enzyme cuts the viral DNA at the targeted site of the viral genome. Once inside the system, the resulting complex can attach upstream of the protospacer adjacent motif (PAM) sequence of the target DNA. Cas9 then unzips the DNA and matches it to its target RNA. Cas9 can use two molecular scissors to cut the DNA and create a double-strand cleavage if the match is successful (Garneau et al., 2010). Furthermore, research showed that this method can not only be used to cut the viral DNA but also be employed to detect and edit precisely a location chosen by the guide RNA sequence. Other than its role in bacterial immunity, Cas9 has been exploited by scientists as a powerful tool for genome editing and gene regulation in many eukaryotic organisms. Compared with zinc finger nucleases and transcription activator-like effector nuclease (TALEN) proteins, Cas9 has the ability that it could cleave almost any sequence complementary to the guide RNA, therefore Cas9 is becoming a prominent tool in the field of genome editing. This mechanism can be used as a powerful tool for genetically editing DNA of interest and multiplexing can be used to edit

several sites (Cong et al., 2013). Zhang and his team were the first to harness CRISPR gene editing in eukaryotes. However, one of the drawbacks with CRISPR/Cas9 is the off-target effect of Cas9 (Zhang et al., 2015).

1.3.1 CRISPR/Cas9 structure and mechanism

The Cas9 protein consists of two important domains: HNH and RuvC. The first is HNH domain accountable for the cleavage of the target sequence (DNA strand complementary) to guide RNA sequence. RuvC is the second domain which is composed of irregular sequential subdomains (e.g., RuvC-I, RuvC-II and RuvC-III) that cleave the crRNA non-complementary strand of the target DNA sequence. They also contain an arginine-rich region which mediates nucleic acid binding (Nishimasu et al., 2014) (Figure 7).

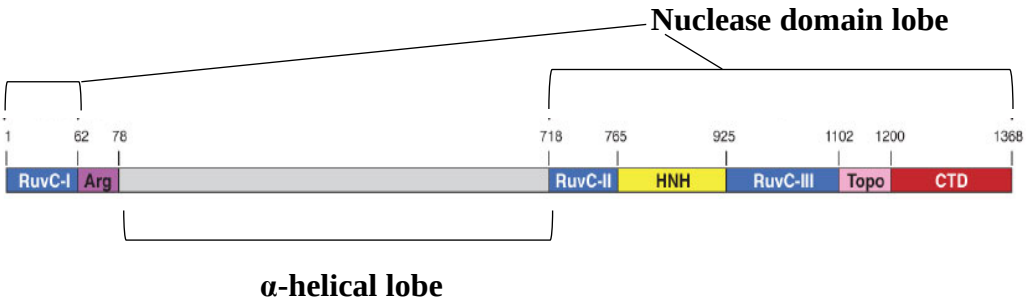

Figure 7: The cas9 protein structure consists of RuvC-I, RuvC-II and RuvC-III domains, an Arg (arginine) region, an HNH domain required for cleavage, followed by Topo and CTD domains (Sinobiological, 2020).

Engineered gene editing using CRISPR/Cas9 is usually composed of guide RNA (gRNA) or synthetic guide RNA (sgRNA) and CRISPR associated Cas9 endonuclease (Barrangou et al., 2007; Garneau et al., 2010; Jiang and Doudna, 2017). The gRNA is a short synthetic RNA which comprises of a scaffold sequence necessary for Cas-binding and a user-defined ~20 nucleotide spacer sequence that defines the part of the target region to be edited (Jinek et al., 2012). The user can design or select the gRNA based on the gene required to be modified. Different variants of the Cas enzyme are utilised, based on the end cleavage

requirement. Cas9 is a class II endonuclease which requires a crRNA and tracer RNA for the target cleavage (Jinek et al., 2012). One of the important requirements of CRISPR editing is that the 20 bp sgRNA sequence should be unique in the genome (Mali et al., 2013a). Also, this sequence should be present adjacent to the PAM sequence and it acts as a signal for Cas enzyme and these vary for different Cas enzymes (Cong et al., 2013; Mali et al., 2013b; Mojica et al., 2009). For *Streptococcus pyogenes* Cas9 (SpCas9), the PAM sequence is NGG (Anders et al., 2014). Over the interaction between the gRNA scaffold and occurrence of surface-exposed positively-charged grooves on Cas9 protein and the gRNA, they tend to form a ribonucleoprotein (RNP) complex instantly (Nishimasu et al., 2014). Interestingly, after this interaction, Cas9 endures a conformational change in its structure, that shifts the molecule into an active DNA-binding conformation from an inactive, non-DNA binding confirmation. However, to interact with the target DNA, the gRNA spacer region remains unobstructed (Barrangou et al., 2007; Garneau et al., 2010; Jinek et al., 2012).

Cas9 has an exception that it can only cleave a specific locus, if ample homology is shared with the target DNA by the selected gRNA spacer sequence. As soon as these conformational variations occur RNP binds target DNA, the seed sequence which is 8-10 bases at the 3′ end of the gRNA targeting sequence starts to anneal towards the target DNA. If the sequence of seed and target DNA matches together, the gRNA will continue annealing towards the target DNA in a 3′ to 5′ direction (Nishimasu et al., 2014). However, if there is a presence of any mismatches between the target sequence and seed sequence in 3′ direction it will completely abolish target cleavage. In contrast, if this mismatch occurs towards the 5′ end to the PAM it will still often allow target cleavage (Anders et al., 2014; Garneau et al., 2010; Jiang and Doudna, 2017; Mali et al., 2013a; Mali et al., 2013b; Mojica et al., 2009). Subsequently, on target binding, the Cas9 undergoes a second conformational shift. This leads to cleavage opposite strands of the target DNA and finally due to the Cas9-mediated DNA cleavage an

introduction of double-strand break (DSB) approximately 3 to 4 nucleotides upstream of the PAM sequence in target DNA site occurs (Jiang and Doudna, 2017).

1.3.2 CRISPR/Cas9 gene knockout

The advantage of CRISPR/Cas9 gene editing is that it can be readily employed to knockout the gene of interest. CRISPR gene knockout is a technique where the function of a gene is set out of action. This is usually performed to study the loss of function of a gene of interest. Figure 8 shows the RNP formation mechanism and overview of DNA editing using DNA free CRISPR/Cas9 editing. CRISPR/Cas9 technology has been recently applied for the development of new crop varieties.

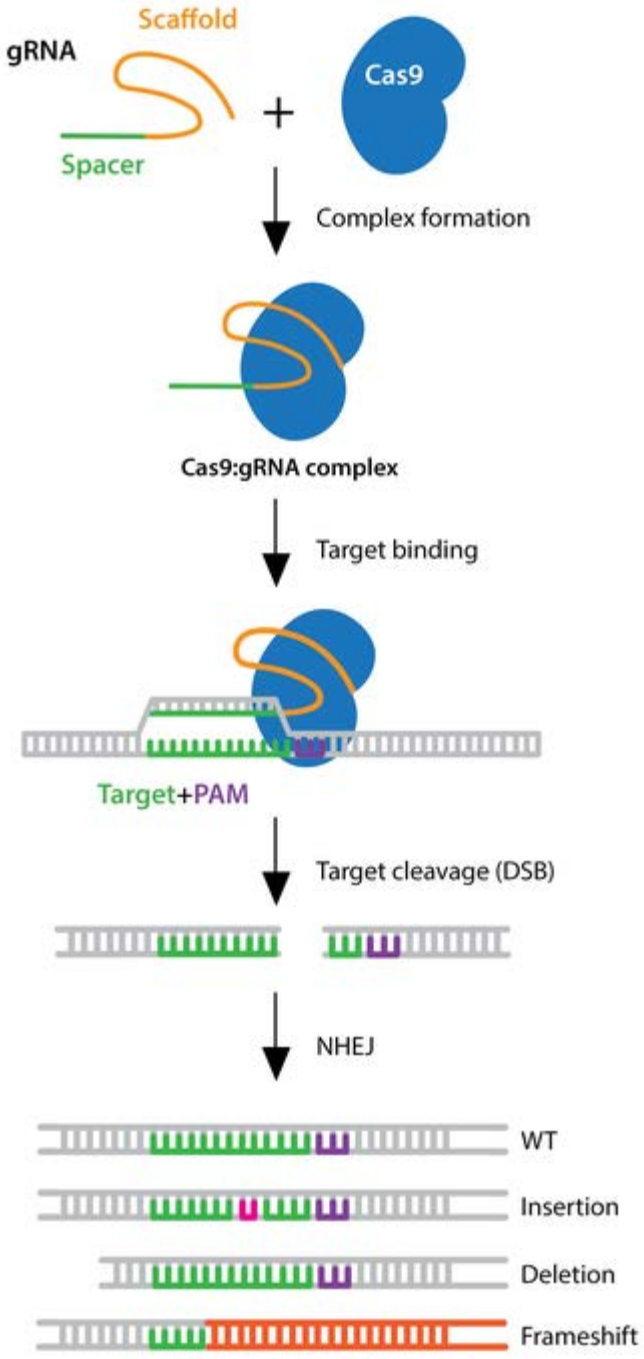

Figure 8: Overview of RNP complex formation and editing. Under optimal conditions, the cas9 enzyme and gRNA will form a complex called Cas9: gRNA complex. This complex will look for the PAM sequence in the target DNA and bind to it. Once the complex is activated, it will introduce DSB at the target site. The cleavage will be usually repaired by the non-homologous end joining (NHEJ) pathway which will further result in mutations due to indel or frameshift in the target sequence (Addgene, 2020).

1.3.3 DNA free editing

Changes in global climate will significantly impact the agriculture industry. There is also a requirement to increase productivity due to the expanding population. This has led scientists to discover new technologies to face these upcoming food security challenges. There is a great need for DNA-free gene editing because there is always the possibility of the integration of foreign DNA in plasmid-mediated delivery of gene editing (Cho et al., 2013). Another advantage of the RNP based delivery is that it can bypass the steps for vector construction and cloning. Subsequently, the RNP complexes provide transient editing without the integration of CRIPSR reagents into the host genome (Banakar et al., 2020).

3.4 CRISPR/Cas9 gene editing in crops

CRISPR/Cas9 gene-editing tools have been utilized by agricultural scientists for gene activation, repression, knockout, knockdown, repression and for altering epigenetic modifications in several plants crops such as *Arabidopsis* (Feng et al., 2014), apple (Osakabe et al., 2018), citrus, carrot (Klimek-Chodacka et al., 2018), grape, tomato, rice (Zhang et al., 2014), sorghum (Liu et al., 2019), maize and soybean (Chilcoat et al., 2017) and wheat (Zhang et al., 2016). However, gene-free editing has been effectively achieved in only a few crop varieties such as *Arabidopsis* (Gao et al., 2016), bread wheat (Liang et al., 2017), grapevines and apple (Malnoy et al., 2016). CRISPR/Cas9 genome editing has been considered as an emerging genome editing tool which has broad application in crop improvement and can be used to develop designer genetically edited non-GM crops. The current focus of agriculture scientists is implementation of this approach in plant breeding to develop new varieties of crops with higher tolerance to environmental constraints (Khatodia et al., 2016). Scientists used CRISPR/Cas9 genome editing to identify abiotic stress response in *Arabidopsis* plants. The results suggested that *OST2* (proton pump), a mutant allele obtained from editing had altered

stomatal closing under environmental stress (Osakabe et al., 2018). A recent study in maize used CRISPR/Cas9 system to produce novel allelic variations which could be used for breeding drought tolerant crops. *ARGOS8*, whose overexpression can lead to reduced ethylene sensitivity, was genetically modified by this system and field studies revealed that *ARGOS8* variants had increased grain yield under drought stress and no loss in yield was recorded under well-watered condition (Shi et al., 2017). The CRISPR/Cas9 DNA free mediated gene editing in crop protoplasts are presented in Table 2. The whole plants regeneration from edited protoplasts has been successfully achieved for many plants. These include wheat (Liang et al., 2017), maize (Svitashev et al., 2016), apple (Malnoy et al., 2016), and soybean (Kim et al., 2017); in coming years this list is expected to expand because of the merits CRISPR DNA free editing technology (Park and Choe, 2019). Currently, the CRISPR/Cas9 platform is clearly on its way to changing the pace and direction of agricultural research. With the emergence of this technology, it will no longer be impossible to develop ideal plants with high yield, quality and resistance to any abiotic/biotic stresses (Leong et al., 2018).

Table 2: Overview of CRISPR/Cas9 mediated gene editing in crop protoplasts.

Year	Study	Crops	Reference
2015	DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins	Arabidopsis thaliana, tobacco, lettuce and rice	(Woo et al., 2015)
2016	DNA-Free genetically edited grapevine and apple protoplast using CRISPR/Cas9 Ribonucleoproteins	Apple	(Malnoy et al., 2016)
2016	Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes	Maize	(Svitashev et al., 2016)
2017	Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes	Bread wheat	(Liang et al., 2017)
2017	CRISPR/Cpf1-mediated DNA-free plant genome editing	Soybean	(Kim et al., 2017)
2018	Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery	Potato	(Andersson et al., 2018)
2019	DNA-Free Genome Editing via Ribonucleoprotein (RNP) Delivery of CRISPR/Cas in Lettuce	Lettuce	(Park et al., 2019)
2020	A streamlined protocol for wheat (Triticum aestivum) protoplast isolation and transformation with CRISPR-cas ribonucleoprotein complexes	Wheat	(Brandt et al., 2020)

The biggest concern for agricultural scientists currently is the need for developing new chickpea varieties which can survive the changing climatic conditions. One of the aims of PBA (Pulse Breeding Australia) is to have improved abiotic stress tolerance in pulses. Two candidates selected for this study are described below.

1.4. Introduction to candidate genes for DNA-free gene editing in chickpea genotype.

1.4.1 4 Coumarate Ligase (*4CL*) gene

The 4-coumatrate: CoA ligase gene codes for coumarate ligase enzyme which is well known for its role in the biosynthesis of plant secondary compounds during phenylpropanoid metabolism and in major branch pathways (Ehlting et al., 1999; Lee et al., 1997; Liu et al., 2016). This phenylpropanoid enzyme is ranked important to activate the hydroxycinnamic acids for the biosynthesis of lignin. A study in *Arabidopsis* showed that a decrease in *4CL* activity correlated with a decrease in thioglycolic acid extractable lignin (Lee et al., 1997). Increased expression of the lignin biosynthesis gene was observed in watermelon during the water stress (Yoshimura et al., 2008).

1.4.2 Reveille (*RVE7*) gene

Reveille 7 is a gene which encodes for a transcription factor involved in circadian rhythm and phytochrome A-mediated cotyledon opening. The regulation of this is controlled by *LHY* and *CCA1* central oscillator mediation. It is known to regulate its expression and belongs to part of circadian feedback (Rawat et al., 2009). *RVE7* has been identified to be involved in the regulation of downstream processes such as hypocotyl growth and flowering of the circadian clock (Kuno et al., 2003). *RVE7* transcription factor was identified to be highly differentially expressed in drought tolerant chickpea genotype, ICC8261 (Bhaskarla et al., 2020).

Aims and objectives

Aim:1

- To conduct RNA sequencing of leaf tissues from drought-tolerant and drought-sensitive chickpea genotypes and identify candidate genes and pathways associated with drought tolerance/sensitivity.

Research questions

- ❖ Which genes/pathways are differentially expressed under drought stress in the leaves of drought-tolerant and sensitive genotypes?

Aim:2

- To identify DNA methylation patterns which potentially regulate drought tolerance/sensitivity of selected chickpea genotypes.

Research question

- ❖ How can the results obtained from RNA-Seq and DNA methylation studies under drought stress help chickpea crop improvement to produce new cultivars that are more tolerant to drought stress?
- ❖ Is differential expression of genes associated with differential DNA methylation patterns in the tolerant and sensitive genotypes? What is the role of epigenetic responses in response to drought stress?

Aim:3

- To perform DNA free gene editing in chickpea protoplasts using the modern gene knockout technique, CRISPR/Cas9 system.

Research question

- ❖ Will any of the candidate genes selected for knockout/gene editing improve drought tolerance? What mechanism could be possibly associated with tolerance?

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Chapter 2

RNA sequencing of leaf tissues from two contrasting chickpea genotypes reveals mechanisms for drought tolerance

Plant Physiology and Biochemistry 129 (2018) 295–304

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

RNA sequencing of leaf tissues from two contrasting chickpea genotypes reveals mechanisms for drought tolerance

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ARTICLE INFO

Keywords:
Chickpea
Drought stress
Drought tolerance/responsive genes
RNA sequencing
Transcription factors
Phytohormones

ABSTRACT

Chickpea (*Cicer arietinum* L.) is the second most important winter crop which is consumed globally due to its high nutritional value. Chickpea as one of the leguminous crop is important in crop rotation with cereal crops like wheat and barley. The main constraints for chickpea production are abiotic stresses such as drought, salinity, and heat. Among these, drought is a major cause of the decline in chickpea production in worldwide. Studies conducted so far have provided a limited insight into different genetic pathways associated with drought tolerance/response. In this study, the leaf tissue from shoots apical meristem stage of drought tolerant (ICC8261) and drought sensitive (ICC283) genotypes were analysed using RNA sequencing to identify genes/pathways associated with drought tolerance/sensitivity in both genotypes. It was observed that genes related to ethylene response, MYB-related protein, xyloglucan endotransglycosylase, alkane hydroxylase MAH-like, BON-1 associated, peroxidase 3, cysteine-rich and transmembrane domain, vignain and mitochondrial uncoupling were specifically up-regulated in the tolerant genotype whereas, same genes were down-regulated in sensitive genotype. The crosstalk between the different hormones and transcriptional factors involved in drought tolerance and sensitivity in both genotypes make them great candidates for future research.

1. Introduction

Chickpea (*Cicer arietinum* L.) is the second most important legume crop grown in Australia. Chickpea is rich in proteins, amino acids and provides other dietary nutrition. As a legume chickpea plays an important role in crop rotation, as it aids in soil fertility by nitrogen fixation and in disease break, and weed control for the cereal crops (Gunes et al., 2007). The protein percentage of chickpea is 20–25% which is comparatively higher than wheat and rice. Most of the chickpea produced in Australia is exported to other countries, which makes it an important industrial crop for Australian farmers. India is the largest buyer of Australian grown chickpeas (www.daf.qld.gov.au). The main concern for chickpea production in Australia is abiotic stresses such as drought, heat, salinity and cold. Among these abiotic stresses, drought is the most common stress, which is limiting crop productivity worldwide. The drought has been considered as a crucial environmental limitation factor frequently experienced by plants, ultimately leading to a loss in crop yield (Shanker et al., 2014). Over 90% of the Australian chickpea growing area is the arable land of the northern grains region. This includes some parts of central and southern Queensland and the northern part of NSW. These parts are climatically highly variable, resulting in the exposure of chickpea to frequent drought and high temperatures (Chauhan et al., 2017).

In order to have a deeper insight into the different regulatory mechanisms involved in the drought stress scientists have used different scientific approaches. The difference in expression of genes coding for functional and regulatory proteins in tolerant and susceptible genotypes under abiotic stress conditions has been previously studied (Mantri et al., 2007). The study confirmed the role of certain genes in the abiotic stress response of chickpea. Other previous work has provided limited insight into the role of different genetic pathways involved in drought tolerance; different genomic resources have been used in past for drought tolerance analysis including Subtractive suppressive hybridization (SSH), SuperSAGE, cDNA libraries, DNA microarray, cDNA Microarray and Next Generation Sequencing (Jha et al., 2014). Next-generation sequencing coupled with bioinformatics tools and software has provided a route to investigate key metabolic pathways involved in stress response and adaptation. In 2013, the draft sequence of desi type chickpea genome was generated using next-generation sequencing (Varshney et al., 2013). Gene expression analysis performed using RNA-

Abbreviations: APX, Ascorbate peroxidase; DS, drought-stressed; JA, Jasmonic acid; GA, Gibberellic acid; GO, Gene ontology; LEA, Late embryogenesis abundant; ROS, reactive oxygen species; UCPs, uncoupling proteins; XTH, Xyloglucan endotransglucosylase/Hydrolase; WW, Well-watered

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https://doi.org/10.1016/j.plaphy.2018.06.007
Received 4 April 2018; Received in revised form 7 June 2018; Accepted 7 June 2018
Available online 13 June 2018
0981-9428/

Seq identified many genes activated under stress conditions (Jain et al., 2013). Further, RNA-Seq analysis in chickpea root and shoot tissue subjected to desiccation, cold and salinity was performed (Garg et al., 2014), providing a comprehensive view of the dynamic transcriptional response of chickpea tissues to different abiotic stress conditions. Differential expression analysis identified 11,640 chickpea transcripts which showed a response to at least one of the stress condition imposed (Garg et al., 2014). It was concluded that extensive transcriptional programming of many genes associated with important plant metabolic pathways occurs under stress conditions.

Recent RNA-Seq analysis of chickpea roots of drought and salinity tolerant genotypes identified 4954 and 5545 genes which were exclusively regulated in drought tolerant and salinity tolerant genotypes, respectively (Garg et al., 2016). Although studies have been conducted with different chickpea tissues research has been limited to one developmental stage or single genotypes. Most studies to date have focussed on the different traits of root, flower and seed in chickpea under drought stress. Very few have focused on the mechanism of leaf stomatal conductance, density and stomatal closure in terms of drought stress tolerance (Blackman et al., 2009). However leaf senescence is a highly regulated process which under drought stress conditions leads to leaf death, thereby contributing to plant survival under drought stress. Drought-induced leaf senescence leads to nutrient accumulation which can be used by the rest of plant under stress. In this study, leaf tissue from the shoot apical meristem developmental stage was analysed using next-generation sequencing and RNA-Seq differential expression analysis. The aim of differential expression analysis was to identify genes involved in metabolic pathways under control and drought stress conditions in drought sensitive and drought tolerant chickpea genotypes. This study will help to develop strategies to mitigate abiotic stress in chickpea cultivars. The identification of differentially expressed genes will help to develop new chickpea cultivars which will be more tolerant to abiotic stress condition using recent biotechnology techniques and bioinformatics tools.

2. Materials and methods

2.1. Plant material and total RNA isolation

In this study, two different chickpeas (*Cicer arietinum* L.) genotypes ICC8261 (Drought tolerant) and ICC283 (Drought sensitive) seeds were grown in a glasshouse under controlled conditions. The ICC 8261 possesses larger root length density and deep root system. Comparatively, ICC283 have a characteristic of shorter root system. The genotypic and phenotypic variance between the two genotypes is in regard to their root traits and transpiration efficiency traits (Kashiwagi et al., 2005; Varshney et al., 2014). The field capacity of soils for control samples was 80% and 20% for drought. Plants were grown in 20 cm polypropylene pots containing 4 kg of soil under controlled environmental conditions with air temperature regulated between 23 °C and 28 °C (night/day). The experiment was a 3 × 2 X 2 completely randomised block design one-time point (Shoot apical meristem), two genotypes, and two treatment conditions). For each genotype, 6 pots were used and randomly designated to one of two treatments: Well-watered (WW) control and drought-stressed (DS). The WW pots were maintained at the optimum water level of 80% field capacity by gravimetric analysis. The DS pots were also maintained at 80% FC at sowing. However, 15 days before the sampling, a regulated dry down was conducted by covering the pot surface with polythene sheets to prevent evaporation of water from the soil surface. Pots were weighed daily to ensure they lost only a fixed % of water leading to a gradual decrease in water content from 80% to 20% field capacity in 15 days. Any excess water lost on a particular day was replenished. Plant tissues at different developmental stages were sampled when the soil water content and leaf water potential of drought-stressed plants reached 15–20% FC and −0.7 to −0.85 Mpa, respectively.

Leaf tissues were harvested at the shoot apical meristem stage and stored by freezing in liquid nitrogen for further use. For each sample, three biological replicates were collected and further analysis was carried out.

2.2. RNA sequencing and differential expression study

Total RNA was extracted from leaf tissues using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. The quality and quantity of isolated RNA were confirmed by using Nanodrop spectrophotometer (NanoDrop Technologies) and Agilent Bioanalyzer.

For RNA sequencing cDNA libraries were prepared from leaf tissue samples at the shoot apical meristem stage. The sequencing for leaf samples was performed by Illumina HiSeq 3000 in one lane each to generate 2 × 150 base pair paired-end reads. The raw Fasta files generated by sequencing were obtained and were cropped and trimmed using trimmomatic command line tool to remove low-quality reads and adaptor sequences.

Tophat (v2.0.0) (Kim et al., 2013) was used to align the filtered high-quality reads to chickpea reference genome for mapping (Varshney et al., 2013). To analyse gene expression, a consensus reference-guided assembly of the transcriptome data from all samples will be generated using Cufflinks (v2.0.2) and the genes exhibiting a significant difference between treatment and control samples (at least two-fold change with P-value ≤ 0.05) considered to be differentially expressed. To process data from high-throughput sequencing the read count was performed by HTseq python package (Anders et al., 2015). To analyse the gene expression of leaf tissue samples two different R programming analysis methods were used, DESeq2 (Love et al., 2014b) and EdgeR (Robinson et al., 2010). A stringent cut-off (q –value ≤ 0.001 and $\log_2$ fold change ≥ 1.0), was used for selecting differentially expressed genes. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE104609 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104609).

2.3. GO enrichment analysis

GO enrichment analysis was performed on gene sets to find out which gene ontologies were enriched in tolerant and sensitive genotypes. Blast2Go was used for the functional annotation and gene ontology analysis (Conesa and Götz, 2008). The parameters for Fisher statistical test method were set as FDR cutoff (≤0.05) of significance value.

2.4. Real-Time PCR validation

To validate the expression of genes Real-Time PCR was carried out using randomly selected genes. cDNA synthesis was performed using 1 µg sample to get cDNA for Real-Time PCR analysis using iScript™ cDNA synthesis kit (BioRAD) followed by Qubit Quantification fluorometer (Thermo Fisher Scientific) to quantify the cDNA according to manufacturer standard protocol. The primers sets were designed specifically to the gene of interest. A BioRAD SSO fast™ Eva green kit was used and reaction mixtures were prepared according to the instructions provided with 5 ng sample template, 0.5 µl primer sequence forward and reverse each, 10 µl buffer mixture, making total volume to 20 µl. Real-time PCR was performed using a Rotor-Gene Q (Qiagen) and results were analysed using Rotor-Gene Q series software. The qPCR parameters were adjusted as follows: hold temperature 95 °C, cycling 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s for 40 cycles. Elongation was set at 72 °C for 5 min. Melt curve analysis was performed at 50–90 °C, with 1 °C increments in temperature. Three technical replicates and three biological replicates were used for Real-time PCR analysis. Elongation factor 1-alpha (*EF1α)* was used as a normalizer

gene for all the selected genes and the Delta Delta CT method was used to calculate the fold change. Pearson's correlation coefficient was used to correlate the values. (Note: List of primers used is provided in supplementary material 3).

3. Results and discussion

3.1. Identification of differentially expressed genes

To understand the mechanism of drought stress in chickpea, two chickpea genotypes were used in this study, ICC 283 (Drought sensitive) and ICC 8261 (Drought tolerant). Leaf tissue samples were collected at the shoot apical meristem stage from both chickpea genotypes to compare the expression of transcripts under drought stress and control conditions. DESeq2 (Love et al., 2014a) and EdgeR (Robinson et al., 2010) were used to analyse differentially expressed genes. Only genes which were common between the two were used for further analysis. After applying stringent cut-off (q-value ≤ 0.001, log2 fold change ≥ −1.0), 2640 (DESeq2) and 2650 (EdgeR) genes were found to be differentially expressed in the sensitive genotypes and 1566 (DESeq2) and 1626 (EdgeR) in the tolerant genotype under stress and control conditions. Common genes were identified Venny2 (http://bioinfogp.cnb.csic.es/tools/venny/). A total of 1562 genes were differentially expressed in tolerant genotypes, comprising 693 up-regulated and 869 down-regulated genes. In comparison, a total of 2592 differentially expressed genes were identified in sensitive genotypes including 837 up-regulated and 1755 down-regulated genes under drought stress conditions (Fig. 1). Overall at drought stress led to significant change in gene expression in both sensitive and tolerant genotypes. Venny was used to identify changes in gene regulation in the two genotypes (Fig. 2). Note: The list of all the genes differentially expressed genes in both genotypes is provided in supplementary material 1.

3.2. GO enrichment analysis of differentially expressed genes and KEGG pathway

Blast2Go, a comprehensive gene ontology enrichment tool was used for functional annotation and enrichment analysis (Conesa and Götz, 2008; Conesa et al., 2005). The genes which were differentially expressed in leaf tissue belonged to the different type of GO classes, which were involved in distinct biological processes, cellular and molecular processes. GO enrichment analysis of both induced and repressed genes in tissue samples led to the recognition of many over-represented and under-represented GO terms in biological processes under drought stress in both tolerant versus sensitive genotype (Fig. 3). A total of 815 statistically significant enriched GO terms were observed in the ICC283 (Drought sensitive) genotype and 187 in ICC8281 (Drought tolerant)

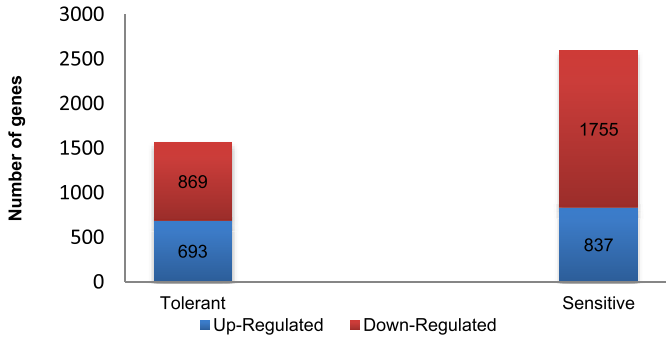

Fig. 1. The number of differentially expressed genes (red = down-regulated; blue = up-regulated in both tolerant and sensitive genotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

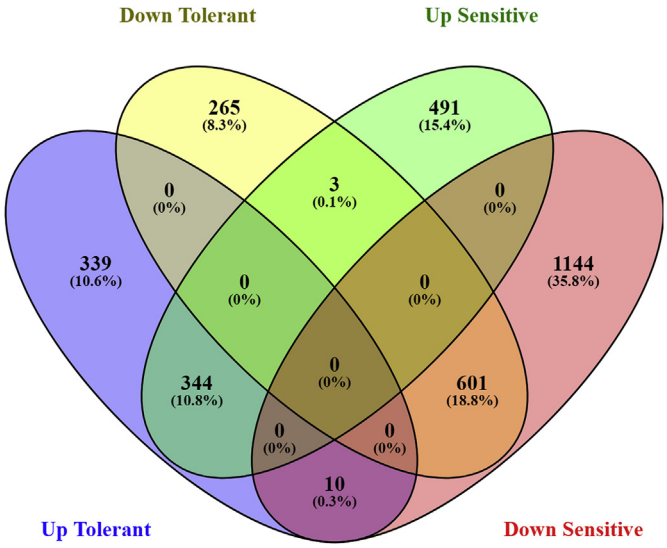

Fig. 2. Venn diagram showing commonly expressed genes between the two genotypes. Venny was used to examine the common up-regulated and down-regulated genes between two genotypes under drought stress.

genotype. In the tolerant genotype, gene ontologies relating to phytohormones and steroids were significantly enriched, for example 'regulation of brassinosteroid mediated signalling pathway' (Go: 1900457), 'response to salicylic acid' (GO: 0009751), 'cellular response to steroid hormone stimulus' (GO: 0071383). GO terms relating to transcription activity, including (GO: 000988) 'transcription activity and proteins', 'sphingolipid metabolic process' (GO: 0006665), 'membrane lipid biosynthesis process' (GO: 0046467) 'related to lipid synthesis', membrane signalling and cell wall modification/adjustment Apart from these 'protein transmembrane transporter activity' (GO: 0008321), and 'molecular transmembrane transporter activity' (GO: 0022884) were also enriched. In addition to this 'axis specification' (GO: 0009758), 'negative regulation of response to biotic stimulus' (GO: 0002832) were also significantly enriched in the tolerant genotype. The other enriched GO terms are shown in (Supplementary material Table.1). In the sensitive genotype 'negative regulation of abscisic acid-activated signalling pathway' (GO: 0009788), 'regulation of protein dephosphorylation' (GO: 00035304), 'jasmonic acid metabolic process' (GO: 0009694), 'negative regulation of protein cell growth' (GO: 0030308) and 'leaf senescence' (GO: 0010150) were among the top enriched gene ontologies.). KEGG pathway map analysis showed differentially expressed genes, mainly in steroid hormone biosynthesis, fatty acid biosynthesis, flavonoid biosynthesis, and metabolism of xenobiotic by cytochrome p450 and carbon fixation in photosynthetic organisms.

3.3. Validation of differential gene expression

To validate the results of differential gene expression obtained from RNA-Seq data quantitative Real-Time PCR analysis was performed. The expression of 11 randomly selected genes, based on expression under control and stress conditions were validated in leaf tissue samples. A similar pattern of expression was observed between both RT-PCR and RNA-Seq data. However, the overall correlation coefficient value between RT-PCR and RNA-Seq data for all the transcripts between the two genotypes was 0.85 for ICC283 and 0.83 for ICC8261. These results confirmed compatibility between the two sets of results obtained from RT-PCR and RNA-Seq data analysis (Fig. 4). Primer list is provided in supplementary material 3.

3.4. Differentially expressed genes in response to drought stress

The genes which were differentially expressed at the shoot apical

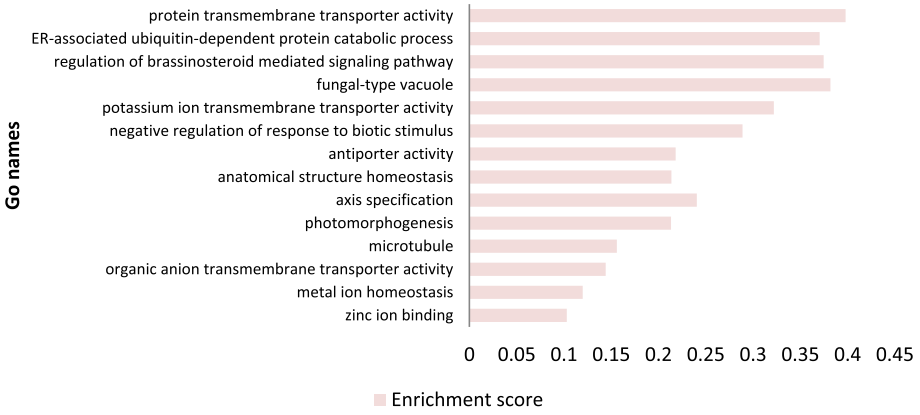

Fig. 3. Graph shows the GO terms which were significantly enriched in tolerant genotype based on enrichment score after gene set enrichment analysis performed by Blast2go.

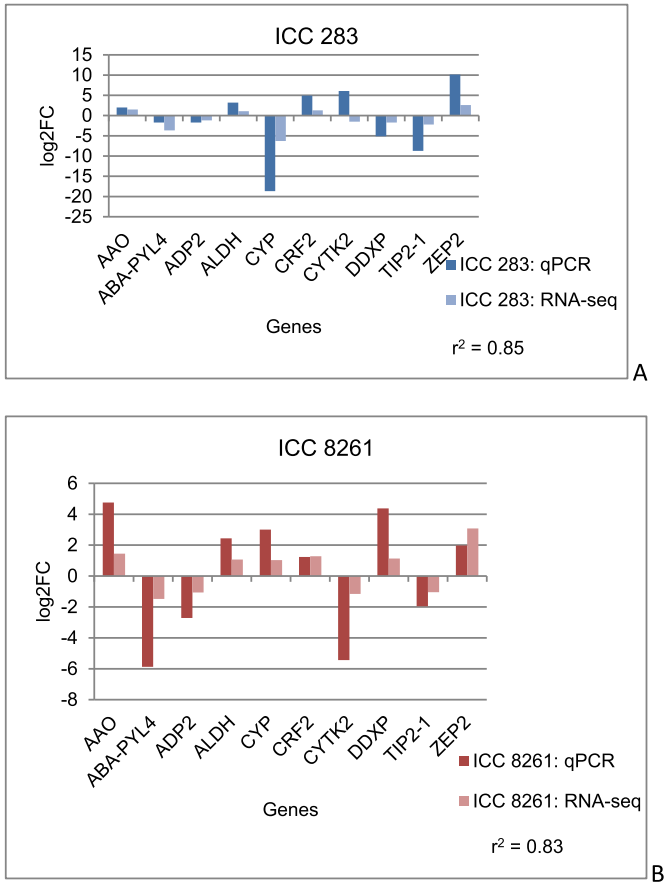

Fig. 4. The above graph shows validation results of selected genes used for qPCR. Ten random genes were selected to validate the RNA Seq results. Results showed similar expression patterns in both the techniques. The correlation value for ICC238 was 0.85 and 0.83 for ICC8261. **A)** Differential expression level of selected genes between Q PCR and RNA Seq in sensitive genotype. **B)** Differential expression level of selected genes between Q PCR and RNA Seq in tolerant genotype.

meristem stage across both the genotypes were exclusively associated with stomatal regulation, photosynthesis, cell wall modification, oxidative stress, transmembrane transport and signal transduction, phytohormone signalling, transcriptional activity and lipid biosynthesis. Genes specifically involved in abiotic stress response and adaptation were up-regulated in the tolerant genotype; whereas, genes related to cell senescence and DNA damage/repair were differentially expressed in the sensitive genotype (Supplementary material 4). In addition, genes related to ethylene response, MYB-related protein, xyloglucan endotransglycosylase, alkane hydroxylase MAH-like, BON-1 associated, peroxidase 3, cysteine-rich and transmembrane domain, vignain and mitochondrial uncoupling were up-regulated in the tolerant genotype and were found to be down-regulated in sensitive genotype (Fig. 5).

3.4.1. Cell redox homeostasis

Plants being sessile organisms have evolved defence mechanisms which allow them to adapt and survive in times of drought stress. One of the major consequences of drought stress is increased production of reactive oxygen species (ROS) in different cellular compartments. In plants a major hydrogen peroxide detoxifying system is the ascorbate-glutathione cycle, in which, ascorbate peroxidase (APX) enzymes play a key role catalysing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor (Caverzan et al., 2012).

Increased ROS levels (such as H_2O_2) have been reported in plants exposed to osmotic stress, thereby matching the demand for the co-substrate of peroxidases (Miller et al., 2010). Peroxidases 3-like was found to be induced in the tolerant genotype (LOC101499357; FC 2↑) but repressed in the sensitive genotype (LOC101499357; FC -2.1↓). A study related to cell wall peroxidases in tomato plants confirmed that under stress conditions cell wall-associated peroxidase plays an important role in the biochemical inhibition of leaf and fruit growth (Bacon et al., 1997). Another enzyme which helps in cell detoxification, L-ascorbate oxidase homolog was found to be less repressed in the tolerant genotype (LOC101515454; FC -8.1↓). Overall, the activation of cell detoxification machinery in the tolerant genotype suggests that the gene products play a key role in drought tolerance and cell growth in tolerant chickpea genotype.

Mitochondria have uncoupling proteins (UCPs) that uncouple electron transport from ATP synthesis. There is evidence that UCPs play a role in alleviating stress caused by ROS overproduction. However, direct evidence is still lacking regarding their role in abiotic stress protection (Begcy et al., 2011). Mitochondrial uncoupling protein 5-like was shown to be induced in the tolerant genotype (LOC101493136; FC 2.5↑) whereas it was down-regulated in the sensitive genotype (LOC101493136; FC -2.8↓). Overexpression of UCP from *Arabidopsis* was analysed in the transgenic Tobacco plant, which resulted in reduced accumulation of hydrogen peroxide in stressed leaves compared to the wild-type (Begcy et al., 2011). Further, the plants showed a better response to drought. The manipulation of UCP expression in mitochondria is a new avenue for crop improvement and may lead to the generation of crops with higher drought tolerance.

3.4.2. Cell wall modification and remodelling

The plant cell wall responses to abiotic stress differ between the plant species, the genotype, the age of the plant, the timing and

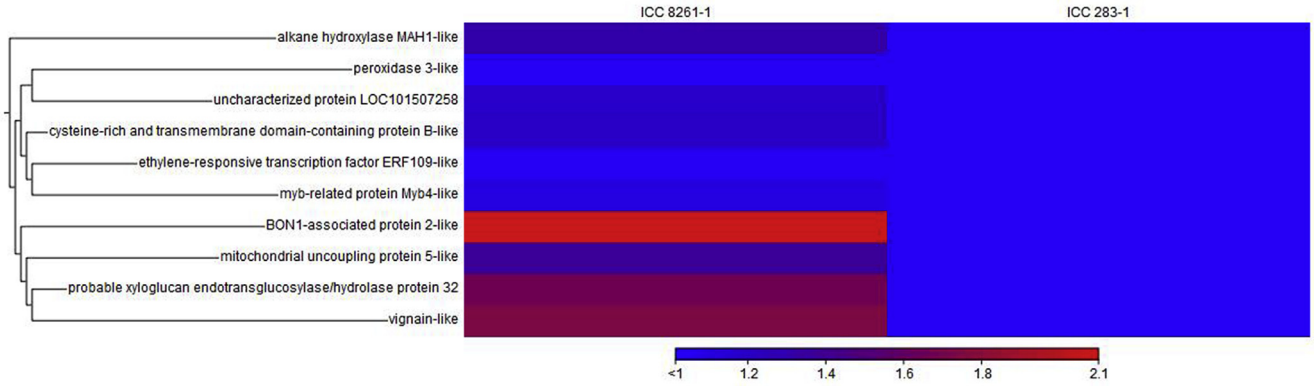

Fig. 5. Heat map displaying hierarchical clustering of genes which were up-regulated in the tolerant genotype (ICC8261) and down-regulated (ICC283) in sensitive genotype based upon respective log2FC values. Scale bar represents the log2 fold change values for respective genotypes.

intensity of stress application (Gall et al., 2015). This makes it harder to identify the pattern of stress response in plant cell walls. The key players in differential cell synthesis and remodelling under drought stress are the formation of ROS and peroxidases. In addition, xyloglucan modifying enzymes and expansins help in cell wall loosening which allows the growth of stressed organs (Tenhaken, 2015). Xyloglucan is a major hemicellulose molecule, which can strengthen the rigidity of the cell wall by forming a skeletal network with cellulose fibrils (Han et al., 2015). Probable xyloglucan endotransglycosylase/hydrolase protein 32 was significantly up-regulated in the tolerant genotype (LOC101508375; FC 3.1↑) while down-regulated in the sensitive genotype (LOC101508375; FC -2.9↓). Cho et al. (2006) reported that the hot pepper xyloglucan endo-trans-gluco-sylase/hydrolase (CaXTH3) gene which was induced via drought stress enhanced the plant's tolerance to drought and high salinity in transgenic Arabidopsis plants (Cho et al., 2006). Later, the same gene was expressed in tomato, where authors suggested an important role of the XTH activity in remodelling the cell wall of stomata possibly by preventing excess water loss (Choi et al., 2011). In addition, rice plants exposed to abiotic stress (cold, heat, drought) showed a strong increase in transcripts for *OsXET9*, a xyloglucan modifying enzyme that might serve as a general stress marker gene (Jiali, 2011).

Expansins are cell wall loosening proteins which permit the micro fibril matrix network to slide in growing plant cell walls, therefore enabling the plant wall to expand and grow (Han et al., 2012). Expansin-B3 was less repressed in the tolerant genotype (LOC101497655; FC -2.8↓) and down-regulated in the sensitive genotype (LOC101497655; FC -9.8↓). In *Arabidopsis* the expression of expansin B3 was repressed by PFTI (MEDIATOR25/PHYTOCHROME AND FLOWERING TIME 1) in hsr81 (High response sugar 8-1 mutant). The difference in expression levels in both genotypes suggests that expansins helped in restructuring cell wall in tolerant genotype which helped in maintaining growth at state steady.

3.4.3. Photosynthesis

One of the crucial effects of drought is the reduction in photosynthesis which arises by a decrease in leaf expansion, damaged photosynthetic system and leaf senescence. Previous studies suggest that the role of drought-induced stomatal closure, which leads to the limitation of CO_2 uptake by leaves, is significant in drought stress tolerance (Pang et al., 2017). The first response of plants to water deficit is preventing transpiration from leaves by closing stomata.

Photosynthesis-related gene coding for β-carbonic anhydrase 5, chloroplastic (LOC101501758; FC 2.2↑) was only induced in the tolerant genotype. Carbonic anhydrase is a zinc-containing metalloenzyme, and the specific association between carbonic anhydrase and RuBisCO enables CO_2 to interact with RuBisCO and maintains the functional machinery of RuBisCO (Das et al., 2016). The results suggest that carbonic anhydrase may have a role in drought stress response in the tolerant chickpea.

3.4.4. Negative regulation of stomatal complex development

Drought stress activates several signalling cascades, regulates gene expression and promotes the biosynthesis of several proteins in leaves. Stomata play a crucial role in water retention and movement in leaves in response to environmental conditions. Very few studies have been conducted on the role played by water deficit in stomatal development in legumes. Leaves that developed under water deficit conditions have been reported to exhibit lower stomatal indices than leaves that were developed under well-watered conditions (Hamanishi et al., 2012). Recently, Tripathi et al. suggested that drought-stressed soybean leaves showed reduced mRNA levels of stomatal development genes. In addition, leaves which were formed after drought stress experienced a reduction in stomatal density of 22.34% and stomatal index of 17.56% (Tripathi et al., 2016).

In the present study, different expression levels of stomatal development genes in both genotypes were observed; two different isoforms of subtilisin-like protease were significantly repressed in only the tolerant genotype SBT1.2 isoform X1 (LOC101504577; FC -2.2↓), subtilisin-like protease SBT1.7 (LOC101493089; FC - 2.6↓). Subtilisin-like protease SBT1.2 is a serene protease involved in the negative regulation of stomatal density and distribution (Berger and Altmann, 2000). Interestingly, SBT3.3 (LOC101497314; FC -3.1↓) and SBT4.14 (LOC101511407; FC -3.1↓) were only repressed in the sensitive genotype. The differences in expression levels of these proteases under stress condition make them an interesting candidate for further research to elucidate their individual role in the plant response to drought stress.

3.4.5. Effects of drought response on phytohormone signalling

Plant growth hormones are substances that effect the growth and development of plants. Phytohormones are multidisciplinary and extensive interplay occurs between all phytohormones, resulting in maintenance of plant growth, other metabolic processes and developing drought stress responses in plants (Wani et al., 2016).

3.4.5.1. Brassinosteroid signalling. Brassinosteroids are plant steroid hormones that play a significant role in plant growth and development. Although their role in plant development is well established, it is unclear which growth traits can be simultaneously influenced by BR and how these growth traits can be related to abiotic stress (Sahni et al., 2016). Few studies have been conducted on the mechanism of BR in conferring drought tolerance in chickpea. Regulation of brassinosteroid mediated signalling pathway (GO: 1900457) was among the top significantly enriched GO terms in the tolerant genotype. BRI1 a brassinosteroid LRR receptor kinase was differentially down-regulated in the two genotypes. Studies have

suggested that BRI1, is a ubiquitously expressed leucine rich-repeat receptor that plays a role in the BR signalling pathway through serine/threonine phosphorylation (Friedrichsen et al., 2000). Here, the serine-threonine kinase gene PBL5 was only induced in the tolerant genotype (LOC101497114; FC 2.8↑). The differential down-regulation of transcript related to the BR pathway is a clear indication that BR pathways were regulated differently in both genotypes.

3.4.5.2. Abscisic acid (ABA). Abscisic acid (ABA) plays a crucial role in plant response to drought stress as its function is to control stomatal closure to minimise the water loss from plants (de Ollas and Dodd, 2016).

Four of the 14 members of PYRABACTIN-LIKE genes were differentially expressed in our study, PYL8 (LOC101505121; FC 3.0↑) was up-regulated in the tolerant genotype and PYL2 (LOC101510806: FC -3.4↓) was repressed in the sensitive genotype, whereas, PYL1 was repressed less in the tolerant genotype (LOC101500611; FC -3.4↓) than in the in sensitive genotype (LOC101500611; FC -6.964405↓). The function of some of the ABA receptors remains unknown. However, a study confirmed the role of the ABA receptor Pyrabactin resistance-like protein 8 (Pyl8) in dehydration conditions. Overexpression of Pyl8 increased ABA sensitivity in guard cells, leading to reduced water loss under drought stress condition. PYL1 was also involved in stomatal closure and inhibition of germination (Miyazono et al., 2009). Up-regulation of PYL8 in the tolerant genotype suggests that the drought defence mechanism only occurs in the tolerant genotype. The elaborative hormone signalling networks in plants and their ability to crosstalk make phytohormones ideal candidates for mediating drought responses (Verma et al., 2016). ABA accumulates upon occurrence of osmotic stresses because expression levels of several ABA biosynthesis genes, such as ZEAXANTHIN EPOXIDASE gene (*ZEP*; also known as LOS6 (LOW EXPRESSION OF OSMOTIC STRESS-RESPONSIVE *gene 6]/* ABA1) are up-regulated by salt and drought stress (see review by (Zhu, 2002); here we observed that ZEP was induced only in the tolerant genotype (LOC101492490; FC 3.2.

3.4.5.3. Jasmonic acid. Synthesis of Jasmonic acid (JA) occurs via the octadecanoid pathway under biotic and abiotic stress conditions, associated with resistance to abiotic stress and wounding. As JA is accumulated during drought stress and has a regulatory role in stomatal closure, JA along with ABA is proposed to play an important role in stomatal closure during drought stress (de Ollas and Dodd, 2016).

In this study genes for JA signalling pathway were differentially expressed in both genotypes with more genes up-regulated in the tolerant compared to the sensitive genotype. One isoform, LOX21 lipoxygenase 2-1 was highly induced in the tolerant genotype (LOC101499921; FC 8.0↑) and the other isoform repressed less in the tolerant genotype (LOC101492220; FC -3.2↓). Further, AOS3 Allene oxide cyclase was highly induced in the tolerant genotype (LOC101512113; FC 8.1↑) compared to the sensitive genotype (LOC101512113; FC 3.4↑). Other genes, PLDA (LOC101495559); (LOC101506781), AOS (LOC101496543), LOX14 (LOC101502001) were specifically down-regulated in the sensitive genotype. A study of the roots of tolerant and sensitive chickpea genotypes demonstrated that the drought-tolerant genotype reacts to drought with sustained and early activation of a specific lipoxygenase (MtLOX1), an allene oxide synthase (MtAOS), hydroperoxidase lyases and oxophytodienoate (De Domenico et al., 2012). The up-regulation of these genes in the tolerant variety suggests a role for jasmonates in early signalling of drought stress, thereby playing a significant role in drought tolerance mechanism in chickpeas.

3.4.5.4. Auxins and gibberellins. Auxins are synthesised by tryptophan-dependent and tryptophan independent pathways. Auxins are multifunctional phytohormones, which have a role in plant growth and development. Additionally, auxins function in mediating and governing plant growth under drought stress (Kazan, 2013). We found that auxin responsive IAA8 (LOC101495287; FC -2.2↓) and genes coding for Auxin transporter AUX3 (LOC101505554; FC -2.0↓) and AUX2 (LOC101505743; FC -2.0↓) were also repressed in the sensitive genotype. In contrast, predicted auxin-binding protein: ABP19a-like was less repressed in the tolerant genotype (LOC101493286; FC -9.3↓). ABP function as an auxin receptor, which is involved in many plant development processes but very little is known about their function in drought response and tolerance (Wang et al., 2016).

Gibberellins are tetracyclic diterpenoid carboxylic acids, which have a positive effect on seed germination, stem elongation, flower and fruit development and leaf expansion (Yamaguchi, 2008). GID1 (LOC101501795; FC 2.1↑) gibberellin receptor and CIGR1 (LOC101491796; FC 2.0↑) were specifically up-regulated in the tolerant genotype. The GA signal is perceived by the GA receptor GID1 (for GA INSENSITIVE DWARF1), which is a soluble protein localised in both cytoplasm and nucleus. DELLA proteins are nuclear transcriptional regulators which have a function in repressing GA signalling and restricting plant growth, presumably by causing transcriptional reprogramming (Murase et al., 2008). The binding of GA to GID1 enhances the interaction between GID1 and DELLA, resulting in rapid degradation of DELLAs via the ubiquitin-proteasome pathway (Sun, 2010). GAI (DELLA GAI) gibberellic acid insensitive (LOC101507839; FC -3.1↓) and GAOX2 gibberellin 20 oxidase 2 (LOC101492441; FC -3.4↓) was specifically down-regulated in sensitive genotype. Gibberellin (GA) 2-oxidases play an important role in the GA catabolic pathway through 2β-hydroxylation. A study in rice and *Arabidopsis* plants revealed that plants overexpressing *OsGA2ox5* were more resistant to high-salinity stress than wild-type plants. These results suggest that *OsGA2ox5* plays an important role in GAs homoeostasis, development, gravity responses and stress tolerance in rice (Shan et al., 2014). The results of this current study suggest that down-regulation of these genes may have the reverse impact on the growth of sensitive plant under drought stress.

3.4.6. Transcriptional factors

Plant drought responses are regulated by multiple signalling pathways that activate a series of transcription factors. It is well known that drought stress regulation in chickpeas is modulated by many genes, including transcription factors (TFs) that help plants withstand highly unfavourable conditions such as drought stress. The signalling pathways of drought stress in plants consist of several proteins such as enzymes, proteins, molecular chaperones, secondary metabolites and most importantly transcriptional factors. Differential expression of transcription factors belonging to AREB/ABF, AP2/ERF, NAC and ZIP families were observed, which have been studied before in drought stress.

3.4.6.1. NAC. The NAC family of transcription factors is the largest and specific to plants TFs. They mainly comprise of a highly conserved DNA binding NAC domain in the N-terminal and a variable C-terminal transcriptional regulation domain (Puranik et al.). Corresponding genes for NAC were seen differentially expressed in both genotypes.

An increased number of NAC transcription factor transcripts were differentially expressed in the tolerant genotype compared to the sensitive genotype. NAC29 was exclusively induced in the tolerant genotype (LOC101506671; FC 5.2↑); (LOC101506671; FC 4.9↑). Interestingly, another gene, probably coding for NAC29 (LOC101514169; FC -5.6↓) was exclusively down-regulated only in the sensitive genotype. TaNAC29 transcription factor from wheat has been reported to enhance salt tolerance in wheat (Xu et al., 2015) and both drought and salt tolerance in *Arabidopsis* plant (Huang et al., 2015). Up-regulation of NAC29 isoforms in the tolerant genotype suggests their role in drought tolerance through regulating drought responsive signalling pathways.

3.4.6.2. AP2/ERF. The AP2/ERF ethylene response element binding factors family covers the large group of plant-specific transcriptional factors. They are characterised by the presence of a conserved AP2/ERF DNA-binding domain, which further binds to the GCC box (DNA sequence which has the role in ethylene responsive transcription) (Rashid et al., 2012; Song et al., 2013). ERFs are subdivided into two main groups in *Arabidopsis*: dehydration-responsive element binding proteins (DREBs) and the ERFs ethylene response factors.

In this study, most of the ethylene response transcription factors were up-regulated in the tolerant ICC8261 genotype. ERF109 was induced in the tolerant genotype (LOC101499805; FC 4.2↑) but highly repressed in the sensitive genotype (LOC101499805; FC -6.7↓). it has been reported that ERF109 have roles in mediating crosstalk between jasmonic acid and auxin biosynthesis (Cai et al., 2014), retardation of programmed cell death and improved salt tolerance (Bahieldin et al., 2016). ERF16 was significantly induced in the tolerant genotype (LOC1014941882; FC 6.1↑). Generally, ERF18 transcript genes (LOC101496910; FC 5.6↑), (LOC101496258; FC 5.6↑) and (LOC101496587; FC 3.7↑) were significantly up-regulated only in the tolerant genotype. In addition (LOC101498533; FC -1.07↓) AP2L1, (LOC101501959; FC -2.6↓) PLET2 and (LOC101497871; FC -2.042024↓) were down-regulated in the tolerant genotype only. ERF80, ERF71, ERF4 and ERF80 exhibited differential expression while genes (LOC101501427; FC 3.4↑), (LOC101523681; FC 2.8↑), (LOC101504527; FC 3.0↑) and (LOC101502158; FC 3.4↑) were up-regulated in the sensitive genotype only. The up-regulation of ERF109 in the tolerant genotype suggests that the tolerant genotype has a well-established mechanism for drought tolerance compared to the sensitive chickpea genotype.

3.4.6.3. MYB/MYC. Previous studies have provided evidence that MYB and MYC transcription factors also regulate gene expression in response to ABA under drought stress. MYB TFs are one of the plant's transcription factor families have a characteristic MYB domain in their DNA binding region (Lindemose et al., 2013). In *Arabidopsis,* 51% of MYB genes were up-regulated and 41% of genes were down-regulated under drought stress (Baldoni et al., 2015). The myb-related protein Myb4-like was highly induced in the tolerant genotype (LOC101501566: FC 2.1↑) whereas it was repressed in the (LOC101501566; FC -2.3↓) sensitive genotype. It has been reported previously that MYB4 has induced drought tolerance in transgenic *Arabidopsis* plant (Mattana et al., 2005).

MYB48 (LOC101507623: FC 4↑), MY1R1 (LOC101502280; FC 2.4↑), MYB39 (LOC1011495806; FC 5.2↑) and PHL9 (LOC101512896; FC 3.4↑) were up-regulated in only the tolerant genotype, while LHY (LOC101500635; FC -2.4↓), MYB26 (LOC101493467; FC -2.8↓), AMYB (LOC101498026; FC -3.7↓) and MYB86 (LOC101497837; FC -3.4↓) were down-regulated in the sensitive genotype; MYB06 (LOC101498747; FC 12.1↓) was up-regulated only in the sensitive genotype. In addition, MYC2 (LOC101508717; FC -2.8↓), RD21B (LOC101488968; FC -4.5↓) and RD21 (LOC101500447; FC -2.0↓) were specifically down-regulated in the sensitive genotype.

3.4.6.4. WRKY. WRKY transcription factors are among the largest transcriptional regulator family in plants. They have many diverse biological functions in plant biotic stress management, abiotic stress responses, cell senescence, embryogenesis, development and hormone controlled processes. In addition, they act as both regulators and repressors of signalling networking in plants (Banerjee and Roychoudhury, 2015; Eulgem and Somssich, 2007; Rushton et al., 2010). WRKY TFs: WRKY18, WRKY40 and WRKY60 function as regulators of ABA signalling in seed germination and post-germination growth (Chen et al., 2010; Shang et al., 2010). WRKY40 has inhibited the expression of the important ABA response genes ABF4, AB14, AB15, DREBIA, MYB2 and RAB18 by binding directly to the W-box Cis-acting element of these promoters (Chen et al., 2010; Shang et al., 2010). WRKY40 (LOC101512877; FC -2.1↓), WRK70 (LOC101500302; FC -5.7↓), WRK41 (LOC101489116; FC -2.4↑), WRKY3 (LOC101496076; FC -2.0↓), WRK15 (LOC101496569; FC -8.8↓), WRK35 (LOC101501992; FC -3.5↓), WRK18 (LOC101512217; FC -2.5↓) were all down-regulated in response to drought in the sensitive genotype. In contrast WRKY24 (LOC101503578; FC 4.0↑: LOC101511198; FC 2.2↑) and WRK23 (LOC101495104; FC 2.0↑) were up-regulated in the tolerant genotype. This result again confirms that a drought response mechanism is not active in the sensitive genotype. Chen et al., proposed that WRKY TFs not only have the ability to modulate gene expression in response to plant stress but also in the plant defence mechanism as they form a highly interacting regulatory network (Chen et al., 2010).

3.4.7. Regulation of genes for functional proteins in drought tolerance

3.4.7.1. LEA (late embryogenesis abundant) proteins. The LEA late embryogenesis abundant proteins have been found to be expressed in a wide range of plant species in response to water deficit resulting from abiotic stresses. Late embryogenesis abundant protein 2 is a water-soluble protein synthesised in desiccation tolerant plants in large amounts (Battaglia and Covarrubias, 2013). In this study, we observed that LEA2 genes were more induced in tolerant (LOC101504724; FC 27.2↑), compared to sensitive genotypes (LOC101504724; FC 8.5↑). However, LEA14 desiccation protectant LEA 14 homolog (LOC101498243; FC 2.6↑) was up-regulated only in the tolerant genotype. Dehydrin, a type of LEA protein has been shown to improve plant growth under stress by reducing the harmful effect of ROS-reactive oxygen species (Ahmad et al., 2016). DHN1 was more induced in tolerant (LOC101504337; FC 33.3↑) compared to sensitive genotypes (LOC101504337; FC 31.3↑). Very few studies have been conducted on the role of LEA in legumes, but available data suggests their role in drought response (Battaglia and Covarrubias, 2013). The results of a study on the carLEA4 gene of chickpea showed that carLEA4 encodes for a protein LEA group 4, which may be involved in various plant development and abiotic stress responses (Gu et al., 2012). Also, the transcripts of this gene were detected in many organs of chickpea.

3.5. Aquaporins

Aquaporins belong to a highly conserved family of major intrinsic membrane proteins. They have the ability to facilitate and maintain cellular water homoeostasis (Zargar et al., 2017) although the mechanisms involved require further elucidation. Most of the TIP1/NIP/PIP4 gene transcripts were down-regulated in our study with different fold change between drought tolerant and sensitive genotypes. NIP61 in was more repressed in tolerant (LOC101497446; FC 2.4↓) compared to sensitive genotype (LOC101497446; FC - 4.2↓). In addition, NIP12 was also less repressed in tolerant (LOC101490316; FC -3.0↓) compared to in sensitive (LOC101490316; FC -5.1↓). However, TIP1 was only repressed in the sensitive genotype (LOC101508956; FC -3.2↓).

The drought-induced expression pattern of aquaporin genes was determined using RT-PCR in two plasma membrane genes PvPIP4 and PvPIP2 and two tonoplast intrinsic proteins PvTIP1 and PvTIP4 in the leaves of common bean. Interestingly, the expression level of genes was down-regulated under stress but, the down-regulation of PvPIP2 and PvTIP4 during the drought was cultivar specific; these genes were more down-regulated in the tolerant cultivar after rehydration (Zupin et al., 2017), suggesting that the ability of plants to conserve water during stress conditions involves timely and down-regulation of specific aquaporins. The down-regulation of these genes at different levels in both genotypes makes them interesting candidates for further understanding their underlying mechanism in chickpea genotypes.

3.5.1. Lipid biosynthesis pathways

Under drought stress different plant defence mechanisms are activated, one of which is involved in fatty acid biosynthesis. Plant cell

membranes are major targets of abiotic stress. As lipids are one of the essential components of cell membrane, changes in their composition can help maintain cell wall integrity in response to drought stress. *Arabidopsis* leaf membranes showed resistance to water deficit, by maintaining their polar lipid contents and the stability of their lipid composition under severe drought conditions (Gigon et al., 2004). Gene ontology studies in chickpea under drought and salinity stress showed significant enriched GO terms related to lipid biosynthesis and ion transport (Garg et al., 2016). Genome-wide transcriptional analysis of old physic nut roots and leaves showed genes related to unsaturated fatty acid biosynthesis were down-regulated and polyunsaturated fatty acids were significantly reduced in leaves 7 days after withholding irrigation (Zhang et al., 2014). Studies conducted on these stress-inducible lipid species have demonstrated that each represented lipid class has specific biological relevance, biosynthetic mechanisms and signalling cascades, which activate defence reactions at the transcriptional level. In addition, to their roles in signalling, lipids also function as stress mitigators to reduce the intensity of different stress factors (Hou et al., 2016; Okazaki and Saito, 2014). In this study it was observed that Go terms related to membrane lipid biosynthetic pathways (GO: 00464667) and sphingolipid metabolic process (GO: 0006665) were significantly enriched in the tolerant genotype. DES1L sphingolipid delta desaturase-like was less repressed in tolerant (LOC101512972; FC -2.2↓) compared to sensitive (LOC101512972; FC -4.3↓). Other genes related to lipid biosynthesis were only suppressed in the sensitive genotype; alkaline ceramidase 3 (LOC101490839; FC -2.1↓), delta (8)-fatty-acid desaturase-like (LOC101502873; FC -2.5↓) and fatty acid 2-hydroxylase 2 (LOC101501399; FC -2.5↓). These results suggest that lipid-mediated defence helps chickpea to resist water stress. We observed that MGDG, the main thylakoid membrane lipid was down-regulated (LOC101515157; FC -2.0↓) in sensitive compared to tolerant genotypes. Drought-induced changes in monogalactosyldiacylglycerol (MGDG) concentration in the chloroplast envelope and in thylakoid membranes in cowpea leafs *(Vigna unguiculata*) was thought to stabilise and maintain lamellar bilayer structure and thus the function of chloroplasts under drought stress (Torres-Franklin et al., 2007). Furthermore, changes in MGDG in the drought-tolerant resurrection plant *Craterostigma plantagineum* during desiccation were reported to contribute to membrane stabilisation and the maintenance of photosynthetic energy supply (Gasulla et al., 2013). Similarly, up-regulation of MGDG was observed in the leaves of tolerant sorghum genotype under drought stress conditions compared to the sensitive genotype (Fracasso et al., 2016).

3.5.2. Cuticle wax biosynthesis

Aerial parts of plants are covered with cuticular waxes which inhibit non-stomatal water loss and gaseous exchange. These are generally composed of long chain fatty acids and their derivatives such as alkanes, aldehydes, primary and secondary alcohols and wax esters (Lee and Suh, 2013). Alkane hydroxylase 1 (MAH1) plays a role in cuticular wax biosynthesis, catalysing the hydroxylation of alkanes into secondary alcohols and playing a role in the oxidation of secondary alcohols into ketones (Greer et al., 2007). In this study it was observed that the alkane hydroxylase was induced in the tolerant genotype (LOC101495087; FC 2.4↑). It has been reported previously in *Arabidopsis* that mid-chain alkane hydroxylases were up-regulated under drought conditions (Seo et al., 2011). In this study the up-regulation of alkane hydroxylase in tolerant genotype may lead to the synthesis of ketones which have been previously reported to play a role in drought tolerance in wheat through changes in cuticle permeability and wax synthesis (Zhang et al., 2013).

3.5.3. Drought defence mechanism

BON1-associated protein 2-like is a negative regulator of cell death and defence responses and it also exhibits calcium-dependent phospholipid binding properties (Yang et al., 2006). BON1-associated protein 2-like transcript was significantly induced in the (LOC101502816; FC 4.2↑) tolerant genotype whereas, it was highly repressed in the (LOC101502816; FC -2.4↓) sensitive genotype. A study in arabidopsis proposed that the BON/CPN family regulates cell death associated with defence responses by repressing a number of R (Disease resistance) genes (Yang et al., 2006). Up-regulation of this gene in the tolerant genotype may help in maintaining cell size and controlled cell death under drought conditions. BON1 is reported to be required for maintaining cellular homeostasis and cell size regulation by cell wall remodelling under external constraints (Jambunathan, 2003; Yang et al., 2006).

Vignain is a cysteine endopeptidase belonging to the papain family of endopeptidases which exhibits low reactivity towards iodoacetate. It acts as an endopeptidase which is involved in mobilising storage proteins in response to water deprivation (Li et al., 2016). Vignain like was highly induced in the tolerant genotype (LOC101497939; FC 3.3↑) and repressed in the sensitive genotype (LOC101497939; FC -2.4↓). Here, up-regulation of Vignain like gene in the tolerant genotype suggests a role in drought tolerance in chickpea, perhaps by the degradation of storage proteins for use in plant growth, as reported for other legumes (Brzin and Kidrič, 1996).

In summary, RNA sequencing was employed to identify drought-responsive genes in chickpea leaf tissue of two genotypes ICC8261 and ICC283 to identify biological, cellular and molecular pathways related to drought tolerance under drought stress. The significant difference observed between the two genotypes in shoot apical meristem leaves indicates how drought stresses affect the different mechanisms in both genotypes. In this study the emphasis was on how the regulation of transcription factors and phytohormone signalling transcripts were influenced by drought stress in the two genotypes. We confirm that sensitive genotype responded more to drought compared to the tolerant genotype, perhaps indicating that the tolerant genotype is less susceptible to water deficit. It is possible that the tolerant genotype, ICC 8261 can be used as a genetic donor to further improve the drought-tolerant traits of the chickpea germplasm. This study will be instrumental in understanding the putative role of genes involved in drought stress response in chickpea. Future investigations should use more robust experimental approaches to identify the individual role of these genes, their mechanisms, pathways involved and application in a field study for promising drought-responsive genes.

Conflicts of interest

The authors declared that they have no competing interest.

Author's contributions

NM conceived the experiments, helped with analysis and writing the manuscript. SB performed the experiments, analysis and writing. PK helped with stress treatment and analysis. AB helped with analysis and manuscript writing.

Acknowledgements

Authors thank Vijay Bhaskarla for helping with cultivation of plants and sequencing.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.06.007.

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Chapter 3

Reduced representation bisulphite sequencing of two chickpea genotypes to identify differential DNA methylation patterns associated with drought tolerance and sensitivity

Reduced representation bisulphite sequencing of two chickpea genotypes to identify differential DNA methylation patterns associated with drought tolerance and sensitivity

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Abstract

Chickpea (*Cicer arietinum* L.) is an important cool season food legume which is consumed worldwide due to its high nutritional value. DNA methylation is an epigenetic modification which is passed at a cellular level without any change of DNA sequence. This study aimed to obtain a clear picture of the drought mechanism involved in two contrasting chickpea genotypes in terms of their drought tolerance. The leaf tissues from the shoot apical meristem of drought-sensitive and drought-tolerant genotypes were used for Reduced representation bisulphite sequencing (RRBS) under drought stress. The results showed a more significant number of differentially methylated regions and bases in the sensitive genotype compared to the tolerant genotype. The hypermethylation in the upstream/promoter region in the sensitive genotype correlated to downregulation of gene expression. In contrast, in the tolerant genotype, hypomethylation in the upstream/promoter region was associated with upregulation of gene expression. Gene ontologies (GO) terms related to cellular metabolic processes, biosynthetic processes, oxidation-reduction processes, regulation of gene expression, regulation of transcription, DNA-template, oxidation-reduction processes, signal transduction, lipid metabolic processes and intracellular signal transduction were enriched in both genotypes. This is the first study to analyse Differentially methylated cytosines (DMCs) and Differentially methylated regions (DMRS) associated with Differentially expressed genes (DEGs) in these

two essential chickpea genotypes. The difference in the DMRs and genes related to the DMRs in contrasting genotypes is an indication of the association of DNA methylation with gene expression under drought stress.

Keywords: Chickpea, Reduced representation bisulphite sequencing (RRBS), Drought stress, Differentially methylated regions (DMRs), Differentially methylated cytosines (DMCs), DNA methylation, Abiotic stress

3.1. Introduction

Epigenetic modifications represent a process whereby an organism can alter the expression of specific genes in response to environmental stress. Among all other epigenetic modification's DNA methylation is most prevalent (Zhang et al., 2018) . DNA methylation is well known for altering the gene expression pattern in such a way that cells can recall their cell type by removing the necessity for continuous external signalling or stimulation. Even more importantly, DNA methylation modification's is a trait possessed throughout the cell-cycle and thus inherited through cell division (Moore et al., 2013). It is referred to as the incorporation of a methyl (CH_3) group to the DNA strand; in most instances, this addition occurs at the fifth carbon atom of a cytosine ring. DNA methyltransferases (DNMTs) are responsible for catalyzing the conversion of cytosine bases to 5-methylcytosine (Goll and Bestor, 2005). In *Arabidopsis* Methylation of CG cytosine is preserved by METHYLTRANSFERASE 1 (MET1). Whereas, CHG methylation maintenance is catalyzed by CHROMETHYLASE 3 (CMT3) DNA methyltransferase and to a much lesser degree by CMT2. Lastly, Methylation of CHH is maintained via DRM2 or CMT2 (Zhang et al., 2018). These reformed cytosine residues are usually found side by side to a guanine base (CpG methylation) and result in two methylated cytosines positioned diagonally to each other on opposite strands of DNA. However, DNA-methylation manifests itself in two distinct forms, hypermethylation (an

increase in methylation) compared to normal tissue and hypomethylation (a loss/reduction of methylation). Plants being sessile are more susceptible to changing environmental conditions compared to other organisms. DNA methylation can be eliminated by active demethylation process, which is triggered by DNA demethylases due to the failure in maintaining the methylation after DNA replication (Penterman et al., 2007; Zhang et al, 2018). The occurrences of these divergent epigenetic modifications help them cope with adverse environmental conditions (Fei et al., 2017; Karan et al., 2012; Liu et al., 2018; Wang et al., 2014; Xu et al., 2015). Recent advances in high-throughput technologies such as next-generation sequencing (NGS) enable genome-wide DNA methylation prediction studies. The outcome of these studies has provided novel insights into the functional consequences of variation in DNA methylation in many plant species. The different approaches used to study methylation are methylation-sensitive amplified polymorphism (MSAP) (Dong et al., 2006; Shan et al., 2013), methylated DNA immunoprecipitation sequencing (Wang et al., 2016), and bisulphite sequencing (Garg et al., 2015).

A study of the root tips of pea suggested that water stress-induced cytosine hypermethylation in the pea genome and activities related to methylation and demethylation in control and stressed plants were consigned to specific DNA sequences (Labra et al., 2002). Further, Wang et al. (2011) proposed that induction of epigenetic changes in the genome of rice could be seen as a very effective regulatory mechanism for the adaptation of rice plants to drought and other environmental stresses (Wang et al., 2011). A study carried out in the rice germplasm also suggested that specific epigenetic changes may play an essential role in identifying and regulating salt stress in the expression network of a cascade of genes involved in tolerance mechanisms (Karan et al., 2012). Diverse DNA methylation patterns were associated with gene expression in rice cultivars in response to drought and salinity. The results provided insight into the interplay between DNA methylation and gene expression in rice

cultivars (Garg et al., 2015). Recently, a DNA methylation study in cold-tolerant and cold-susceptible genotype suggested that DNA methylation were higher compared to demethylation in the tolerant genotype (Rakei et al., 2016). A study in rice showed that DMRs associated changes in DNA methylation in genotypes were gene-specific (Wang et al., 2016). Previous recent studies in legumes such as soybean (Chilcoat et al., 2017), mungbean (Kang et al., 2017a) and chickpea (Bhatia et al., 2018) provided evidence of a significant relationship between DNA methylation and gene expression under controlled conditions. However, there remains a requirement to comprehensively understand the role of these epigenetic modifications in abiotic stress tolerance/sensitivity in leguminous crop plants. Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid leguminous crop with eight chromosomes (2n=16) belonging to the Fabaceae family. Chickpea is a vital winter crop which is grown worldwide due to its high nutritional value and role in crop rotation (Varshney et al., 2013). One of the significant abiotic constraints in chickpea production worldwide is drought stress (Jha et al., 2014). DNA methylation studies have been carried out in other plants species rather than chickpea; there is much yet to uncover in this legume crop. Critically, we remain unaware of the relationship between gene regulation and differential methylation patterns under drought stress in these two chickpea genotypes: ICC8261 (Drought tolerant) and ICC283 (Drought-sensitive).

RRBS is a sequencing technique which leads to the generation of single base resolution DNA methylation (5-methyl) information covering the genomic sample of interest. By analyzing a reduced representation of the particular genome, the amount of sequencing required is efficiently reduced relative to whole-genome bisulphite sequencing, as only the specific targets will be formed, based on the particular restriction enzyme used in the sequencing process (Meissner et al., 2005). The RRBS method was firstly proposed in 2005 and was actively adopted by many researchers to analyse DNA methylation in plants. RRBS has

become a widely used method for detecting DNA methylation because of its high single-base resolution and acceptable cost. Currently, RRBS is considered a useful technology for the analysis of epigenetic profiles of many plant crops.

In this study, DNA methylation patterns which potentially regulate drought tolerance/sensitivity of two contrasting genotypes were analysed. Chickpea leaf tissues from the shoot apical meristem were used for RRBS analysis using Illumina Hiseq3000. The main objective of this study was to evaluate whether drought-sensitive genotype has different differential methylation patterns compared to drought-tolerant genotype under drought. Furthermore, to assess whether drought will lead to hypermethylation or hypomethylation of drought-related genes. Finally, to analyse the relationship between drought-related hypomethylation/hypermethylation and gene expression. The study will help to find the cross-link between DNA methylation and gene expression in these contrasting genotypes. This will assist in utilizing molecular and biological approaches to make more drought-tolerant chickpea varieties.

3.2. Materials and Methods

3.2.1 Chickpea cultivation and genomic DNA isolation

The drought-sensitive (ICC283) and the drought-tolerant (ICC8261) chickpea genotype were used in this study for RRBS under drought stress. Both chickpea genotypes vary in root density and deep root system. ICC8261 is kabuli type chickpea and its origin is Turkey. Whereas, ICC283 is a Desi type chickpea originated in India. Experimental plants were grown in 20 cm polypropylene pots containing 4 kg of soil (Potting soil and sand) under controlled environmental conditions with air temperature regulated between 23˚C and 28˚C (night/day). The experimental design was a 3 X 2 X 2 wholly randomized block design with leaf tissue from one-time point (shoot apical meristem), two genotypes (ICC8261 and ICC283), and two

treatment conditions (control/stress). For each genotype, six pots were assigned and randomly designated to one of two treatments as well-watered (WW) control and drought-stressed (DS). The drought stress and plant growth conditions were as previously described (Badhan et al., 2018). The moisture field capacity of soils for control samples was 80% and 20% for drought. Chickpea leaf tissues from the shoot apical meristem developmental stage for both ICC8261 and ICC283 genotypes were harvested. SAM stage is key organizing centre of stem cells and controls most developmental traits contributing to seed yield in crop plants. For each sample three biological replicates were collected for further studies. The genomic DNA for respective samples was isolated from frozen leaf tissues using Qiagen DNeasy Minikit (Qiagen). Quality check and quantification of genomic DNA were performed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific) and Qubit Fluorimeter (Life Technologies). Agarose gel electrophoresis was also used to determine the quality of the respective genomic DNA samples.

3.2.2 Bisulphite conversion and Reduced Representation bisulphite sequencing

The genomic DNA isolated from chickpea leaf tissue at shoot apical meristem stage was further processed for RRBS. To perform bisulphite sequencing, Ovation® RRBS Methyl-Seq Library System with Msp1 enzyme restriction digestion was used according to the manufacturer's protocol. This approach utilizes the methylation insensitive restriction enzyme Msp1, which recognizes the CCGG site. Due to partial fragmentation during bisulfite conversion, PCR and efficiency of cluster generation, only a subset of these fragments, typically under 300 bp in length are sequenced. The Ovation® RRBS Methyl-Seq Library System requires 100 ng of DNA for RRBS. The respective genomic DNA samples for both genotypes were fragmented via sonication a size of approximately 100-300 bp, which were further end-repaired and TruSeq-methylated adapters were ligated to the DNA fragments. For bisulphite conversion of unmethylated C's EZ DNA Methylation-GoldTM kit (Zymo Research

Corporation, CA, USA), approximately 500 ng of adapter-ligated DNA fragments were used according to the manufacturer's protocol. Library quality was analysed after desalting, size selection and PCR amplification. The qualified bisulphite-converted libraries were sequenced on the HiSeq 3000 system (Illumina Inc) for 150 cycles in the paired-end mode to achieve 3X genome coverage for each sample and further processed after removal of reads containing adaptor sequences and low-quality reads.

3.2.3 Quality control read alignment and identification of methylated cytosine's

The sequencing reads were trimmed prior to alignment to remove adaptor sequences, low-quality reads and diversity bases using Trim Galore (Martin, 2011). It uses Cutadapt and FastQC to reliably apply consistency and adapter trimming to FastQ files, with some extra features for RRBS MspI-digested libraries. Here, the stringent parameters used for the quality control were -q 30 --phred33 --rrbs --non_directional --paired. The quality-filtered 150 base pair paired-end reads from each sample were mapped to the chickpea genome *Cicer arietinum* L (assembly ASM33114v1) (Varshney et al., 2013) using Bismark v0.20.1 (Krueger and Andrews, 2011). The output mapping files were operated by bismark_methylation_extractor to extract the methylation call for every single C analysed in both genotypes in test and control samples. This generated the methylation call in all three contexts CpG, CHG and CHH, cytosine report for whole-genome methylation, biography and Mbias reports. To obtain more reliable results and to correct methylation counts, the sequencing reads from all samples were mapped to the chickpea chloroplast genome. This was further used to calculate the error rate of the bisulphite sequencing conversion method. The error rate was applied to calculate the true methylation status for both genotypes using a binomial test. The R package, methylkit v1.7.8 (Akalin et al., 2012) process BismarkAln function was used to obtain methylation percentage per base from sorted bam files of both genotypes respectively in all the contexts.

3.2.4 Identification and distribution of differentially methylated regions in ICC8261 and ICC283 genotypes

The R package, methylkit v1.7.8 was utilized to identify DMRs and DMCs. Apart from the identification of DMRs, we also investigated differential DNA methylation at base level in both genotypes, which provided DMCs in both genotypes using Methylkit. The reads covered by ≥ 5 were considered for further analysis. The tile MethylCounts function was used to summarize methylated and unmethylated base counts over the tilling window bin size of 1000 and size step of 1000 across the chickpea genome to perform analysis of differentially methylated regions. To calculate the differential methylation, calculateDiffMeth function in Methylkit was used. Fisher's exact test was used on each set to calculate the P-values. Further, P-values were adjusted to Q-values using SLIM method. After q-value calculation, the differentially methylated regions/bases based on q-value and percent methylation difference cutoffs were selected as hypermethylated or hypomethylated the bases with q-value <0.01 and percent methylation difference more massive than 25%. Pearl script was used to annotate differentially methylated regions in the respective genotypes. The DMRs were annotated with gene-body/Inside, upstream/Promoter (5 kb above the coding gene) and downstream (2 kb flanking region to the gene) to obtain hypermethylated and hypomethylated regions in all three sequence contexts.

3.2.5 Gene ontology enrichment analysis

GO enrichment analysis of the DMRs related genes for hypermethylated and hypomethylated DMRs associated genes in ICC8261, and ICC283 genotypes were performed. GO terms which were significantly enriched in tolerant and sensitive genotype were identified. The hypermethylated and hypomethylated genes were mapped to Uniprot using batch ID retrieval to retrieve all the GO terms. Subsequently, the p-value for differential methylation

was matched to corresponding genes. This was provided as input to Revigo to extract all GO enrichment.

3.3. Results

3.3.1 DNA methylation patterns in contrasting genotypes

Two chickpea genotypes, drought-tolerant (ICC8261) and drought-sensitive (ICC283) with contrasting response and adaptability to drought were used for RRBS, to analyse different DNA methylation patterns associated with drought tolerance/sensitivity. The quality trimming and grooming of sequenced reads (Paired-end 150 bp) were performed using TrimGalore to remove adaptor sequences. High quality filtered reads following quality assurance were used for mapping. The reads were mapped to the chickpea genome *Cicer arietinum* L. (assembly ASM33114v1) (Varshney et al., 2013) using Bismark_v0.20.1 which produced approximately 69 million, 150 base pairs reads from ICC8261 and approximately 97 million reads from the ICC283 genotype. After deduplication, duplicate reads created due to PCR bias were removed, and a mapping efficiency of 40 to 45% was achieved. A total of approximately 14 million reads in ICC8261 and 19 million reads in ICC283 were uniquely mapped to the chickpea genome. These unique best hits were subsequently used for obtaining methylation calls in different samples. The raw reads were also mapped to the chloroplast genome to calculate the frequency of methylated cytosines and error rate.

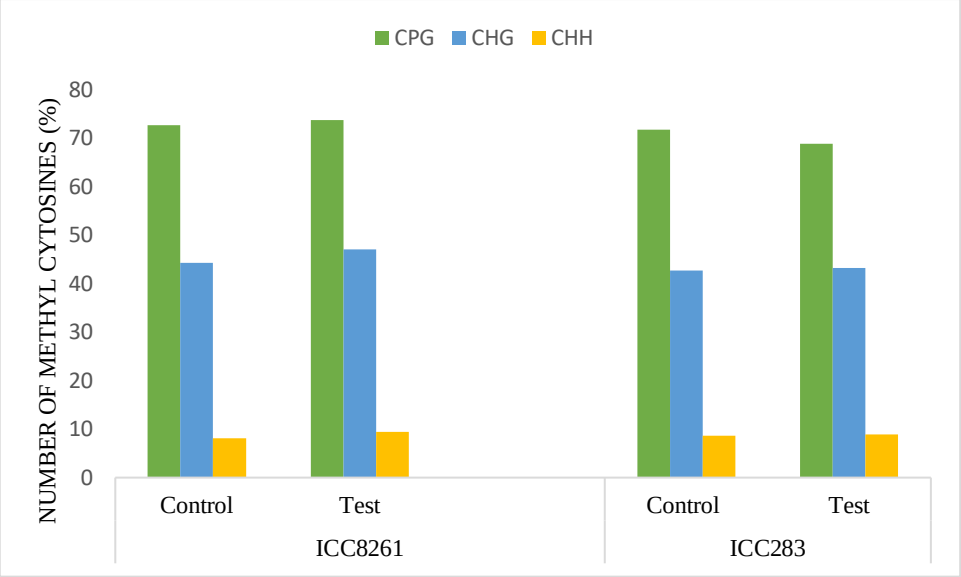


Figure 1: Percentage of methylcytosines in CpG, CHG and CHH (where H correspond to A, T or C) sequence context in ICC8261 and ICC283 genotypes, control and test samples.

The distribution of methylcytosines in ICC8261 control samples was highest in the CpG (72%) context, followed by CHG (44.2%) and CHH (8.1%) context (Figure 1). A similar pattern of methylcytosines in different sequence context was observed in test samples and the ICC283 genotype, with the highest number of methylated cytosines in the CpG context, followed by CHG and CHH. A table highlighting the methylome data obtained from RRBS for the number of methylated cytosines present at different sites in control and treated samples for respective genotypes are provided (Appendix chapter 3: Supplementary data 1).

3.3.2 Differential DNA methylation at the base level

Apart from the identification of differentially methylated regions, we looked for base-level methylation differences between the control and treated samples using methylkit, resulting in the generation of methylation statistics per base level defined as DMCs (Figure 2). Higher numbers of differentially methylated bases were identified in drought-sensitive genotype compared to the tolerant genotype. The sequence context of methylation was more

elevated in CpG, followed by CHH and CHG; however, a more significant number of bases were hypermethylated in ICC283 compared to hypomethylated bases. A similar pattern was observed in ICC8261 with lesser number of bases in total. In addition, the CHH context showed a significant number of hypermethylated bases in both genotypes compared to hypomethylated bases.

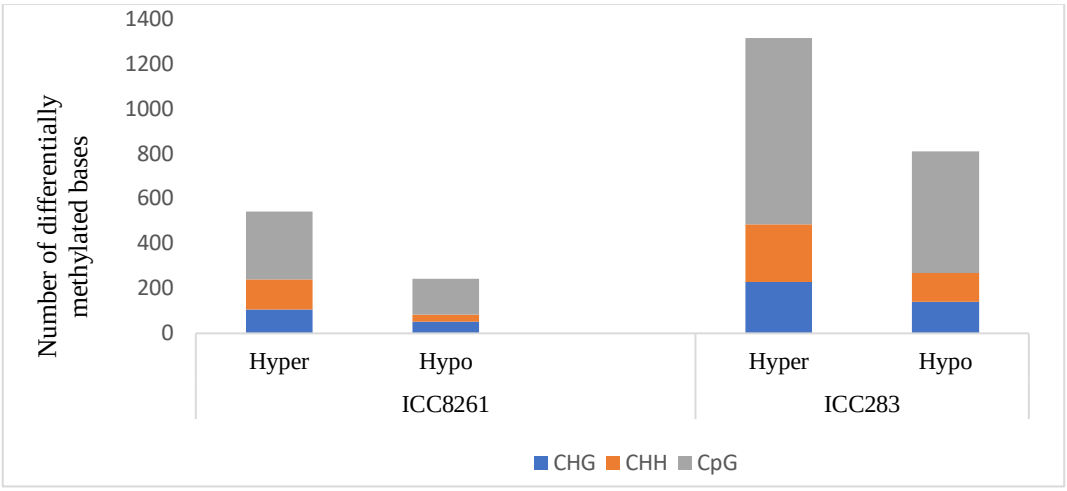


Figure 2: Number of differentially methylated bases in ICC8261 and ICC283 under drought stress. Hypermethylated and hypomethylated bases in different methylation contexts: CpG, CHG and CHH.

3.3.3 Identification of differentially methylated regions in the tolerant and sensitive genotype under drought stress

Methylkit was used to determine the number of DMCs and DMRs in the two contrasting chickpea genotypes. To determine the number of hypermethylated and hypomethylated DMRs under drought stress in both genotypes, the Fisher exact test with SLIM window approach was used with q-value ≤ 0.01. It was observed that the number of DMRs were higher in ICC283 compared to ICC8261 (Figure 3).

In ICC8261, a total of 278 hyper DMRs were identified from which 170 were differentially methylated in CpG context followed by 90 in CHG and 18 in CHH context. In comparison, 196 DMRs were hypomethylated with 114 (CpG), 46 (CHG) and 6 (CHH)

methylations. In contrast, in ICC283 a more significant number of 786 DMRs were hypermethylated, 465 (CpG), 151 (CHG) and 20 (CHH) compared with 423 hypomethylated regions in ICC8261, with 321 (CpG), 90 (CHG) and 12 (CHH) methylations (Figure 3).

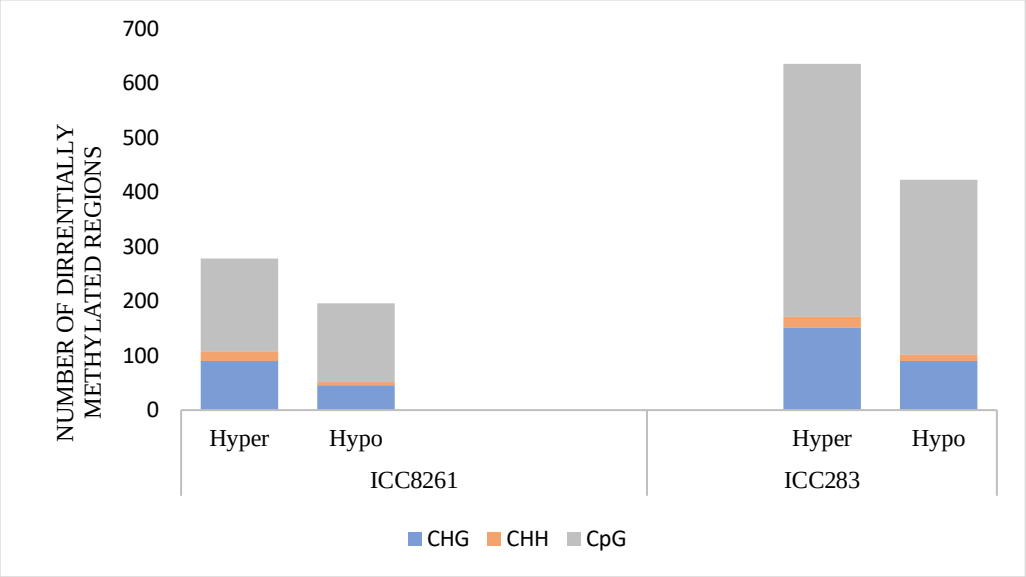
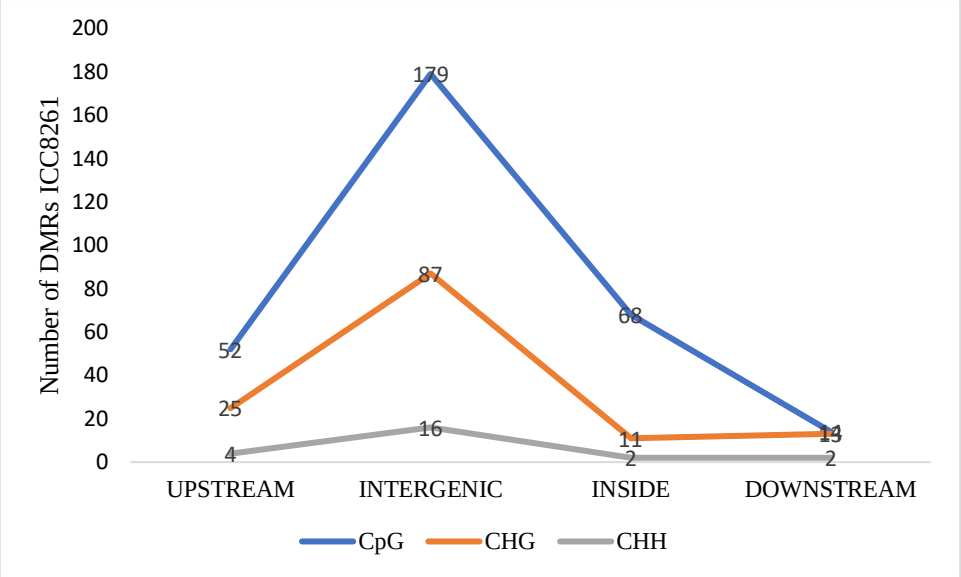
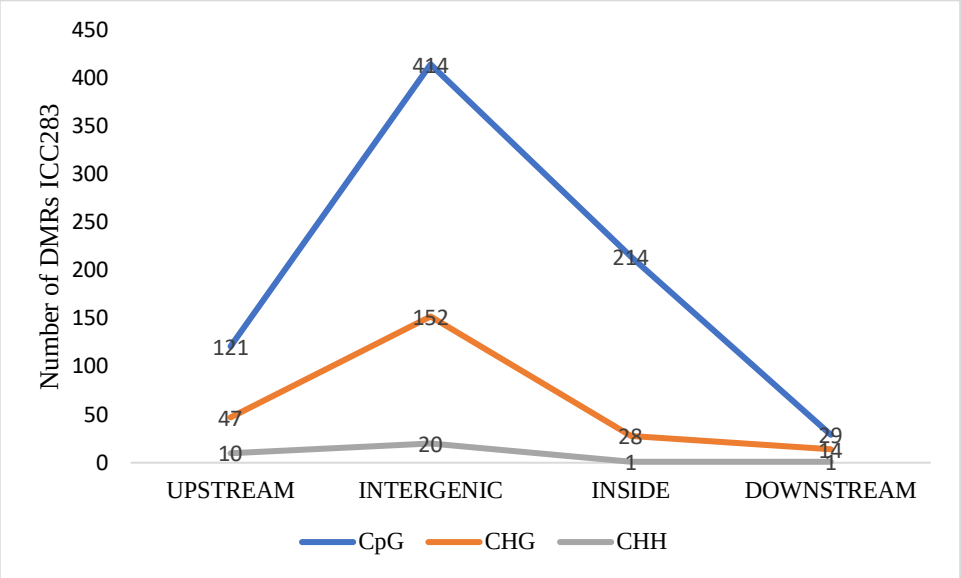


Figure 3: Number of Hyper- and Hypo-methylated regions in all context in ICC8261 and ICC283.

The presence of DMRs was relatively higher in upstream (5kb upstream of the gene) and intergenic regions near/inside the gene-body compared to downstream (2kb downstream of the gene) of the gene-body in both genotypes. This number was most generous in the CpG sequence context followed by the CHG and CHH contexts (Figure 4). Figure 5a shows the hypermethylated and hypomethylated regions in the different contexts (CpG, CHH and CHG) in both genotypes. It was observed that 445 DMRs were exclusively hypermethylated in the sensitive genotype whereas 126 DMRs were hypermethylated in the tolerant genotype. In comparison, 310 DMRs were hypomethylated in sensitive genotype versus 95 in the tolerant.



a)

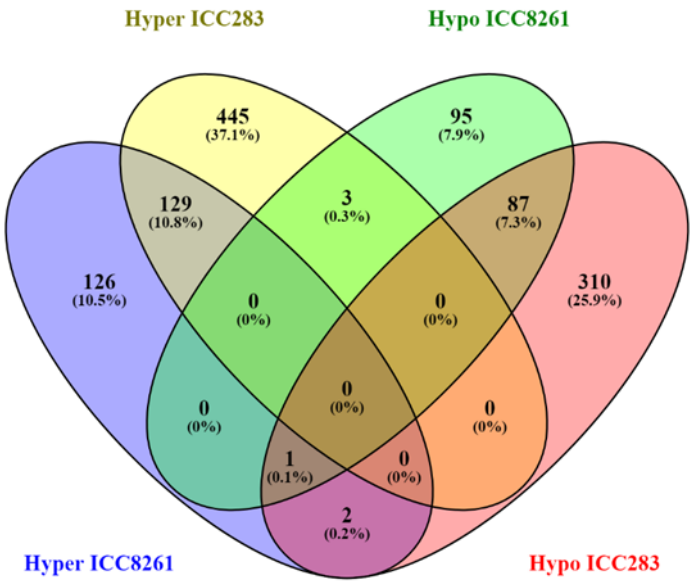


b)

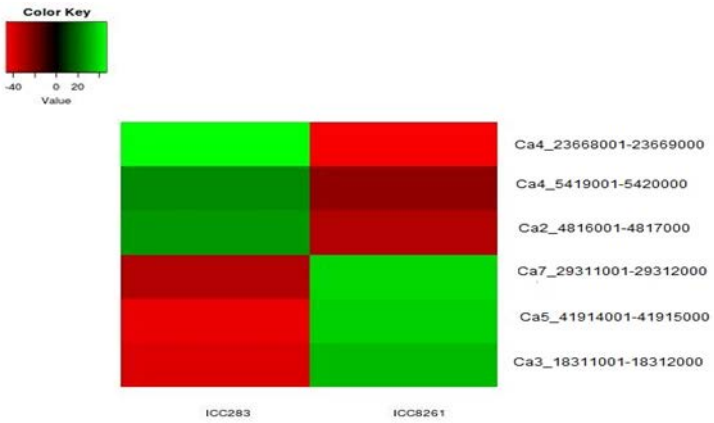
Figure 4: Number of differentially methylated regions falling within upstream, intergenic, inside/gene-body and downstream in different methylation context in ICC8261**(a)** and ICC283 genotype **(b)**.

Further, 87 DMRs were commonly hypomethylated in both genotypes, whereas 129 were commonly hypermethylated. Six different DMRs which were differentially hypermethylated or hypomethylated are shown in the heat map (Figure 5b). The three hypomethylated regions

which were differentially methylated in the CpG sequence context were Ca3_18311001-18312000 (intergenic region), Ca5_41914001-41915000 annotated as acetylserotonin O-methyltransferase-like falling in the promoter region was hypomethylated in ICC8261 and hypermethylated in ICC283. As previously reported, the unmethylated region in the promoter allows gene expression (Bartels et al., 2018). The role of acetylserotonin O-methyltransferase-like has been studied previously in the synthesis of melatonin. Melatonin function has been defined very well in many crop plants previously in the context of protecting plants from salt and drought stresses. A study in soybean showed increased melatonin helped in the upregulation of genes related to abiotic stress tolerance and hence alleviated the inhibitory effects of stress (Wei et al., 2015).



a)



ICC283 **ICC8261**

b)

Figure 5: a) Venn diagram showing differentially hypermethylated and hypomethylated regions in both genotypes in all contexts. **b)** Heatmap showing DMRs which were hypermethylated in ICC8261 and hypomethylated in ICC283 genotype under drought stress.

Further, Ca4_5419001-5420000 (receptor-like cytosolic serine/threonine-protein kinase RBK2), with a role in protein phosphorylation was hypermethylated in the promoter region of ICC8261 and hypomethylated in ICC283 in CpG context. The extracellular stress signal in plants is mostly performed by the receptors present on the cell wall and cell membranes. The hypermethylation of this region in the promoter of the tolerant genotype and hypomethylation in the sensitive genotype may be related to its role in drought tolerance as the hypomethylation of this receptor does not allow for immediate abiotic stress response in the sensitive genotype. In addition, Ca4_23668001-23669000 and Ca2_4816001-4817000 intergenic regions were also hypermethylated in ICC8261 and hypomethylated in ICC283.

In this study, the extent of differential methylation and pattern of occurrence of these methylations were distinct in both genotypes. The top hypomethylated DMRs related genes identified in upstream region in the tolerant genotype were related to plant hormones, oxidative stress and transcription factors. These were auxin-responsive, protein SAUR72 (LOC101490217), trafficking protein particle complex subunit 4%2C transcript variant X1

(LOC101491504), chromatin modification-related protein MEAF6-like (LOC101501790), protoporphyrinogen oxidase chloroplastic transcript variant X1 (LOC101514774), putative B3 domain-containing protein At1g78640 (LOC101505348), uncharacterized LOC101506858 transcript variant X1 (LOC101506858), receptor-like kinase TMK4 (LOC101497374) and probable nucleoredoxin 2 (LOC101498505).

In comparison, top hypermethylated regions in the tolerant genotype were in downstream region of NEDD8 ultimate buster 1 (LOC101490332) and zinc finger-containing ubiquitin peptidase 1-like transcript variant X1 (LOC101506150). Top hypermethylated and hypomethylated DMR-associated genes in both genotypes are provided in (Supplementary data 4).

In the sensitive genotype, uncharacterized gene (LOC101493876), sulfoquinovosidase-like (LOC101503817), coenzyme Q-binding protein COQ10 homolog mitochondrial (LOC101515676), uncharacterized gene (LOC101488675), chaperone protein dnaJ transcript variant X1 (LOC101512023) and probable protein phosphatase transcript variant X1 (LOC101505781) were the top hypomethylated DMRs within the gene body.. Also, probable methionine-tRNA ligase (LOC101492339), putative B3 domain-containing protein At1g78640 (LOC101505348), 40S ribosomal protein S13-like (LOC101492487), small nucleolar RNA Z122 (LOC113785206) and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit transcript variant X1 (LOC101513965) were the top upstream/promoter region hypomethylated DMRs associated genes in the sensitive genotype. The small nucleolar RNA Z122 has been identified to have a regulatory role under drought stress in plants by stabilizing rRNAs and splicing small nuclear RNAs. Among the top hypermethylated DMR- associated genes in upstream were ankyrin repeat domain-containing protein 13C-like (LOC101512834), RNA polymerase II C-terminal domain phosphatase-like 2%2C transcript variant X1 (LOC101507570), protein TIC chloroplastic (LOC101493321), uncharacterized protein

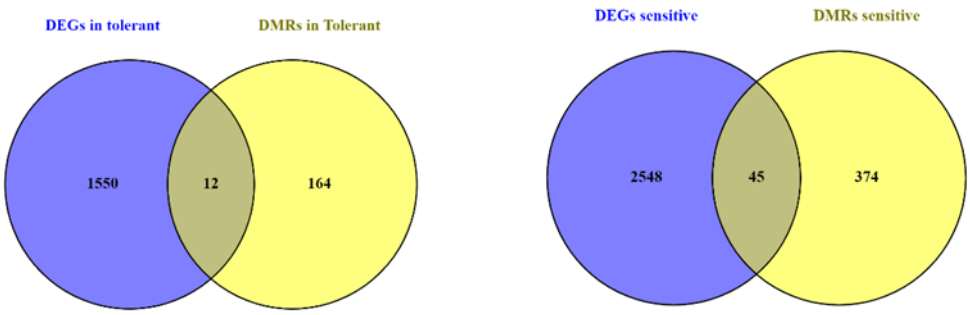
(LOC113787620), actin-interacting protein 1-2 (LOC101490046), homeobox-leucine zipper protein HAT9-like (LOC101491148), DELLA protein RGL2-like (LOC101494454) and AP2-like ethylene-responsive transcription factor At1g7970 (LOC101488375).

3.3.4 GO analysis of DMRs associated with gene

To analyse the gene ontologies or GO terms in relation to hypermethylated and hypomethylated DMRs in both genotypes GO analysis was conducted. The hypermethylated and hypomethylated genes were mapped to Uniprot using the batch ID to retrieve all the GO terms. After that, the p-value for differential methylation was matched to corresponding genes. This was provided as input to Revigo to get all GO enrichment. It was noticed that GO terms related to biological processes such as cellular metabolic process (GO:0044237), biosynthetic process (GO:0009058), oxidation-reduction process (GO:0055114), regulation of gene expression (GO:0010468), regulation of transcription, DNA-templated (GO:0006355) were highly enriched in the sensitive genotype. In contrast in the tolerant genotype oxidation-reduction process (GO:0055114), regulation of transcription, DNA-templated (GO:0006355), signal transduction (GO:0007165), translation (GO:0006412), carbohydrate metabolic process (GO:0005975), lipid metabolic process (GO:0006629) and intracellular signal transduction (GO:0035556) were among the top biological enriched GO terms (Appendix chapter 3: Supplementary data 3). The abundance of the GO terms related to oxidation-reduction process, regulation of transcription and intracellular signal transduction in ICC8261 is a clear indication of the presence of active machinery for methylation modification in the tolerant genotype to tolerate/adapt to drought stress.

3.3.5 Comparison of DMRs with differential gene expression under drought stress

To analyse the association of DMRs with differential expression of genes under drought stress, data from our previous study (Badhan et al., 2018) were compared with this study. The tissue samples for both studies were harvested at the same time from the same experiment and can, therefore, be directly compared. To obtain a clear picture of association of DMRs in different context (CpG, CHH and CHG) and position in the gene body or flanking regions with gene expression, we investigated the common methylated DMRs associated with the gene body, upstream /downstream region and compared their expression levels. The DNA methylation has a diverse effect on the gene expression of specific gene sets in both the contrasting genotypes. In ICC8261, 12 common DEGs with differential methylation were observed, whereas, in ICC283, 45 common genes were identified between DEGs and associated DMRs (Figure 6). The expression values and methylation percentage are provided in (Appendix chapter :Supplementary data 4).



a) DEGs in ICC8261 DMRs in ICC8261 b) DEGs in ICC283 DMRs in ICC283

Figure 6: Venn diagram showing common differentially expressed genes in the tolerant and differentially methylated regions associated with genes in the upstream, gene body and downstream regions in contrasting **a)** ICC8261 (tolerant) and **b)** ICC283 (sensitive) genotype.

3.4. Discussion

Recent studies conducted in legumes have shown that the DNA methylation plays a crucial role in the regulation of abiotic stress response (Bhatia et al., 2018; Yaish et al., 2018b). Here, in this study, the total amount of methylated C's obtained was higher in the CpG context in both ICC8261 and ICC283, followed by CHG and CHH (Appendix chapter 3: Supplementary data1). The main reason behind this may be that in RRBS, use of MSP1 enzyme produces a cut at CCGG site and captures methylation mainly at the CpG site in the genome. Similar patterns were observed in shotgun bisulphite sequencing in *Arabidopsis*, showing the highest methylation in the CpG context followed by CHG and CHH (Cokus et al., 2008). Similarly, genome-wide DNA methylation study in mung bean showed that genes that were differentially expressed between the two mung bean cultivars also displayed diverse DNA methylation patterns (Kang et al., 2017b). Further, methylation transformations that were recognized during the early peanut pod development stage provided insightful information for understanding the role of epigenetic regulation in peanut pod development (Wang et al., 2018). In context to the DMRs, it was observed that in the sensitive genotype, more DMRs were differentially methylated compared to tolerant genotype under drought stress. The highest number of DMRs was in a CpG context, followed by CHG and CHH context. A previous study showed definite methylation patterns between different cell types in response to heat stress in soybean root hair (Hossain et al., 2017). In ICC8261 acetylserotonin O-methyltransferase-like was hypomethylated, whereas it was hypermethylated in ICC283. As previously reported, the unmethylated region in the promoter allows gene expression (Bartels et al, 2018). The role of acetylserotonin O-methyltransferase-like has been studied previously in the synthesis of melatonin. Melatonin function has been defined very well in many crop plants previously in the context of protecting plants from salt and drought stresses. A study in soybean showed

increased melatonin helped in the upregulation of genes related to abiotic stress tolerance and hence alleviated the inhibitory effects of stress (Wei et al, 2015).

Genome-wide epigenetic changes play an important regulatory mechanism in terms of the response of fababean to drought and other abiotic stresses (Abid et al, 2017). Six DMRs were identified related to the drought response including lipoxygenase (LOX), calcium-dependent protein kinase (CDPK), ABC transporter family (ABC), glycosyl hydrolase (GH) and phosphoenolpyruvate carboxylase (PEPC) (Abid et al, 2017). A study in sesame under two abiotic stresses showed that DNA methylation was associated with an increase of differentially accumulated transcripts levels while induced de novo methylation was correlated with a decrease of differentially accumulated transcripts levels (Komivi et al, 2018).

DMRs related to plant hormone, oxidative stress and transcription factors were among the top hypermethylated in tolerant genotype. The auxin-responsive protein SAUR72 is well studied for its role in auxin transport and cell expansion during its expression in hypocotyls and stele of young roots (Qiu et al, 2013). Similarly, receptor-like kinase TMK4 helps in cell expansion and cell proliferation as an essential component of auxin pathway signalling. The gene expression of chromatin modification-related protein MEAF6-like has been reported to be repressed under water stress (Acevedo et al, 2016). Whereas, the hypermethylated in downstream region were NEDD8 ultimate buster 1 and zinc finger-containing ubiquitin peptidase 1-like transcript variant X1. The NEDD8 ultimate buster 1 long (NUB1L) is reported to suppress atypical NEDDylation and promotion of the degradation of misfolded proteins (Li et al, 2015). Recently, a study highlighted the role of NUB1L in NEDDylation and how it promotes nuclear protein aggregation to assist in the defence mechanism against proteotoxic stress (Maghames et al, 2018). Therefore, the hypermethylation of these genes could be related to drought tolerance in the tolerant genotype. In the upstream region, tRNA threonyl carbamoyl adenosine dehydratase 2-like (LOC101504123) known to have ubiquitin-like modifier enzyme

activity in catalysing small proteins, ubinuclein-1-like (LOC101501927), RNA polymerase II C-terminal domain phosphatase-like transcript variant X1 (LOC101507570), homeobox-leucine zipper protein HAT9-like (LOC101491148), and protein STICHEL-like transcript variant X1 (LOC101498627) were hypermethylated, and gene expression was downregulated (Ilgenfritz et al, 2003b; Wang et al, 2015). AP2-like ethylene-responsive transcription factor was also among the top hypermethylated. The role of these AP2 transcription factors is well studied in controlling plant developmental processes (Dietz et al, 2010). The lysM domain receptor-like kinase 3 has been reported to enhance abiotic stress tolerance by chitin induced signalling (Brotman et al, 2012).

In contrast for sensitive genotype the sulfoquinovosidase-like, coenzyme Q-binding protein COQ10 homolog mitochondrial, uncharacterized gene, chaperone protein dnaJ transcript variant X1 and probable protein phosphatase transcript variant X1 were the top hypomethylated DMRs within gene body. The chaperone protein dnaJ transcript variant X1 and other genes were found to be downregulated under water stress in peanuts (Akkasaeng et al, 2007; Zhang et al, 2017). Hypermethylation of DELLA protein RGL2-like (LOC101494454), a gibberellic acid regulator and AP2-like ethylene-responsive transcription factor At1g7970 (LOC101488375) in the stress-sensitive genotype may be associated with its drought sensitivity. In contrast, within the gene body, heavy metal-associated isoprenylated plant protein 47-like (LOC101491732), known for homeostasis, detoxification and abiotic stress responses (de Abreu-Neto et al, 2013) was hypermethylated. The protein FORGETTER 1-like transcript variant X1 (LOC101509353) plays a vital role in the regulation of transcription (Brzezinka et al, 2016). The uncharacterized LOC101495587 transcript variant X1 (LOC101495587) and RPM1-interacting protein 4-like (LOC101488549) related to plant immunity (Liu et al, 2009) were also among the top hypermethylated genes. Previous studies confirmed the existence of two DNA methylation mechanisms regulating gene expression in

both a positive and negative manner (Wan et al, 2015). Studies have shown that DNA methylation in the promoter region can repress transcription by inhibiting the binding of transcription activators or by promoting binding of repressors (Zhang et al, 2018). Connection of hypomethylation of promoter regions and upregulation of gene expression may be positively related to drought tolerance in the tolerant genotype. We observed that a smaller number of drought-induced DMRs were identified in the tolerant genotype compared to the sensitive genotype. This suggests that the tolerant genotype methylation pattern in the tolerant chickpea was more stable under stress conditions than the sensitive genotype.

A recent study identified a higher number of CG DMRs in wild chickpea and CHH DMRs in cultivated chickpea. The number of DMRs were relatively higher in upstream and genic region in comparison to downstream regions in both genotypes in this study. Interestingly, an increased number of hypermethylated DMRs were recognized in the promoter regions and hypomethylated DMRs in the genic regions of cultivated chickpea (Bhatia et al., 2018). In addition, a change in DNA methylation/demethylation patterns was considered significant factors in preserving cells against cold-induced oxidative stress in chickpea under cold stress (Rakei et al., 2016).

Moreover, a recent study in soybean focusing on the distinct continuous cropping comprehensive stress adaptability, documented increase in DNA methylation enzymes (Liang et al., 2019). It was noted that demethylation in CpG and CHG context was mainly present in gene regulatory regions. In *Medicago trancatula,* salinity stress caused plants to rebuild the landscape of the 5-methylcytosine nucleotide (5-mC) in the DNA over gene structures (Yaish et al., 2018a). The remodelling was observed to be different not only in the methylation context but also in gene regions.

The genes associated with differential methylation either in the promoter region, gene body or in the downstream region have been related to their potential function in plants in relation to drought tolerance or sensitivity in respective genotypes. The differential methylation patterns may lead to inhibition of transcription activators or repressors, thus leading to differential expression of genes. Usually, in plants, methylation in the promoter region is related to inhibition of gene transcription leading to failure of gene expression (Zhang et al., 2018). These genes are discussed in the following section to understand their role in drought tolerance or sensitivity in both genotypes.

3.4.1 Cell wall modification

The plant cell wall plays an essential role in plant structure but also acts as the first line of defence in response to both, abiotic and biotic stresses by remodelling (Houston et al., 2016). Members of the expansin and XTH gene families are observed to show differential expression under abiotic stress (Kaashyap et al., 2018; Tenhaken, 2015). The formin-like protein 20%2C transcript variant X1 (LOC101505448), located in the gene body was hypermethylated in the tolerant genotype, and its expression was downregulated. The actin cytoskeleton in plants is regulated by external stimuli; as formins belong to a class of actin nucleator they may be responsible for the plant growth response to abiotic stress (Yi et al., 2005). Downregulation of this gene may help in drought stress avoidance by controlling growth under stress. Xyloglucan endotransglucosylase/hydrolase enzymes are known to play a role in the reconstruction and modification of cell walls which helps in the extensibility of cell wall during growth (Kaashyap et al., 2018). In the sensitive genotype, probable xyloglucan endotransglucosylase/hydrolase protein 6 (LOC101498505) was hypomethylated in the downstream region and its expression was also downregulated. The role of xyloglucan endotransglucosylase/hydrolase has been established before in other plant species under abiotic stress (Cho et al., 2006; Zhu et al., 2007). Repression of this gene in the sensitive genotype may be responsible for drought sensitivity as

it was not able to undergo cell wall remodelling under stress conditions. In the tolerant genotype, glucan endo-1%2C3-beta-glucosidase 14 (LOC101494403) was distributed in the downstream region where it was hypomethylated, and its expression was also repressed. Gene encoding for different enzymes which play roles in cell wall component synthesis regulates differentially under stress conditions (Houston et al., 2016). Moreover, in the sensitive genotype, protein REDUCED WALL ACETYLATION 2%2C transcript variant X1(LOC101493129) was hypermethylated, and protein REDUCED WALL ACETYLATION 3 (LOC101496987) was hypomethylated, but gene expression for both was upregulated. It has been reported that *in vitro* acetyl groups tend to influence the sensitivity of pectin and xylans to enzymatic degradation, and therefore cell wall acetylation can act as a barrier to cell wall deconstruction (Tenhaken, 2015). Differential expression of these genes in the sensitive genotype may be correlated to a lack of flexibility towards cell wall modification and remodelling in response to drought stress. Similarly, protein KINESIN LIGHT CHAIN-RELATED 2 (LOC101503717) was also hypermethylated in the sensitive genotype, and its expression was downregulated. Plants with overexpression of the kinesin light chain-related gene, which is involved in microtubule movement together with a cytoskeletal role were observed with enhanced sensitivity to drought stress in *Arabidopsis* (Li et al., 2019).

The remorin 4.1-like (LOC101513770) was hypermethylated, and its expression was downregulated in the sensitive genotype. The function of remorin proteins has been identified earlier in plasmodesmatal conductance through interaction with actin. Remorins may oligomerize and act as scaffold proteins during early signalling events in potato (Jarsch and Ott, 2011). An increase in transcripts number was noted under stress conditions in mulberry, which associated remorin to signalling pathway transduction (Checker and Khurana, 2013). However, the downregulation of genes related to cell wall remodelling and construction in the

sensitive genotype and differential methylation in the gene body/flanking regions should be further investigated to understand their role and correlation to tolerance/sensitivity.

3.4.2 Cell redox homeostasis and detoxification

Stress condition always leads to cellular accumulation of reactive oxygen species in plants. Kneeshaw and others suggested that oxidoreductase nucleoredoxin protects antioxidant enzymes under oxidative stress (Kneeshaw et al., 2017). The probable nucleoredoxin 2 (LOC101498505) was upregulated in the tolerant genotype and was extremely hypomethylated in the upstream region whereas in the sensitive genotype it was hypomethylated, and gene expression was downregulated. The nucleoredoxin 2 isoform X is known for its function in cell redox homeostasis and intracellular signal transduction (Kneeshaw et al., 2017). The differential expression of this gene in both genotypes may be associated with drought tolerance of the tolerant genotype. Here, the effect of hypomethylation can be positively and negatively related to their role in expression regulation in both genotypes under stress condition.

In the sensitive genotype, aldehyde oxidase GLOX-like (LOC101511561) was hypermethylated, and its expression was downregulated. Plants produce toxic aldehydes naturally and in response to stress conditions. A study in *Arabidopsis* confirmed that aldehyde oxidase plays a vital role in delaying senescence by catalyzing aldehyde detoxification (Srivastava et al., 2017).

Further, probable serine/threonine-protein kinase PBL5 (LOC101497114) was hypomethylated in the upstream region, and its expression was upregulated in the tolerant genotype. In contrast, in the sensitive genotype, G-type lectin S-receptor-like serine/threonine-protein kinase At4g2729 (LOC101495047) was hypermethylated in the downstream region, and its expression was upregulated. Different types of protein kinases are well known for phosphorylation/dephosphorylation to mediate and regulate signal transduction pathways

under environmental stresses (Mizoguchi et al., 1996). Probable serine/threonine-protein kinase PBL5 have been previously identified to have a role in plant immunity in *Arabidopsis* (Rao et al., 2018); its upregulation may, therefore, be associated with drought tolerance.

3.4.3 Stomatal complex development

The signalling peptides of the epidermal patterning factor are responsible for stomatal development in plants. EPIDERMAL PATTERNING FACTOR-like protein 5 (LOC101489249) was hypermethylated in the intragenic region and upregulated in the tolerant genotype. The EPF has been reported to be involved in stomatal complex development, and its overexpression led to complete inhibition of stomatal development (Lu et al., 2019). Niwa et al. suggested that EPF5 represses stomatal development in leaves and cotyledons by inhibiting maintenance of meristemoid activity (Niwa et al., 2013). The upregulation of this gene in the tolerant genotype may provide an advantage to the tolerant genotype by helping to control the development of stomata under drought stress; smaller/fewer stomata may prevent water loss.

Stomatal development is regulated by basic helix loop helix transcription factors in *Arabidopsis*, which controls cell division and cell state transition to promote the formation of stomata (Lu et al., 2019). In plants, basic helix loop helix transcription factors have been known to play a vital role in many biological processes. In this study, the transcription factor bHLH68-like%2C transcript variant X1 (LOC101514717) was hypermethylated, and its expression was repressed in the sensitive genotype. A recent study in tomato suggested that by enhancing ROS scavenging system, increasing the osmotic potential and increased the accumulation of secondary metabolites was due to the overexpression of SlbHLH22 which improved tomato plant stress resistance (Waseem et al., 2019). Therefore, the sensitive genotype may be deprived due to hypermethylation and repression of this gene.

4.4.4 Phytohormone signalling

Plant hormones and growth regulators play a central role in stress signalling pathways. The adagio protein 3 (LOC101491262) was hypermethylated, and its expression was upregulated in tolerant genotype which can be associated with ABA signalling transduction. A soybean study suggested that adagio protein 3 may be responsible for the ABA signal transduction and removal of degraded proteins under abiotic stress (Yin et al., 2017). Adagio protein 3 is an essential component of the E3 ubiquitin ligase complex which is involved in the regulation of circadian clock-dependent processes which includes the transition to hypocotyl elongation, flowering time, cotyledons and leaf movement rhythms.

4.4.5 Cell regulation and proliferation

Plants undergo significant changes in cell growth and proliferation under drought stress. The lamin-like protein (LOC101503881) was hypomethylated in the upstream region and downregulated in the tolerant genotype. Plant lamins have been identified to have divergent functions in terms of regulating chromatin positioning (Hu et al., 2019). Lamin-like proteins are also known to negatively control plant immunity (Guo et al., 2017). The association of this protein was studied prior to cleavage *in vivo* and *in vitro* apoptosis during heat shock (Chen et al., 2000). This indicates that the downregulation of this gene may be related to drought tolerance in the tolerant genotype. Further, it can be proposed that downregulation of this protein in the tolerant genotype leads to alternative chromatin organization as part of the stress response.

The protein STICHEL-like (LOC101498627) was highly hypermethylated, and its expression was downregulated in the tolerant genotype. The function of this gene has been studied before in *Arabidopsis*, where it plays a significant role in the regulation of branch number of trichomes (Ilgenfritz et al., 2003a). This study proved that the STI gene is a regulator

of branch number rather than a requirement for branching in STI mutant plants. Differential expression of this gene in the tolerant genotype is an indication that tolerant genotype may be trying to regulate the growth of trichomes to avoid drought stress. However, another critical positive regulator of trichome development is transcription factor bHLH68-like%2C transcript variant X1 (LOC101514717) which was observed to be hypermethylated in upstream and expression was downregulated in sensitive genotype (Kasili et al., 2011). Previous studies have shown the role of trichomes in drought tolerance which can be further exploited to enhance water efficiency under stressed conditions (Galdon-Armero et al., 2018; Piritta et al., 2010). Further investigation of the relationship between the trichome density and lengths under drought stress is required to understand its complete role in drought tolerance and sensitivity.

The GDSL esterase/lipase (LOC101513587) was hypermethylated, and its expression was downregulated in the sensitive genotype. This protein has been identified to function in pollen development in Brassica and *Arabidopsis* (Dong et al., 2016; Lai et al., 2017). It has also been reported to affect the germination and early growth of the seedling. Since we analysed the shoot apical meristem tissues, the downregulation of this gene in the sensitive genotype may affect pollen development leading to drought sensitivity.

4.4.6 Starch and sucrose metabolism

Sucrose synthase 6-like (LOC101514095) was also hypomethylated, and its expression was downregulated in the tolerant genotype and sensitive genotype. The abundance of this protein increased in a drought-tolerant wheat variety when compared to the sensitive type under drought stress (Faghani et al., 2015). Sucrose synthase 6-like which was reported as a sucrose-cleaving enzyme that provides the UDP-glucose and fructose for different metabolic pathways was noticed to be differentially methylated in both genotypes under drought stress (Thalmann and Santelia, 2017). The role of sucrose synthase has been studied in soybean and chickpea

under salt and cold stress where its function was directly correlated to drought stress tolerance (Arrese-lgor et al., 1995). The importance of the sucrose metabolism in the tolerant compared to sensitive genotype has been studied before under drought stress in *Triticum aestivum* L. (Nemati et al., 2018). The expression of this gene was more downregulated in the sensitive genotype compared to the tolerant genotype in our study. It could be proposed that drought-related downregulation of sucrose synthase helps to enhance energy saving by slowing cell division and adjusting cellular metabolism due to insufficient production of UDP-glucose. Therefore, the downregulation of this gene maybe helpful for stress adjustment in both the genotypes by effectively utilizing energy.

4.4.7 Uncharacterized proteins

It was observed that many genes which were differentially methylated and differentially expressed are still uncharacterized proteins. The uncharacterized proteins LOC101491825 and LOC101506193 were hypomethylated, and their gene expression was upregulated in ICC8261. The hypomethylation of the uncharacterized protein LOC101491825 and uncharacterized protein LOC101506193 in the upstream/promoter region and upregulation of gene expression may be associated with the drought tolerance traits in the ICC8261 genotype. The uncharacterized protein LOC101510228 was hypermethylated in both tolerant genotype and the sensitive genotype. The expression was more downregulated in sensitive genotype compared to tolerant genotype. Further investigation of these genes can help to understand the role of methylation in gene expression regulation in these genotypes.

The abundance of the GO terms related to oxidation-reduction process, regulation of transcription and intracellular signal transduction in ICC8261 is a clear indication of the presence of active machinery for methylation modification in the tolerant genotype to tolerate/adapt to drought stress.

Nevertheless, in the sensitive genotype, more genes were hypermethylated, and expression levels were downregulated. Some genes which were hypomethylated were repressed. In contrast in the tolerant genotype hypomethylation in the upstream region may be related to an increase in expression, whereas hypermethylation may be associated to downregulation of gene expression. Further investigation of these differential methylation patterns of individual genes is required to understand this correlation.

The present study is the first comparative analysis of DNA methylation and its correlation with gene expression in two contrasting chickpea genotypes under drought stress. In this study, the RRBS genome methylation profile was used for the first time in two contrasting chickpea genotypes ICC8261 and ICC283 under drought stress. The outcome of the study produced informative datasets for both genotypes, which will contribute to future studies aimed at unmasking the role of these powerful epigenetic regulatory mechanisms involved in drought tolerance and sensitivity. RRBS has delivered genome-wide quantitative DNA methylation information at a single base resolution in this study, and this can be further exploited to uncover the basis of abiotic stress responses in chickpea. To elucidate the relationship between DNA methylation and gene regulation, further investigation of the DMRs and associated genes needs to be performed.

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Chapter 4

CRISPR/Cas9 mediated DNA-free editing of *4CL* and *RVE7* genes in chickpea protoplasts

First report of CRISPR/Cas9 mediated DNA-free editing of *4CL* and *RVE7* genes in chickpea protoplasts

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Abstract

The current genome editing system Clustered Regularly Interspaced Short Palindromic Repeats Cas9 (CRISPR/Cas9) has already confirmed its proficiency, adaptability and simplicity in several plant-based applications. Together with the availability of a vast amount of genome data and transcriptome data, CRISPR/Cas9 presents a massive opportunity for plant breeders and researchers. The successful delivery of ribonucleoprotein (RNPs), which are composed of Cas9 enzyme and a synthetically designed single guide RNA (sgRNA), are used in combination with various transformation methods or lately available novel nanoparticle-based delivery approaches, allows targeted mutagenesis in plants species. Even though this editing technique is limitless, it is still not have been employed in many plant species. Chickpea is the second most crucial winter grain crop cultivated worldwide; there are currently no reports on CRISPR/Cas9 gene editing in chickpea. Here, we selected the *4-coumarate ligase* (*4CL*) and *Reveille 7* (*RVE7*) genes, both associated with drought tolerance for CRISPR/Cas9 editing in chickpea protoplast. The *4CL* represents a key enzyme involved in phenylpropanoid metabolism in the lignin biosynthesis pathway. It regulates the accumulation of lignin under stress conditions in several plants. The *RVE7* is a MYB transcription factor which is part of regulating circadian rhythm in plants. The knockout of these selected genes in the chickpea protoplast using DNA-free CRISPR/Cas9 editing represents a novel approach for achieving

targeted mutagenesis in chickpea. Results showed high-efficiency editing was achieved for *RVE7* gene *in vivo* compared to the *4CL* gene. This study will help unravel the role of these genes under drought stress and understanding the complex drought stress mechanism pathways. This is the first study in chickpea protoplast utilizing CRISPR/Cas9 DNA free gene editing of drought tolerance associated genes.

4.1 Introduction

CRISPR/Cas9 based gene editing has revolutionized targeted gene editing in plants in recent years (Baek et al., 2016; Johansen et al., 2019; Kim et al., 2018; Liang et al., 2017; Lin et al., 2018; Malnoy et al., 2016; Murovec et al., 2018; Osakabe et al., 2018; Petersen et al., 2019; Woo et al., 2015). The CRISPR/Cas9 method of gene editing is based on a natural immune system used by bacteria to prevent infection by viruses (Mojica et al., 2000). However, recently this mechanism was used for genetically editing DNA of interest. CRISPR/Cas9 genome editing has broad application in crop improvement and can be used to develop designer genetically edited non-GM crops. A key focus of agriculture scientists is the implementation of this approach to plant breeding for the development of new varieties of crops with higher tolerance to environmental constraints (Khatodia et al., 2016; Noman et al., 2016). The most recent approach to genetic engineering is genome editing by programmable endonucleases. In CRISPR/Cas9 gene editing, Cas9 endonuclease enzyme is explicitly used for inducing double strand breaks in target genes of interest. However, to restore the damage by the double-strand break, the cellular DNA repair pathway then acts through non-homologous end joining (NHEJ) or homology-directed repair (HDR) systems. Insertions, deletions, base substitutions, and DNA recombination can occur in the repair mechanism process, leading to a frameshift of the sequence (Puchta, 2005; Puchta et al., 1996).

CRISPR/Cas9 gene-editing tools have been utilized for gene activation, repression, knockout, knockdown, repression and for altering epigenetic modifications in several plants

crops such as *Arabidopsis* (Feng et al., 2014), apple (Osakabe et al., 2018), citrus, carrot (Klimek-Chodacka et al., 2018), grape (Nakajima et al., 2017), tomato (Wang et al., 2019), rice (Zhang et al., 2014), sorghum (Liu et al., 2019), maize and soybean (Chilcoat et al., 2017), and wheat (Zhang et al., 2016). However, gene free editing has effectively been achieved in only a few crop varieties such as *Arabidopsis* (Gao et al., 2016), bread wheat (Liang et al., 2017), grapevine and apple (Malnoy et al., 2016). For example, CRISPR/Cas9 genome editing was used to identify abiotic stress response in *Arabidopsis* plants; the results suggested that OST2 (proton pump), a mutant allele obtained from editing, altered stomatal closing under environmental stress (Osakabe et al., 2018). Another recent study in maize used the CRISPR/Cas9 system to produce novel allelic variations which could be used for breeding drought-tolerant crops. ARGOS8, whose overexpression can lead to reduced ethylene sensitivity, was genetically modified by this system and field studies revealed that ARGOS8 variants had increased grain yield under drought stress; further, no loss in yield was recorded under well-watered conditions (Shi et al., 2017).

Global climate change and population increase have put pressure on the agriculture industry to improve productivity, leading to the development of new, improved technologies associated with enhancing the ability of crops to continue to be productive under sub-optimal conditions such as elevated temperature and reduced moisture availability. There remains an urgent need for DNA-free gene editing because there are always chances of foreign DNA integration using plasmid-mediated approaches to gene editing (Cho et al., 2013). Current genetically modified organisms (GMO) regulations are stringent, making it challenging to produce commercial genetically modified food crops (Woo et al., 2015).

Due to its high nutritional value chickpea is the second largest internationally produced cool-season food legume. To date, a low degree of intraspecific genetic diversity has limited success in increasing the yield and quality of cultivated chickpeas. Croser et al. proposed that

increased genetic diversity can be achieved from within the forty-three species of the *Cicer* genus by hybridizing the cultivated species with unimproved 'wild' relatives to integrate beneficial traits from the eight species that share an annual growth habit and chromosome number with cultivated chickpea. Furthermore, the morphological characteristics and tolerance to a variety of abiotic and biotic stresses of potential advantage to chickpea improvement programmes have been identified in the screening process (Croser et al., 2003). Consequently, for the development of transgenic chickpea plantlets derived from embryonic axis co-cultivation, Agrobacterium-mediated transformation and other standard protocols have been developed (Anwar et al., 2010; Krishnamurthy et al., 2000). However, only a few reports on the use of genetic transformation/transgene (s) for the development of abiotic stress tolerant transgenic chickpea plants have been published. Transgenic chickpeas have been developed based on the insertion of the abiotic stress-tolerant bacterial *codA* gene. Anwar et al. concluded that the application of genomic techniques for the analysis of the chickpea genome and improvement of chickpea would be significantly facilitated by the development of a robust and reproducible genetic transformation method (Anwar et al., 2010). Chickpea CarNac3 transgene showed increased drought tolerance in poplar plants. Hajyzadeh et al. identified that the overexpression of miR408 leads to drought tolerance in chickpea One of the significant constraints in terms of chickpea production is drought stress. Many previous studies have focussed on this issue using genetic engineering of chickpea cultivars (Anbazhagan et al., 2015; Bhatnagar-Mathur et al., 2009; Sanyal et al., 2005). However, recent innovations in *in vitro* culture and gene technology give unique opportunities to the full potential of the cultivation of chickpeas using these new technologies. Regrettably, no transgenic chickpea variety in the world has been approved for cultivation (Kumar et al., 2018).

In this study, two potential drought tolerance genes were selected for transformation in a commercial *Kabuli* chickpea genotype. The *4-coumatrate: CoA ligase* gene codes for

coumarate ligase enzyme which is well known for its role in the biosynthesis of secondary plant metabolites during phenylpropanoid metabolism and in major branch pathways (Ehlting et al., 1999; Lee et al., 1997; Liu et al., 2016). This phenylpropanoid enzyme is essential for the activation of the hydroxycinnamic acids during lignin biosynthesis. A study in *Arabidopsis* showed that a decrease in *4CL* activity was correlated with a reduction in thioglycolic acid extractable lignin (Lee et al., 1997). Further, the increased expression of the lignin biosynthesis gene was observed in watermelon during water stress (Yoshimura et al., 2008).

RVE7 is a gene that encodes the transcription factor involved in circadian rhythm and the opening of cotyledon mediated by phytochrome A. The regulation of the *RVE7* gene is controlled by LHY and CCA1 central oscillator mediation. It is known to regulate its expression and belongs to part of the circadian feedback (Rawat et al., 2009). *RVE7* is active in controlling the circadian clock's downstream processes such as hypocotyl growth and flowering (Kuno et al., 2003). Transcription factors such as *REVEILLE 2* (*RVE2*), *RVE7*, *RVE8*, and *MYB HYPOCOTYL ELONGATION-RELATED* (*MYBH*) act in ways like *CCA1* and *LHY1.1,3,4,6,9-14* on the circadian clock. *RVE7 / EARLY-PHYTOCHROME-RESPONSIVE1* (*EPR1*) was found to be phytochrome A and phytochrome B regulated and to work as a slave oscillator part (Kuno et al., 2003; Nguyen and Lee, 2016). Studies of other clock related *MYB* proteins demonstrated that these variables have a less prime role in regulating *TOC1* expression. *RVE7 / EPR1* does not control the expression of *CCA1, LHY* or *TOC1*, although these oscillator components are involved in the circadian regulation of *RVE7 / EPR1* (Farinas and Mas, 2011).

Here, a pilot study was conducted to knockout the *4CL* and *RVE7* genes in the chickpea protoplast to help understand the drought response mechanism in chickpea. The main aim of this study was to conduct CRISPR/Cas9 for the polygenic adaptive trait i.e. drought tolerance and propose the method for the use of DNA free gene editing in recalcitrant species. Figure 1

provides an overview of CRISPR/Cas9 gene-editing process and how it was employed in this study.

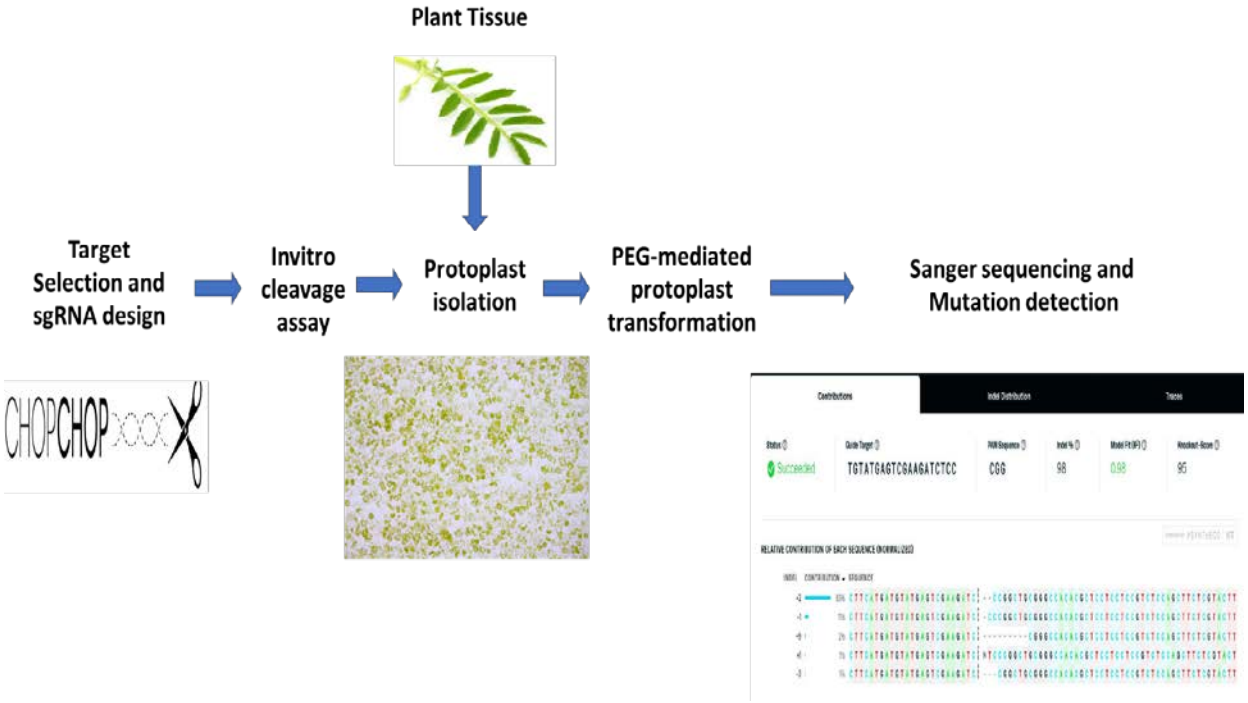


Figure 1: Graphical abstract: An overview of the CRISPR/Cas9 DNA free gene editing in the chickpea protoplast. The protoplasts of young leaves were isolated and transformed with CRISPR RNP complex under optimum conditions. The DNA was extracted from the protoplast control and test samples and sent for Sanger sequencing to detect the mutations. The transformation efficiency and knockout score were identified using ICE bioinformatics tool (Synthego).

4.2 Materials and Methods

4.2.1 Chickpea plant material and Cas9 protein

Commercial *Kabuli* chickpea plants were grown in a glasshouse facility at RMIT Bundoora campus. The 1.5 g leaf tissue from young chickpea plants were collected for the protoplast isolation from 4 to 5 weeks old plants. The recombinant *S. pyogenes* Cas9 nuclease (Integrated DNA Technologies, IDT), purified from an *E. coli* strain expressing the nuclease was used in this study. It consisted of a nuclear localization sequence (NLS) and C-terminal 6-

His tag, which is provided in solution at 10 µg/µL. The components of the crRNA:tracrRNA duplex were Alt-R® CRISPR-Cas9; crRNA contained the target-specific sequence for guiding Cas9 protein to the genomic location of the selected target sequences, and Alt-R® CRISPR-Cas9 had tracrRNA which hybridizes to crRNA to activate the Cas9 enzyme. The required dilution was performed according to the manufacturer's protocol under controlled conditions.

4.2.2 Target site selection and sgRNA design

Two potential drought tolerance associated genes (*4CL* and *RVE7*) were selected for knockout based on their expression levels in ICC283 (Drought sensitive genotype) and ICC8261 (Drought tolerant genotype) under drought stress. The expression levels for the selected genes are shown in Table 1.

Table 1: The gene expression values of the *RVE7* and *4CL* in both genotypes. Shoot apical meristem (SAM), Flowering bud (FB), Fully opened flower (FOF), young pod (YP), Partially open flower (POF).

Gene Name	ICC8261 (Drought tolerant genotype) Expression levels	ICC283 (Drought sensitive genotype) Expression levels
RVE7	SAM 2.69, FB-3.4, POF0, FOF-1.29, YP1.9	SAM 3.4, FB-3.02, POF0, FOF-5.9, YP 0
4CL	FB 10.39	Not Differentially expressed

The sgRNA targets were designed using CHOPCHOP tool (Labun et al., 2019). CHOPCHOP can predict the frameshift rate of each gRNA which are target sites for high-efficiency editing. All the recommended parameters were selected for the range of sgRNA sequences; for traditional Cas9 (20bp-NGG), preferred features for effective sgRNA include: gRNA has no off-targets; gRNA is expected to target all isoforms of the target gene and gRNA

downstream of any in-frame ATG to avoid the expression of truncated proteins. Based on these selection criteria, the top sgRNA were selected for the knockout experiments. The sgRNA used in this study are shown in Table 2 below.

Table 2: List of sgRNAs designed to target *4CL* and *RVE7* genes in chickpea. 20 bp sequence of crRNA/tracrRNA with PAM in red.

Gene Name	sgRNA Name	sgRNA sequence
4-coumarate—CoA ligase-like NW_004516753.1_162798-165892	*4CL*sgRNA1	TATGTCACCGTCTAGTTCATTGG
	*4CL*sgRNA2	GTTTAGGTTACCGAACGAAGAGG
REVEILLE 7-like NW_004516329.1_420654-4253847	*RVE7*sgRNA1	GTGGAGGATTGAATGTAAGACGG
	*RVE7*sgRNA2	AGTGTGCAGCTGATGTATCGAGG

4.2.3 *In vitro* cleavage assay

The 5Kb DNA fragments for selected targets genes containing the target site were amplified using PCR, purified and eluted with RNase-free water. The RNP complex was prepared according to manufacturer's instructions and 1 µM RNP were mixed with the purified target DNA (100–150 ng) in 10 X Cas9 reaction buffer (20 mM HEPES, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT) in a total volume of 10 µl, followed by digestion at 37 °C for 60 min. Further, to release the DNA substrate from the complex, 1µl proteinase K was added to reaction and incubated for 10 mins at 56 °C (https://sg.idtdna.com/pages/support/guides-and-protocols). The digested DNA products for respective samples were separated on a 2% agarose gel immediately after the digestion process, and cleavage activity was measured by the number of digested products over the total amount of input target DNA. The obtained DNA bands were quantified using Gel

quantification software (ChemiDoc imaging system, BIO-RAD). The *in vitro* digestion assay was performed before every transformation to confirm that the RNP complex was functional and capable of editing the respective genes.

4.2.4 Protoplast isolation

Leaf tissue from *Kabuli* chickpea was collected to produce protoplasts using a Plant Media Protoplast Isolation kit (PlantMedia™, a division of bioWORLD, United States, Catalog no. SKU# 30210002-1) using a slightly modified protocol. Briefly, 1.5 g fresh plant leaf tissue was surface sterilized by 2% bleach followed by three distilled water washes for 5 min each. The leaf tissue was sliced thinly into approximately 0.5–1.0 mm strips using a sterile razor blade and resuspended in distilled water in 50 ml tubes and centrifuged at 300x *g* in a swinging bucket rotor for 10 min at room temperature. Suspended cells were washed with 5 ml cell wall wash buffer. Pellets were resuspended in 5 ml of ice-cold protoplast enzyme solution and transferred to a 25 ml flask. The flasks were kept at room temperature for 5 hours in the dark with gentle agitation at 100-150 rpm. The protoplast formation was checked in using 10 µl of suspension on a cell counter and microscope. The protoplast suspension was centrifuged in 50 ml tubes at 300x *g* for 5 min at 4 ˚C. The protoplasts were washed twice by resuspending the pellet in 5 ml of ice-cold protoplast wash solution and centrifuged at 300x *g* for 10 min at 4˚C. Finally, BSA (0.5mg/ml) was added to the final protoplast preparation for transformation. The viability of the protoplast was calculated via a cell counter (Invitrogen™ Countess™ Automated Cell Counter) and $2x10^5$ cells used for transformation.

4.2.5 Protoplast transformation with RNP complex

A polyethylene glycol (PEG4000)-mediated transformation was used to transform respective RNP complexes into chickpea protoplast cells. The PEG transformation protocol for the efficient targeted knockout in both genes was adapted from previous studies (Brandt et al.,

2020; Malnoy et al., 2016; Subburaj et al., 2016; Woo et al., 2015). The $2x10^5$ resuspended protoplasts were transformed with Cas9 protein and sgRNA in a ratio of 1:1. Protoplasts (200 µl, 2×10^5 cells) and RNPs for example, 1:1 is Cas9 30 µg (stock 10 µg/µl) and sgRNA 30 µg (stock 10 µg/µl) used for transformation. Before the transformation experiment, Cas9 and sgRNA were pre-mixed to form the RNP complex and incubated at room temperature for 10 min. Further, all the components: protoplast, Cas9 and sgRNA mix were combined, and an equal volume of freshly prepared PEG 4000 added (40% PEG [w/v] PEG 4000, 0.2 M mannitol, 0.1 M $CaCl_2$), with immediate mixing to prevent aggregation. This mixture was incubated at room temperature for 20 min. An aliquot (400 µl) of W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl) was added, mixed and incubated at room temperature for 10 min. An additional 800 µl of filter sterilized W5 solution was added to the tubes, mixed gradually and incubated at room temperature for 10 min. The tubes were centrifuged at $50 \times g$ for 5 min; the supernatant was discarded, and another 1 ml of W5 solution was added to the tubes. Subsequently, the tubes were incubated overnight at room temperature in the dark. The lower sediments were collected for genomic DNA isolation from the protoplast cells. Three biological replicates of the protoplast transformation were performed for each set of genes. Genomic DNA was isolated from protoplast cells for Sanger sequencing (Macrogen, Geumcheon-gu, Seoul). The sample treated only with the Cas9 enzyme was used as a negative control. The transformation experiments were repeated twice, and similar results were obtained both times.

4.2.6 DNA extraction and PCR amplification of target regions

Genomic and protoplast DNA were extracted using Isolate II Genomic DNA kits (Catalog number: BIO-52069, Bioline (Aust) Pty Ltd). The DNA concentration was determined with a Nanodrop spectrophotometer (Thermo Scientific); the concentration of the

samples was around 25-30 ng/µl. The PCR reaction to amplify the 5kb genomic region is provided in (Appendix chapter 4: supplementary Table 1). The PCR reaction for sequencing samples are also provided in (Appendix chapter 4: supplementary Table 2). The 5 kb fragment was amplified using GoTaq® Long PCR Master Mix (Catalog number: M4021, Promega, Australia). The samples for sequencing the target regions were amplified using MyTaq™ Red Mix, 2 x (Catalog number: BIO-25043, Bioline (Aust) Pty Ltd).

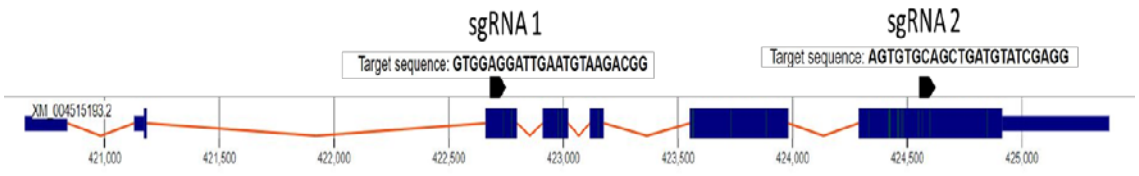
Table 3. The sequences of primer used for amplification of CRISPR target loci of *4CL* and *RVE7*.

4CL **Primer Set1**	**Sequence**	**Expected Product Size**
4CL Primer Set1	Forward: ACAATACCAATGAACTAGACGGTGA Reverse: TCCCTAACAAAATCCAACACATCT	592
4CL Primer Set2	Forward: ACAATACCAATGAACTAGACGGTG Reverse: TCCCTAACAAAATCCAACACATC	590
RVE7 Primer Set1	Forward: AACATGCTGCTGCTTGGTTG Reverse: GACGAAGAGAGGGACTAATTTCA	398
RVE7 Primer Set2	Forward: GGAAGCAGGTTTCATCCGTC Reverse: TGATGAAAGAAATTGATGCTCACTA	420

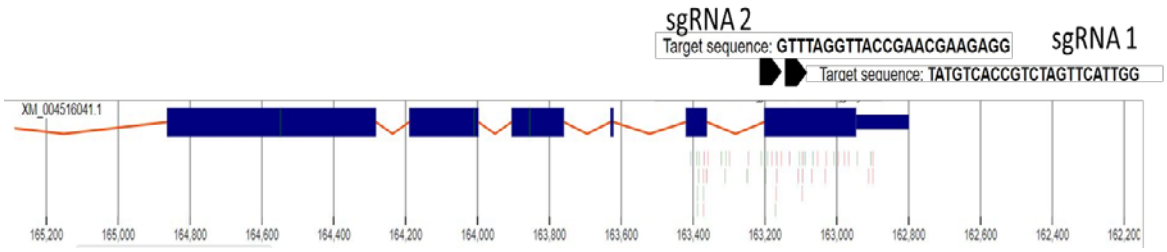
4.3 Results

4.3.1 sgRNA selection and design

Two candidate genes (*4CL* and *RVE7*) which are potentially associated with drought tolerance in chickpea were selected for the knockout in chickpea protoplasts. The sgRNA for the target location were designed using CHOPCHOP and verified by other tools such as CCTop using the genome sequence for *Kabuli* chickpea. The preferred sgRNA based on the target selection algorithm were selected for further studies. The illustration of the location of sgRNA target sites in *RVE7* and *4CL* nucleotide sequences is shown in Figure 2.



a)

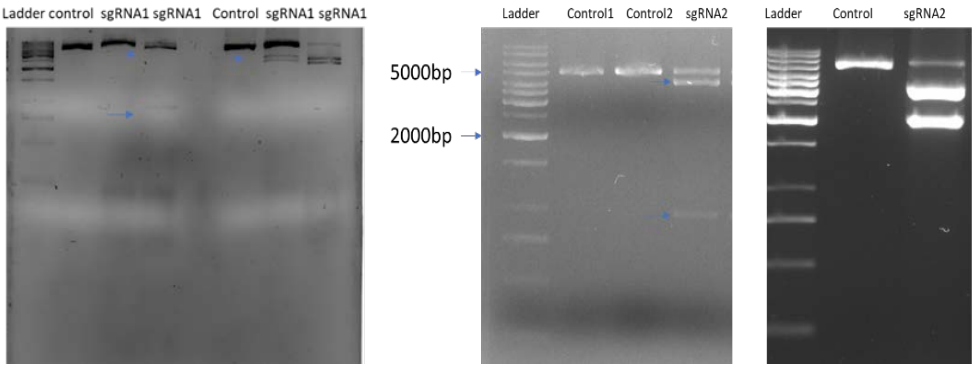


b)

Figure 2: Systematic design to show the location of sgRNA target sites in *RVE7* and *4CL* nucleotide sequences. **a)** Systematic illustration of the nucleotide sequence of *RVE7* gene locus **b)** Systematic diagram of the nucleotide sequence of *4CL* gene locus. The blue boxes represent exons and orange connecting lines represent introns.

4.3.2 *In vitro* digestion assay

The primary step for the CRISPR/Cas9 gene editing is the selection of sgRNA sequences for target knockout. Two sets of sgRNA were designed using CHOPCHOP and were tested *in vitro* for their efficacy. In order to determine the specificity of the target sgRNA sequences, a 5kb region around the target regions were amplified. Primers were designed to amplify the target region for both target genes and *in vitro* cleavage assay was conducted using the respective purified PCR products and preassembled RNPs. The *in vitro* digestion shows that individual band sizes were achieved with both sgRNA sets in *RVE7* and *4CL* amplified fragments. The sequences used for the amplification of DNA and position of the sgRNA sequences are provided in (Appendix chapter 4: Supplementary material 3).



a) sgRNA 1 for *4CL* and *RVE7* **b) sgRNA 2 for *4CL* and *RVE7***

Figure 3: Results of in vitro digestion cleavage assay of both sgRNA sets for *4CL* and *RVE7* gene. (**a**) sgRNA 1 for *4CL* and *RVE7*. The DNA of 5kb PCR-amplified fragments for gene *4CL* and *RVE7* were treated with preassembled RNPs (sgRNAs (crRNA/tracrRNA) + Cas9) and in vitro cleavage assay was performed. For in vitro cleavage assay non-treated samples were used as negative controls in gel electrophoresis. (**b**) sgRNA 2 for *4CL* and *RVE7*. The digested samples for *4CL* left side and *RVE7* at right side. Above lane 2,3,4 correspond to *4Cl* and lane 6,7,8 for *RVE7* samples. The expected band size for sgRNA 1 *RVE7* were approximatly 2298 and 3543; sgRNA 2 4469 and 1363. For *4CL* the expected band size after digestion for sgRNA1 were 4931 and 1170; sgRNA 2 approximately 4477 and 1428.

4.3.3 Protoplast Isolation and Transformation

Protoplasts were isolated using the BioWORLD kit with minor modifications. The protoplast was isolated from young chickpea leaves from 4 to 5-week-old plants. It was noted that protoplast isolated from older leaves had reduced protoplast yield compared to younger leaves (data not shown). However, the viability and protoplast number were lower initially but optimized by changing the leaf maturity, amount of leaves used, centrifugation speed and time for the enzymatic digestion.

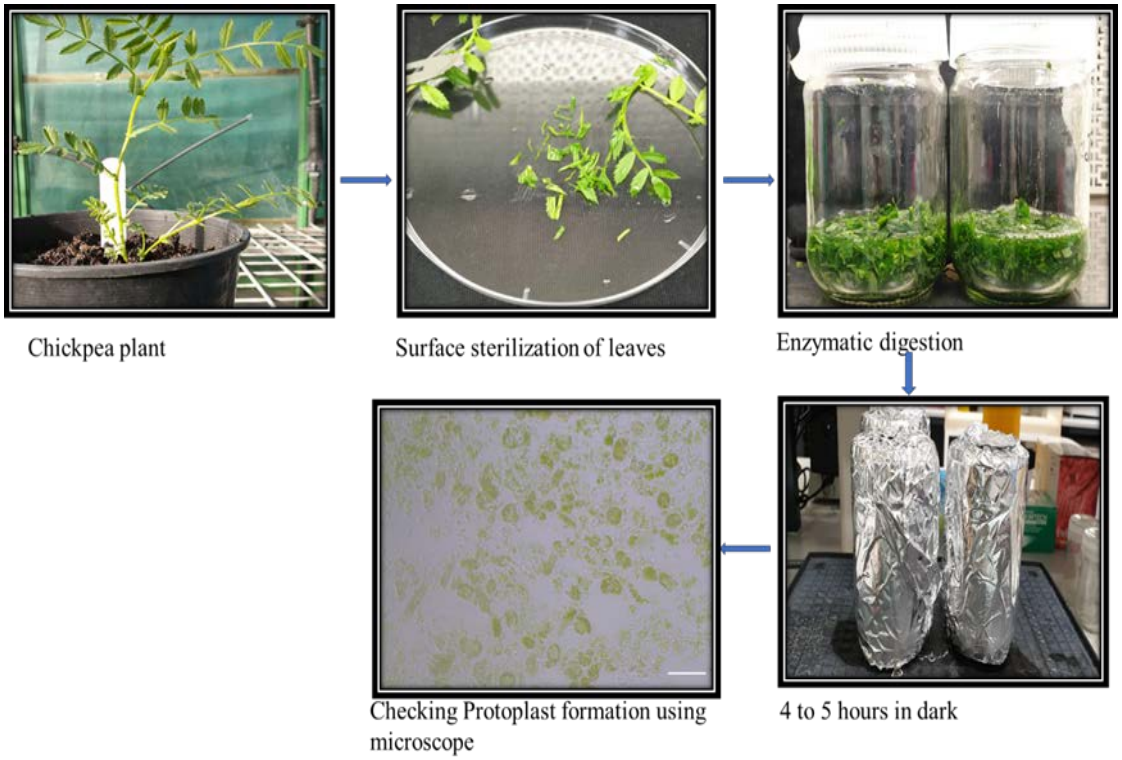


Figure 4: Flow chart of the steps performed during the protoplast isolation from leaf tissue of chickpea plants. The leaves from the 4 to 5 weeks old plants were collected and surface sterilized. The leaves were cut into small pieces. The enzyme solution was prepared and cut leaf sections were kept in the dark for 4 to 5 hours digestion resulting in protoplast formation. After 2 hours, samples for protoplast formation were collected to check the protoplast formation stage, and once the protoplast were isolated, they were used for the PEG mediated transfections.

4.3.4 Mutation Detection

Sanger sequencing was performed to detect mutations in the transfected protoplasts. The DNA was isolated from the transformed protoplast, and target regions were amplified using primer sets to conduct sequencing. The results obtained from Sanger sequencing are classically given as nucleotide occurrence chromatograms for respective samples, represented as distinct coloured peaks. Usually, the biggest drawback in protoplast mutation identification is that mutated protoplast DNA chromatograms do not provide any apparent indication of the mutation due to the difference in editing forms in each protoplast cells and the presence of

unmutated protoplast cell DNA in each sample. To overcome these challenges, Inference of CRISPR Edits (ICE), a bioinformatics tool by Synthego, was used for mutation detection (Hsiau et al., 2018). The *RVE7* sgRNA 1 results showed 76%, 77% and 79% indel percentage for the treated protoplast samples compared to the 99% for control sample, with average of 77.3% and standard deviation of 0.81 for all treated samples (Figure 5) and (Figure 6). Indel Percentage is the editing efficiency (percentage of the pool with non-wild type sequence) as determined by comparing the edited trace to the control trace. The Knockout Score represents the proportion of cells that have either a frameshift or 21+ bp indel. The knockout score is used in an understanding number of the contributing indels expected to result in a functional Knockout of the target gene.

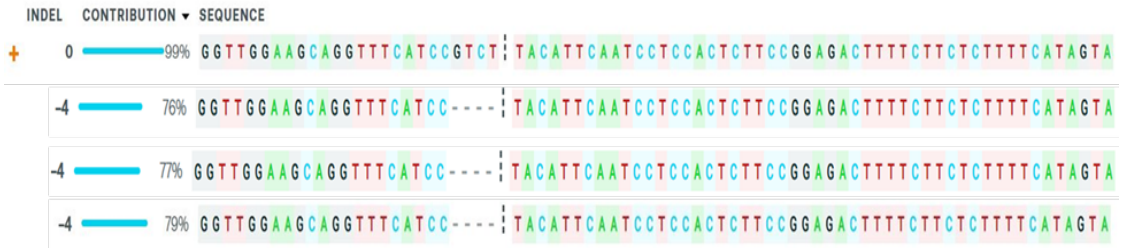


Figure 5: Illustration of ICE Analysis of *RVE7* sgRNA1 (GTGGAGGATTGAATGTAAGACGG) RNP complex edited protoplast cells in chickpea. The respective protoplast samples were edited and sequenced with Sanger sequencing (Macrogen, Korea) and further analyzed with the ICE (Synthego) online tool to deconvolute the pooled protoplast DNA. The contributions show the sequences inferred in the edited protoplast population and their relative proportions. The "+" sign is for the wild type sample. The target cut sites are denoted by black vertical dotted lines. The trace plots for the edited samples below show the cut site and PAM sequence labelled in the edited sample and control sample. The sgRNA 2 sequencing samples had low quality before the cut site and could not be analyzed using the ICE tool.

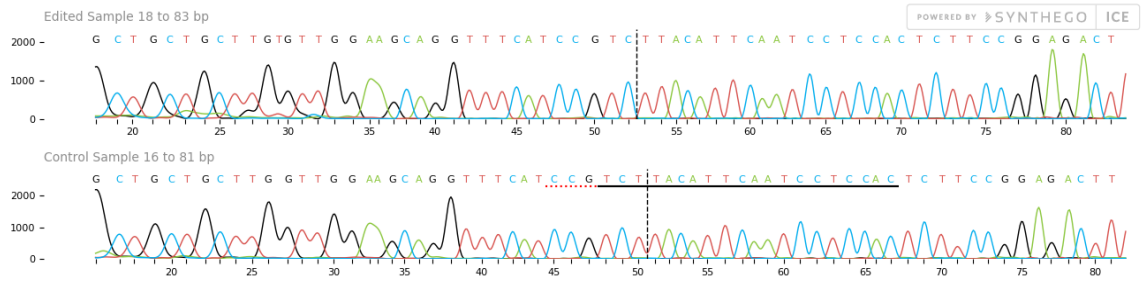
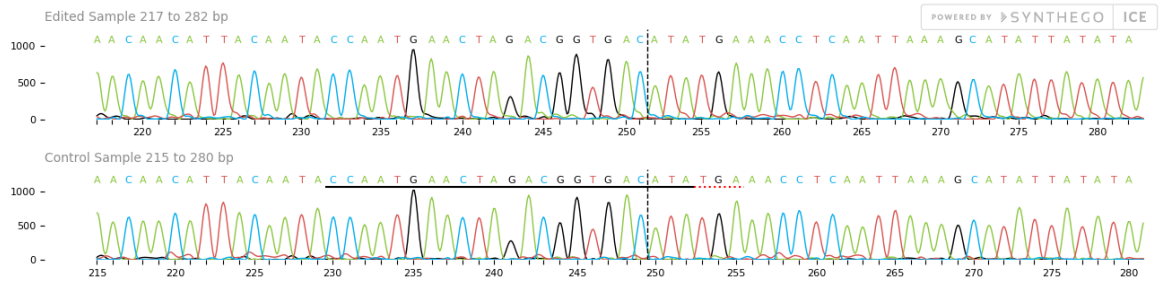


Figure 6: Traces of the edited and control population of the protoplast as shown by the Sanger series, showing edited and control sequences around the guide sequence in the field. This shows sequence base calls from both the .ab1 files control and the experimental study, which contain mixed base call. The region underlined in horizontal black represents the guide sequence. The PAM-site is represented as the horizontal red focus. The vertical dotted black line represents the site of the actual break. Cutting and fixing which is sensitive to errors usually results in mixed sequencing bases after cutting.

The sequencing samples for *4CL* sgRNA 1 were also analyzed using the ICE tool. However, in *4CL* samples only one sample showed +1 indel with knockout score of 2%, shown in sequence and trace plot in (Figure 7). Nevertheless, all other *4CL* test samples showed no indel percentage. The sgRNA 2 sequencing results were low quality and did not fit the threshold values for ICE software (Data not presented here).

a)



b)

Figure 7: ICE results for 4CL a) The sequence plot represents the ICE Analysis of *4CL* sgRNA1 Target 1 (sgRNA:CCAATGAACTAGACGGTGACATA) RNP edited protoplast cells in the chickpea. The contributions show the sequences inferred in the edited protoplast population and their relative proportions. The cut sites are represented by black vertical dotted lines and "+" far left side denotes the control sample. **b)** The trace plot of the *4CL* sample 4 edited and control population of the protoplast showing the PAM sequence and cut site.

4.4 Discussion

The simplicity of the CRISPR/Cas9 system offers agriculture scientists an opportunity to edit a gene of interest at almost any laboratory-based facility effectively and economically. Successful DNA-free editing with the CRISPR/Cas9 system is important for crops such as chickpea, due to the crops high demand and export value, but low productivity because of challenging climatic conditions. The main drawback of plasmid-mediated delivery in genome editing is the possibility of random integration of plasmid sequence in the host genome. In addition, the current GMO regulations and the complication of commercialization of genetically edited varieties makes DNA-free gene editing more suitable compared to traditional gene editing techniques (Malnoy et al., 2016). It will help to grow crops that are resistant to different biotic and abiotic stresses if the CRISPR/Cas9 technique can gain social acceptance and still comply with the strict regulations set by global and local authorities. Moreover, other techniques used for gene editing due to their random nature of the gene integration such as

observed that even if the sgRNA selected for the knockout works effectively during the *in vitro* digestion assay, it does not necessarily translate to reliable *in vivo* editing of the gene. Out of the two sgRNAs selected for the knockout, only one showed *in vivo* effectiveness. Recently, a study in *N. benthamiana* to evaluate sgRNA efficiency using different online tools found no significant correlation between the predicted rankings and the editing frequencies *in vivo* in the transformation of *N. benthamiana* compared to *in vitro* CRISPR digestion (Naim et al., 2020). Further, a study in wheat showed that the PinB-D gene had 0% editing efficiency in wheat protoplasts; however, the sgRNA showed optimum results during *in vitro* digestion (Brandt et al., 2020). Here, the *4CL* protoplast sample only showed a 2% knockout score which may be due to sequencing artefacts or the presence of a target site in the conserved domain of the gene which made access to the RNP complex difficult. Therefore, the most likely reason for unedited protoplast cells could be the inaccessibility of the target site by the RNP complex in the *in vivo* environment. Amongst the various limitations of the CRISPR/Cas9 gene editing in plants, the most significant is a recalcitrant sgRNA/target site due to chromatin conformation (Naim et al., 2020; Wu et al., 2014). A study in cucumber reported the occurrence of chromatin structural rearrangements during the de-differentiation of protoplasts with no obvious change in the expression profile (Ondřej et al., 2009). Studies have confirmed that certain sgRNAs may show low efficiencies or may even fail to work in many setups, which may occur due to the chromatin states of the target loci, unwanted hairpin structures of selected sgRNA or additional unidentified factors (Shan et al., 2014). In this study, the main objectives were to set up a CRISPR/Cas9 system for chickpea to knockout two potential drought tolerance candidates using protoplast cells *in vivo*. In mammalian cells, many studies have shown that conformation of the chromatin surrounding a sgRNA/Cas9 target site may significantly affect the site's accessibility to the RNP complex (Li et al., 2020; Wu et al., 2014).

Interestingly, in plants, the tools used to design the sgRNA does not take the chromatin conformation into account when predicting sgRNAs because of the lack of knowledge of the epigenetic profiling in many plant genomes (Naim et al., 2020). In addition, the secondary structures of sgRNAs are proposed to have a critical consequence for Cas9/sgRNA effectiveness in many studies (Jensen et al., 2017; Kocak et al., 2019; Makarova et al., 2011). A study in rice reported relatively higher editing efficiencies in target sequences with greater GC content. It is therefore desirable to pick targets with a GC content between 50 and70%, and those with less or no base pairing with the sgRNA sequence. The use of higher GC content target sequences may theoretically contribute to a greater risk of off targeting (Tsai et al., 2015). A study in rice reported reduced editing efficiency of the *Os07g0261200* gene due to the low GC content (35%) of the target sequence (Ma et al., 2015).

Further, the inaccessibility of the RNP complex in the *4CL* target site may also be due to the biological function of this gene. Generally, the *4CLs* are known to play a vital role in generating Coenzyme A (CoA) esters within the phenylpropanoid pathway. This channels carbon flow into various secondary phenolic metabolism branching pathways, producing different groups of secondary natural phenolic products, including flavonoids, lignin, suberins and coumarins, which play an essential role in the growth of plants and environmental interactions (Cao et al., 2016; Dixon and Paiva, 1995). Hu et al. proposed that the lignin content in maize leaf can play an important role in drought tolerance and needs to be further explored as a molecular marker for drought tolerance traits (Hu et al., 2009). A recent study in cotton by Sun and others suggested that *Gh4CL7*-silencing amplified sensitivity to drought stress. However, the overexpression enhanced drought tolerance, which indicates the function of *4CL* genes and their potential role in drought tolerance (Sun et al., 2020). Another recent study in chickpea identified metabolic pathways that are differentially regulated in different genotypes; for example, phenylpropanoid metabolism, and biological processes such as stomatal

development with potential consequences on drought tolerance (Moenga et al., 2020). *RVE 7* and other family members are well known for their role in the circadian clock, which coordinates biological processes related to daily and seasonal environmental changes (Kuno et al., 2003; Lu et al., 2009). The information available so far suggests the importance of exploring the roles of these candidate genes to establish the basis of drought tolerance in chickpeas. The drought tolerance pathways a play a significant role in the activation of signalling mechanisms and differentially expressed genes. The manipulation of the novel candidate genes identified may help to achieve the drought resilient chickpea varieties. Climate change has already affected many crops worldwide, but it is expected to go beyond the drought tolerance will impact mineral nutrient availability and accumulation to the plants and soil. It is predicted that the microorganisms will be affected by global climate which will further effect the plant-soil interactions (Cavicchioli et al., 2019). The protoplast transformation method presented here in chickpea can be utilized as a screening protocol for *in vivo* efficiency of the selected sgRNAs for a target site. This will save not only time but also resources, avoiding the selection of sgRNAs which may not be accessible for *in vivo* editing. Confirmation of the target editing in the protoplast can be set closer to the actual editing in plants. The RNP complex for *RVE7* designed in this study can be used in future for producing CRISPR-edited chickpea plants and validate the physiological role of this gene in chickpea.

Conclusions

This study is the first proof of concept study, which shows that CRISPR/Cas9 gene editing can be employed to achieve targeted genome editing in chickpea protoplasts. The results obtained from this study could help in developing new traits and understanding the drought mechanism in chickpea plants by knocking out the desired gene, followed by protoplast regeneration or using the plant tissue for transformation. In several plant species the transient expression systems have been demonstrated to be helpful in rapid high-throughput

screening and systematic characterization of interesting genes and proteins. Such transient expression systems are particularly important in plants where stable transformation protocols are time-consuming and inefficient. The transient chickpea expression system using protoplasts presented in this study can be used as an effective method to help harness the cumulative amounts of existing genomic data resources and can also be used to speed up the discovery of genes that survive drought and improve crop characteristics in this essential legume crop. The functional validation of the genes studied in this study should be performed to understand their individual role in the drought tolerant genotypes. The effect of the successful knockout under drought stress imposition needs to be further studied. As less studies have been conducted on the role of these genes in the drought mechanism, establishing more stringent experiments using NGS and qPCR for expression profiles and microscopy to examine the structural changes will be of great importance for development of new chickpea varieties.

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Chapter 5

Summary and General Discussion

Summary and General Discussion

The most recent United Nations study estimates that by 2050, the world population is projected to exceed 9.8 billion (DESA, 2019), suggesting that food security will be the biggest challenge that the humans will face in the coming years. As the global climate is expected to change over the coming years, temperatures will increase. Any rise in temperature will impact agriculture as a result of reduced water availability for irrigation which, coupled with less rainfall, would seriously impact farming (Elliott et al., 2014; Haddeland et al., 2014; Osborne, Rose, & Wheeler, 2013; Pastor et al., 2019). We therefore need alternative crops that are tolerant to abiotic stresses (Hasegawa et al., 2018; Raza et al., 2019). In chickpeas drought decreases plant growth at young plant levels, leading to stunting and decreased accumulation of biomass (Siddique, Loss, Regan, & Jettner, 1999). To ensure enough nutritious food for everyone there is an urgent need to have food crops which are resilient to these climatic variations (Dhankher & Foyer, 2018).

Chickpea is exceptional due to its high level of protein content that accounts for nearly 40 percent of its weight (Siddique et al., 2000). With an average annual production of more than 11.5 million tonnes, production ranks third after beans; India leads chickpea production. In recent years, the land area allocated for chickpea cultivation has grown and now stands at an estimated 14.56 million hectares. Production per unit area has increased slowly but steadily since 1961 at around 6 kg ha^{-1} $year^{-1}$. More than 2.3 million tonnes of chickpea per year reach world markets to supplement the needs of countries unable to meet demand from domestic production especially India (Merga & Haji, 2019).

Drought stress is one of the major constraints for the global chickpeas production (Nadeem et al., 2019; Thudi et al., 2014). Chickpea experiences drought stress at various growth stages; terminal drought, along with heat stress at flowering and seed filling can reduce

yields by 40–45% (Rani et al., 2020). It is observed that drought at young plant stages reduces plant growth leading to stunting and reduced biomass accumulation (Siddique et al., 1999). Water deficit during podding stage in chickpea augmented ABA that may impair pod set and cause pod abscission ultimately cause significant yield losses (Pang et al., 2017). The latest genomics-based research has laid the foundation to delve further into understanding the different genes activated during drought tolerance and sensitivity. Recent advancement in this path comprise the application of the RNA-Seq approaches to understand the differential regulation of genes in *Desi* and *Kabuli* chickpea genotypes. These studies highlighted biological pathways functioning under drought stress in contrasting types (Bhaskarla et al., 2020; Kudapa, Garg, Chitikineni, & Varshney, 2018; Mashaki et al., 2018; Varshney et al., 2014). However, no single study has yet collected genome-wide data on gene expression profiles in many drought-related genotypes, including all major plant organs at different developmental stages, resulting in a major gap in information between gene sequence datasets and phenotypes. Furthermore, the studies conducted so far have been limited to a single genotype only, and only one growth stage. In addition, previous work failed to encompass comparative studies with consideration to the role of epigenetics, which may have major part in the gene regulation. The gaps in the previous research allowed us to develop strategies for chickpea genetic analysis. Multidimensional approaches for functional assessment allowed scientists to steadily bridge the gap between physio-biochemical and molecular events that emerge in the development of drought tolerance.

This study aimed to contribute to the existing knowledge about drought tolerance in chickpea by examining the correlation between DNA methylation patterns and their effect on gene expression profiles under drought stress. Here, a standardised DNA-free CRISPR/Cas9 editing method for knockout of important candidate genes in chickpea is presented. The validated candidates may be used to increase drought stress tolerance and productivity in the

future. The information from this study can be employed by the chickpea breeders to produce new drought tolerant variety in coming years.

In this study, for the first-time, drought sensitive (ICC283) and drought tolerant (ICC8261) genotypes were used for elucidation of the relationship between gene expression and DNA methylation patterns using RNA sequencing and RRBS under drought stress. Further, chickpea protoplasts were edited using CRISPR/Cas9 DNA free gene editing to achieve *in vivo* knockout of two potential genes *RVE7* and *4CL*.

5.1 RNA sequencing of ICC8261 and ICC283 genotypes under drought stress

The objective of gene expression analysis in chapter 2 was to identify genes which were differentially expressed in control and drought stress conditions in leaf tissue of drought sensitive and drought tolerant chickpea genotypes. The drought tolerant chickpea genotypes analysed here possess enormous differences in root length and density. Their comparative differences in phenotype is a result of changes in root traits and transpiration efficiency (Kashiwagi et al., 2005; Varshney et al., 2014). The purpose of documentation of differentially expressed genes in this study, was to identify candidates for the development of new chickpea cultivars, that are tolerant to abiotic stress conditions. The information presented here will contribute to the chickpea breeding program established by Grains Research and Development Corporation (GRDC) for overcoming fluctuations in chickpea production that have occurred over recent years in Australia. Furthermore, this will contribute to the GRDC's Research, Development and Extension plan for 2018-2023 in grains, which aims for crop protection and increased yield at lower cost.

In tolerant genotype, a total of 1,562 differentially expressed genes, comprising 693 up-regulated and 869 down-regulated genes, were identified. In contrast, in sensitive genotype, including 837 up-regulated and 1755 down-regulated genes, a total of 2,592 differentially

expressed genes were identified under drought stress conditions. The significant difference observed between the two genotypes in shoot apical meristem leaves indicates how drought stress differently affects these contrasting genotypes. In order to further develop the drought-tolerant characteristics of the resilient chickpeas, the information from this study will enable plant breeders to use ICC8261 as a genetic donor of selected genes for improvement. Next-generation sequencing (NGS) has started a new era of transcriptomics-based sequencing of legumes to identify different genes and pathways related to drought sensitivity and tolerance in many crops (Nadeem et al., 2019). To understand how the underlying genome sequence results in specific plant phenotypes, information on gene expression patterns is important. An atlas of gene expression could predict gene clusters expressed at different plant developmental stages in each tissue (Kudapa et al., 2018).

5.1.1 Potential role of differentially expressed genes in drought tolerance and sensitivity

Ethylene response-related genes, MYB-related protein, xyloglucan endotransglycosylase, alkane hydroxylase MAH-like, BON-1-related genes, peroxidase 3, cysteine-rich and transmembrane domain, vignain and mitochondrial uncoupling were specifically up-regulated in the tolerant genotype, while the same genes were down-regulated in the sensitive genotype. However, probable xyloglucan endotransglycosylase/hydrolase protein 32 related to cell wall modification and remodelling, was significantly up-regulated in the tolerant genotype (LOC101508375; FC 3.1↑) while down-regulated in the sensitive genotype (LOC101508375; FC -2.9↓). Cho et al., demonstrated that in transgenic *Arabidopsis* this gene was induced under drought and salinity, resulting in enhancement of drought tolerance. These results show that CaXTH3 is functional in heterologous Arabidopsis cells, thus effectively altering cell growth and the response to abiotic stresses. Although the physiological function of CaXTHs is not yet clear, there are several possibilities to combat dehydration and high salinity stresses in a subset of physiological responses in transgenic

Arabidopsis plants (Cho, Kim, Park, Eom, & Kim, 2006). Cuticular wax covers plant leaves and prevents stomatal water loss and gas exchange, thus helping in dealing with water scarcity.

Interestingly, it was observed that the tolerant genotype had (LOC101495087; FC 2.4↑) induced alkane hydroxylase expression. This led to the impression that the upregulation of alkane hydroxylase in the tolerant genotype may play a key role in ketone synthesis. The role of this gene in drought tolerance in wheat has previously been established via changes in cuticle permeability and wax synthesis (Zhang, Wang, & Li, 2013).

Two important cell redox homeostasis genes were also upregulated in the tolerant genotype: Peroxidases 3-like and Mitochondrial uncoupling protein 5-like. In the tolerant genotype, Peroxidases 3-like expression was up-regulated (LOC101499357; FC 2↑), but repressed in the sensitive genotype (LOC101499357; FC -2.1↓). Similarly, the Mitochondrial uncoupling protein 5-like was also up-regulated in the tolerant genotype (LOC101493136; FC 2.5↑) and down-regulated in the sensitive genotype (LOC101493136; FC -2.8↓). Plants are sessile organisms and have established defensive mechanisms that allow them to adapt and thrive in times of stressful drought. One of the key implications of drought stress is the increased production of reactive oxygen species (ROS) in different cellular compartments. Overexpression of UCP from Arabidopsis was studied in transgenic tobacco plants, resulting in decreased accumulation of hydrogen peroxide in stressed leaves compared to the wild form, contributing to an improved drought response (Begcy et al., 2011). As there is little knowledge of the role of Mitochondrial uncoupling protein 5-like in drought stress tolerance, this is certainly an important area for further studies.

A negative regulator of cell death and defence responses, BON1-associated protein 2-like also exhibits calcium-dependent phospholipid binding properties (Yang et al., 2006). In the tolerant genotype, the BON1-associated protein 2-like transcript was significantly induced

(LOC101502816; FC 4.2↑) but it was highly repressed in the sensitive genotype (LOC101502816; FC -2.4↓). It is reported that in legumes, the Vignain like proteins are involved in mobilizing storage proteins and help in drought tolerance (Li, Shi, Leng, & Zhou, 2016). In this study, Vignain like was highly induced in the tolerant genotype (LOC101497939; FC 3.3↑) and repressed in the sensitive genotype (LOC101497939; FC -2.4↓). The differential expression study thus provided some promising candidates for future studies and contributed to existing genomic data available for chickpea improvement.

Following on from the differential expression studies in the leaf tissue at the shoot apical meristem stage, the next step was to investigate the methylation patterns between the control and test samples for both genotypes using reduced representative bisulphite sequencing.

5.2 DNA methylation plays a key role in regulation of the gene expression under drought stress

Chapter 3 focused on the analysis of differential DNA methylation patterns in both the contrasting genotypes using Reduced Representation Bisulphite Sequencing (RRBS). This provided insight into the role epigenetic mechanisms driving drought tolerance and sensitivity in chickpea. RRBS technique offers genome-wide quantitative DNA methylation information at a single base resolution that can be further exploited to reveal the basis for chickpea abiotic stress responses (Meissner et al., 2005; Paun, Verhoeven, & Richards, 2019; Schmidt et al., 2017). The interaction between gene regulation and DNA methylation patterns was investigated in these two contrasting chickpea genotypes. The results presented in Chapter 3 showed that compared to the tolerant genotype, a larger number of differentially methylated regions and bases were present in the sensitive genotype. A Fababean study indicated that in terms of photosynthesis and water status in plants, the drought-tolerant Bachar genotype was less affected by water deficiency compared to drought-sensitive F177 (Abid et al., 2017). Rakei

et al. proposed that the level of methylation during cold stress was higher compared to demethylation (29.05 vs 19.79 %) in the tolerant genotype and (27.92 vs 22.09 %) in the susceptible genotype. However, shifts in demethylated bands were higher in the resistant genotype than in the susceptible genotype for prolonged periods of cold stress (9.24 vs 4.13 percent), indicating a higher propensity for activation of cold stress tolerant genes (Rakei, Maali-Amiri, Zeinali, & Ranjbar, 2016). Here, the hypermethylation in the upstream/promoter region in the sensitive genotype was correlated to downregulation of its gene expression. In plants, methylation in the promoter region is typically associated with inhibition of gene transcription, leading to gene expression failure (Zhang, Lang, & Zhu, 2018). In contrast, in the tolerant genotype, upstream/promoter region hypomethylation was associated with upregulation of gene expression. This is the first study to examine differentially expressed genes (DEGs) associated differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in these two significant chickpea genotypes. In contrasting genotypes, the disparity in the differential methylated regions and genes associated with the DMRs is an indicator of the relationship between DNA methylation and gene expression under drought stress. The study provided insightful datasets for both genotypes that will lead to future studies aimed at unmasking the role of these important epigenetic regulatory mechanisms involved in drought tolerance and sensitivity. This study will enhance the understanding of the various adaptation methods of drought stress in chickpea and the mechanisms which maybe behind these responses. This information can be further used to develop drought resistant chickpea varieties. Further investigation of the DMRs and related genes needs to be performed to elucidate the relationship between DNA methylation and gene regulation.

It is important to utilise the information gathered from the genomic approaches to further understand their actual function in the genotype and phenotype for implementation into the breeding processes. CRISPR/Cas9 editing is the best approach to explore the functional status

of the gene of interest. There have been many studies which have listed candidate genes which may play an important role in drought tolerance. However, the immediate need is to further investigate these genes. With this aim I used chickpea protoplasts to perform *in vivo* knockout of the *RVE7* and *4CL* genes.

5.3 CRISPR/Cas9 DNA free editing offers a solution to the development of resilient chickpea varieties

The final aim of this research was to perform knockout of selected genes in chickpea protoplasts to test the functionality of the CRISPR/Cas9 system in this crop. Due to chickpea's recalcitrant response to *in vitro* regeneration and genetic transformation, there is an urgent need to develop stringent transformation methods to improve this crop. This is the first proof of concept research demonstrating that CRISPR/Cas9 gene editing can be used in chickpea protoplasts to achieve selective genome editing. By knocking out the desired gene, followed by protoplast regeneration or using the plant tissue for transformation, the results obtained from this study could aid in the development of new characteristics and an understanding of the drought response mechanism in chickpea.

For many important food crops, DNA-free CRISPR editing has already been successfully applied (Andersson et al., 2018; Brandt, Gunn, Moretti, & Zemetra, 2020; Kim et al., 2017; Liang et al., 2017; Malnoy et al., 2016; Jongjin Park & Choe, 2019; Svitashev, Schwartz, Lenderts, Young, & Mark Cigan, 2016; Woo et al., 2015). Woo et al. first employed DNA-free genome editing in protoplasts of four plants with preassembled CRISPR/Cas9 ribonucleoproteins. The protoplasts of *Arabidopsis thaliana*, tobacco, lettuce and rice were transformed and regenerated with a mutagenesis frequency of up to 46% (Woo et al., 2015). In 2016, by editing the MLO-7 gene to increase resistance to powdery mildew in grapevine cultivars, and DIPM-1, DIPM-2, and DIPM-4 in apples to increase resistance to fire blight

disease, Malony et al. achieved DNA-free genome editing in apples and grapevines (Malnoy et al., 2016). Svitashev et al. performed similar experiments with maize embryos using biolistic delivery, targeting LIG, ALS2, MS26 and MS45 genes (Svitashev et al., 2016). Liang and team achieved completely transgene-free mutants in bread wheat using DNA free genome editing in wheat embryos (Liang et al., 2017). Additionally, Anderson et al. reported DNA-free editing in potato in 2018 (Andersson et al., 2018); Park et al. conducted similar research in lettuce (Park et al., 2019). Recently, Brand et al. published a streamlined protocol for wheat protoplasts for DNA-free editing using polyethylene glycol (PEG)-mediated transformation, achieving a variable transformation efficiency for selected gene and knockout scores of 0 to 43 (Brandt et al., 2020).

Interestingly, in many plants, transient expression systems have proven to be helpful in rapid high-throughput screening and systematic gene and protein characterisation. Such transient expression systems are of high importance in plants where stable transformation protocols take time and/or are inefficient. The transient system of expression of chickpea presented in this study can be used as a tool to help leverage the increasing quantities of genomic data available and will be helpful in speeding up the discovery of genes associated with drought tolerance and improving crop characteristics in chickpea.

In this study, two important drought tolerance-related genes were selected based on their expression levels in the tolerant and sensitive genotype under drought stress. *RVE7* is a MYB transcription factor involved in regulatory circadian rhythm in plants (Kuno et al., 2003; Lu, Knowles, Andronis, Ong, & Tobin, 2009). The *RVE7* gene was found to be highly upregulated at the reproductive stage in the drought tolerant genotype compared to the sensitive genotype (Bhaskarla et al., 2020). In this study the *RVE7* sgRNA 1 results showed 77.3% of indel percentage for the treated protoplast samples compared to control sample. The *4-Coumarate ligase* (*4CL*) is a central enzyme in the lignin biosynthesis pathway for phenylpropanoid

metabolism. This enzyme is known to manage the aggregation of lignin in other plants under stress conditions (Ehlting et al., 1999; Liu et al., 2016). A study in maize proposed that leaf lignin content can impact drought tolerance, and further investigation of this trait should be a priority (Hu et al., 2009). This research will help unravel the function of these genes under drought stress and bring us a step closer to understanding the mechanisms of the complicated drought stress mechanism. This is the first research using CRISPR/Cas9 DNA free gene editing of candidate genes linked to drought in chickpea protoplast studies.

5.4 Contribution to new knowledge

- In this study, for the first-time, drought sensitive (ICC283) and drought tolerant (ICC8261) genotypes were used for elucidation of the relationship between gene expression and DNA methylation patterns using RNA sequencing and reduced representative bisulphite sequencing under drought stress. Both chickpea genotypes possess enormous differences in root length and density. Their comparative difference in phenotype is a result of a changes in root traits and transpiration efficiency. The genotypes selected in the study are used as parents for selective traits in plant breeding due to their importance in drought tolerance and yield.

- The results from Chapters 2 and 3 provide insight into the different drought tolerance mechanisms and pathways related to the differentially expressed genes. The genes which were differentially regulated in both genotypes were found to be related to differentially methylated regions and associated genes. This knowledge can be further used for the functional analysis of these candidate genes and help in designing strategies to mitigate drought stress in chickpea.

- Additionally, the gene candidates selected for CRISPR/Cas9 gene free editing will assist the understanding of the role of these candidate genes in drought

tolerance/sensitivity which would further result in the development of a drought tolerant variety with higher yield under diverse climatic conditions. This is the first study in chickpea utilising CRISPR/Cas 9 gene free editing of selected candidate genes. For the *RVE7* gene a high editing efficiency was achieved in this study. Streamlined protocols for achieving high editing efficiency for the chickpea protoplast were developed and presented.

5.5 Future Research

The results of this study have added to existing knowledge on the drought tolerance mechanism and provided insight into the potential candidate genes which may play major role in combatting drought stress. Future research should explore the individual role of these genes, their mechanisms, and the pathways involved in field studies for promising drought tolerant chickpea varieties using more robust experimental approaches. Recommended areas for future research based on the findings of this study are:

1) The differentially expressed genes which were highly differentially expressed under drought stress in the SAM stage needs to be studied individually to gain insight into their specific roles in chickpea drought tolerance. A similar study can be conducted with the ICC4958 genotype, as it is the most drought tolerant known genotype and widely used cultivar in chickpea breeding programmes. A study with more contrasting genotypes in their drought tolerance can be explored using RNA sequencing. Genes such as: ethylene response (LOC101499805), MYB-related protein (LOC10150156), xyloglucan endotransglycosylase (LOC101508375), alkane hydroxylase MAH-like (LOC101495087), BON-1 associated, peroxidase 3 (LOC101502816), cysteine-rich and transmembrane domain (LOC101497939;), vignain (LOC101497939;) and mitochondrial uncoupling (LOC101493136), which

were specifically up-regulated in the tolerant genotype and down-regulated in sensitive should be considered as potential candidates for the further examination. A comparative study and functional validation of these genes should be conducted.

2) DNA methylation data has provided knowledge about the correlation between the differential methylation patterns and differential expressions of genotypes used in this study. However, future studies should not only take this information into consideration but also validate the relation using qPCR-based methods. Interestingly, using the CRISPR/Cas9 system, even epigenetic editing has become possible (Khan et al., 2018). Fused enzymes such as DNA methylases, histone acetyltransferases, and deacetylases, using inactive dCas9 as a DNA-binding domain platform, can be targeted to modify the epigenetic state at exact locations within the genome. For genome-wide screening, Cas9 epigenetic effectors (epiCas9s) may also be used to discover novel connections between epigenetic changes, chromatin states and phenotypes such as cellular differentiation or progression of disease. A study to investigate the epigenetic changes following CRISPR/Cas9 editing can also be performed (Lee et al., 2020).

3) In the current scenario, it is right to comment that the future will bring more challenges in terms of food security. CRISPR /Cas9 gene technology offer a way to meet many of these challenges. Setting up successful *in vitro* work for future experiments will not only save time and resources but also help to select the right targets for the modification, which will result in transgenic plants. An optimised method for protoplast isolation, target selection, and delivery method presented here for chickpea protoplasts will speed up the process for future experiments. Functional validation of the genes which were selected in this study must be conducted to understand their role in drought tolerant chickpea genotypes. The effect of knockout on drought stress imposition in plants should be further exploited. For future studies, prior to the

regeneration of protoplasts and the transformation of plant tissue, it is recommended to perform *in vivo* experiments with all target sgRNA sets. *RVE7* and *4CL* represent important candidates for further studies and delving further into their roles and functions may be useful for future breeding approaches. Currently, there is very little known about the individual function of these genes in terms of the drought mechanism; establishing core experiments to resolve their role by knockout in plant tissue using CRISPR/Cas9, microscopy to examine structural changes, and qPCR and NGS for expression profiles may lead to significant progress in the development of drought tolerant chickpea varieties.

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Appendices

Appendix Chapter 2

1) Supplementary material 1

Description: (Tables of differentially expressed genes in both genotypes)

Table1 : Differentially expressed genes in tolerant genotype	
Gene ID	LOG2FC
LOC101513086	3.376259
LOC101500742	3.513102
LOC101508200	5.472741
LOC101503652	3.180212
LOC101490752	4.153101
LOC101510690	5.29337
LOC101504337	4.304191
LOC101497005	4.732265
LOC101507529	4.496636
LOC101504724	4.477872
LOC101507843	4.183602
LOC101507599	4.20817
LOC101488329	3.407542
LOC101511679	3.916201
LOC101490264	3.971419
LOC101506718	3.839207
LOC101507211	3.810023
LOC101509884	2.416141
LOC101505722	3.690322
LOC101495485	3.903372
LOC101515720	3.621229
LOC101498747	2.717669
LOC101511010	3.51385
LOC101490948	3.606648
LOC101497905	3.59096
LOC101498338	2.754931
LOC101489405	1.54728
LOC101488636	3.346419
LOC101505009	3.308424
LOC101500182	3.542944
LOC101502259	3.076405
LOC101492033	2.218907
LOC101511433	3.486939

LOC101489280	3.249415
LOC101502697	3.239106
LOC101504255	3.059624
LOC101506193	3.599588
LOC101496038	3.14057
LOC101500798	3.124218
LOC101496712	2.917794
LOC101503775	3.043667
LOC101500690	2.979353
LOC101495357	3.02197
LOC101512113	3.019369
LOC101499921	3.011351
LOC101512341	2.480687
LOC101488851	2.097973
LOC101498843	2.950885
LOC101488254	2.943907
LOC101512579	3.069469
LOC101498505	2.881936
LOC101510725	2.69061
LOC101505051	3.240761
LOC101497133	2.667658
LOC101513770	2.751417
LOC101497534	2.624777
LOC101504428	2.593302
LOC101510050	2.704078
LOC101501469	2.794279
LOC101498024	2.57118
LOC101514664	2.698429
LOC101514679	2.672119
LOC101508158	2.7771
LOC101513489	2.647935
LOC101511785	2.714036
LOC101494182	1.742044
LOC101497260	2.154265
LOC101510953	2.607159
LOC101488313	2.604034
LOC101488360	2.556239
LOC101506993	2.441387
LOC101496910	1.475124
LOC101491264	2.477727
LOC101504686	2.371675
LOC101514390	2.505246
LOC101491309	2.425997
LOC101514919	2.471463
LOC101491559	2.442366
LOC101495806	2.335313

LOC101494875	2.400126
HP3	2.427136
LOC101513498	2.425265
LOC101514838	2.413827
LOC101503752	2.38654
LOC101488655	2.394726
LOC101514284	2.388121
LOC101503480	2.57564
LOC101507557	2.312537
LOC101509643	2.345457
LOC101488582	2.201733
LOC101503220	2.27465
LOC101491129	2.333786
LOC101497664	2.3519
LOC101497376	2.233391
LOC101494020	2.308721
LOC101495126	2.306982
LOC101508211	2.3022
LOC101490870	2.196101
LOC101503656	2.322193
LOC101494910	2.208171
LOC101514764	2.28265
LOC101501288	2.29925
LOC101501710	2.251675
LOC101499787	2.283645
LOC101492365	2.145554
LOC101513181	2.152307
LOC101510445	2.229838
LOC101507107	2.2401
LOC101506614	2.218982
LOC101508772	2.205303
LOC101511317	2.193482
LOC101503922	2.175237
LOC101492587	2.17454
LOC101507763	2.113662
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2) Supplementary material 2

Description: (List of GO terms and categories for both genotypes (GSEA RESULTS)

GO	GO NAME	GO CATERGORY	SIZE	ES	NES	PVALUE	FDR
GO:0008406	gonad development	BIOLOGICAL_PROCESS	62	-0.21	-3.44756	0	0
GO:0006898	receptor-mediated endocytosis	BIOLOGICAL_PROCESS	71	-0.24	-2.72636	0	0
GO:0097190	apoptotic signaling pathway	BIOLOGICAL_PROCESS	55	-0.15	-2.64879	0	0
GO:1900457	regulation of brassinosteroid mediated signaling pathway	BIOLOGICAL_PROCESS	20	0.375	2.586066	0	0
GO:0006897	endocytosis	BIOLOGICAL_PROCESS	138	-0.14	-2.56145	0	0
GO:0003697	single-stranded DNA binding	MOLECULAR_FUNCTION	21	-0.4	-2.42643	0	0
GO:0009798	axis specification	BIOLOGICAL_PROCESS	56	0.241	2.406326	0	0
GO:0009640	photomorphogenesis	BIOLOGICAL_PROCESS	72	0.213	2.350722	0	0
GO:0002832	negative regulation of response to biotic stimulus	BIOLOGICAL_PROCESS	29	0.289	2.337291	0	0
GO:0015297	antiporter activity	MOLECULAR_FUNCTION	46	0.218	2.314418	0	0
GO:0060249	anatomical structure homeostasis	BIOLOGICAL_PROCESS	46	0.214	2.270591	0	0
GO:0046661	male sex differentiation	BIOLOGICAL_PROCESS	15	-0.45	-2.25684	0	0.045224
GO:0034101	erythrocyte homeostasis	BIOLOGICAL_PROCESS	16	0.331	2.243504	0	0.014929
GO:0010639	negative regulation of organelle organization	BIOLOGICAL_PROCESS	46	-0.19	-2.23098	0	0.051741
GO:0022832	voltage-gated channel activity	MOLECULAR_FUNCTION	16	-0.31	-2.22504	0	0.045274
GO:0090068	positive regulation of cell cycle process	BIOLOGICAL_PROCESS	40	-0.23	-2.19194	0	0.040243
GO:0008514	organic anion transmembrane transporter activity	MOLECULAR_FUNCTION	89	0.144	2.177182	0	0.026698

GO:0015079	potassium ion transmembrane transporter activity	MOLECULAR_FUNCTION	23	0.322	2.166983	0	0.023732
GO:0034708	methyltransferase complex	CELLULAR_COMPONENT	15	-0.32	-2.15879	0	0.036219
GO:0000324	fungal-type vacuole	CELLULAR_COMPONENT	21	0.382	2.15019	0	0.032327
GO:0030433	ER-associated ubiquitin-dependent protein catabolic process	BIOLOGICAL_PROCESS	19	0.371	2.139283	0	0.029388
GO:0030324	lung development	BIOLOGICAL_PROCESS	19	-0.31	-2.13647	0	0.041191
GO:0008270	zinc ion binding	MOLECULAR_FUNCTION	203	0.103	2.13039	0	0.026939
GO:0048732	gland development	BIOLOGICAL_PROCESS	44	-0.26	-2.12441	0	0.037759
GO:0005874	microtubule	CELLULAR_COMPONENT	82	0.156	2.124357	0	0.033378
GO:0000322	storage vacuole	CELLULAR_COMPONENT	34	0.255	2.11965	0	0.030994
GO:0008320	protein transmembrane transporter activity	MOLECULAR_FUNCTION	18	0.398	2.111115	0	0.028928
GO:0008213	protein alkylation	BIOLOGICAL_PROCESS	34	-0.26	-2.08295	0	0.034854
GO:0055065	metal ion homeostasis	BIOLOGICAL_PROCESS	118	0.12	2.073193	0	0.040863
GO:0010623	programmed cell death involved in cell development	BIOLOGICAL_PROCESS	23	-0.28	-2.06921	0	0.038854
GO:0044772	mitotic cell cycle phase transition	BIOLOGICAL_PROCESS	77	-0.19	-2.06072	0	0.036263
GO:0031570	DNA integrity checkpoint	BIOLOGICAL_PROCESS	34	-0.23	-2.05912	0	0.033997
GO:0000082	G1/S transition of mitotic cell cycle	BIOLOGICAL_PROCESS	43	-0.25	-2.03297	0	0.03759
GO:0061630	ubiquitin protein ligase activity	MOLECULAR_FUNCTION	55	-0.21	-2.02392	0	0.040518
GO:0043067	regulation of programmed cell death	BIOLOGICAL_PROCESS	175	-0.12	-2.02375	0	0.038385
GO:0009259	ribonucleotide metabolic process	BIOLOGICAL_PROCESS	104	-0.11	-2.01049	0	0.050054
GO:0006873	cellular ion homeostasis	BIOLOGICAL_PROCESS	108	0.128	2.005542	0	0.089467
GO:0015893	drug transport	BIOLOGICAL_PROCESS	31	0.268	2.000383	0	0.090562
GO:0000075	cell cycle checkpoint	BIOLOGICAL_PROCESS	42	-0.2	-1.99867	0	0.0648
GO:0048527	lateral root development	BIOLOGICAL_PROCESS	144	0.143	1.985436	0	0.091619
GO:0046942	carboxylic acid transport	BIOLOGICAL_PROCESS	95	0.157	1.983344	0	0.087038
GO:2000027	regulation of organ morphogenesis	BIOLOGICAL_PROCESS	29	0.206	1.978388	0	0.088092

GO:0006479	protein methylation	BIOLOGICAL_PROCESS	34	-0.26	-1.96696	0	0.078267
GO:0042330	taxis	BIOLOGICAL_PROCESS	77	-0.12	-1.96282	0	0.074864
GO:0006302	double-strand break repair	BIOLOGICAL_PROCESS	37	-0.21	-1.96127	0	0.071744
GO:0006928	movement of cell or subcellular component	BIOLOGICAL_PROCESS	153	-0.11	-1.95513	0	0.07974
GO:0051336	regulation of hydrolase activity	BIOLOGICAL_PROCESS	82	-0.13	-1.95485	0	0.076673
GO:0019787	ubiquitin-like protein transferase activity	MOLECULAR_FUNCTION	109	-0.14	-1.95078	0	0.087201
GO:2000122	negative regulation of stomatal complex development	BIOLOGICAL_PROCESS	15	0.345	1.949464	0	0.094036
GO:2000030	regulation of response to red or far red light	BIOLOGICAL_PROCESS	23	0.299	1.933294	0	0.123409
GO:0034248	regulation of cellular amide metabolic process	BIOLOGICAL_PROCESS	135	0.13	1.924915	0	0.127451
GO:0010976	positive regulation of neuron projection development	BIOLOGICAL_PROCESS	30	-0.23	-1.92318	0	0.106615
GO:0030003	cellular cation homeostasis	BIOLOGICAL_PROCESS	98	0.151	1.918726	0	0.130883
GO:0021700	developmental maturation	BIOLOGICAL_PROCESS	203	0.098	1.915743	0	0.125849
GO:0022884	macromolecule transmembrane transporter activity	MOLECULAR_FUNCTION	18	0.398	1.915213	0	0.121188
GO:0006954	inflammatory response	BIOLOGICAL_PROCESS	22	-0.25	-1.91354	0	0.106028
GO:0022804	active transmembrane transporter activity	MOLECULAR_FUNCTION	192	0.097	1.913274	0	0.11686
GO:0009612	response to mechanical stimulus	BIOLOGICAL_PROCESS	39	-0.26	-1.91264	0	0.102494
GO:1905114	cell surface receptor signaling pathway involved in cell-cell signaling	BIOLOGICAL_PROCESS	54	0.143	1.909569	0	0.120434
GO:0099513	polymeric cytoskeletal fiber	CELLULAR_COMPONENT	94	0.146	1.905443	0	0.116419
GO:0006022	aminoglycan metabolic process	BIOLOGICAL_PROCESS	16	0.315	1.904421	0	0.112664
GO:0055082	cellular chemical homeostasis	BIOLOGICAL_PROCESS	159	0.104	1.8978	0	0.109143
GO:1903047	mitotic cell cycle process	BIOLOGICAL_PROCESS	202	-0.09	-1.89481	0	0.105185
GO:0006665	sphingolipid metabolic process	BIOLOGICAL_PROCESS	22	0.347	1.893211	0	0.115653
GO:0009975	cyclase activity	MOLECULAR_FUNCTION	18	-0.35	-1.89269	0	0.101898

GO:0005657	replication fork	CELLULAR_COMPONENT	16	-0.34	-1.89251	0	0.09881
GO:0050769	positive regulation of neurogenesis	BIOLOGICAL_PROCESS	41	-0.22	-1.89112	0	0.095904
GO:0008559	xenobiotic-transporting ATPase activity	MOLECULAR_FUNCTION	16	0.245	1.887109	0	0.125143
GO:0019216	regulation of lipid metabolic process	BIOLOGICAL_PROCESS	72	-0.14	-1.88636	0	0.100864
GO:0044770	cell cycle phase transition	BIOLOGICAL_PROCESS	79	-0.19	-1.88187	0	0.098063
GO:0009636	response to toxic substance	BIOLOGICAL_PROCESS	64	-0.17	-1.87122	0	0.097868
GO:0098754	detoxification	BIOLOGICAL_PROCESS	21	-0.28	-1.86841	0	0.095292
GO:0022836	gated channel activity	MOLECULAR_FUNCTION	23	-0.31	-1.86537	0	0.102244
GO:0009821	alkaloid biosynthetic process	BIOLOGICAL_PROCESS	20	-0.3	-1.86512	0.166667	0.101949
GO:0002262	myeloid cell homeostasis	BIOLOGICAL_PROCESS	16	0.331	1.86491	0	0.146429
GO:0032102	negative regulation of response to external stimulus	BIOLOGICAL_PROCESS	33	0.239	1.864326	0	0.142362
GO:0009880	embryonic pattern specification	BIOLOGICAL_PROCESS	53	0.232	1.863099	0	0.138514
GO:0000329	fungal-type vacuole membrane	CELLULAR_COMPONENT	15	0.372	1.862911	0	0.134869
GO:0009875	pollen-pistil interaction	BIOLOGICAL_PROCESS	15	-0.33	-1.86287	0	0.101781
GO:0010948	negative regulation of cell cycle process	BIOLOGICAL_PROCESS	41	-0.2	-1.85974	0	0.099358
GO:0090332	stomatal closure	BIOLOGICAL_PROCESS	19	-0.29	-1.85918	0	0.097047
GO:0010074	maintenance of meristem identity	BIOLOGICAL_PROCESS	37	0.193	1.858484	0	0.137038
GO:0050770	regulation of axonogenesis	BIOLOGICAL_PROCESS	16	-0.29	-1.8516	0	0.098958
GO:0050660	flavin adenine dinucleotide binding	MOLECULAR_FUNCTION	30	-0.29	-1.84695	0	0.098752
GO:1900376	regulation of secondary metabolite biosynthetic process	BIOLOGICAL_PROCESS	41	0.218	1.832608	0	0.15555
GO:0055081	anion homeostasis	BIOLOGICAL_PROCESS	41	0.173	1.821526	0	0.157071
GO:0045137	development of primary sexual characteristics	BIOLOGICAL_PROCESS	62	-0.21	-1.81954	0	0.11627
GO:0070993	translation preinitiation complex	CELLULAR_COMPONENT	18	0.326	1.819298	0	0.155921

GO:1900459	positive regulation of brassinosteroid mediated signaling pathway	BIOLOGICAL_PROCESS	17	0.345	1.815204	0	0.16246
GO:0007018	microtubule-based movement	BIOLOGICAL_PROCESS	49	-0.2	-1.81114	0	0.121519
GO:0000272	polysaccharide catabolic process	BIOLOGICAL_PROCESS	64	0.168	1.809421	0	0.163754
GO:0071453	cellular response to oxygen levels	BIOLOGICAL_PROCESS	46	0.206	1.803248	0	0.172305
GO:0098771	inorganic ion homeostasis	BIOLOGICAL_PROCESS	144	0.108	1.800829	0	0.173303
GO:0006816	calcium ion transport	BIOLOGICAL_PROCESS	39	0.159	1.799274	0	0.169616
GO:0005813	centrosome	CELLULAR_COMPONENT	46	-0.23	-1.79925	0.166667	0.128403
GO:0015718	monocarboxylic acid transport	BIOLOGICAL_PROCESS	41	0.217	1.796888	0	0.170645
GO:0007032	endosome organization	BIOLOGICAL_PROCESS	17	-0.33	-1.79581	0	0.129492
GO:0010008	endosome membrane	CELLULAR_COMPONENT	68	-0.16	-1.79528	0	0.126902
GO:0061640	cytoskeleton-dependent cytokinesis	BIOLOGICAL_PROCESS	78	-0.17	-1.79062	0	0.135034
GO:0019725	cellular homeostasis	BIOLOGICAL_PROCESS	206	0.091	1.787214	0	0.169296
GO:0035567	non-canonical Wnt signaling pathway	BIOLOGICAL_PROCESS	22	0.288	1.78664	0	0.16591
GO:0031346	positive regulation of cell projection organization	BIOLOGICAL_PROCESS	37	-0.25	-1.78418	0	0.137686
GO:0010015	root morphogenesis	BIOLOGICAL_PROCESS	242	0.091	1.78342	0	0.166938
GO:0006112	energy reserve metabolic process	BIOLOGICAL_PROCESS	22	0.273	1.782027	0	0.16584
GO:1902115	regulation of organelle assembly	BIOLOGICAL_PROCESS	15	-0.39	-1.77926	0	0.138513
GO:0032784	regulation of DNA-templated transcription, elongation	BIOLOGICAL_PROCESS	15	-0.35	-1.77627	0	0.137613
GO:0061659	ubiquitin-like protein ligase activity	MOLECULAR_FUNCTION	55	-0.21	-1.77418	0	0.136755
GO:0000578	embryonic axis specification	BIOLOGICAL_PROCESS	37	0.258	1.773686	0	0.172868
GO:0042910	xenobiotic transporter activity	MOLECULAR_FUNCTION	16	0.245	1.770938	0	0.173653
GO:0071375	cellular response to peptide hormone stimulus	BIOLOGICAL_PROCESS	24	0.263	1.764055	0	0.184518
GO:0048585	negative regulation of response to stimulus	BIOLOGICAL_PROCESS	262	0.098	1.763779	0	0.183173

GO:0004857	enzyme inhibitor activity	MOLECULAR_FUNCTION	43	-0.2	-1.76361	0	0.145738
GO:0071383	cellular response to steroid hormone stimulus	BIOLOGICAL_PROCESS	91	0.117	1.758103	0	0.189574
GO:0007265	Ras protein signal transduction	BIOLOGICAL_PROCESS	24	-0.22	-1.75578	0	0.151187
GO:0071482	cellular response to light stimulus	BIOLOGICAL_PROCESS	86	0.141	1.75452	0	0.195663
GO:0048825	cotyledon development	BIOLOGICAL_PROCESS	63	0.171	1.750795	0	0.197911
GO:0071824	protein-DNA complex subunit organization	BIOLOGICAL_PROCESS	50	0.108	1.750226	0	0.194612
GO:0035295	tube development	BIOLOGICAL_PROCESS	81	-0.15	-1.74826	0	0.157986
GO:0010243	response to organonitrogen compound	BIOLOGICAL_PROCESS	214	0.104	1.746513	0	0.195001
GO:0072503	cellular divalent inorganic cation homeostasis	BIOLOGICAL_PROCESS	45	0.2	1.746162	0	0.191856
GO:0046873	metal ion transmembrane transporter activity	MOLECULAR_FUNCTION	66	0.152	1.745617	0	0.188811
GO:0071774	response to fibroblast growth factor	BIOLOGICAL_PROCESS	15	0.317	1.744674	0	0.18756
GO:0010101	post-embryonic root morphogenesis	BIOLOGICAL_PROCESS	91	0.147	1.744522	0	0.184674
GO:0098602	single organism cell adhesion	BIOLOGICAL_PROCESS	22	0.256	1.744021	0	0.181876
GO:0071367	cellular response to brassinosteroid stimulus	BIOLOGICAL_PROCESS	75	0.145	1.741664	0	0.179161
GO:0010259	multicellular organism aging	BIOLOGICAL_PROCESS	48	0.144	1.738991	0	0.179767
GO:0044272	sulfur compound biosynthetic process	BIOLOGICAL_PROCESS	111	0.127	1.73661	0	0.180371
GO:1903825	organic acid transmembrane transport	BIOLOGICAL_PROCESS	40	0.205	1.736223	0	0.177794
GO:0030865	cortical cytoskeleton organization	BIOLOGICAL_PROCESS	34	-0.24	-1.7362	0	0.167666
GO:0006875	cellular metal ion homeostasis	BIOLOGICAL_PROCESS	86	0.152	1.735195	0	0.17529
GO:0016482	cytosolic transport	BIOLOGICAL_PROCESS	28	0.131	1.73504	0	0.172856
GO:0031331	positive regulation of cellular catabolic process	BIOLOGICAL_PROCESS	50	0.215	1.732233	0	0.173501
GO:0050661	NADP binding	MOLECULAR_FUNCTION	25	-0.26	-1.72485	0	0.181479

GO:0048764	trichoblast maturation	BIOLOGICAL_PROCESS	90	0.111	1.721671	0	0.185963
GO:0006417	regulation of translation	BIOLOGICAL_PROCESS	132	0.129	1.715206	0	0.196657
GO:0042435	indole-containing compound biosynthetic process	BIOLOGICAL_PROCESS	33	0.229	1.714395	0	0.194069
GO:0032259	methylation	BIOLOGICAL_PROCESS	83	-0.16	-1.71192	0	0.190469
GO:0009751	response to salicylic acid	BIOLOGICAL_PROCESS	194	0.102	1.703134	0	0.20147
GO:0071462	cellular response to water stimulus	BIOLOGICAL_PROCESS	22	-0.19	-1.70249	0	0.204898
GO:0070482	response to oxygen levels	BIOLOGICAL_PROCESS	102	0.096	1.700636	0	0.200287
GO:0030001	metal ion transport	BIOLOGICAL_PROCESS	144	0.131	1.700252	0	0.199074
GO:1903510	mucopolysaccharide metabolic process	BIOLOGICAL_PROCESS	15	0.329	1.699901	0	0.19795
GO:2001234	negative regulation of apoptotic signaling pathway	BIOLOGICAL_PROCESS	20	-0.28	-1.69977	0	0.205952
GO:0015491	cation:cation antiporter activity	MOLECULAR_FUNCTION	15	0.347	1.69905	0	0.196854
GO:0009696	salicylic acid metabolic process	BIOLOGICAL_PROCESS	26	-0.25	-1.69453	0	0.20696
GO:0019843	rRNA binding	MOLECULAR_FUNCTION	72	0.108	1.692246	0	0.203774
GO:0099080	supramolecular complex	CELLULAR_COMPONENT	129	0.108	1.691206	0	0.202634
GO:0000323	lytic vacuole	CELLULAR_COMPONENT	91	0.151	1.690057	0	0.200222
GO:0050801	ion homeostasis	BIOLOGICAL_PROCESS	167	0.101	1.689551	0	0.197866
GO:0006874	cellular calcium ion homeostasis	BIOLOGICAL_PROCESS	32	0.291	1.689088	0	0.195566
GO:0055074	calcium ion homeostasis	BIOLOGICAL_PROCESS	32	0.291	1.688506	0	0.194581
GO:0010183	pollen tube guidance	BIOLOGICAL_PROCESS	38	-0.18	-1.68846	0	0.216524
GO:1900425	negative regulation of defense response to bacterium	BIOLOGICAL_PROCESS	25	0.278	1.682767	0	0.203559
GO:0031348	negative regulation of defense response	BIOLOGICAL_PROCESS	58	0.197	1.682696	0	0.201272
GO:0000228	nuclear chromosome	CELLULAR_COMPONENT	90	-0.13	-1.68263	0.166667	0.231074
GO:0009694	jasmonic acid metabolic process	BIOLOGICAL_PROCESS	26	-0.22	-1.68224	0	0.227625
GO:0071822	protein complex subunit organization	BIOLOGICAL_PROCESS	229	-0.05	-1.67817	0	0.234954
GO:0006400	tRNA modification	BIOLOGICAL_PROCESS	15	-0.41	-1.67788	0	0.234187

GO:0016571	histone methylation	BIOLOGICAL_PROCESS	29	-0.24	-1.67263	0	0.237354
GO:0030203	glycosaminoglycan metabolic process	BIOLOGICAL_PROCESS	16	0.315	1.672174	0	0.21479
GO:0060071	Wnt signaling pathway, planar cell polarity pathway	BIOLOGICAL_PROCESS	17	0.26	1.671547	0	0.213646
GO:0043434	response to peptide hormone	BIOLOGICAL_PROCESS	35	0.243	1.671053	0.166667	0.211324
GO:0000988	transcription factor activity, protein binding	MOLECULAR_FUNCTION	68	0.11	1.670611	0	0.210242
GO:0048509	regulation of meristem development	BIOLOGICAL_PROCESS	105	0.117	1.66718	0	0.213843
GO:0030496	midbody	CELLULAR_COMPONENT	19	-0.2	-1.66617	0	0.248072
GO:0043112	receptor metabolic process	BIOLOGICAL_PROCESS	21	-0.29	-1.66389	0	0.248398
GO:0009820	alkaloid metabolic process	BIOLOGICAL_PROCESS	28	-0.25	-1.66358	0	0.244995
GO:0097479	synaptic vesicle localization	BIOLOGICAL_PROCESS	26	-0.19	-1.66203	0	0.242895
GO:0046467	membrane lipid biosynthetic process	BIOLOGICAL_PROCESS	19	0.269	1.66061	0	0.223023
GO:0071446	cellular response to salicylic acid stimulus	BIOLOGICAL_PROCESS	69	0.13	1.65988	0	0.221833
GO:0046149	pigment catabolic process	BIOLOGICAL_PROCESS	16	-0.36	-1.65911	0	0.244553
GO:0000226	microtubule cytoskeleton organization	BIOLOGICAL_PROCESS	138	-0.11	-1.65907	0.166667	0.241336
GO:1901699	cellular response to nitrogen compound	BIOLOGICAL_PROCESS	70	0.154	1.658751	0	0.224075
GO:1901652	response to peptide	BIOLOGICAL_PROCESS	35	0.243	1.657881	0	0.221789
GO:0005815	microtubule organizing center	CELLULAR_COMPONENT	69	-0.18	-1.65762	0	0.239366
GO:0048870	cell motility	BIOLOGICAL_PROCESS	92	-0.14	-1.65688	0	0.238682
GO:0006826	iron ion transport	BIOLOGICAL_PROCESS	24	0.27	1.654476	0	0.222817
GO:0043085	positive regulation of catalytic activity	BIOLOGICAL_PROCESS	121	-0.12	-1.65407	0	0.237949
GO:0030243	cellulose metabolic process	BIOLOGICAL_PROCESS	53	0.131	1.653699	0	0.221696
GO:0008757	S-adenosylmethionine-dependent methyltransferase activity	MOLECULAR_FUNCTION	57	-0.18	-1.65285	0	0.236094
GO:0000041	transition metal ion transport	BIOLOGICAL_PROCESS	39	0.22	1.650098	0	0.229181

GO:0044440	endosomal part	CELLULAR_COMPONENT	70	-0.17	-1.64834	0	0.239882
GO:0071806	protein transmembrane transport	BIOLOGICAL_PROCESS	36	0.216	1.646276	0	0.232224
GO:0071363	cellular response to growth factor stimulus	BIOLOGICAL_PROCESS	43	0.216	1.645926	0	0.22997
GO:0012506	vesicle membrane	CELLULAR_COMPONENT	87	0.148	1.641922	0	0.234048
GO:0042058	regulation of epidermal growth factor receptor signaling pathway	BIOLOGICAL_PROCESS	18	0.223	1.640531	0	0.234974
GO:0009567	double fertilization forming a zygote and endosperm	BIOLOGICAL_PROCESS	16	0.321	1.637504	0	0.238967

Go terms related to ICC283 genotype

GO ID	GO name	GO category	SIZE	ES	NES
GO:0071669	plant-type cell wall organization or biogenesis	BIOLOGICAL_PROCESS	237	0.210115	4.988905
GO:0042546	cell wall biogenesis	BIOLOGICAL_PROCESS	199	0.217466	4.434249
GO:0044550	secondary metabolite biosynthetic process	BIOLOGICAL_PROCESS	201	0.155177	4.004488
GO:0010393	galacturonan metabolic process	BIOLOGICAL_PROCESS	99	0.2786	3.513796
GO:0051253	negative regulation of RNA metabolic process	BIOLOGICAL_PROCESS	213	-0.13767	-3.5019
GO:0016757	transferase activity, transferring glycosyl groups	MOLECULAR_FUNCTION	174	0.172025	3.492442
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	MOLECULAR_FUNCTION	126	0.212251	3.490417
GO:0044264	cellular polysaccharide metabolic process	BIOLOGICAL_PROCESS	181	0.182377	3.312904
GO:0048285	organelle fission	BIOLOGICAL_PROCESS	149	-0.19444	-3.22983
GO:0033365	protein localization to organelle	BIOLOGICAL_PROCESS	142	-0.198	-3.21502
GO:0008361	regulation of cell size	BIOLOGICAL_PROCESS	49	0.364205	3.196557

GO:0009832	plant-type cell wall biogenesis	BIOLOGICAL_PROCESS	167	0.210872	3.140556
GO:0016458	gene silencing	BIOLOGICAL_PROCESS	104	-0.20698	-3.08034
GO:0033692	cellular polysaccharide biosynthetic process	BIOLOGICAL_PROCESS	130	0.195701	3.067376
GO:0034637	cellular carbohydrate biosynthetic process	BIOLOGICAL_PROCESS	151	0.19516	3.043807
GO:0009698	phenylpropanoid metabolic process	BIOLOGICAL_PROCESS	161	0.193626	3.032111
GO:0030104	water homeostasis	BIOLOGICAL_PROCESS	20	0.539514	3.026625
GO:0006412	translation	BIOLOGICAL_PROCESS	145	-0.2341	-3.0076
GO:0044451	nucleoplasm part	CELLULAR_COMPONENT	123	-0.22349	-2.99232
GO:0016758	transferase activity, transferring hexosyl groups	MOLECULAR_FUNCTION	153	0.189389	2.989792
GO:0000956	nuclear-transcribed mRNA catabolic process	BIOLOGICAL_PROCESS	48	-0.28942	-2.95497
GO:0031225	anchored component of membrane	CELLULAR_COMPONENT	113	0.218491	2.928907
GO:0006417	regulation of translation	BIOLOGICAL_PROCESS	77	-0.19635	-2.92374
GO:0010191	mucilage metabolic process	BIOLOGICAL_PROCESS	75	0.230838	2.907675
GO:0042254	ribosome biogenesis	BIOLOGICAL_PROCESS	101	-0.20501	-2.89353
GO:0044036	cell wall macromolecule metabolic process	BIOLOGICAL_PROCESS	124	0.224458	2.891782
GO:0016722	oxidoreductase activity, oxidizing metal ions	MOLECULAR_FUNCTION	18	0.60937	2.851489
GO:0016798	hydrolase activity, acting on glycosyl bonds	MOLECULAR_FUNCTION	142	0.188099	2.850641
GO:0033043	regulation of organelle organization	BIOLOGICAL_PROCESS	159	-0.19892	-2.84566
GO:0000398	mRNA splicing, via spliceosome	BIOLOGICAL_PROCESS	67	-0.30424	-2.83477
GO:0015077	monovalent inorganic cation transmembrane transporter activity	MOLECULAR_FUNCTION	52	-0.2261	-2.79908
GO:0009834	plant-type secondary cell wall biogenesis	BIOLOGICAL_PROCESS	113	0.236433	2.792019

GO:0009451	RNA modification	BIOLOGICAL_PROCESS	118	-0.1803	-2.79086
GO:0008380	RNA splicing	BIOLOGICAL_PROCESS	95	-0.23503	-2.78325
GO:0005694	chromosome	CELLULAR_COMPONENT	144	-0.17732	-2.76511
GO:0000271	polysaccharide biosynthetic process	BIOLOGICAL_PROCESS	136	0.21066	2.764758
GO:0016876	ligase activity, forming aminoacyl-tRNA and related compounds	MOLECULAR_FUNCTION	15	-0.51782	-2.75545
GO:0009699	phenylpropanoid biosynthetic process	BIOLOGICAL_PROCESS	141	0.189845	2.710647
GO:0034248	regulation of cellular amide metabolic process	BIOLOGICAL_PROCESS	80	-0.2059	-2.692
GO:0009741	response to brassinosteroid	BIOLOGICAL_PROCESS	127	0.171897	2.689139
GO:0034660	ncRNA metabolic process	BIOLOGICAL_PROCESS	141	-0.20837	-2.68254
GO:0043043	peptide biosynthetic process	BIOLOGICAL_PROCESS	150	-0.22985	-2.6808
GO:0018193	peptidyl-amino acid modification	BIOLOGICAL_PROCESS	135	-0.15618	-2.67801
GO:0010345	suberin biosynthetic process	BIOLOGICAL_PROCESS	29	0.349709	2.668956
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	BIOLOGICAL_PROCESS	78	-0.27422	-2.66755
GO:0017038	protein import	BIOLOGICAL_PROCESS	52	-0.2669	-2.65793
GO:0008194	UDP-glycosyltransferase activity	MOLECULAR_FUNCTION	114	0.154359	2.647269
GO:0009739	response to gibberellin	BIOLOGICAL_PROCESS	147	0.201389	2.645179
GO:0051222	positive regulation of protein transport	BIOLOGICAL_PROCESS	26	-0.24823	-2.62383
GO:0009627	systemic acquired resistance	BIOLOGICAL_PROCESS	69	0.239164	2.619169
GO:0016051	carbohydrate biosynthetic process	BIOLOGICAL_PROCESS	225	0.142274	2.611648
GO:0016875	ligase activity, forming carbon-oxygen bonds	MOLECULAR_FUNCTION	15	-0.51782	-2.61023
GO:0000785	chromatin	CELLULAR_COMPONENT	81	-0.20593	-2.59602
GO:0035304	regulation of protein dephosphorylation	BIOLOGICAL_PROCESS	15	0.456229	2.589486

GO:0019900	kinase binding	MOLECULAR_FUNCTION	106	0.199277	2.587831
GO:0034212	peptide N-acetyltransferase activity	MOLECULAR_FUNCTION	18	-0.36719	-2.58358
GO:0010410	hemicellulose metabolic process	BIOLOGICAL_PROCESS	92	0.255413	2.55643
GO:0016298	lipase activity	MOLECULAR_FUNCTION	51	0.26877	2.556263
GO:0022890	inorganic cation transmembrane transporter activity	MOLECULAR_FUNCTION	77	-0.21348	-2.55622
GO:0051169	nuclear transport	BIOLOGICAL_PROCESS	69	-0.22958	-2.54553
GO:0045491	xylan metabolic process	BIOLOGICAL_PROCESS	65	0.247938	2.544622
GO:1902679	negative regulation of RNA biosynthetic process	BIOLOGICAL_PROCESS	209	-0.13443	-2.54413
GO:0060968	regulation of gene silencing	BIOLOGICAL_PROCESS	33	-0.26325	-2.54353
GO:0006281	DNA repair	BIOLOGICAL_PROCESS	119	-0.16662	-2.54069
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	BIOLOGICAL_PROCESS	24	-0.39192	-2.53727
GO:0051028	mRNA transport	BIOLOGICAL_PROCESS	27	-0.34953	-2.53579
GO:0006397	mRNA processing	BIOLOGICAL_PROCESS	116	-0.2396	-2.53368
GO:0052689	carboxylic ester hydrolase activity	MOLECULAR_FUNCTION	89	0.212012	2.523194
GO:0009787	regulation of abscisic acid-activated signaling pathway	BIOLOGICAL_PROCESS	77	0.252383	2.522788
GO:1902600	hydrogen ion transmembrane transport	BIOLOGICAL_PROCESS	16	-0.45589	-2.51862
GO:0042981	regulation of apoptotic process	BIOLOGICAL_PROCESS	95	-0.17178	-2.51462
GO:0010639	negative regulation of organelle organization	BIOLOGICAL_PROCESS	53	-0.26647	-2.51077
GO:0016570	histone modification	BIOLOGICAL_PROCESS	87	-0.22367	-2.50845
GO:0006325	chromatin organization	BIOLOGICAL_PROCESS	140	-0.1602	-2.50828
GO:0098687	chromosomal region	CELLULAR_COMPONENT	51	-0.25475	-2.49536
GO:0071215	cellular response to abscisic acid stimulus	BIOLOGICAL_PROCESS	163	0.165232	2.490457

GO:0045892	negative regulation of transcription, DNA-templated	BIOLOGICAL_PROCESS	207	-0.13277	-2.49044
GO:0010214	seed coat development	BIOLOGICAL_PROCESS	75	0.221602	2.488291
GO:0072594	establishment of protein localization to organelle	BIOLOGICAL_PROCESS	108	-0.19702	-2.48391
GO:0006972	hyperosmotic response	BIOLOGICAL_PROCESS	74	0.237362	2.480948
GO:0097306	cellular response to alcohol	BIOLOGICAL_PROCESS	168	0.155865	2.464468
GO:1903047	mitotic cell cycle process	BIOLOGICAL_PROCESS	167	-0.15107	-2.46404
GO:0006638	neutral lipid metabolic process	BIOLOGICAL_PROCESS	18	-0.39737	-2.4565
GO:0046527	glucosyltransferase activity	MOLECULAR_FUNCTION	91	0.214174	2.451861
GO:0006366	transcription from RNA polymerase II promoter	BIOLOGICAL_PROCESS	183	-0.14181	-2.44544
GO:0009664	plant-type cell wall organization	BIOLOGICAL_PROCESS	124	0.216168	2.444129
GO:0009740	gibberellic acid mediated signaling pathway	BIOLOGICAL_PROCESS	65	0.252174	2.444084
GO:1903829	positive regulation of cellular protein localization	BIOLOGICAL_PROCESS	30	-0.35516	-2.44358
GO:0051168	nuclear export	BIOLOGICAL_PROCESS	31	-0.36363	-2.43475
GO:0042538	hyperosmotic salinity response	BIOLOGICAL_PROCESS	61	0.260024	2.430423
GO:0080167	response to karrikin	BIOLOGICAL_PROCESS	153	0.137032	2.423678
GO:0016569	covalent chromatin modification	BIOLOGICAL_PROCESS	94	-0.22078	-2.41934
GO:0033044	regulation of chromosome organization	BIOLOGICAL_PROCESS	68	-0.21025	-2.41356
GO:1901419	regulation of response to alcohol	BIOLOGICAL_PROCESS	77	0.252383	2.398594
GO:0022613	ribonucleoprotein complex biogenesis	BIOLOGICAL_PROCESS	139	-0.17466	-2.3971
GO:0000375	RNA splicing, via transesterification reactions	BIOLOGICAL_PROCESS	78	-0.27422	-2.39466
GO:0040029	regulation of gene expression, epigenetic	BIOLOGICAL_PROCESS	108	-0.18189	-2.39105
GO:0032270	positive regulation of cellular protein metabolic process	BIOLOGICAL_PROCESS	107	-0.18988	-2.3907
GO:0031175	neuron projection development	BIOLOGICAL_PROCESS	75	-0.18749	-2.3885

GO:0006405	RNA export from nucleus	BIOLOGICAL_PROCESS	24	-0.37257	-2.38536
GO:0006342	chromatin silencing	BIOLOGICAL_PROCESS	57	-0.21975	-2.38353
GO:0071370	cellular response to gibberellin stimulus	BIOLOGICAL_PROCESS	68	0.26798	2.38339
GO:0003735	structural constituent of ribosome	MOLECULAR_FUNCTION	31	-0.34485	-2.37823
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	BIOLOGICAL_PROCESS	230	-0.13653	-2.36955
GO:0044087	regulation of cellular component biogenesis	BIOLOGICAL_PROCESS	96	-0.20719	-2.36736
GO:0043254	regulation of protein complex assembly	BIOLOGICAL_PROCESS	43	-0.24193	-2.36607
GO:0015078	hydrogen ion transmembrane transporter activity	MOLECULAR_FUNCTION	32	-0.30015	-2.36505
GO:0034708	methyltransferase complex	CELLULAR_COMPONENT	17	-0.43241	-2.35925
GO:1902589	single-organism organelle organization	BIOLOGICAL_PROCESS	194	-0.14097	-2.34956
GO:0031226	intrinsic component of plasma membrane	CELLULAR_COMPONENT	216	0.158958	2.348425
GO:0048858	cell projection morphogenesis	BIOLOGICAL_PROCESS	55	-0.21978	-2.34683
GO:0006275	regulation of DNA replication	BIOLOGICAL_PROCESS	23	-0.28831	-2.3467
GO:0071166	ribonucleoprotein complex localization	BIOLOGICAL_PROCESS	25	-0.37768	-2.34538
GO:0044744	protein targeting to nucleus	BIOLOGICAL_PROCESS	28	-0.31081	-2.34348
GO:0030201	heparan sulfate proteoglycan metabolic process	BIOLOGICAL_PROCESS	15	0.416097	2.340223
GO:0000791	euchromatin	CELLULAR_COMPONENT	15	-0.41838	-2.33878
GO:0010476	gibberellin mediated signaling pathway	BIOLOGICAL_PROCESS	68	0.26798	2.330635
GO:0031123	RNA 3'-end processing	BIOLOGICAL_PROCESS	24	-0.34361	-2.31819
GO:0035303	regulation of dephosphorylation	BIOLOGICAL_PROCESS	16	0.468874	2.315926
GO:0006639	acylglycerol metabolic process	BIOLOGICAL_PROCESS	18	-0.39737	-2.30817
GO:0051247	positive regulation of protein metabolic process	BIOLOGICAL_PROCESS	114	-0.1816	-2.30767

GO:0010383	cell wall polysaccharide metabolic process	BIOLOGICAL_PROCESS	105	0.235921	2.307297
GO:0006402	mRNA catabolic process	BIOLOGICAL_PROCESS	53	-0.25116	-2.30605
GO:0010029	regulation of seed germination	BIOLOGICAL_PROCESS	76	0.216197	2.302647
GO:0042545	cell wall modification	BIOLOGICAL_PROCESS	134	0.167015	2.30077
GO:0006401	RNA catabolic process	BIOLOGICAL_PROCESS	58	-0.23694	-2.29743
GO:1902275	regulation of chromatin organization	BIOLOGICAL_PROCESS	43	-0.29264	-2.29533
GO:0004812	aminoacyl-tRNA ligase activity	MOLECULAR_FUNCTION	15	-0.51782	-2.27234
GO:0006833	water transport	BIOLOGICAL_PROCESS	23	0.37031	2.268342
GO:0070201	regulation of establishment of protein localization	BIOLOGICAL_PROCESS	48	-0.18887	-2.26732
GO:0048359	mucilage metabolic process involved in seed coat development	BIOLOGICAL_PROCESS	45	0.284301	2.265349
GO:0005681	spliceosomal complex	CELLULAR_COMPONENT	53	-0.30158	-2.26224
GO:0000280	nuclear division	BIOLOGICAL_PROCESS	138	-0.18984	-2.26202
GO:0043543	protein acylation	BIOLOGICAL_PROCESS	37	-0.29614	-2.25777
GO:0000030	mannosyltransferase activity	MOLECULAR_FUNCTION	15	0.393899	2.2571
GO:0007059	chromosome segregation	BIOLOGICAL_PROCESS	65	-0.20892	-2.25421
GO:0006612	protein targeting to membrane	BIOLOGICAL_PROCESS	25	-0.38873	-2.24764
GO:0051592	response to calcium ion	BIOLOGICAL_PROCESS	16	0.328959	2.24212
GO:0043288	apocarotenoid metabolic process	BIOLOGICAL_PROCESS	30	0.321681	2.23654
GO:0006606	protein import into nucleus	BIOLOGICAL_PROCESS	28	-0.31081	-2.23653
GO:0044038	cell wall macromolecule biosynthetic process	BIOLOGICAL_PROCESS	62	0.260206	2.236371
GO:0045814	negative regulation of gene expression, epigenetic	BIOLOGICAL_PROCESS	58	-0.22041	-2.23326
GO:0010638	positive regulation of organelle organization	BIOLOGICAL_PROCESS	77	-0.21297	-2.21825
GO:0043038	amino acid activation	BIOLOGICAL_PROCESS	18	-0.42248	-2.21472
GO:0034504	protein localization to nucleus	BIOLOGICAL_PROCESS	41	-0.30164	-2.2036
GO:0045488	pectin metabolic process	BIOLOGICAL_PROCESS	98	0.275799	2.201363

GO:1901420	negative regulation of response to alcohol	BIOLOGICAL_PROCESS	45	0.292761	2.201103
GO:0003729	mRNA binding	MOLECULAR_FUNCTION	111	-0.16001	-2.20069
GO:1902644	tertiary alcohol metabolic process	BIOLOGICAL_PROCESS	30	0.321681	2.199856
GO:0032535	regulation of cellular component size	BIOLOGICAL_PROCESS	68	0.21524	2.198318
GO:0016573	histone acetylation	BIOLOGICAL_PROCESS	32	-0.29161	-2.19701
GO:0009694	jasmonic acid metabolic process	BIOLOGICAL_PROCESS	28	0.282729	2.196196
GO:0048813	dendrite morphogenesis	BIOLOGICAL_PROCESS	19	-0.43923	-2.19492
GO:0015665	alcohol transmembrane transporter activity	MOLECULAR_FUNCTION	19	0.410738	2.192433
GO:1902582	single-organism intracellular transport	BIOLOGICAL_PROCESS	109	-0.16307	-2.18961
GO:0006310	DNA recombination	BIOLOGICAL_PROCESS	47	-0.22628	-2.18537
GO:0071427	mRNA-containing ribonucleoprotein complex export from nucleus	BIOLOGICAL_PROCESS	21	-0.40222	-2.18408
GO:0071668	plant-type cell wall assembly	BIOLOGICAL_PROCESS	17	0.431528	2.179284
GO:0032990	cell part morphogenesis	BIOLOGICAL_PROCESS	57	-0.22085	-2.17836
GO:0098655	cation transmembrane transport	BIOLOGICAL_PROCESS	99	-0.153	-2.1768
GO:0010192	mucilage biosynthetic process	BIOLOGICAL_PROCESS	60	0.25193	2.175786
GO:0015144	carbohydrate transmembrane transporter activity	MOLECULAR_FUNCTION	43	0.226949	2.173052
GO:0051960	regulation of nervous system development	BIOLOGICAL_PROCESS	58	-0.19573	-2.1727
GO:1902580	single-organism cellular localization	BIOLOGICAL_PROCESS	122	-0.15798	-2.16402
GO:0043241	protein complex disassembly	BIOLOGICAL_PROCESS	37	-0.28409	-2.1607
GO:0019901	protein kinase binding	MOLECULAR_FUNCTION	100	0.197079	2.15858
GO:0070592	cell wall polysaccharide biosynthetic process	BIOLOGICAL_PROCESS	60	0.268263	2.153296
GO:0061024	membrane organization	BIOLOGICAL_PROCESS	111	-0.16201	-2.15058
GO:0048812	neuron projection morphogenesis	BIOLOGICAL_PROCESS	55	-0.21978	-2.1402

GO:0016241	regulation of macroautophagy	BIOLOGICAL_PROCESS	23	-0.29137	-2.13878
GO:0050767	regulation of neurogenesis	BIOLOGICAL_PROCESS	53	-0.19955	-2.13791
GO:0070589	cellular component macromolecule biosynthetic process	BIOLOGICAL_PROCESS	62	0.260206	2.136715
GO:0030031	cell projection assembly	BIOLOGICAL_PROCESS	18	-0.30114	-2.13478
GO:0006418	tRNA aminoacylation for protein translation	BIOLOGICAL_PROCESS	15	-0.51782	-2.13463
GO:0045492	xylan biosynthetic process	BIOLOGICAL_PROCESS	48	0.285873	2.133444
GO:1900140	regulation of seedling development	BIOLOGICAL_PROCESS	86	0.196096	2.131403
GO:0009765	photosynthesis, light harvesting	BIOLOGICAL_PROCESS	21	0.380732	2.130911
GO:0099643	signal release from synapse	BIOLOGICAL_PROCESS	19	-0.29234	-2.12459
GO:0004722	protein serine/threonine phosphatase activity	MOLECULAR_FUNCTION	40	-0.25161	-2.12301
GO:0098662	inorganic cation transmembrane transport	BIOLOGICAL_PROCESS	83	-0.1765	-2.12246
GO:0045490	pectin catabolic process	BIOLOGICAL_PROCESS	26	0.340186	2.116919
GO:0090693	plant organ senescence	BIOLOGICAL_PROCESS	139	0.146868	2.113561
GO:0006406	mRNA export from nucleus	BIOLOGICAL_PROCESS	21	-0.40222	-2.1134
GO:0016762	xyloglucan:xyloglucosyl transferase activity	MOLECULAR_FUNCTION	16	0.506644	2.111975
GO:0007005	mitochondrion organization	BIOLOGICAL_PROCESS	101	-0.19816	-2.11112
GO:0045489	pectin biosynthetic process	BIOLOGICAL_PROCESS	44	0.310535	2.109902
GO:0030599	pectinesterase activity	MOLECULAR_FUNCTION	16	0.41663	2.109665
GO:1903827	regulation of cellular protein localization	BIOLOGICAL_PROCESS	42	-0.27314	-2.10765
GO:0016829	lyase activity	MOLECULAR_FUNCTION	128	0.126201	2.107543
GO:0006968	cellular defense response	BIOLOGICAL_PROCESS	16	-0.25474	-2.10574
GO:0071472	cellular response to salt stress	BIOLOGICAL_PROCESS	22	-0.41485	-2.0993
GO:0045321	leukocyte activation	BIOLOGICAL_PROCESS	24	-0.34736	-2.09914
GO:0006605	protein targeting	BIOLOGICAL_PROCESS	118	-0.20648	-2.0963

GO:1901699	cellular response to nitrogen compound	BIOLOGICAL_PROCESS	70	-0.19866	-2.09412
GO:0034622	cellular macromolecular complex assembly	BIOLOGICAL_PROCESS	186	-0.13392	-2.09265
GO:0071426	ribonucleoprotein complex export from nucleus	BIOLOGICAL_PROCESS	23	-0.36702	-2.08778
GO:0002520	immune system development	BIOLOGICAL_PROCESS	66	-0.23233	-2.08459
GO:0032880	regulation of protein localization	BIOLOGICAL_PROCESS	69	-0.18539	-2.0837
GO:0019866	organelle inner membrane	CELLULAR_COMPONENT	96	-0.16966	-2.07913
GO:0048354	mucilage biosynthetic process involved in seed coat development	BIOLOGICAL_PROCESS	24	0.371041	2.0779
GO:0010143	cutin biosynthetic process	BIOLOGICAL_PROCESS	16	0.369293	2.07653
GO:0015992	proton transport	BIOLOGICAL_PROCESS	31	-0.32748	-2.07449
GO:0009615	response to virus	BIOLOGICAL_PROCESS	101	-0.1323	-2.07329
GO:0070726	cell wall assembly	BIOLOGICAL_PROCESS	19	0.363322	2.066669
GO:0017148	negative regulation of translation	BIOLOGICAL_PROCESS	45	-0.19041	-2.0638
GO:0044427	chromosomal part	CELLULAR_COMPONENT	116	-0.19831	-2.06196
GO:0035770	ribonucleoprotein granule	CELLULAR_COMPONENT	56	-0.24032	-2.06166
GO:0043039	tRNA aminoacylation	BIOLOGICAL_PROCESS	18	-0.42248	-2.05829
GO:0009738	abscisic acid-activated signaling pathway	BIOLOGICAL_PROCESS	138	0.164088	2.050144
GO:0006974	cellular response to DNA damage stimulus	BIOLOGICAL_PROCESS	153	-0.16598	-2.04858
GO:0034470	ncRNA processing	BIOLOGICAL_PROCESS	100	-0.19202	-2.04671
GO:0006818	hydrogen transport	BIOLOGICAL_PROCESS	31	-0.32748	-2.04558
GO:0008285	negative regulation of cell proliferation	BIOLOGICAL_PROCESS	34	-0.25925	-2.04273
GO:0044454	nuclear chromosome part	CELLULAR_COMPONENT	81	-0.20622	-2.04139
GO:0006913	nucleocytoplasmic transport	BIOLOGICAL_PROCESS	69	-0.22958	-2.04101
GO:0009645	response to low light intensity stimulus	BIOLOGICAL_PROCESS	21	0.247074	2.040109
GO:0032182	ubiquitin-like protein binding	MOLECULAR_FUNCTION	17	-0.34753	-2.03823

GO:0031047	gene silencing by RNA	BIOLOGICAL_PROCESS	60	-0.2062	-2.03679
GO:1903507	negative regulation of nucleic acid-templated transcription	BIOLOGICAL_PROCESS	209	-0.13443	-2.03666
GO:0006306	DNA methylation	BIOLOGICAL_PROCESS	30	-0.25406	-2.03545
GO:0022853	active ion transmembrane transporter activity	MOLECULAR_FUNCTION	35	-0.26401	-2.03529
GO:0019829	cation-transporting ATPase activity	MOLECULAR_FUNCTION	21	-0.30463	-2.03271
GO:0046351	disaccharide biosynthetic process	BIOLOGICAL_PROCESS	17	0.367851	2.029243
GO:0009523	photosystem II	CELLULAR_COMPONENT	25	0.278453	2.028441
GO:1990837	sequence-specific double-stranded DNA binding	MOLECULAR_FUNCTION	117	0.126175	2.025917
GO:0080135	regulation of cellular response to stress	BIOLOGICAL_PROCESS	73	-0.15628	-2.02067
GO:0010150	leaf senescence	BIOLOGICAL_PROCESS	139	0.146868	2.016075
GO:0051493	regulation of cytoskeleton organization	BIOLOGICAL_PROCESS	42	-0.22205	-2.01524
GO:0051726	regulation of cell cycle	BIOLOGICAL_PROCESS	163	-0.14949	-2.01499
GO:0000793	condensed chromosome	CELLULAR_COMPONENT	26	-0.30305	-2.01262
GO:1901990	regulation of mitotic cell cycle phase transition	BIOLOGICAL_PROCESS	48	-0.20128	-2.01221
GO:0015168	glycerol transmembrane transporter activity	MOLECULAR_FUNCTION	16	0.401053	2.009948
GO:0000380	alternative mRNA splicing, via spliceosome	BIOLOGICAL_PROCESS	17	-0.37937	-2.00935
GO:0006364	rRNA processing	BIOLOGICAL_PROCESS	75	-0.21683	-2.00482
GO:0031124	mRNA 3'-end processing	BIOLOGICAL_PROCESS	18	-0.37104	-2.00064
GO:0000819	sister chromatid segregation	BIOLOGICAL_PROCESS	38	-0.22563	-1.99582
GO:0071013	catalytic step 2 spliceosome	CELLULAR_COMPONENT	30	-0.37419	-1.99575
GO:0009827	plant-type cell wall modification	BIOLOGICAL_PROCESS	43	0.284467	1.994597
GO:0006413	translational initiation	BIOLOGICAL_PROCESS	30	-0.2885	-1.99008
GO:0017145	stem cell division	BIOLOGICAL_PROCESS	16	0.439182	1.98574

GO:0061733	peptide-lysine-N-acetyltransferase activity	MOLECULAR_FUNCTION	18	-0.36719	-1.98571
GO:0003682	chromatin binding	MOLECULAR_FUNCTION	90	-0.186	-1.98277
GO:0036464	cytoplasmic ribonucleoprotein granule	CELLULAR_COMPONENT	56	-0.24032	-1.97984
GO:0043414	macromolecule methylation	BIOLOGICAL_PROCESS	64	-0.1947	-1.97918
GO:0016072	rRNA metabolic process	BIOLOGICAL_PROCESS	84	-0.19738	-1.97513
GO:0000781	chromosome, telomeric region	CELLULAR_COMPONENT	33	-0.27654	-1.97369
GO:0009788	negative regulation of abscisic acid-activated signaling pathway	BIOLOGICAL_PROCESS	45	0.292761	1.973634
GO:0050826	response to freezing	BIOLOGICAL_PROCESS	23	0.281403	1.973383
GO:0016874	ligase activity	MOLECULAR_FUNCTION	67	-0.20809	-1.97227
GO:0044770	cell cycle phase transition	BIOLOGICAL_PROCESS	67	-0.15007	-1.97159
GO:0051130	positive regulation of cellular component organization	BIOLOGICAL_PROCESS	119	-0.17555	-1.97146
GO:0006457	protein folding	BIOLOGICAL_PROCESS	65	-0.1456	-1.96983
GO:0090406	pollen tube	CELLULAR_COMPONENT	81	0.202211	1.966063
GO:0005507	copper ion binding	MOLECULAR_FUNCTION	79	0.146369	1.965681
GO:0006611	protein export from nucleus	BIOLOGICAL_PROCESS	30	-0.35273	-1.96404
GO:0007050	cell cycle arrest	BIOLOGICAL_PROCESS	19	-0.19235	-1.96383
GO:0015630	microtubule cytoskeleton	CELLULAR_COMPONENT	172	-0.12938	-1.96349
GO:0045786	negative regulation of cell cycle	BIOLOGICAL_PROCESS	66	-0.20011	-1.96077
GO:0006357	regulation of transcription from RNA polymerase II promoter	BIOLOGICAL_PROCESS	167	-0.11772	-1.96058
GO:1902645	tertiary alcohol biosynthetic process	BIOLOGICAL_PROCESS	21	0.302348	1.95982
GO:0097190	apoptotic signaling pathway	BIOLOGICAL_PROCESS	32	-0.26239	-1.95437
GO:0006475	internal protein amino acid acetylation	BIOLOGICAL_PROCESS	33	-0.2703	-1.94978
GO:0065004	protein-DNA complex assembly	BIOLOGICAL_PROCESS	43	-0.20447	-1.94934
GO:0006403	RNA localization	BIOLOGICAL_PROCESS	64	-0.21741	-1.94471
GO:0016192	vesicle-mediated transport	BIOLOGICAL_PROCESS	181	-0.12064	-1.94421

GO:0009141	nucleoside triphosphate metabolic process	BIOLOGICAL_PROCESS	74	-0.12599	-1.93994
GO:0010608	posttranscriptional regulation of gene expression	BIOLOGICAL_PROCESS	109	-0.18739	-1.93985
GO:0000794	condensed nuclear chromosome	CELLULAR_COMPONENT	15	-0.30232	-1.93583
GO:0009814	defense response, incompatible interaction	BIOLOGICAL_PROCESS	163	0.139869	1.922425
GO:1902593	single-organism nuclear import	BIOLOGICAL_PROCESS	33	-0.26646	-1.91692
GO:0048667	cell morphogenesis involved in neuron differentiation	BIOLOGICAL_PROCESS	50	-0.19533	-1.91608
GO:0030030	cell projection organization	BIOLOGICAL_PROCESS	97	-0.14965	-1.91441
GO:0034655	nucleobase-containing compound catabolic process	BIOLOGICAL_PROCESS	67	-0.18824	-1.90773
GO:1903046	meiotic cell cycle process	BIOLOGICAL_PROCESS	109	-0.12668	-1.90769
GO:0044391	ribosomal subunit	CELLULAR_COMPONENT	29	-0.26316	-1.90671
GO:0008374	O-acyltransferase activity	MOLECULAR_FUNCTION	44	0.227424	1.906135
GO:0010411	xyloglucan metabolic process	BIOLOGICAL_PROCESS	35	0.272647	1.905573
GO:0015925	galactosidase activity	MOLECULAR_FUNCTION	18	0.427724	1.904689
GO:0042762	regulation of sulfur metabolic process	BIOLOGICAL_PROCESS	28	-0.23983	-1.90221
GO:0010648	negative regulation of cell communication	BIOLOGICAL_PROCESS	128	0.123565	1.900561
GO:2001252	positive regulation of chromosome organization	BIOLOGICAL_PROCESS	29	-0.27315	-1.89615
GO:0015629	actin cytoskeleton	CELLULAR_COMPONENT	29	-0.2751	-1.89599
GO:0044042	glucan metabolic process	BIOLOGICAL_PROCESS	138	0.144284	1.894713

GO:0043066	negative regulation of apoptotic process	BIOLOGICAL_PROCESS	63	-0.20585	-1.89265
GO:0005759	mitochondrial matrix	CELLULAR_COMPONENT	44	-0.26401	-1.89151
GO:0050829	defense response to Gram-negative bacterium	BIOLOGICAL_PROCESS	25	0.357127	1.890943
GO:0005635	nuclear envelope	CELLULAR_COMPONENT	64	-0.21368	-1.88969
GO:0042274	ribosomal small subunit biogenesis	BIOLOGICAL_PROCESS	33	-0.25511	-1.88851
GO:0044728	DNA methylation or demethylation	BIOLOGICAL_PROCESS	31	-0.27143	-1.88759
GO:0009311	oligosaccharide metabolic process	BIOLOGICAL_PROCESS	40	0.207927	1.884609
GO:0018393	internal peptidyl-lysine acetylation	BIOLOGICAL_PROCESS	32	-0.29161	-1.88371
GO:0044430	cytoskeletal part	CELLULAR_COMPONENT	185	-0.13032	-1.88279
GO:0005740	mitochondrial envelope	CELLULAR_COMPONENT	106	-0.10383	-1.88228
GO:0009789	positive regulation of abscisic acid-activated signaling pathway	BIOLOGICAL_PROCESS	29	0.266099	1.880873
GO:0018022	peptidyl-lysine methylation	BIOLOGICAL_PROCESS	26	-0.25177	-1.87954
GO:0016410	N-acyltransferase activity	MOLECULAR_FUNCTION	40	-0.25543	-1.87744
GO:0009808	lignin metabolic process	BIOLOGICAL_PROCESS	80	0.204334	1.877079
GO:0009835	fruit ripening	BIOLOGICAL_PROCESS	15	0.359427	1.87476
GO:0006721	terpenoid metabolic process	BIOLOGICAL_PROCESS	115	0.11374	1.873028
GO:0006714	sesquiterpenoid metabolic process	BIOLOGICAL_PROCESS	33	0.266951	1.871983
GO:0033293	monocarboxylic acid binding	MOLECULAR_FUNCTION	29	0.298896	1.870043
GO:0034605	cellular response to heat	BIOLOGICAL_PROCESS	72	-0.14947	-1.86595
GO:0008080	N-acetyltransferase activity	MOLECULAR_FUNCTION	20	-0.35475	-1.86535
GO:0004386	helicase activity	MOLECULAR_FUNCTION	58	-0.22282	-1.86426
GO:0090066	regulation of anatomical structure size	BIOLOGICAL_PROCESS	70	0.204241	1.862595
GO:0006022	aminoglycan metabolic process	BIOLOGICAL_PROCESS	16	0.375441	1.862163
GO:0005924	cell-substrate adherens junction	CELLULAR_COMPONENT	28	-0.23618	-1.86198
GO:0044429	mitochondrial part	CELLULAR_COMPONENT	131	-0.1151	-1.86044
GO:0051607	defense response to virus	BIOLOGICAL_PROCESS	65	-0.16856	-1.85902
GO:0031969	chloroplast membrane	CELLULAR_COMPONENT	87	0.145554	1.853032
GO:0016209	antioxidant activity	MOLECULAR_FUNCTION	41	0.212058	1.852466
GO:0071824	protein-DNA complex subunit organization	BIOLOGICAL_PROCESS	52	-0.2171	-1.85068
GO:2001251	negative regulation of chromosome organization	BIOLOGICAL_PROCESS	34	-0.27373	-1.84887
GO:0000228	nuclear chromosome	CELLULAR_COMPONENT	88	-0.19138	-1.84731
GO:0004601	peroxidase activity	MOLECULAR_FUNCTION	29	0.257366	1.845493
GO:0022008	neurogenesis	BIOLOGICAL_PROCESS	137	-0.15038	-1.84487
GO:0003697	single-stranded DNA binding	MOLECULAR_FUNCTION	29	-0.27167	-1.84293
GO:0043062	extracellular structure organization	BIOLOGICAL_PROCESS	70	0.175446	1.842476
GO:0000288	nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	BIOLOGICAL_PROCESS	25	-0.31204	-1.84214
GO:0048471	perinuclear region of cytoplasm	CELLULAR_COMPONENT	67	-0.17365	-1.84132

GO:0018205	peptidyl-lysine modification	BIOLOGICAL_PROCESS	72	-0.20954	-1.84047
GO:0051783	regulation of nuclear division	BIOLOGICAL_PROCESS	33	-0.27169	-1.83958
GO:0043434	response to peptide hormone	BIOLOGICAL_PROCESS	28	-0.23629	-1.83781
GO:0022411	cellular component disassembly	BIOLOGICAL_PROCESS	85	-0.14585	-1.83751
GO:0015931	nucleobase-containing compound transport	BIOLOGICAL_PROCESS	60	-0.18764	-1.83238
GO:0071826	ribonucleoprotein complex subunit organization	BIOLOGICAL_PROCESS	61	-0.20119	-1.83236
GO:0009856	pollination	BIOLOGICAL_PROCESS	183	0.095317	1.832229
GO:0019904	protein domain specific binding	MOLECULAR_FUNCTION	47	0.129441	1.827836
GO:0098798	mitochondrial protein complex	CELLULAR_COMPONENT	19	-0.33154	-1.82736
GO:0009845	seed germination	BIOLOGICAL_PROCESS	156	0.112805	1.826501
GO:0072330	monocarboxylic acid biosynthetic process	BIOLOGICAL_PROCESS	171	0.109959	1.825214
GO:0090329	regulation of DNA-dependent DNA replication	BIOLOGICAL_PROCESS	20	-0.28157	-1.82519
GO:0016556	mRNA modification	BIOLOGICAL_PROCESS	55	-0.20071	-1.82393
GO:0071695	anatomical structure maturation	BIOLOGICAL_PROCESS	18	0.307983	1.821636
GO:1901987	regulation of cell cycle phase transition	BIOLOGICAL_PROCESS	49	-0.18929	-1.82107
GO:1990204	oxidoreductase complex	CELLULAR_COMPONENT	29	-0.27165	-1.82073
GO:0009250	glucan biosynthetic process	BIOLOGICAL_PROCESS	81	0.160792	1.819613
GO:0010212	response to ionizing radiation	BIOLOGICAL_PROCESS	22	-0.30762	-1.81815
GO:1990234	transferase complex	CELLULAR_COMPONENT	134	-0.13618	-1.81582
GO:0030243	cellulose metabolic process	BIOLOGICAL_PROCESS	54	0.19152	1.814995
GO:0003678	DNA helicase activity	MOLECULAR_FUNCTION	26	-0.27719	-1.81279
GO:0035251	UDP-glucosyltransferase activity	MOLECULAR_FUNCTION	73	0.161006	1.812046
GO:0010508	positive regulation of autophagy	BIOLOGICAL_PROCESS	20	-0.3021	-1.81162
GO:0050684	regulation of mRNA processing	BIOLOGICAL_PROCESS	24	-0.28879	-1.81141
GO:0060341	regulation of cellular localization	BIOLOGICAL_PROCESS	69	-0.21382	-1.8086
GO:0048806	genitalia development	BIOLOGICAL_PROCESS	25	0.290608	1.807312
GO:0016604	nuclear body	CELLULAR_COMPONENT	56	-0.21434	-1.80493
GO:0000272	polysaccharide catabolic process	BIOLOGICAL_PROCESS	62	0.221178	1.804351
GO:0051129	negative regulation of cellular component organization	BIOLOGICAL_PROCESS	75	-0.21125	-1.80407
GO:0006305	DNA alkylation	BIOLOGICAL_PROCESS	30	-0.25406	-1.80288
GO:0051193	regulation of cofactor metabolic process	BIOLOGICAL_PROCESS	30	-0.23083	-1.80217
GO:0018394	peptidyl-lysine acetylation	BIOLOGICAL_PROCESS	33	-0.2703	-1.80211
GO:0015399	primary active transmembrane transporter activity	MOLECULAR_FUNCTION	69	-0.14874	-1.79752
GO:0002229	defense response to oomycetes	BIOLOGICAL_PROCESS	51	0.190626	1.797252
GO:0009687	abscisic acid metabolic process	BIOLOGICAL_PROCESS	30	0.321681	1.795653
GO:0045017	glycerolipid biosynthetic process	BIOLOGICAL_PROCESS	34	-0.25246	-1.79466
GO:0048534	hematopoietic or lymphoid organ development	BIOLOGICAL_PROCESS	61	-0.21571	-1.79155
GO:0016052	carbohydrate catabolic process	BIOLOGICAL_PROCESS	110	0.137019	1.788827
GO:0000278	mitotic cell cycle	BIOLOGICAL_PROCESS	176	-0.14848	-1.7867
GO:0000077	DNA damage checkpoint	BIOLOGICAL_PROCESS	29	-0.24094	-1.786
GO:0008026	ATP-dependent helicase activity	MOLECULAR_FUNCTION	41	-0.20682	-1.78506

GO:0016835	carbon-oxygen lyase activity	MOLECULAR_FUNCTION	45	0.163571	1.784314
GO:0009644	response to high light intensity	BIOLOGICAL_PROCESS	54	0.200862	1.780344
GO:0006029	proteoglycan metabolic process	BIOLOGICAL_PROCESS	16	0.378556	1.779318
GO:0006073	cellular glucan metabolic process	BIOLOGICAL_PROCESS	137	0.146923	1.779286
GO:0090316	positive regulation of intracellular protein transport	BIOLOGICAL_PROCESS	17	-0.33631	-1.77546
GO:0016032	viral process	BIOLOGICAL_PROCESS	86	-0.16332	-1.77531
GO:0080168	abscisic acid transport	BIOLOGICAL_PROCESS	21	0.273497	1.774764
GO:0003724	RNA helicase activity	MOLECULAR_FUNCTION	25	-0.33768	-1.77262
GO:0042110	T cell activation	BIOLOGICAL_PROCESS	19	-0.28972	-1.77078
GO:0000987	core promoter proximal region sequence-specific DNA binding	MOLECULAR_FUNCTION	24	0.304445	1.768946
GO:0004402	histone acetyltransferase activity	MOLECULAR_FUNCTION	18	-0.36719	-1.7688
GO:0042285	xylosyltransferase activity	MOLECULAR_FUNCTION	16	0.350546	1.768035
GO:0016571	histone methylation	BIOLOGICAL_PROCESS	34	-0.22948	-1.76515
GO:0099537	trans-synaptic signaling	BIOLOGICAL_PROCESS	43	-0.19504	-1.76049
GO:0030308	negative regulation of cell growth	BIOLOGICAL_PROCESS	26	0.279178	1.75987
GO:0006302	double-strand break repair	BIOLOGICAL_PROCESS	50	-0.21267	-1.75973
GO:1901476	carbohydrate transporter activity	MOLECULAR_FUNCTION	43	0.226949	1.758933
GO:0035097	histone methyltransferase complex	CELLULAR_COMPONENT	17	-0.43241	-1.75859
GO:0009226	nucleotide-sugar biosynthetic process	BIOLOGICAL_PROCESS	18	0.32105	1.758584
GO:0046034	ATP metabolic process	BIOLOGICAL_PROCESS	63	-0.1594	-1.75858
GO:0009968	negative regulation of signal transduction	BIOLOGICAL_PROCESS	121	0.133956	1.758483
GO:0030016	myofibril	CELLULAR_COMPONENT	28	0.217935	1.757859
GO:0007276	gamete generation	BIOLOGICAL_PROCESS	123	-0.13274	-1.75754
GO:0010506	regulation of autophagy	BIOLOGICAL_PROCESS	36	-0.27517	-1.75419
GO:2001233	regulation of apoptotic signaling pathway	BIOLOGICAL_PROCESS	22	-0.28698	-1.75305
GO:1903311	regulation of mRNA metabolic process	BIOLOGICAL_PROCESS	43	-0.24208	-1.75219
GO:0006109	regulation of carbohydrate metabolic process	BIOLOGICAL_PROCESS	78	-0.16041	-1.75159
GO:0009696	salicylic acid metabolic process	BIOLOGICAL_PROCESS	30	0.202876	1.751486
GO:0080190	lateral growth	BIOLOGICAL_PROCESS	16	0.358486	1.747989
GO:0000963	mitochondrial RNA processing	BIOLOGICAL_PROCESS	17	-0.32351	-1.74445
GO:0032388	positive regulation of intracellular transport	BIOLOGICAL_PROCESS	23	-0.25017	-1.744
GO:0048639	positive regulation of developmental growth	BIOLOGICAL_PROCESS	23	-0.30703	-1.74341
GO:0030097	hemopoiesis	BIOLOGICAL_PROCESS	57	-0.21814	-1.74264
GO:0010822	positive regulation of mitochondrion organization	BIOLOGICAL_PROCESS	24	-0.34204	-1.7404
GO:0015672	monovalent inorganic cation transport	BIOLOGICAL_PROCESS	80	-0.19161	-1.7382
GO:0007067	mitotic nuclear division	BIOLOGICAL_PROCESS	75	-0.21251	-1.73794
GO:0006909	phagocytosis	BIOLOGICAL_PROCESS	33	-0.22949	-1.73683
GO:0005198	structural molecule activity	MOLECULAR_FUNCTION	86	-0.15688	-1.73343

GO:0031331	positive regulation of cellular catabolic process	BIOLOGICAL_PROCESS	41	-0.16646	-1.73069
GO:0006520	cellular amino acid metabolic process	BIOLOGICAL_PROCESS	159	-0.10576	-1.72575
GO:0022618	ribonucleoprotein complex assembly	BIOLOGICAL_PROCESS	59	-0.20194	-1.72552
GO:0030532	small nuclear ribonucleoprotein complex	CELLULAR_COMPONENT	21	-0.32413	-1.72292
GO:0016462	pyrophosphatase activity	MOLECULAR_FUNCTION	250	-0.09242	-1.72233
GO:0030424	axon	CELLULAR_COMPONENT	43	-0.18803	-1.72184
GO:0010208	pollen wall assembly	BIOLOGICAL_PROCESS	46	0.165399	1.72073
GO:0000075	cell cycle checkpoint	BIOLOGICAL_PROCESS	36	-0.26159	-1.72025
GO:0044764	multi-organism cellular process	BIOLOGICAL_PROCESS	119	-0.13939	-1.72024
GO:0051983	regulation of chromosome segregation	BIOLOGICAL_PROCESS	19	-0.33171	-1.71873
GO:0031966	mitochondrial membrane	CELLULAR_COMPONENT	95	-0.14688	-1.71774
GO:0031056	regulation of histone modification	BIOLOGICAL_PROCESS	33	-0.25024	-1.71657
GO:0031497	chromatin assembly	BIOLOGICAL_PROCESS	31	-0.20922	-1.71551
GO:0008135	translation factor activity, RNA binding	MOLECULAR_FUNCTION	20	-0.34625	-1.71528
GO:0007517	muscle organ development	BIOLOGICAL_PROCESS	19	-0.33913	-1.71404
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	BIOLOGICAL_PROCESS	57	-0.18782	-1.71257
GO:0004518	nuclease activity	MOLECULAR_FUNCTION	134	-0.13754	-1.71126
GO:1905268	negative regulation of chromatin organization	BIOLOGICAL_PROCESS	17	-0.31432	-1.71023
GO:0008170	N-methyltransferase activity	MOLECULAR_FUNCTION	21	-0.31633	-1.70935
GO:0044743	protein transmembrane import into intracellular organelle	BIOLOGICAL_PROCESS	21	-0.35637	-1.70869
GO:0008340	determination of adult lifespan	BIOLOGICAL_PROCESS	28	-0.26858	-1.70636
GO:0060548	negative regulation of cell death	BIOLOGICAL_PROCESS	101	-0.14063	-1.7048
GO:0051236	establishment of RNA localization	BIOLOGICAL_PROCESS	39	-0.23491	-1.70425
GO:0031401	positive regulation of protein modification process	BIOLOGICAL_PROCESS	67	-0.17332	-1.70421
GO:0008284	positive regulation of cell proliferation	BIOLOGICAL_PROCESS	41	-0.26529	-1.70354
GO:0002181	cytoplasmic translation	BIOLOGICAL_PROCESS	16	-0.44596	-1.70305
GO:0034728	nucleosome organization	BIOLOGICAL_PROCESS	38	-0.22153	-1.70256
GO:0002682	regulation of immune system process	BIOLOGICAL_PROCESS	169	-0.1112	-1.7015
GO:0042625	ATPase coupled ion transmembrane transporter activity	MOLECULAR_FUNCTION	24	-0.3256	-1.7006
GO:0071478	cellular response to radiation	BIOLOGICAL_PROCESS	70	-0.17634	-1.69892
GO:0043044	ATP-dependent chromatin remodeling	BIOLOGICAL_PROCESS	16	-0.3168	-1.69784
GO:0010232	vascular transport	BIOLOGICAL_PROCESS	19	0.293766	1.696931
GO:0048699	generation of neurons	BIOLOGICAL_PROCESS	110	-0.16661	-1.69544
GO:0030198	extracellular matrix organization	BIOLOGICAL_PROCESS	70	0.175446	1.693444

GO:0098660	inorganic ion transmembrane transport	BIOLOGICAL_PROCESS	95	-0.142	-1.68978
GO:0034599	cellular response to oxidative stress	BIOLOGICAL_PROCESS	73	-0.1405	-1.68909
GO:0009901	anther dehiscence	BIOLOGICAL_PROCESS	32	0.197765	1.688703
GO:0050769	positive regulation of neurogenesis	BIOLOGICAL_PROCESS	34	-0.17704	-1.68831
GO:0008645	hexose transport	BIOLOGICAL_PROCESS	15	0.382212	1.687545
GO:0044448	cell cortex part	CELLULAR_COMPONENT	39	-0.19161	-1.68683
GO:0019058	viral life cycle	BIOLOGICAL_PROCESS	42	-0.21259	-1.68602
GO:0009199	ribonucleoside triphosphate metabolic process	BIOLOGICAL_PROCESS	69	-0.1519	-1.68546
GO:0034968	histone lysine methylation	BIOLOGICAL_PROCESS	26	-0.25177	-1.68491
GO:0006304	DNA modification	BIOLOGICAL_PROCESS	36	-0.20399	-1.68445
GO:0098869	cellular oxidant detoxification	BIOLOGICAL_PROCESS	18	0.276258	1.683382
GO:0098793	presynapse	CELLULAR_COMPONENT	33	-0.20364	-1.68041
GO:0005743	mitochondrial inner membrane	CELLULAR_COMPONENT	65	-0.15987	-1.67981
GO:1903959	regulation of anion transmembrane transport	BIOLOGICAL_PROCESS	23	0.188502	1.67928
GO:0016168	chlorophyll binding	MOLECULAR_FUNCTION	17	0.327688	1.679021
GO:0030178	negative regulation of Wnt signaling pathway	BIOLOGICAL_PROCESS	20	-0.23477	-1.67844
GO:0006338	chromatin remodeling	BIOLOGICAL_PROCESS	35	-0.19247	-1.67696
GO:0043005	neuron projection	CELLULAR_COMPONENT	71	-0.16958	-1.67631
GO:0010927	cellular component assembly involved in morphogenesis	BIOLOGICAL_PROCESS	60	0.177468	1.676015
GO:0052546	cell wall pectin metabolic process	BIOLOGICAL_PROCESS	44	0.224953	1.675105
GO:1902531	regulation of intracellular signal transduction	BIOLOGICAL_PROCESS	95	-0.14386	-1.67386
GO:0016887	ATPase activity	MOLECULAR_FUNCTION	177	-0.10308	-1.67209
GO:0010091	trichome branching	BIOLOGICAL_PROCESS	25	0.22674	1.669379
GO:0043069	negative regulation of programmed cell death	BIOLOGICAL_PROCESS	86	-0.1526	-1.66862
GO:0099568	cytoplasmic region	CELLULAR_COMPONENT	53	-0.20199	-1.66828
GO:0008276	protein methyltransferase activity	MOLECULAR_FUNCTION	16	-0.32196	-1.66805
GO:0010439	regulation of glucosinolate biosynthetic process	BIOLOGICAL_PROCESS	22	-0.25582	-1.6678
GO:0016679	oxidoreductase activity, acting on diphenols and related substances as donors	MOLECULAR_FUNCTION	18	0.306218	1.665889
GO:0042181	ketone biosynthetic process	BIOLOGICAL_PROCESS	15	-0.24954	-1.66462
GO:0005615	extracellular space	CELLULAR_COMPONENT	76	0.157601	1.663578
GO:0019080	viral gene expression	BIOLOGICAL_PROCESS	16	-0.33778	-1.66222
GO:0016791	phosphatase activity	MOLECULAR_FUNCTION	82	-0.11581	-1.66162
GO:0043624	cellular protein complex disassembly	BIOLOGICAL_PROCESS	32	-0.27101	-1.66118
GO:1901421	positive regulation of response to alcohol	BIOLOGICAL_PROCESS	29	0.266099	1.659845
GO:0046906	tetrapyrrole binding	MOLECULAR_FUNCTION	68	0.195685	1.659694
GO:0004003	ATP-dependent DNA helicase activity	MOLECULAR_FUNCTION	19	-0.27676	-1.65812

GO:0060249	anatomical structure homeostasis	BIOLOGICAL_PROCESS	45	-0.19896	-1.65783
GO:0006836	neurotransmitter transport	BIOLOGICAL_PROCESS	19	-0.29234	-1.65699
GO:0042440	pigment metabolic process	BIOLOGICAL_PROCESS	133	0.114748	1.656109
GO:0033157	regulation of intracellular protein transport	BIOLOGICAL_PROCESS	20	-0.29812	-1.65452
GO:0030141	secretory granule	CELLULAR_COMPONENT	25	-0.18829	-1.65437
GO:0043130	ubiquitin binding	MOLECULAR_FUNCTION	17	-0.34753	-1.65429
GO:0000922	spindle pole	CELLULAR_COMPONENT	18	0.310606	1.654187
GO:1903530	regulation of secretion by cell	BIOLOGICAL_PROCESS	25	-0.2674	-1.65357
GO:0046688	response to copper ion	BIOLOGICAL_PROCESS	21	0.309487	1.649666
GO:0009813	flavonoid biosynthetic process	BIOLOGICAL_PROCESS	107	0.137192	1.649478
GO:0071822	protein complex subunit organization	BIOLOGICAL_PROCESS	203	-0.10101	-1.64935
GO:0050792	regulation of viral process	BIOLOGICAL_PROCESS	21	-0.25973	-1.64849
GO:0030017	sarcomere	CELLULAR_COMPONENT	26	0.23718	1.645706
GO:0031399	regulation of protein modification process	BIOLOGICAL_PROCESS	132	-0.10233	-1.64075
GO:1900055	regulation of leaf senescence	BIOLOGICAL_PROCESS	37	0.214168	1.639656
GO:0034250	positive regulation of cellular amide metabolic process	BIOLOGICAL_PROCESS	16	-0.30919	-1.63852
GO:0014706	striated muscle tissue development	BIOLOGICAL_PROCESS	16	-0.32584	-1.63736
GO:1904951	positive regulation of establishment of protein localization	BIOLOGICAL_PROCESS	29	-0.26575	-1.63704
GO:0005777	peroxisome	CELLULAR_COMPONENT	94	-0.12197	-1.63695
GO:0030244	cellulose biosynthetic process	BIOLOGICAL_PROCESS	47	0.202768	1.636674
GO:0043244	regulation of protein complex disassembly	BIOLOGICAL_PROCESS	21	-0.28277	-1.63657
GO:0000792	heterochromatin	CELLULAR_COMPONENT	18	-0.26903	-1.63526
GO:0010090	trichome morphogenesis	BIOLOGICAL_PROCESS	46	0.196173	1.634442
GO:2000377	regulation of reactive oxygen species metabolic process	BIOLOGICAL_PROCESS	36	0.212462	1.634238
GO:0042537	benzene-containing compound metabolic process	BIOLOGICAL_PROCESS	52	0.151178	1.63383
GO:0042548	regulation of photosynthesis, light reaction	BIOLOGICAL_PROCESS	19	0.295346	1.632509
GO:0043289	apocarotenoid biosynthetic process	BIOLOGICAL_PROCESS	21	0.302348	1.632102
GO:0080156	mitochondrial mRNA modification	BIOLOGICAL_PROCESS	39	-0.21037	-1.62988
GO:0015405	P-P-bond-hydrolysis-driven transmembrane transporter activity	MOLECULAR_FUNCTION	64	-0.16626	-1.62886
GO:0016763	transferase activity, transferring pentosyl groups	MOLECULAR_FUNCTION	21	0.268492	1.628571
GO:0090332	stomatal closure	BIOLOGICAL_PROCESS	23	0.247673	1.626109
GO:0010286	heat acclimation	BIOLOGICAL_PROCESS	37	0.17175	1.625655
GO:0048024	regulation of mRNA splicing, via spliceosome	BIOLOGICAL_PROCESS	21	-0.30757	-1.62363
GO:0001012	RNA polymerase II regulatory region DNA binding	MOLECULAR_FUNCTION	59	0.171658	1.623294

GO:0050658	RNA transport	BIOLOGICAL_PROCESS	39	-0.23491	-1.6207
GO:0071806	protein transmembrane transport	BIOLOGICAL_PROCESS	29	-0.24367	-1.61939
GO:0006839	mitochondrial transport	BIOLOGICAL_PROCESS	37	-0.19639	-1.6193
GO:0009566	fertilization	BIOLOGICAL_PROCESS	43	0.180685	1.61864
GO:0070997	neuron death	BIOLOGICAL_PROCESS	25	-0.21945	-1.61726
GO:0006090	pyruvate metabolic process	BIOLOGICAL_PROCESS	43	-0.20465	-1.61669
GO:0016358	dendrite development	BIOLOGICAL_PROCESS	25	-0.2747	-1.61554
GO:0032868	response to insulin	BIOLOGICAL_PROCESS	19	-0.29144	-1.61314
GO:0098754	detoxification	BIOLOGICAL_PROCESS	25	0.216354	1.611788
GO:0007140	male meiosis	BIOLOGICAL_PROCESS	26	-0.22569	-1.61083
GO:0051053	negative regulation of DNA metabolic process	BIOLOGICAL_PROCESS	18	-0.29602	-1.60922
GO:1990538	xylan O-acetyltransferase activity	MOLECULAR_FUNCTION	17	0.324029	1.608913
GO:0048510	regulation of timing of transition from vegetative to reproductive phase	BIOLOGICAL_PROCESS	47	0.161349	1.608271
GO:0009524	phragmoplast	CELLULAR_COMPONENT	28	-0.22368	-1.60632
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	MOLECULAR_FUNCTION	59	0.171658	1.606229
GO:0001708	cell fate specification	BIOLOGICAL_PROCESS	20	-0.26865	-1.60554
GO:0050877	neurological system process	BIOLOGICAL_PROCESS	59	-0.15179	-1.60444
GO:0006259	DNA metabolic process	BIOLOGICAL_PROCESS	238	-0.10878	-1.60378
GO:0019897	extrinsic component of plasma membrane	CELLULAR_COMPONENT	21	0.24413	1.603384
GO:0001817	regulation of cytokine production	BIOLOGICAL_PROCESS	25	-0.2453	-1.60173
GO:0034976	response to endoplasmic reticulum stress	BIOLOGICAL_PROCESS	27	-0.26923	-1.60128
GO:0010319	stromule	CELLULAR_COMPONENT	26	-0.25973	-1.60009
GO:0042044	fluid transport	BIOLOGICAL_PROCESS	25	0.358232	1.6
GO:0000245	spliceosomal complex assembly	BIOLOGICAL_PROCESS	18	-0.29511	-1.59891
GO:0000790	nuclear chromatin	CELLULAR_COMPONENT	53	-0.20942	-1.59672
GO:0009567	double fertilization forming a zygote and endosperm	BIOLOGICAL_PROCESS	19	0.27295	1.596684
GO:0043010	camera-type eye development	BIOLOGICAL_PROCESS	19	-0.26068	-1.59627
GO:0071453	cellular response to oxygen levels	BIOLOGICAL_PROCESS	35	0.184053	1.596031
GO:0009816	defense response to bacterium, incompatible interaction	BIOLOGICAL_PROCESS	53	0.187383	1.595058
GO:0060070	canonical Wnt signaling pathway	BIOLOGICAL_PROCESS	19	-0.29512	-1.59283
GO:0032869	cellular response to insulin stimulus	BIOLOGICAL_PROCESS	17	-0.2851	-1.59268
GO:0007444	imaginal disc development	BIOLOGICAL_PROCESS	22	-0.23769	-1.59191
GO:0044089	positive regulation of cellular component biogenesis	BIOLOGICAL_PROCESS	35	-0.21041	-1.58863
GO:0099504	synaptic vesicle cycle	BIOLOGICAL_PROCESS	19	-0.24169	-1.58725
GO:0070647	protein modification by small protein conjugation or removal	BIOLOGICAL_PROCESS	140	-0.15363	-1.58665
GO:0010564	regulation of cell cycle process	BIOLOGICAL_PROCESS	97	-0.15632	-1.58497
GO:0098791	Golgi subcompartment	CELLULAR_COMPONENT	180	0.104475	1.583735
GO:0016830	carbon-carbon lyase activity	MOLECULAR_FUNCTION	49	0.198019	1.581897

GO:0009828	plant-type cell wall loosening	BIOLOGICAL_PROCESS	21	0.286377	1.581868
GO:0010027	thylakoid membrane organization	BIOLOGICAL_PROCESS	19	-0.20574	-1.58086
GO:0044439	peroxisomal part	CELLULAR_COMPONENT	34	-0.19712	-1.58069
GO:1901618	organic hydroxy compound transmembrane transporter activity	MOLECULAR_FUNCTION	22	0.293106	1.580463
GO:0009792	embryo development ending in birth or egg hatching	BIOLOGICAL_PROCESS	88	-0.13275	-1.57912
GO:0034249	negative regulation of cellular amide metabolic process	BIOLOGICAL_PROCESS	47	-0.1924	-1.57822
GO:0060537	muscle tissue development	BIOLOGICAL_PROCESS	16	-0.32584	-1.57751
GO:0019843	rRNA binding	MOLECULAR_FUNCTION	24	-0.22645	-1.57713
GO:0010119	regulation of stomatal movement	BIOLOGICAL_PROCESS	82	0.118741	1.575775
GO:0000976	transcription regulatory region sequence-specific DNA binding	MOLECULAR_FUNCTION	104	0.154352	1.574645
GO:0042127	regulation of cell proliferation	BIOLOGICAL_PROCESS	106	-0.16191	-1.57443
GO:0042547	cell wall modification involved in multidimensional cell growth	BIOLOGICAL_PROCESS	21	0.238537	1.57381
GO:0040012	regulation of locomotion	BIOLOGICAL_PROCESS	29	-0.24946	-1.57237
GO:0006511	ubiquitin-dependent protein catabolic process	BIOLOGICAL_PROCESS	83	-0.14404	-1.56943
GO:0040035	hermaphrodite genitalia development	BIOLOGICAL_PROCESS	23	0.276496	1.569182
GO:0042579	microbody	CELLULAR_COMPONENT	94	-0.12197	-1.56914
GO:0070918	production of small RNA involved in gene silencing by RNA	BIOLOGICAL_PROCESS	21	-0.26827	-1.56869
GO:0030182	neuron differentiation	BIOLOGICAL_PROCESS	98	-0.16635	-1.56853
GO:0004721	phosphoprotein phosphatase activity	MOLECULAR_FUNCTION	49	-0.19846	-1.56678
GO:0090351	seedling development	BIOLOGICAL_PROCESS	172	0.111862	1.566756
GO:0010821	regulation of mitochondrion organization	BIOLOGICAL_PROCESS	25	-0.3505	-1.56642
GO:0072331	signal transduction by p53 class mediator	BIOLOGICAL_PROCESS	24	-0.19957	-1.56603
GO:2000145	regulation of cell motility	BIOLOGICAL_PROCESS	24	-0.2333	-1.56529
GO:0000209	protein polyubiquitination	BIOLOGICAL_PROCESS	29	-0.23014	-1.56473
GO:0051223	regulation of protein transport	BIOLOGICAL_PROCESS	43	-0.20871	-1.5602
GO:0009809	lignin biosynthetic process	BIOLOGICAL_PROCESS	68	0.188112	1.559257
GO:0009612	response to mechanical stimulus	BIOLOGICAL_PROCESS	29	0.24739	1.557471
GO:0000725	recombinational repair	BIOLOGICAL_PROCESS	26	-0.25486	-1.55706
GO:0015748	organophosphate ester transport	BIOLOGICAL_PROCESS	27	-0.25584	-1.55665
GO:0006461	protein complex assembly	BIOLOGICAL_PROCESS	183	-0.07651	-1.55613
GO:0032386	regulation of intracellular transport	BIOLOGICAL_PROCESS	36	-0.27937	-1.55513
GO:0048666	neuron development	BIOLOGICAL_PROCESS	85	-0.15913	-1.55508
GO:1903900	regulation of viral life cycle	BIOLOGICAL_PROCESS	20	-0.267	-1.55496
GO:0042393	histone binding	MOLECULAR_FUNCTION	37	-0.22447	-1.55466
GO:0048002	antigen processing and presentation of peptide antigen	BIOLOGICAL_PROCESS	22	-0.19619	-1.55457

GO:0009201	ribonucleoside triphosphate biosynthetic process	BIOLOGICAL_PROCESS	17	-0.27886	-1.55388
GO:0016628	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	MOLECULAR_FUNCTION	19	0.227021	1.553474
GO:0004004	ATP-dependent RNA helicase activity	MOLECULAR_FUNCTION	21	-0.29337	-1.55309
GO:1901988	negative regulation of cell cycle phase transition	BIOLOGICAL_PROCESS	29	-0.1915	-1.55275
GO:0051054	positive regulation of DNA metabolic process	BIOLOGICAL_PROCESS	15	-0.36714	-1.55221
GO:0020037	heme binding	MOLECULAR_FUNCTION	51	0.169218	1.551384
GO:1900864	mitochondrial RNA modification	BIOLOGICAL_PROCESS	45	-0.19512	-1.55087
GO:0043021	ribonucleoprotein complex binding	MOLECULAR_FUNCTION	17	-0.28533	-1.55066
GO:0035195	gene silencing by miRNA	BIOLOGICAL_PROCESS	25	-0.2347	-1.54999
GO:0048863	stem cell differentiation	BIOLOGICAL_PROCESS	22	-0.25331	-1.54876
GO:0007286	spermatid development	BIOLOGICAL_PROCESS	20	-0.2266	-1.54865
GO:0009688	abscisic acid biosynthetic process	BIOLOGICAL_PROCESS	21	0.302348	1.548533
GO:0010051	xylem and phloem pattern formation	BIOLOGICAL_PROCESS	105	-0.11166	-1.54701
GO:0015662	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	MOLECULAR_FUNCTION	15	-0.38655	-1.54667
GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	MOLECULAR_FUNCTION	30	0.274779	1.546608
GO:0007163	establishment or maintenance of cell polarity	BIOLOGICAL_PROCESS	31	-0.24207	-1.54629
GO:0000469	cleavage involved in rRNA processing	BIOLOGICAL_PROCESS	24	-0.26145	-1.54623
GO:0003714	transcription corepressor activity	MOLECULAR_FUNCTION	15	-0.32782	-1.54603
GO:0005938	cell cortex	CELLULAR_COMPONENT	52	-0.19864	-1.54473
GO:0006915	apoptotic process	BIOLOGICAL_PROCESS	123	-0.14058	-1.54434
GO:0002684	positive regulation of immune system process	BIOLOGICAL_PROCESS	104	-0.10403	-1.54338
GO:0007399	nervous system development	BIOLOGICAL_PROCESS	172	-0.09324	-1.54317
GO:0043067	regulation of programmed cell death	BIOLOGICAL_PROCESS	142	-0.10337	-1.54272
GO:0045088	regulation of innate immune response	BIOLOGICAL_PROCESS	103	-0.1019	-1.54176
GO:0072593	reactive oxygen species metabolic process	BIOLOGICAL_PROCESS	66	0.175695	1.539723
GO:0016831	carboxy-lyase activity	MOLECULAR_FUNCTION	28	0.274104	1.537841
GO:0051276	chromosome organization	BIOLOGICAL_PROCESS	211	-0.13222	-1.53487
GO:0009825	multidimensional cell growth	BIOLOGICAL_PROCESS	53	0.19885	1.532621
GO:0071482	cellular response to light stimulus	BIOLOGICAL_PROCESS	66	-0.16228	-1.53188
GO:0006479	protein methylation	BIOLOGICAL_PROCESS	38	-0.2109	-1.52825
GO:0016036	cellular response to phosphate starvation	BIOLOGICAL_PROCESS	37	0.225601	1.527523
GO:0097193	intrinsic apoptotic signaling pathway	BIOLOGICAL_PROCESS	19	-0.28199	-1.52662
GO:0009642	response to light intensity	BIOLOGICAL_PROCESS	93	0.126448	1.526222

	core promoter proximal region				
GO:0001159	DNA binding	MOLECULAR_FUNCTION	25	0.276022	1.525031
	metal ion transmembrane				
GO:0046873	transporter activity	MOLECULAR_FUNCTION	53	-0.17094	-1.52333
	regulation of cellular				
GO:0010675	carbohydrate metabolic process	BIOLOGICAL_PROCESS	57	-0.17674	-1.52245
GO:0051287	NAD binding	MOLECULAR_FUNCTION	28	0.229544	1.521994
GO:0031425	chloroplast RNA processing	BIOLOGICAL_PROCESS	41	-0.18258	-1.5213
	modulation of synaptic				
GO:0050804	transmission	BIOLOGICAL_PROCESS	28	-0.23894	-1.52012
GO:0001816	cytokine production	BIOLOGICAL_PROCESS	28	-0.22966	-1.51939
GO:0043484	regulation of RNA splicing	BIOLOGICAL_PROCESS	26	-0.26636	-1.51891
GO:0051301	cell division	BIOLOGICAL_PROCESS	183	0.085553	1.518141
	jasmonic acid biosynthetic				
GO:0009695	process	BIOLOGICAL_PROCESS	19	0.250183	1.517794
	nuclear-transcribed mRNA				
GO:0000289	poly(A) tail shortening	BIOLOGICAL_PROCESS	19	-0.27781	-1.51694
	ATPase activity, coupled to				
GO:0043492	movement of substances	MOLECULAR_FUNCTION	55	-0.16161	-1.51574
	reactive nitrogen species				
GO:2001057	metabolic process	BIOLOGICAL_PROCESS	25	0.239116	1.515103
GO:0016607	nuclear speck	CELLULAR_COMPONENT	28	-0.27786	-1.51431
GO:0010183	pollen tube guidance	BIOLOGICAL_PROCESS	24	0.225862	1.514145
GO:0043424	protein histidine kinase binding	MOLECULAR_FUNCTION	17	0.280401	1.512883
	polyol transmembrane				
GO:0015166	transporter activity	MOLECULAR_FUNCTION	18	0.404776	1.512597
GO:0050657	nucleic acid transport	BIOLOGICAL_PROCESS	39	-0.23491	-1.51007
GO:0007126	meiotic nuclear division	BIOLOGICAL_PROCESS	72	-0.20389	-1.509
	cullin-RING ubiquitin ligase				
GO:0031461	complex	CELLULAR_COMPONENT	24	-0.24796	-1.50786
	regulation of shoot system				
GO:0048831	development	BIOLOGICAL_PROCESS	159	0.097892	1.506674
GO:0097525	spliceosomal snRNP complex	CELLULAR_COMPONENT	17	-0.2937	-1.50663
GO:0050661	NADP binding	MOLECULAR_FUNCTION	24	0.184655	1.505743
	nucleotide-sugar metabolic				
GO:0009225	process	BIOLOGICAL_PROCESS	28	0.213954	1.502796
GO:0051273	beta-glucan metabolic process	BIOLOGICAL_PROCESS	60	0.17487	1.502791
GO:0042054	histone methyltransferase activity	MOLECULAR_FUNCTION	15	-0.36369	-1.50273
	cellular monovalent inorganic				
GO:0030004	cation homeostasis	BIOLOGICAL_PROCESS	21	-0.24428	-1.50208
	intracellular protein				
GO:0065002	transmembrane transport	BIOLOGICAL_PROCESS	23	-0.2941	-1.5017
GO:0009894	regulation of catabolic process	BIOLOGICAL_PROCESS	67	-0.17501	-1.50039
	cellular response to auxin				
GO:0071365	stimulus	BIOLOGICAL_PROCESS	97	0.104396	1.50025
GO:0045296	cadherin binding	MOLECULAR_FUNCTION	26	0.180813	1.499542
	nuclear chromosome, telomeric				
GO:0000784	region	CELLULAR_COMPONENT	23	-0.26268	-1.49927
GO:0051170	nuclear import	BIOLOGICAL_PROCESS	38	-0.23223	-1.49897
	protein localization to				
GO:0070585	mitochondrion	BIOLOGICAL_PROCESS	24	-0.22866	-1.49843
GO:0046865	terpenoid transport	BIOLOGICAL_PROCESS	21	0.273497	1.498085

GO:0008186	RNA-dependent ATPase activity	MOLECULAR_FUNCTION	22	-0.29262	-1.49748
GO:0044772	mitotic cell cycle phase transition	BIOLOGICAL_PROCESS	66	-0.15577	-1.49746
GO:0034284	response to monosaccharide	BIOLOGICAL_PROCESS	66	0.107811	1.496856
GO:0006766	vitamin metabolic process	BIOLOGICAL_PROCESS	31	0.195718	1.496493
GO:0005802	trans-Golgi network	CELLULAR_COMPONENT	160	0.102634	1.496264
GO:0009798	axis specification	BIOLOGICAL_PROCESS	44	-0.1789	-1.49589
GO:0006473	protein acetylation	BIOLOGICAL_PROCESS	34	-0.25509	-1.49588
GO:0010413	glucuronoxylan metabolic process	BIOLOGICAL_PROCESS	27	0.265917	1.495521
GO:0071359	cellular response to dsRNA	BIOLOGICAL_PROCESS	21	-0.26827	-1.49538
GO:0090501	RNA phosphodiester bond hydrolysis	BIOLOGICAL_PROCESS	36	-0.1981	-1.4951
GO:0030036	actin cytoskeleton organization	BIOLOGICAL_PROCESS	55	-0.14734	-1.49388
GO:0042278	purine nucleoside metabolic process	BIOLOGICAL_PROCESS	82	-0.13596	-1.49321
GO:0099531	presynaptic process involved in chemical synaptic transmission	BIOLOGICAL_PROCESS	19	-0.29234	-1.49085
GO:0080050	regulation of seed development	BIOLOGICAL_PROCESS	20	0.258609	1.488801
GO:0007346	regulation of mitotic cell cycle	BIOLOGICAL_PROCESS	69	-0.18071	-1.48833
GO:0046864	isoprenoid transport	BIOLOGICAL_PROCESS	21	0.273497	1.487948
GO:0010287	plastoglobule	CELLULAR_COMPONENT	28	0.192061	1.487412
GO:0046649	lymphocyte activation	BIOLOGICAL_PROCESS	23	-0.32908	-1.487
GO:0001666	response to hypoxia	BIOLOGICAL_PROCESS	81	0.151471	1.485452
GO:0009639	response to red or far red light	BIOLOGICAL_PROCESS	148	0.113533	1.484915
GO:0002020	protease binding	MOLECULAR_FUNCTION	20	0.251876	1.484872
GO:0000479	endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	BIOLOGICAL_PROCESS	20	-0.24448	-1.48478
GO:1902904	negative regulation of supramolecular fiber organization	BIOLOGICAL_PROCESS	15	-0.24983	-1.48454
GO:0001503	ossification	BIOLOGICAL_PROCESS	22	-0.21497	-1.48389
GO:0045926	negative regulation of growth	BIOLOGICAL_PROCESS	37	0.197064	1.483678
GO:0030029	actin filament-based process	BIOLOGICAL_PROCESS	59	-0.14528	-1.48079
GO:0031346	positive regulation of cell projection organization	BIOLOGICAL_PROCESS	31	-0.19735	-1.48056
GO:0016820	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	MOLECULAR_FUNCTION	55	-0.16161	-1.47886
GO:0098796	membrane protein complex	CELLULAR_COMPONENT	117	-0.14001	-1.47876
GO:0016102	diterpenoid biosynthetic process	BIOLOGICAL_PROCESS	33	0.184267	1.478363
GO:0034293	sexual sporulation	BIOLOGICAL_PROCESS	36	0.197484	1.478158
GO:0070482	response to oxygen levels	BIOLOGICAL_PROCESS	85	0.146723	1.476938
GO:0016441	posttranscriptional gene silencing	BIOLOGICAL_PROCESS	43	-0.2175	-1.47584
GO:0002239	response to oomycetes	BIOLOGICAL_PROCESS	59	0.16908	1.474263
GO:0003779	actin binding	MOLECULAR_FUNCTION	28	-0.22225	-1.47268
GO:0046434	organophosphate catabolic process	BIOLOGICAL_PROCESS	17	0.279	1.472055
GO:0010026	trichome differentiation	BIOLOGICAL_PROCESS	54	0.180226	1.472006

GO:0009751	response to salicylic acid	BIOLOGICAL_PROCESS	189	0.114415	1.4719
GO:0051274	beta-glucan biosynthetic process	BIOLOGICAL_PROCESS	52	0.186194	1.471622
GO:0000726	non-recombinational repair	BIOLOGICAL_PROCESS	18	-0.25027	-1.47035
GO:0030490	maturation of SSU-rRNA	BIOLOGICAL_PROCESS	30	-0.22139	-1.46887
GO:0031012	extracellular matrix	CELLULAR_COMPONENT	54	0.172524	1.46807
GO:0046700	heterocycle catabolic process	BIOLOGICAL_PROCESS	93	-0.12852	-1.46791
GO:1902495	transmembrane transporter complex	CELLULAR_COMPONENT	23	-0.24127	-1.46791
GO:0032984	macromolecular complex disassembly	BIOLOGICAL_PROCESS	45	-0.23182	-1.46733
GO:0010256	endomembrane system organization	BIOLOGICAL_PROCESS	71	-0.13968	-1.46712
GO:0046486	glycerolipid metabolic process	BIOLOGICAL_PROCESS	57	-0.14925	-1.4663
GO:0007265	Ras protein signal transduction	BIOLOGICAL_PROCESS	20	-0.27583	-1.4644
GO:0080117	secondary growth	BIOLOGICAL_PROCESS	16	0.358486	1.463972
GO:0071214	cellular response to abiotic stimulus	BIOLOGICAL_PROCESS	151	-0.09169	-1.46201
GO:0048825	cotyledon development	BIOLOGICAL_PROCESS	73	-0.11372	-1.46165
GO:0005925	focal adhesion	CELLULAR_COMPONENT	28	-0.23618	-1.46085
GO:0009521	photosystem	CELLULAR_COMPONENT	32	0.207849	1.460404
GO:0048514	blood vessel morphogenesis	BIOLOGICAL_PROCESS	17	-0.30637	-1.46008
GO:0006303	double-strand break repair via nonhomologous end joining	BIOLOGICAL_PROCESS	17	-0.24693	-1.45959
GO:0006826	iron ion transport	BIOLOGICAL_PROCESS	21	-0.22404	-1.45925
GO:0072657	protein localization to membrane	BIOLOGICAL_PROCESS	45	-0.22844	-1.45773
GO:0098813	nuclear chromosome segregation	BIOLOGICAL_PROCESS	52	-0.2161	-1.45764
GO:0019319	hexose biosynthetic process	BIOLOGICAL_PROCESS	19	-0.31311	-1.45692
GO:0019761	glucosinolate biosynthetic process	BIOLOGICAL_PROCESS	47	-0.15643	-1.45654
GO:0009269	response to desiccation	BIOLOGICAL_PROCESS	24	0.279865	1.456311
GO:0004312	fatty acid synthase activity	MOLECULAR_FUNCTION	16	0.304229	1.456221
GO:0019941	modification-dependent protein catabolic process	BIOLOGICAL_PROCESS	84	-0.14845	-1.45512
GO:0031050	dsRNA fragmentation	BIOLOGICAL_PROCESS	21	-0.26827	-1.45335
GO:0072655	establishment of protein localization to mitochondrion	BIOLOGICAL_PROCESS	23	-0.21586	-1.45304
GO:0052325	cell wall pectin biosynthetic process	BIOLOGICAL_PROCESS	17	0.235709	1.451639
GO:0032508	DNA duplex unwinding	BIOLOGICAL_PROCESS	24	-0.25692	-1.45163
GO:0072507	divalent inorganic cation homeostasis	BIOLOGICAL_PROCESS	48	-0.13818	-1.45073
GO:0000070	mitotic sister chromatid segregation	BIOLOGICAL_PROCESS	29	-0.19449	-1.45041
GO:0005887	integral component of plasma membrane	CELLULAR_COMPONENT	120	0.119846	1.450041
GO:0009742	brassinosteroid mediated signaling pathway	BIOLOGICAL_PROCESS	70	0.132366	1.449878
GO:0043620	regulation of DNA-templated transcription in response to stress	BIOLOGICAL_PROCESS	21	-0.26871	-1.44967
GO:0044802	single-organism membrane organization	BIOLOGICAL_PROCESS	94	-0.16183	-1.44951

GO:0010359	regulation of anion channel activity	BIOLOGICAL_PROCESS	19	0.222804	1.449099
GO:0006633	fatty acid biosynthetic process	BIOLOGICAL_PROCESS	101	0.106162	1.44848
GO:0004674	protein serine/threonine kinase activity	MOLECULAR_FUNCTION	210	0.072043	1.447159
GO:0016925	protein sumoylation	BIOLOGICAL_PROCESS	16	-0.26662	-1.44635
GO:0010162	seed dormancy process	BIOLOGICAL_PROCESS	19	0.225499	1.44599
GO:0019725	cellular homeostasis	BIOLOGICAL_PROCESS	172	0.089007	1.445318
GO:0009312	oligosaccharide biosynthetic process	BIOLOGICAL_PROCESS	26	0.293103	1.44413
GO:0004519	endonuclease activity	MOLECULAR_FUNCTION	68	-0.15007	-1.4417
GO:0043966	histone H3 acetylation	BIOLOGICAL_PROCESS	22	-0.27959	-1.43978
GO:0006261	DNA-dependent DNA replication	BIOLOGICAL_PROCESS	49	0.124258	1.438986
GO:2000012	regulation of auxin polar transport	BIOLOGICAL_PROCESS	17	0.275937	1.438701
GO:0007600	sensory perception	BIOLOGICAL_PROCESS	35	-0.18095	-1.43737
GO:0036294	cellular response to decreased oxygen levels	BIOLOGICAL_PROCESS	35	0.184053	1.437219
GO:0008270	zinc ion binding	MOLECULAR_FUNCTION	168	-0.0832	-1.43602
GO:0000723	telomere maintenance	BIOLOGICAL_PROCESS	29	-0.23738	-1.43593
GO:0006399	tRNA metabolic process	BIOLOGICAL_PROCESS	47	-0.21836	-1.43537
GO:0010037	response to carbon dioxide	BIOLOGICAL_PROCESS	21	0.2339	1.435303
GO:0016413	O-acetyltransferase activity	MOLECULAR_FUNCTION	25	0.315138	1.434608
GO:0010233	phloem transport	BIOLOGICAL_PROCESS	19	0.293766	1.43382
GO:0036293	response to decreased oxygen levels	BIOLOGICAL_PROCESS	85	0.146723	1.433057
GO:0000422	mitophagy	BIOLOGICAL_PROCESS	16	-0.30183	-1.4317
GO:0001085	RNA polymerase II transcription factor binding	MOLECULAR_FUNCTION	20	-0.31722	-1.43139
GO:0009897	external side of plasma membrane	CELLULAR_COMPONENT	24	0.243887	1.431072
GO:0032392	DNA geometric change	BIOLOGICAL_PROCESS	25	-0.25171	-1.4309
GO:0008022	protein C-terminus binding	MOLECULAR_FUNCTION	23	-0.26103	-1.42973
GO:0003008	system process	BIOLOGICAL_PROCESS	106	-0.09052	-1.42954
GO:0043331	response to dsRNA	BIOLOGICAL_PROCESS	22	-0.28138	-1.42855
GO:0000478	endonucleolytic cleavage involved in rRNA processing	BIOLOGICAL_PROCESS	21	-0.26128	-1.42838
GO:0050660	flavin adenine dinucleotide binding	MOLECULAR_FUNCTION	27	0.210021	1.42697
GO:0030276	clathrin binding	MOLECULAR_FUNCTION	18	-0.22212	-1.42445
GO:0090305	nucleic acid phosphodiester bond hydrolysis	BIOLOGICAL_PROCESS	66	-0.18412	-1.42253
GO:0010498	proteasomal protein catabolic process	BIOLOGICAL_PROCESS	62	-0.14013	-1.41953
GO:0009144	purine nucleoside triphosphate metabolic process	BIOLOGICAL_PROCESS	66	-0.13242	-1.41946
GO:1903320	regulation of protein modification by small protein conjugation or removal	BIOLOGICAL_PROCESS	24	-0.24459	-1.41823
GO:0007268	chemical synaptic transmission	BIOLOGICAL_PROCESS	43	-0.19504	-1.41709
GO:0009606	tropism	BIOLOGICAL_PROCESS	67	-0.11763	-1.41668

GO:0002252	immune effector process	BIOLOGICAL_PROCESS	105	-0.12576	-1.41519
GO:0006508	proteolysis	BIOLOGICAL_PROCESS	211	-0.08727	-1.4129
GO:0051445	regulation of meiotic cell cycle	BIOLOGICAL_PROCESS	18	-0.31281	-1.41143
GO:0035194	posttranscriptional gene silencing by RNA	BIOLOGICAL_PROCESS	39	-0.18784	-1.41065
GO:0046982	protein heterodimerization activity	MOLECULAR_FUNCTION	79	-0.11869	-1.40899
GO:0015934	large ribosomal subunit	CELLULAR_COMPONENT	18	-0.2401	-1.40688
GO:0000959	mitochondrial RNA metabolic process	BIOLOGICAL_PROCESS	57	-0.19208	-1.40547
GO:0009161	ribonucleoside monophosphate metabolic process	BIOLOGICAL_PROCESS	71	-0.12705	-1.4048
GO:0009896	positive regulation of catabolic process	BIOLOGICAL_PROCESS	43	-0.19402	-1.40334
GO:0000462	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	BIOLOGICAL_PROCESS	28	-0.23087	-1.40304
GO:0006119	oxidative phosphorylation	BIOLOGICAL_PROCESS	16	-0.31482	-1.40067
GO:0031334	positive regulation of protein complex assembly	BIOLOGICAL_PROCESS	17	-0.30881	-1.40048
GO:0001568	blood vessel development	BIOLOGICAL_PROCESS	23	-0.23924	-1.39995
GO:0008324	cation transmembrane transporter activity	MOLECULAR_FUNCTION	139	-0.08676	-1.39911
GO:0016553	base conversion or substitution editing	BIOLOGICAL_PROCESS	22	-0.20551	-1.39804
GO:0005856	cytoskeleton	CELLULAR_COMPONENT	225	-0.1304	-1.39604
GO:0007062	sister chromatid cohesion	BIOLOGICAL_PROCESS	15	-0.29768	-1.39586
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	MOLECULAR_FUNCTION	24	-0.21601	-1.39526
GO:0030055	cell-substrate junction	CELLULAR_COMPONENT	28	-0.23618	-1.39494
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	BIOLOGICAL_PROCESS	77	-0.14859	-1.39435
GO:0051701	interaction with host	BIOLOGICAL_PROCESS	42	-0.13287	-1.39383
GO:0017111	nucleoside-triphosphatase activity	MOLECULAR_FUNCTION	236	-0.09923	-1.39336
GO:0045930	negative regulation of mitotic cell cycle	BIOLOGICAL_PROCESS	36	-0.16691	-1.39297
GO:0048872	homeostasis of number of cells	BIOLOGICAL_PROCESS	22	-0.20515	-1.39077
GO:0001775	cell activation	BIOLOGICAL_PROCESS	27	-0.28239	-1.3901
GO:0030522	intracellular receptor signaling pathway	BIOLOGICAL_PROCESS	38	-0.18687	-1.38908
GO:0030111	regulation of Wnt signaling pathway	BIOLOGICAL_PROCESS	28	-0.22656	-1.38902
GO:0032446	protein modification by small protein conjugation	BIOLOGICAL_PROCESS	131	-0.15899	-1.38888
GO:0099536	synaptic signaling	BIOLOGICAL_PROCESS	43	-0.19504	-1.38752
GO:0032940	secretion by cell	BIOLOGICAL_PROCESS	77	-0.1467	-1.38728
GO:0032956	regulation of actin cytoskeleton organization	BIOLOGICAL_PROCESS	21	-0.23316	-1.38719

GO:0040025	vulval development	BIOLOGICAL_PROCESS	15	-0.25241	-1.3849
GO:0045121	membrane raft	CELLULAR_COMPONENT	21	-0.16405	-1.38361
GO:0017156	calcium ion regulated exocytosis	BIOLOGICAL_PROCESS	17	-0.27577	-1.38034
GO:0061061	muscle structure development	BIOLOGICAL_PROCESS	47	-0.14723	-1.37939
GO:0005778	peroxisomal membrane	CELLULAR_COMPONENT	27	-0.19975	-1.37916
GO:0030334	regulation of cell migration	BIOLOGICAL_PROCESS	20	-0.27903	-1.37841
GO:0043618	regulation of transcription from RNA polymerase II promoter in response to stress	BIOLOGICAL_PROCESS	19	-0.29072	-1.3761
GO:0043632	modification-dependent macromolecule catabolic process	BIOLOGICAL_PROCESS	91	-0.16147	-1.37602
GO:0090150	establishment of protein localization to membrane	BIOLOGICAL_PROCESS	37	-0.27371	-1.3741
GO:0006096	glycolytic process	BIOLOGICAL_PROCESS	27	-0.20071	-1.37231
GO:0000151	ubiquitin ligase complex	CELLULAR_COMPONENT	44	-0.19168	-1.37174
GO:0008565	protein transporter activity	MOLECULAR_FUNCTION	26	-0.20756	-1.3716
GO:0061726	mitochondrion disassembly	BIOLOGICAL_PROCESS	16	-0.30183	-1.37033
GO:1900865	chloroplast RNA modification	BIOLOGICAL_PROCESS	38	-0.19477	-1.36765
GO:0045666	positive regulation of neuron differentiation	BIOLOGICAL_PROCESS	29	-0.17889	-1.36715
GO:0006094	gluconeogenesis	BIOLOGICAL_PROCESS	15	-0.35583	-1.36668
GO:0046128	purine ribonucleoside metabolic process	BIOLOGICAL_PROCESS	81	-0.1298	-1.36666
GO:0006458	'de novo' protein folding	BIOLOGICAL_PROCESS	18	-0.2185	-1.36637
GO:0019083	viral transcription	BIOLOGICAL_PROCESS	15	-0.32098	-1.36625
GO:0051261	protein depolymerization	BIOLOGICAL_PROCESS	24	-0.23912	-1.3653
GO:0043596	nuclear replication fork	CELLULAR_COMPONENT	18	-0.28447	-1.3649
GO:0009119	ribonucleoside metabolic process	BIOLOGICAL_PROCESS	89	-0.12947	-1.36402
GO:0009706	chloroplast inner membrane	CELLULAR_COMPONENT	28	-0.25254	-1.36212
GO:0099503	secretory vesicle	CELLULAR_COMPONENT	42	-0.14323	-1.36193
GO:0055029	nuclear DNA-directed RNA polymerase complex	CELLULAR_COMPONENT	21	-0.24553	-1.36117
GO:0031252	cell leading edge	CELLULAR_COMPONENT	23	-0.18751	-1.36023
GO:0008154	actin polymerization or depolymerization	BIOLOGICAL_PROCESS	17	-0.24701	-1.35984
GO:0008356	asymmetric cell division	BIOLOGICAL_PROCESS	17	-0.2563	-1.35833
GO:0051146	striated muscle cell differentiation	BIOLOGICAL_PROCESS	20	-0.22925	-1.3565
GO:0006757	ATP generation from ADP	BIOLOGICAL_PROCESS	27	-0.20071	-1.35603
GO:0032200	telomere organization	BIOLOGICAL_PROCESS	29	-0.23738	-1.35577

Supplementary material 3 (List of primers used for RT-PCR validation)

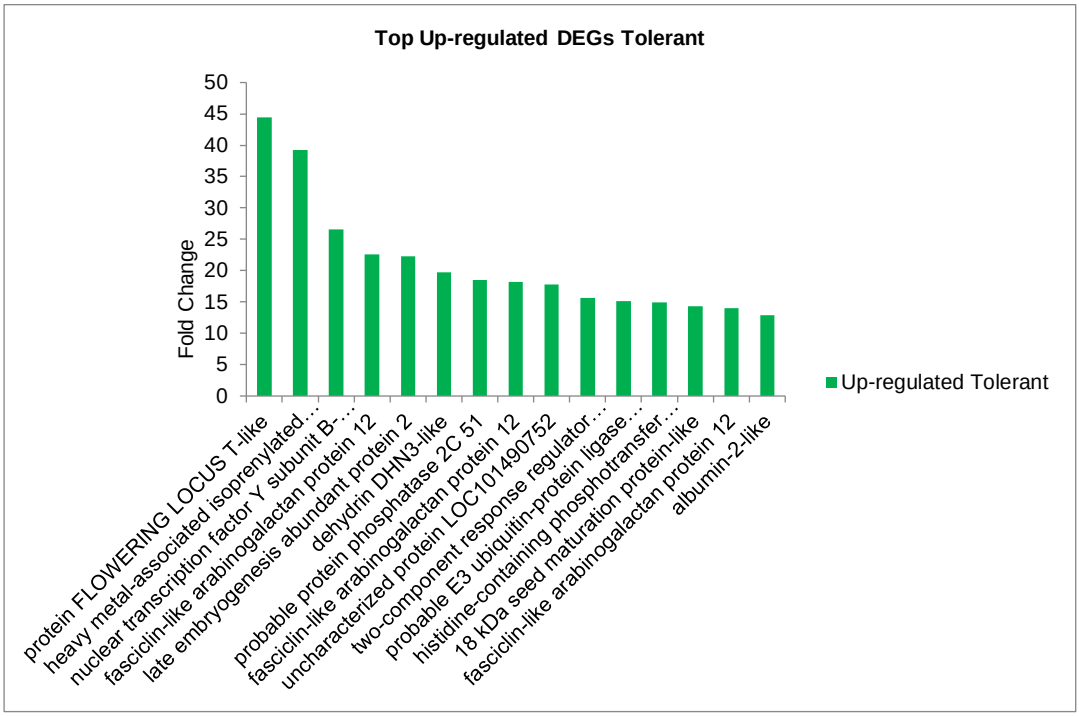
Table S1. List of primers used in qPCR validation

Gene name	Primer sequence	Product size (bp)	tm
ADP 2 F	GTGGCTGCCGAATCCAAGTTGA	125	59.7

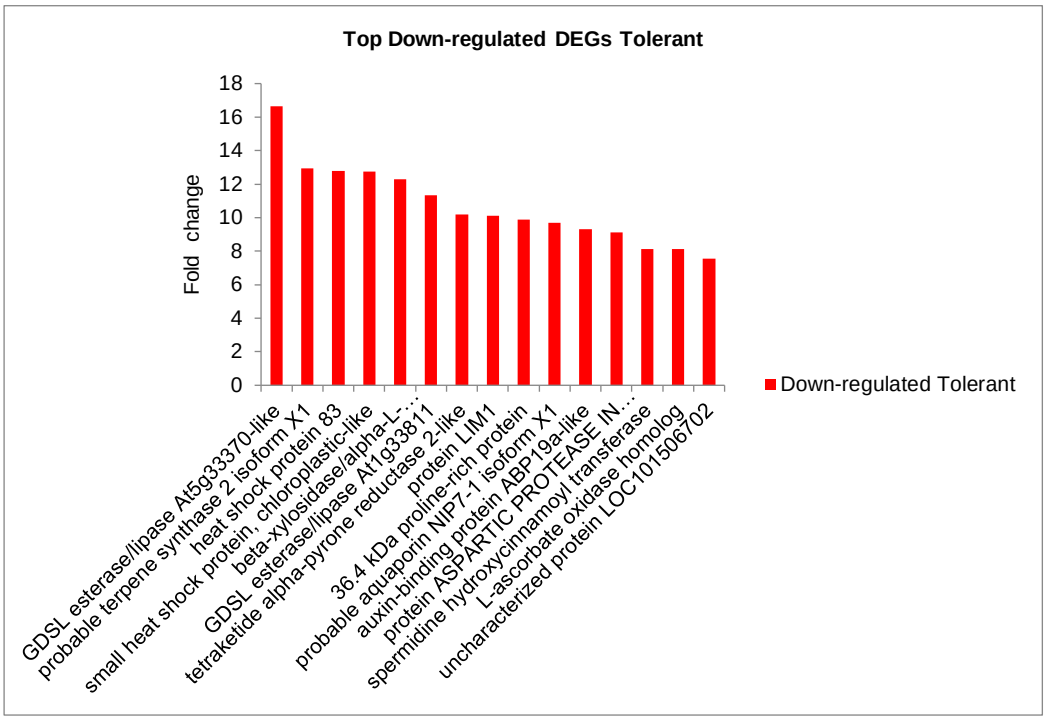
ADP 2 R	TTGGCTCGTCGCTTGGTGAGA		59.7
Cytk 2 F	TTGGATCTGCCGGTGAGTTG	131	60.32
Cytk 2 R	CAGGCCAGAGGCATCTTGAG		60.46
Cytk 15 F	GTGAACGAGAATAACGCCGC	124	59.89
Cytk 15 R	CCCGGTATTTCTTCCCGGAG		59.89
Cytk 18 F	CGTCTGTTGATGTCCCTGCT	145	60.04
Cytk 18 R	ACACAACTTATTAGTGCACCGT		58.26
ABA 1 F	TAACACGGAAGAGGACACGC	139	60.04
ABA 1R	ATTTGCGGTGAAAAGTGGGC		56.97
ZEP 2 F	TTCTTGCTCGTGCAGTAGGG	103	60.04
ZEP 2 R	TGACCATTCTCCAGCACCAC		59.96
NCED 1 F	CCCAAGAAAAAGCCTTGGGC	105	59.96
NCED 1 R	AGAGGGAACGGGCGTAGTAT		60.11
ALDH F	CGGAGAAAGCATTGTTCGCC	101	60.18
ALDH R	CGCCAAAGGAATTGCAGAGG		59.83
AAO 2 F	ATCTGGTGAGCCTCCTTTGC	141	60.03
AAO 2 R	GCAGGTACCCCCAACTGAAA		59.89
CYP 4 F	TGAGCTTGCTAAGCTGGAGAC	138	60.07
CYP 4 R	CAACACCTTACTGGGAGTCCA		59.3
DDXP 3 F	TTTCGATGTCGGGATAGCCG	119	59.97
DDXP 3 R	CCTGATCGTATCCGCGTTGA		59.97

Supplementary material 4 (Graphs of top up-regulated and down-regulated genes in both genotypes).

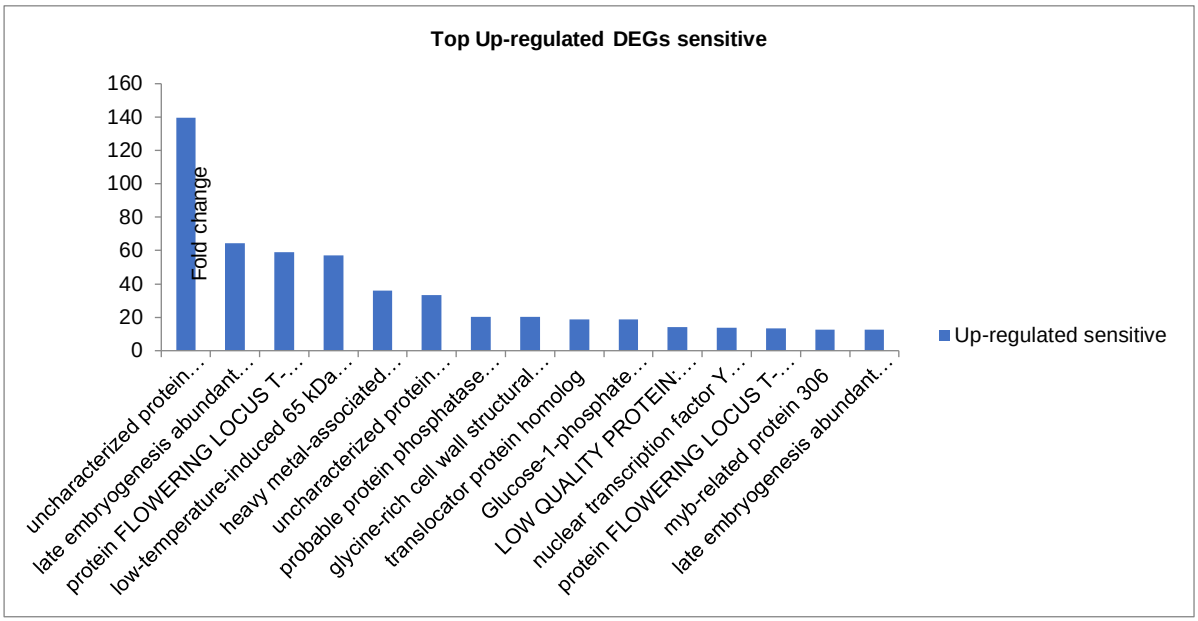
A

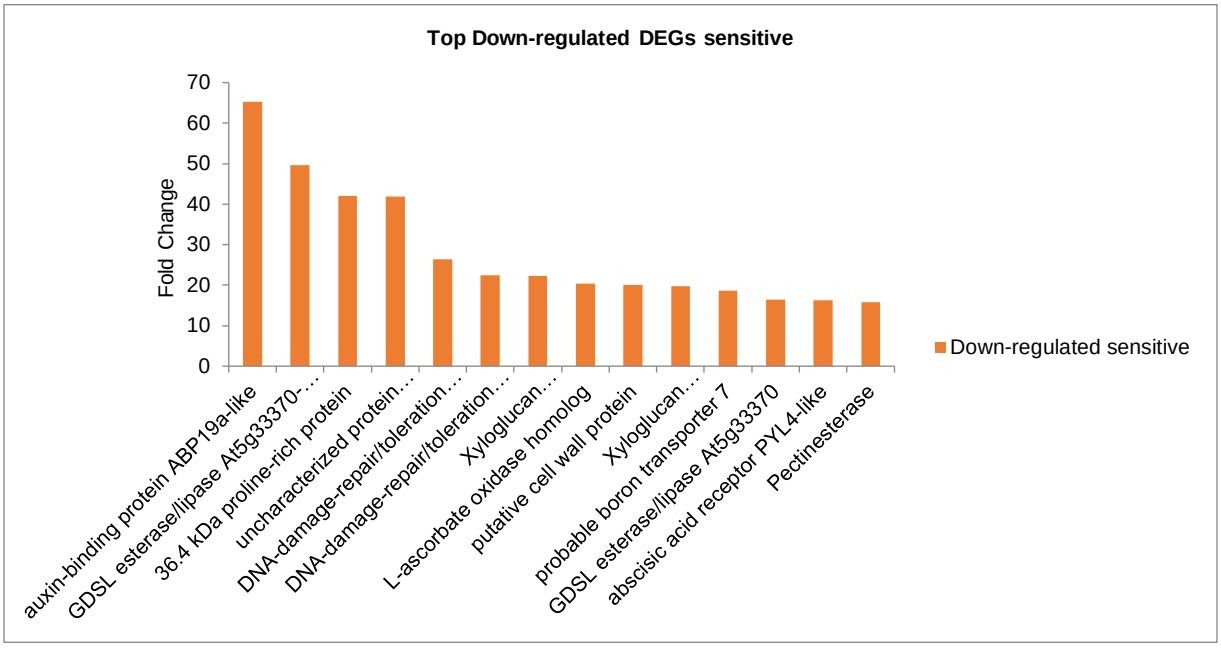


B



C





D

Fig S1. Graphs shows top differentially expressed genes in tolerant and sensitive genotype. The above graph shows top 15 up-regulated and down-regulated genes in tolerant and sensitive genotype. Fold change is represented in different colours. **A)** and **B)** Top up-regulated and down- regulated genes in tolerant genotype. **C)** and **D)** Top up-regulated and down-regulated genes in sensitive genotype.

Appendix Chapter3

Supplementary data1(a): Total number of cytosines methylated, A table highlighting the methylome data obtained from RRBS, Bisulphite conversion for all samples

	Test1	Test2	Test3	Control1	Control2	Control3	Total
ICC8261	s113	111	112	105	106	107	
Sequence pairs analysed in total	4410238	5339847	12717054	18645466	16666338	12083656	69862599
Paired-end alignments with a unique best hit	953022	1123323	2428728	3850253	3809757	2335484	14500567
Pairs without alignments under any condition	3321431	4017932	9886021	14042104	12157984	9293005	52718477
Pairs that did not map uniquely	135785	198592	402305	753109	698597	455167	2643555
fourteen million five hundred thousand five hundred sixty-seven							
	Control1	Control2	Control3	Test1	Test2	Test3	Total
	108	109	116	110	115	114	
ICC283							
Sequence pairs analysed in total	29520585	7583464	17357213	18986625	15490500	8121358	97059745
Paired-end alignments with a unique best hit	5478948	1756508	3616466	3673903	2667357	1855437	19048619
Pairs without alignments under any condition	23129210	5597628	13217449	14746179	12348877	5999330	75038673
Pairs that did not map uniquely	912427	229328	523298	566543	474266	266591	2972453

Table1b: Cytosine after extraction

	Test1	Test2	Test3	Control1	Control2	Control3	Total
ICC8261	s113	111	112	105	106	107	
Sequence pairs analysed in total	4410238	5339847	12717054	18645466	16666338	12083656	69862599
Paired-end alignments with a unique best hit	953022	1123323	2428728	3850253	3809757	2335484	14500567
Pairs without alignments under any condition	3321431	4017932	9886021	14042104	12157984	9293005	52718477
Pairs that did not map uniquely	135785	198592	402305	753109	698597	455167	2643555
fourteen million five hundred thousand five hundred sixty-seven							
	Control1	Control2	Control3	Test1	Test2	Test3	Total
	108	109	116	110	115	114	
ICC283							
Sequence pairs analysed in total	29520585	7583464	17357213	18986625	15490500	8121358	97059745
Paired-end alignments with a unique best hit	5478948	1756508	3616466	3673903	2667357	1855437	19048619
Pairs without alignments under any condition	23129210	5597628	13217449	14746179	12348877	5999330	75038673
Pairs that did not map uniquely	912427	229328	523298	566543	474266	266591	2972453

Table1(c): Total number of methylated C’s

ICC8261						
Total C's analysed	17549400	16184209	33530537	44306478	37886442	28785575
Methylated C's in CpG context	1891970	1781560	3697233	4749296	3922658	3141669
Methylated C's in CHG context	880186	802780	1765590	2269485	1726220	1397211
Methylated C's in CHH context	1308538	924659	2644858	3195399	2006082	1632096
Unmethylated C's in CpG context	678828	668308	1252896	1713902	1584805	1160591
Unmethylated C's in CHG context	992151	970140	1856573	2596369	2473521	1703771
Unmethylated C's in CHH context	11797727	11036762	22313387	29782027	26173156	19750237
Percentage methylation (CpG context)	73.60%	72.70%	74.70%	73.50%	71.20%	73.00%
Percentage methylation (CHG context)	47.00%	45.30%	48.70%	46.60%	41.10%	45.10%
Percentage methylation (CHH context)	10.00%	7.70%	10.60%	9.70%	7.10%	7.60%
ICC283						
Total C's analysed	54784286	25856032	43971186	43138808	35350105	28486764
Methylated C's in CpG context	5687641	2738058	4633870	4441062	3978858	3007851
Methylated C's in CHG context	2555136	1223891	2091491	1944040	1867282	1379355
Methylated C's in CHH context	3639489	1708514	2906282	2275896	2805684	2123679

Unmethylated C's in CpG context	2232443	1039417	1714130	1833616	1300863	1145816
Unmethylated C's in CHG context	3514299	1580980	2636344	2773202	1996248	1704606
Unmethylated C's in CHH context	37155278	17565172	29989069	29870992	23401170	19125457
Percentage methylation (CpG context)	71.80%	72.50%	73.00%	70.80%	75.40%	72.40%
Percentage methylation (CHG context)	42.10%	43.60%	44.20%	41.20%	48.30%	44.70%
Percentage methylation (CHH context)	8.9	8.90%	8.80%	7.10%	10.70%	10.00%

Supplementary table1(d): Conversion rate of samples

total otherC considered (>95% C+T): 383344
average conversion rate = 84.920959485077
total otherC considered (Forward) (>95% C+T): 189675
average conversion rate (Forward) = 84.89432309222
total otherC considered (Reverse) (>95% C+T): 193669
average conversion rate (Reverse) = 84.947046560527
Reading methylation percentage per base for sample: S4-1_S109

total otherC considered (>95% C+T): 921691
average conversion rate = 81.521674940864
total otherC considered (Forward) (>95% C+T): 456101
average conversion rate (Forward) = 81.488176936198
total otherC considered (Reverse) (>95% C+T): 465590
average conversion rate (Reverse) = 81.554490236353
Reading methylation percentage per base for sample: S4-5_S115

total otherC considered (>95% C+T): 1110630
average conversion rate = 84.89460785799
total otherC considered (Forward) (>95% C+T): 546024
average conversion rate (Forward) = 84.963123458782
total otherC considered (Reverse) (>95% C+T): 564606
average conversion rate (Reverse) = 84.828347204708
Reading methylation percentage per base for sample: S4-2_S116

total otherC considered (>95% C+T): 1610292
average conversion rate = 85.389447858648
total otherC considered (Forward) (>95% C+T): 797674
average conversion rate (Forward) = 85.407948711025
total otherC considered (Reverse) (>95% C+T): 812618
average conversion rate (Reverse) = 85.371287235922
Reading methylation percentage per base for sample: S1-6_S108

total otherC considered (>95% C+T): 1152786
average conversion rate = 87.127023071405
total otherC considered (Forward) (>95% C+T): 572381
average conversion rate (Forward) = 87.170451926577
total otherC considered (Reverse) (>95% C+T): 580405
average conversion rate (Reverse) = 87.084194612738
Reading methylation percentage per base for sample: S4-3_S110

total otherC considered (>95% C+T): 523749
average conversion rate = 83.276804915981
total otherC considered (Forward) (>95% C+T): 261228

average conversion rate (Forward) = 83.245589725624
total otherC considered (Reverse) (>95% C+T): 262521
average conversion rate (Reverse) = 83.307866361529
Reading methylation percentage per base for sample: S4-6_S114
total otherC considered (>95% C+T): 215719
average conversion rate = 85.025116260902
total otherC considered (Forward) (>95% C+T): 106678
average conversion rate (Forward) = 85.046599568109
total otherC considered (Reverse) (>95% C+T): 109041
average conversion rate (Reverse) = 85.004098513025
Reading methylation percentage per base for sample: S4-4_S111

total otherC considered (>95% C+T): 798025
average conversion rate = 81.636936386279
total otherC considered (Forward) (>95% C+T): 395143
average conversion rate (Forward) = 81.60676376081
total otherC considered (Reverse) (>95% C+T): 402882
average conversion rate (Reverse) = 81.666529422816
Reading methylation percentage per base for sample: S4-2_S112

total otherC considered (>95% C+T): 205403
average conversion rate = 82.979968047184
total otherC considered (Forward) (>95% C+T): 101789
average conversion rate (Forward) = 82.975568023545
total otherC considered (Reverse) (>95% C+T): 103614
average conversion rate (Reverse) = 82.984290571228
Reading methylation percentage per base for sample: S4-5_S113

total otherC considered (>95% C+T): 1176368
average conversion rate = 83.150232875071
total otherC considered (Forward) (>95% C+T): 586096
average conversion rate (Forward) = 83.109070489487
total otherC considered (Reverse) (>95% C+T): 590272
average conversion rate (Reverse) = 83.191104048938
Reading methylation percentage per base for sample: S1-4_S105

total otherC considered (>95% C+T): 874375
average conversion rate = 86.949485994508
total otherC considered (Forward) (>95% C+T): 435522
average conversion rate (Forward) = 87.031960208746
total otherC considered (Reverse) (>95% C+T): 438853
average conversion rate (Reverse) = 86.867637779426
Reading methylation percentage per base for sample: S1-3_S106

total otherC considered (>95% C+T): 598094
average conversion rate = 84.806527311075
total otherC considered (Forward) (>95% C+T): 298058
average conversion rate (Forward) = 84.746808055329

total otherC considered (Reverse) (>95% C+T): 300036
average conversion rate (Reverse) = 84.865852865105
Reading methylation percentage per base for sample: S1-1_S107

Supplementary data2: Top hypermethylated and hypomethylated DMRs associated genes

Top In Tolerant genotype			
100	LOC101490332	DOWNSTREAM	NEDD8 ultimate buster 1
97.76119	LOC101504123	UPSTREAM	tRNA threonylcarbamoyladenosine dehydratase 2-like
91.66667	LOC101506150	DOWNSTREAM	zinc finger-containing ubiquitin peptidase 1-like%2C transcript variant X1
86.67476	LOC101501927	UPSTREAM	ubinuclein-1-like
86.27451	LOC101507570	UPSTREAM	RNA polymerase II C-terminal domain phosphatase-like 2%2C transcript variant X1
86.03935	LOC101491148	UPSTREAM	homeobox-leucine zipper protein HAT9-like
83.21168	LOC101498627	UPSTREAM	protein STICHEL-like 2%2C transcript variant X1
82.06623	LOC101488375	UPSTREAM	AP2-like ethylene-responsive transcription factor At1g79700
81.47613	LOC101489236	INSIDE	putative Peroxidase 48
79.33294	LOC101507570	UPSTREAM	RNA polymerase II C-terminal domain phosphatase-like 2%2C transcript variant X1
74.95514	LOC101500403	UPSTREAM	uncharacterized LOC101500403
73.23245	LOC101491148	UPSTREAM	homeobox-leucine zipper protein HAT9-like
73.08355	LOC101496727	UPSTREAM	pentatricopeptide repeat-containing protein At5g41170%2C mitochondrial-like
69.59707	LOC101490332	DOWNSTREAM	NEDD8 ultimate buster 1
69.28347	LOC101493971	INSIDE	sodium/hydrogen exchanger 7%2C transcript variant X1
69.24603	LOC101512993	INSIDE	cytochrome P450 714A2-like
69.23077	LOC113788016	DOWNSTREAM	U3 small nucleolar RNA-associated protein 21-like
67.43451	LOC101509967	UPSTREAM	uncharacterized LOC101509967%2C transcript variant X1
65.82118	LOC101502380	INSIDE	hydroxyproline O-galactosyltransferase GALT2
62.93706	LOC101503589	INSIDE	protein COFACTOR ASSEMBLY OF COMPLEX C SUBUNIT B CCB2%2C chloroplastic%2C transcript variant X1
59.1684	LOC101495140	DOWNSTREAM	uncharacterized protein At4g14100-like
58.59609	LOC101489148	INSIDE	lysM domain receptor-like kinase 3

Top Hypo in Tolerant			
-94.5946	LOC101505348	UPSTREAM	putative B3 domain-containing protein At1g78640
-74.2547	LOC101497374	UPSTREAM	receptor-like kinase TMK4
-65.1948	LOC101498505	UPSTREAM	probable nucleoredoxin 2
-62.5127	LOC101490217	UPSTREAM	auxin-responsive protein SAUR72
-52.3364	LOC101514774	UPSTREAM	protoporphyrinogen oxidase 1%2C chloroplastic%2C transcript variant X1
-52.2727	LOC101501790	UPSTREAM	chromatin modification-related protein MEAF6-like
-50.8454	LOC101506858	UPSTREAM	uncharacterized LOC101506858%2C transcript variant X1
-50.1213	LOC101491504	UPSTREAM	trafficking protein particle complex subunit 4%2C transcript variant X1
-46.4815	LOC101493441	UPSTREAM	protein trichome birefringence-like 12%2C transcript variant X1
-45.5663	LOC101503804	UPSTREAM	protein transport protein Sec61 subunit gamma%2C transcript variant X1
-44.9095	LOC101514095	INSIDE	sucrose synthase 6-like
-42.061	LOC101509001	UPSTREAM	acetylserotonin O-methyltransferase-like
-40.6014	LOC101493491	UPSTREAM	putative glucan endo-1%2C3-beta-glucosidase GVI
-40.0194	LOC101501790	UPSTREAM	chromatin modification-related protein MEAF6-like
-38.7097	LOC101502817	INSIDE	receptor-like serine/threonine-protein kinase At2g45590
-38.0552	LOC101491698	UPSTREAM	transcription factor MYB13-like
-37.9015	LOC101496713	UPSTREAM	F-box/FBD/LRR-repeat protein At1g13570-like
-37.9012	LOC101498470	INSIDE	cancer-related nucleoside-triphosphatase homolog%2C transcript variant X1
-37.2549	LOC101497114	UPSTREAM	probable serine/threonine-protein kinase PBL5
-36.3571	LOC101515663	INSIDE	inosine triphosphate pyrophosphatase
-36.1095	LOC101491504	UPSTREAM	trafficking protein particle complex subunit 4%2C transcript variant X1
-35.8014	LOC101505952	INSIDE	serine/threonine-protein kinase HT1-like

Top Hypermethylated in sensitive			
98.59155	LOC101512834	UPSTREAM	ankyrin repeat domain-containing protein 13C-like
98.00307	LOC101507570	UPSTREAM	RNA polymerase II C-terminal domain phosphatase-like 2%2C transcript variant X1
97.82609	LOC101493321	UPSTREAM	protein TIC 40%2C chloroplastic
97.36842	LOC101512834	UPSTREAM	ankyrin repeat domain-containing protein 13C-like
94.80519	LOC101491732	INSIDE	heavy metal-associated isoprenylated plant protein 47-like
92.59259	LOC113787620	UPSTREAM	uncharacterized LOC113787620
91.30435	LOC101507570	UPSTREAM	RNA polymerase II C-terminal domain phosphatase-like 2%2C transcript variant X1

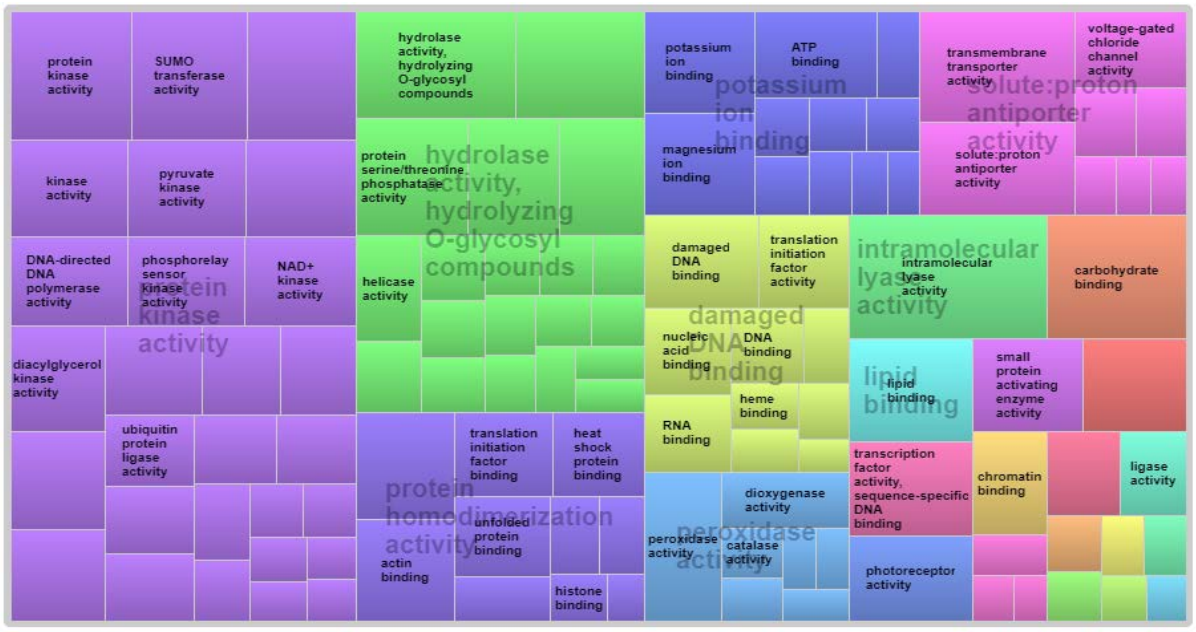
90.47619	LOC101509353	INSIDE	protein FORGETTER 1-like%2C transcript variant X1
89.13043	LOC101488549	INSIDE	RPM1-interacting protein 4-like
87.76447	LOC101504123	UPSTREAM	tRNA threonylcarbamoyladenosine dehydratase 2-like
85.98726	LOC101498910	UPSTREAM	eukaryotic translation initiation factor
82.03766	LOC101490046	UPSTREAM	actin-interacting protein 1-2
81.00566	LOC101491148	UPSTREAM	homeobox-leucine zipper protein HAT9-like
79.4074	LOC101494454	UPSTREAM	DELLA protein RGL2-like
77.12159	LOC101488375	UPSTREAM	AP2-like ethylene-responsive transcription factor At1g79700
75.80177	LOC101511593	UPSTREAM	ribose-phosphate pyrophosphokinase 1
75.53191	LOC101510315	UPSTREAM	uncharacterized LOC101510315
75.43506	LOC101500403	UPSTREAM	uncharacterized LOC101500403
73.3752	LOC101496727	UPSTREAM	pentatricopeptide repeat-containing protein At5g41170%2C mitochondrial-like
73.08203	LOC101495587	INSIDE	uncharacterized LOC101495587%2C transcript variant X1

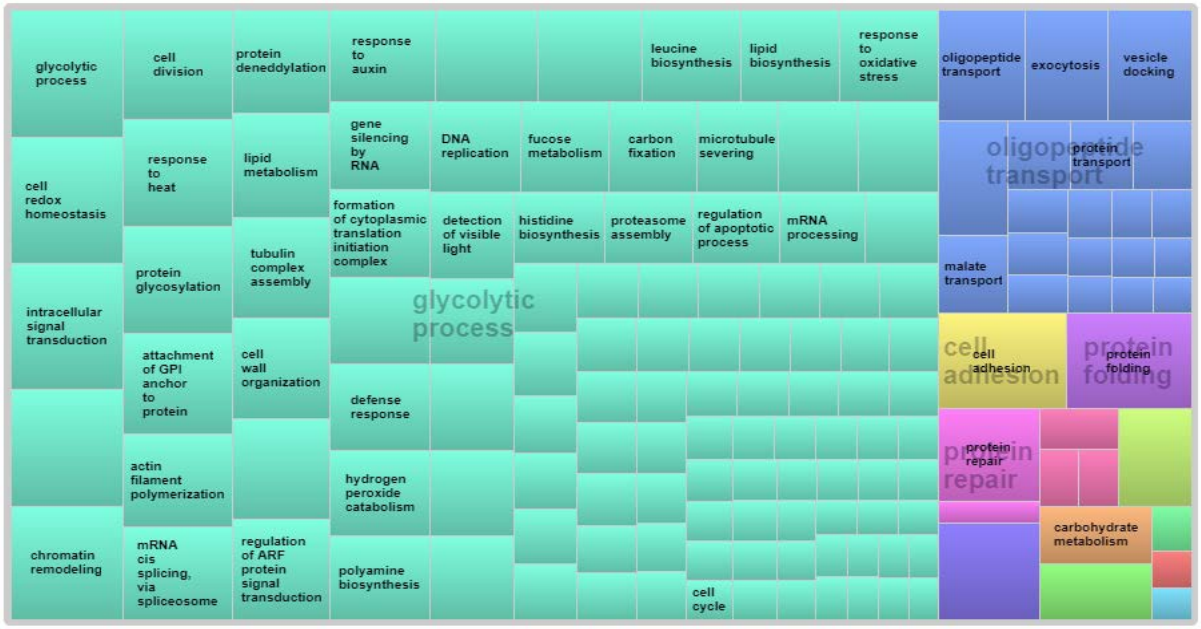
Top Hypomethylated in sensitive			
-100	LOC101493876	INSIDE	uncharacterized LOC101493876
-100	LOC101503817	INSIDE	sulfoquinovosidase-like
-95.1871	LOC101492339	UPSTREAM	probable methionine--tRNA ligase
-95.0915	LOC101515676	INSIDE	coenzyme Q-binding protein COQ10 homolog B%2C mitochondrial
-92.8904	LOC101505348	UPSTREAM	putative B3 domain-containing protein At1g78640
-89.1304	LOC101488675	INSIDE	uncharacterized LOC101488675
-88.9908	LOC101512023	INSIDE	chaperone protein dnaJ 49%2C transcript variant X1
-82.3587	LOC101505781	INSIDE	probable protein phosphatase 2C 55%2C transcript variant X1
-80.9091	LOC101495288	INSIDE	UPF0481 protein At3g47200-like%2C transcript variant X1
-80.2942	LOC101493676	INSIDE	calmodulin-binding transcription activator 5-like%2C transcript variant X1
-79.3103	LOC101490865	INSIDE	polypyrimidine tract-binding protein homolog 3-like%2C transcript variant X1
-79.3025	LOC101492487	UPSTREAM	40S ribosomal protein S13-like
-76.6234	LOC113785206	UPSTREAM	small nucleolar RNA Z122
-74.063	LOC101510091	INSIDE	arogenate dehydratase/prephenate dehydratase 1%2C chloroplastic-like
-71.3289	LOC101493077	INSIDE	putative leucine-rich repeat-containing protein DDB_G0290503%2C transcript variant X1
-70.6897	LOC101508620	INSIDE	WRKY transcription factor 1-like
-69.4211	LOC101504850	INSIDE	uncharacterized LOC101504850
-69.344	LOC101507532	INSIDE	probable 2-oxoglutarate-dependent dioxygenase At3g111800
-69.3112	LOC101500954	INSIDE	perakine reductase-like%2C transcript variant X1

-69.0821	LOC101513965	UPSTREAM	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2-like%2C transcript variant X1

Supplementary data 3a, **b** GO terms in relation to hypermethylated and hypomethylated DMRs in both genotypes

a) Tolerant genotype





b) Sensitive genotype

Supplementary data 4 (a) Correlation between expression and methylation percentage (Tolerant genotype).

DMR associated genes	Expression status	Methylation status	Context			
LOC101497114	1.497133372	-37.25490196	LOC101497114	UPSTREAM	probable serine/threonine-protein kinase PBL5	hypo
LOC101491825	1.28929788	-27.92792793	LOC101491825	UPSTREAM	uncharacterized LOC101491825	hypo
LOC101506193	3.599587588	-28.15694943	LOC101506193	UPSTREAM	uncharacterized LOC101506193	hypo
LOC101505448	-1.172201839	35.18518519	LOC101505448	INSIDE	formin-like protein 20%2C transcript variant X1	hyper
LOC101498627	-1.436100219	83.21167883	LOC101498627	UPSTREAM	protein STICHEL-like 2%2C transcript variant X1	hyper
LOC101503881	-1.276473033	-34.75877193	LOC101503881	UPSTREAM	lamin-like protein	hypo
LOC101489249	1.406856516	30.81515396	LOC101489249	INSIDE	EPIDERMAL PATTERNING FACTOR-like protein 5	hyper
LOC101510228	-1.277810991	42.02247191	LOC101510228	UPSTREAM	uncharacterized protein At5g39865-like	hyper
LOC101498505	2.881935795	-65.19480519	LOC101498505	UPSTREAM	probable nucleoredoxin 2	hypo
LOC101494403	-1.584865341	-29.29824561	LOC101494403	DOWNSTREAM	glucan endo-1%2C3-beta-glucosidase 14	hypo
LOC101514095	-1.596095385	-44.90950226	LOC101514095	INSIDE	sucrose synthase 6-like	hypo
LOC101491262	1.740527895	25.38969795	LOC101491262	DOWNSTREAM	adagio protein 3	hyper

Supplementary data 4 (b) Correlation between expression and methylation percentage (Sensitive genotype)

LOC101495047	2.77467	31.54809	G-type lectin S-receptor-like serine/threonine-protein kinase At4g27290	DOWNSTREAM
LOC101499724	-1.20307	35.64516	uncharacterized LOC101499724	DOWNSTREAM
LOC101503344	-1.97449	36.64596	mavicyanin	DOWNSTREAM
LOC101496067	1.425742	50	phosphomannomutase	DOWNSTREAM
LOC101503717	-1.11222	67.43506	protein KINESIN LIGHT CHAIN-RELATED 2	DOWNSTREAM
LOC101513587	-5.38759	26.1398	GDSL esterase/lipase At5g03610-like	INSIDE
LOC101506002	-1.36962	27.06511	uncharacterized LOC101506002	INSIDE
LOC101493129	2.190022	28.46131	protein REDUCED WALL ACETYLATION 2%2C transcript variant X1	INSIDE
LOC101500380	1.161285	37.75277	cellulose synthase A catalytic subunit 4 [UDP-forming]-like	INSIDE
LOC101513770	-3.5171	41.03012	remorin 4.1-like	INSIDE
LOC101496613	-1.30519	43.89788	vesicle-associated membrane protein 722%2C transcript variant X1	INSIDE
LOC101494422	1.396229	49.75347	phosphoenolpyruvate carboxylase 4-like%2C transcript variant X1	INSIDE
LOC101498101	1.239934	54.93827	DNA repair protein RadA-like	INSIDE
LOC101493562	-1.595	55.89012	biotin carboxyl carrier protein of acetyl-CoA carboxylase	INSIDE
LOC101502146	-3.03985	57.88435	protein PLASTID TRANSCRIPTIONALLY ACTIVE 10	INSIDE
LOC101493661	-1.31627	27.43023	protein NUCLEAR FUSION DEFECTIVE 4-like	UPSTREAM
LOC101507013	-1.75175	28.88889	basic leucine zipper 34-like	UPSTREAM
LOC101515070	-5.38759	29.86111	ribonuclease E/G-like protein%2C chloroplastic%2C transcript variant X1	UPSTREAM
LOC101514717	-5.38759	34.62557	transcription factor bHLH68-like%2C transcript variant X1	UPSTREAM
LOC101510228	-2.07852	37.40602	uncharacterized protein At5g39865-like	UPSTREAM
LOC101513580	-5.38759	39.629	uncharacterized protein At3g50808-like	UPSTREAM
LOC101501552	-1.14637	41.16929	probable inactive receptor kinase At5g58300%2C transcript variant X1	UPSTREAM
LOC101505395	-2.30909	42.1875	ASC1-like protein	UPSTREAM

LOC101497295	-1.5428	43.57317	uncharacterized protein At4g22758	UPSTREAM
LOC101511561	-2.28088	50.23423	aldehyde oxidase GLOX-like	UPSTREAM
LOC101511699	-3.43622	56.86275	uncharacterized LOC101511699%2C transcript variant X1	UPSTREAM
LOC101491148	1.47	72.11476	homeobox-leucine zipper protein HAT9-like	UPSTREAM
LOC101496727	2.009901	73.3752	pentatricopeptide repeat-containing protein At5g41170%2C mitochondrial-like	UPSTREAM
LOC101496849	1.366961	92.1875	protein WVD2-like 3%2C transcript variant X1	UPSTREAM
LOC101505384	-1.81146	-100	pentatricopeptide repeat-containing protein At5g67570%2C chloroplastic%2C transcript variant X1	INSIDE
LOC101503868	-1.3744	-66.2121	putative ABC transporter C family member 15%2C transcript variant X1	INSIDE
LOC101496987	1.2276	-59.2285	protein REDUCED WALL ACETYLATION 3	INSIDE
LOC101498505	-1.80177	-49.5721	probable xyloglucan endotransglucosylase/hydrolase protein 6	DOWNSTREAM
LOC101502093	-1.15585	-48.6277	uncharacterized LOC101502093	INSIDE
LOC101495556	3.002412	-44.051	O-fucosyltransferase 9%2C transcript variant X1	DOWNSTREAM
LOC101498505	-1.30625	-43.4728	probable nucleoredoxin 2	UPSTREAM
LOC101498090	-1.06676	-39.1176	branched-chain-amino-acid aminotransferase 6-like	INSIDE
LOC101492791	2.805491	-37.9303	sulfoquinovosyl transferase SQD2	INSIDE
LOC101492389	1.24	-37.7778	FAD-linked sulfhydryl oxidase ERV1-like	INSIDE
LOC101507516	-1.26758	-35.7092	probable LRR receptor-like serine/threonine-protein kinase IRK	INSIDE
LOC101514095	-2.84863	-35.5384	sucrose synthase 6-like	INSIDE
LOC101497864	-1.04666	-34.7973	cycloartenol-C-24-methyltransferase	UPSTREAM
LOC101492868	1.006065	-32.5253	callose synthase 9%2C transcript variant X1	INSIDE
LOC101502490	-1.60819	-32.5052	DNA replication licensing factor MCM5	INSIDE
LOC101503532	-1.50853	-25.4506	endoglucanase 11%2C transcript variant X1	DOWNSTREAM

Appendix Chapter 4

Supplementary Data: Chapter4

Supplementary Table1: The PCR program to amplify the 5kb genomic region is provided in Supplementary Table 1.

PCR steps	Temperature	Duration
Initial Denaturation	95˚C	2 min
Denaturation	93˚C	30 sec
Annealing	52˚C	30sec
Extension	68˚C	5min
Final Extension	72˚C	10 min

Supplementary Table2: The PCR program for sequencing samples is also provided in Supplementary Table 2.

PCR steps	Temperature	Duration
Initial Denaturation	95˚C	1 min
Denaturation	95˚C	15sec
Annealing	55˚C	30sec
Extension	72˚C	10sec

Supplementary Table3: The PCR reaction to amplify the 5kb genomic region is provided in Supplementary Table 3

Component	Amount(µl)	Final Concentration
GoTaq® Long PCR Master Mix, 2X	25µl	1X
upstream primer	5–50pmol	0.1–1.0µM
downstream primer	5–50pmol	0.1–1.0µM
template DNA	0.1–0.5µg1	<0.5µg/50µl
Nuclease-Free Water to a final volume of	50µl	

Supplementary Table4: The PCR reaction to amplify the sequencing fragment is provided in Supplementary Table 4

Component	Amount(µl)
Template	200 ng
Primers (20 µM each)	1 µL
MyTaq Red Mix, 2x	25 µL
Water (ddH2O) up to 50 µL	

Sequence used for 5kb target amplification for *4CL* gene

4-coumarate--CoA ligase-like 1

NW_004516753.1_162798-165892

Length 5094

ATATATATATATATATACTCTTTTGTTTTGTATATAAAAAAATATTGAGTCAACAAAAATTAATGTATTTGATT
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CAGAGATTAACGACGTTTAAAAATAACATATAAGACATTAATTTTGATATATCTTAATAATATACCAAATATTTT
TATATT

Sequence used for 5kb target amplification for *RVE 7*

RVE 7

NW_004516329.1_420654-425384

Length 5841

CTATTATAATATATACTTTTTTGTTAAAAATATAATCACTTTGTTTAAATAATAATAAAAAATAGCACTTGGACT
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Appendix: List of Publications

1. Badhan, S., Kole, P., Ball, A., and Mantri, N. (2018) RNA sequencing of leaf tissues from two contrasting chickpea genotypes reveals mechanisms for drought tolerance. *Plant Physiol Biochem* 129, 295-304. (Published)
2. Badhan, S., Kole, P., Ball, A., and Mantri, N. Reduced representation bisulphite sequencing of two chickpea genotypes to identify differential DNA methylation patterns associated with drought tolerance and sensitivity. (Submitted)
3. Badhan, S., Ball, A., and Mantri, N. First Report of CRISPR/Cas9 Mediated DNA-Free Editing of 4CL and RVE7 Genes in Chickpea Protoplasts. International journal of molecular sciences, 22,396. https://doi.org/10.3390/ijms22010396. (Published)
4. Badhan, S., Ball, A., and Mantri, N. Future of CRISPR/Cas9 DNA free editing in developing drought tolerant crops. (Manuscript in preparation)

Conference Presentation

1. Badhan, S., Kole, P., Ball, A., and Mantri, N. (2018) RNA sequencing of leaf tissues from two contrasting chickpea genotypes reveals mechanisms for drought tolerance. Australian Society of Plant Scientists Conference. 27th November 2019. (Poster Presentation)