Physiological, Biochemical and Gene Expression Studies of Salinity Stress Response and Tolerance in Local Rice Varieties of Sarawak, Malaysia

By

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Abstract

Rice is a staple food in a wide range of population for an extended amount of time. It is the grain from the grass family *Oryza sativa* (Asian Rice) or *Oryza glaberrima* (African Rice). With the ever-rising population and scarcity of fresh water, it is getting more challenging to maintain rice as an affordable food source. Meanwhile, the traditional rice plantation in Sarawak had allowed many upland rice varieties to flourish. In this study, salt tolerance in 3 Sarawak local rice varieties named Bario, Bajong and Biris, have been assessed. The variations in the plants physiological and biochemical aspects upon exposure to salinity stress were compared to a commercial Malaysian rice variety (MR219). After exposing seedlings to 100mM, 150mM and 200mM of NaCl, no significant differences could be observed between their seedling length, fresh weight, total plant biomass, stem diameter and number of leaves in the seedlings. None of the stated physiological parameters was therefore considered as suitable biomarkers for salt tolerance screening. In the biochemical analysis, Biris appeared to accumulate a significant amount of salt while Bajong was able to limit the amount of salt accumulation in the seedlings. Gene expression analysis of all the varieties revealed that Bajong was reacting positively to the salt stress while all other varieties shown decrease gene expression in several salt-inducible genes after the salt induction. Transcriptomic sequencing of Bajong after the saline stress exposure had revealed a total of 4096 DEGs that showed a high enrichment factor in the secondary metabolite synthesis pathways. A list of 179 salinity-responsive genes had also been identified for future studies.

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Declaration

I, Mr Brandon Yeo Pei Hui, Masters of Science (By Research), Faculty of Engineering, Computing and Science, hereby declare that my project work titled "Physiological, Biochemical and Gene Expression Studies of Salinity Stress Response and Tolerance in Local Rice Varieties of Sarawak, Malaysia". This work is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of candidate's knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors. All the given information is correct to best of my knowledge.

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(BRANDON YEO PEI HUI)

DATE:

As the principal coordinating supervisor, I hereby acknowledge and certify that the statements mentioned above are legitimate to the best of my knowledge.

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(DR HWANG SIAW SAN)

DATE:

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List of Abbreviations

ADC	Arginine Decarboxylase
CIPK	CBL-Interacting Protein Kinase
DEGs	Differentially Expressed Genes
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
GAE	Gallic Acid Equivalent
GO	Gene Ontology
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LEA	Late Embryogenesis Abundant
NGS	Next Generation Sequencing
ODC	Ornithine Decarboxylase
QE	Quercetin Equivalent
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SNP	Single Nucleotide Polymorphism
SOS	Salt Overly Sensitive
SSR	Single Sequence Repeat
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoid Content
TPC	Total Phenolic Content

Chapter 1 Research Background

1.1 Introduction

Human beings are highly dependent on irrigated land as their primary source of food supply. A little over 15% of irrigated land provides one-third of the total food supply in the world (Munns 2002b). Irrigated lands are preferable in modern agriculture, as they are more productive than traditional rain-fed farmland. However, irrigated areas are also more susceptible to salinity problem. Management practice such as "System of Rice Intensification" (Uphoff & Kassam 2009) and improvement of rice cultivars via artificial breeding (Ganguly et al. 2012) have been employed to counter salinity problem, but more research is still needed before salt tolerant rice can be widely adopted.

Therefore, creating an overview of the transcriptomic profile in wild rice varieties upon salinity stress exposure could help us better comprehend the salinity responses in them and, in turn, discover any unique pathway that is superior and had not been observed in common varieties. It will also ease the route of creating salt tolerance rice varieties capable of mass commercialization. In this thesis, the physiological and biochemical traits of several Sarawak local rice varieties upon salinity stress were examined and the best performing varieties were chosen for transcriptomic sequencing. The transcriptome sequence was cross-examined with the physiological and biochemical trait obtained to generate an overview of the salt tolerance mechanism in local rice varieties.

1.2 Definition of Salinity

Salinity can be classified into primary salinity and secondary salinity. Primary salinity occurs naturally in the formation of salt lakes, salt flat, salt marshes and salt pans while secondary salinity is caused by human development and agriculture activities such as intensive point source irrigations, seawater intrusion and land clearing events (USDA-ARS n.d.). Soil salinity is measured based on its electrical conductivity, EC (Hardie & Doyle 2012) and soils that measure more than 3dS/m (deci-Siemens per Meter) are considered as saline (USDA-ARS 2008). Rice, being one of the most salt sensitive cereal crops relative to wheat, barley and sorghum, will not survive to maturity in such conditions (Zeng & Shannon 2000).

1.3 Rice Farming in Malaysia

Extreme droughts event has become increasingly regular in the past decade (Dai 2013). For example, Australia has been suffering from prolonged drought for many years with little interruption (Dijk et al. 2013), Amazon had been hit by a huge drought in 2010 that resulted in a massive depression in fisheries (Lewis et al. 2011) and Europe had been affected by a heat wave in 2003 that took more than 40,000 lives (Beniston 2004). All of these events were atrocious towards the social-economy system and agriculture industries. In Malaysia, weather patterns have become increasingly extreme in the recent years. Major states such as Selangor and Johor have been suffering from insufficient water supplies in the dry seasons, while floods have constantly harassed Kelantan, Terengganu and Pahang states during the monsoon (Dam 2015; Gasim, Toriman & Abdullahi 2014; Martin 2015). All these natural events are detrimental towards the rice plantation that required a consistent supply of fresh water (IRRI 2015).

Currently, rice farming is heavily protected and subsidised in Malaysia. The government has been actively involved in the eradication of poverty among rice farmers, in hope for them to upgrade to better machinery and technology. A whole network of subsidies and agencies has been established to keep farmers above the poverty line. Yield improvement has been observed in the past decade (FAO 2015) but has not been sufficient to attain self-sufficiency in the country. In the year 2015, the production volume of rice Malaysia is only approximately 1.8 million tonnes, 1 million tonne less to attain 100% self-sufficiency of the country (Production 2016). Meanwhile, getting farmers to plant rice while they could be better making money from other crops (such as oil palm) posed a huge challenge to the government.

1.3.1 Challenge of Rice Farming in Sarawak

According to International Rice Research Institute (IRRI), “Upland rice is grown in rain-fed, naturally well-drained soils with bunded or unbunded fields without surface water accumulation” (IRRI 2016). They are more tolerance to abiotic stress but have low yield potential and usually only farm to give a stable yield under adverse environmental conditions. Meanwhile, Aerobic rice is a type of high yielding rice with relatively lower tolerance towards environmental stress, targeted for farmers with access to modern machinery and chemicals.

Aerobic rice in Malaysia constitutes approximately 88% of the rice cultivated and concentrated mainly on 8 granary areas. Meanwhile, upland rice such as those planted in Sarawak represents less than 12% of the total production volume (DOA 2014). Located in the northeast segment on the Borneo Island, Sarawak is gifted with a rich biodiversity. Its unique geographical location and dense tropical forest facilitate the creation of isolated villages and indigenous rice cultivars maintained by the local inhabitants.

One local Sarawak Rice named Bario is originated from Bario Highland, a remote community located in Kelabit highlands of Sarawak at an altitude of around 1200 meters, with an average temperature of the highland ranges from 27ºC to 28ºC and a constant average rainfall ranging from 8 to 15 mm/month (Forecast 2016). Bario rice was widely known for its soft consistency, elongated grains, a pleasing aroma, delicate mouthfeel and its traditional farming methods. The cultivation of Bario rice is conducted without the use of any chemicals or modern machinery and is heavily protected by the government. The Department of Agriculture, Sarawak has established "Bario Rice Certification Scheme (BRCS)" n 2007 as the official certification system for Bario rice to safeguard the quality and quantity of Bario rice produced (Nordin et al. 2007).

The lack of constant water supply has subjected this variety to drought stress from time to time, which might result in descendants with stronger abiotic stress tolerance capability after many generations of farming. Besides Bario, many traditional lowland rice varieties such as Bajong and Biris are also cultivated using traditional methods and thus could possess similar stress tolerances properties as Bario.

1.4 Research Aims and Objective

This project was designed with two aims: the first aim of this work was to examine and test different varieties of local rice towards their salt tolerance capability. To achieve this aim, experiments were planned to accomplish the following objectives:

i. Examine the physiological and biochemical differences in control and salt-stressed samples of each variety
ii. Establish the relationship between physiological and biochemical performances of rice varieties towards salt tolerances capability
iii. Selection of a suitable variety for transcriptome sequencing

The second aim of this work was set out to understand the underlying salt tolerance mechanism at a molecular level using molecular technique:

i. Study of expression level on several salt-inducible pathways such as those transcoding SOS, CIPK, LEA and putrescence after stress treatment
ii. Examine the transcriptomic changes in rice during salinity stress with the use of RNA-Seq technology
iii. Examine the difference between transcriptomic of a selected Sarawak rice variety upon salinity salt stress exposure and assess any correlations between expression variance and salt tolerance

1.5 Contributions to Society

The outcomes of this research are expected to:

i. Promote research on Sarawak rice varieties with higher salt tolerance capability
ii. Enhance international knowledge base on salt tolerance properties on Sarawak rice varieties to a molecular level
iii. Establish supporting data for protection of biodiversity of Sarawak rice varieties due to the presences of beneficial trait
iv. Establish supporting data for further investigation in wild rice varieties

It is hoped that this project will attract further investigation and researches on local Sarawak rice varieties. Additionally, transcriptomic studies might unveil any novel pathways and interactions that have not been discovered in other studies. This might establish a different model of studying salinity stress mechanisms that could be incorporated for the marker-assisted selection (MAS) technique of rice breeding programmes.

Chapter 2 Literature Review

2.1 Growth and Development of Rice

The physiological and biochemical traits in young rice seedlings are commonly used as an indication of plant health and for distinguishing rice seedlings from unwanted weeds. In this chapter, the description of rice physiology is based on work published by Moldenhauer and Slaton (2001). In summary, rice seedlings have round hollow and jointed culms. The leaf blades are narrow, flat and connected to the leaf sheaths via leaf collars. They also have terminal panicles and well-defined, sickle-shaped auricles. The growth and development of rice are divided into 3 agronomic stages as described below.

2.1.1 Vegetative Stage

The vegetative stage describes the period where a gradual increase in plant height and a number of leaves occurs in a steady manner. This period lasts from seed germination to maximum tittering phase.

Seed germination begins when water penetrates the seed coats, making them soft and elastic. Once the rice grain has absorbed enough water, the coleorhiza elongates and emerges, which is closely followed by the development of coleoptile and primary leaf. The optimum temperature for seed germination is around 30ºC, but it can be delayed or physically challenged if the growing environment is not optimum. The radicle then elongates to form the seminal roots while the mesocotyl develops (Figure 1). Next, the seedling emergence phase is defined loosely by the period from the first appearance of mesocotyl through the soil surface right until the emergence of the first leaf (Moldenhauer & Slaton 2001).The pre-tillering phase is marked as the period from the appearance of the first leaf to the full development of the fourth leaf, which is approximately 15 to 25 days old and it is the period where active root growth occurs. Plants can be independent of external nutrient up to this phase (Yoshida 1981).

Tillering begins at the emergence of the fifth leaf and the appearance of the first tiller from the axillary bud on the second leaf (Figure 2). This process continues until the appearance of the sixth leaf and the development of the second tiller. The development of the tillers persists in a synchronous manner with the appearance of the $(n)^{th}$ leaf from the main culm and the tiller surfacing from the axillary bud at the $(n-3)^{th}$ leaf. The maximum tillering phase is defined by the active development of tillers. The

proliferation of tillers continues in a sigmoidal pattern until the maximum number is reached. The highest number of tillers in plants is approximately 2 to 5 if they were grown in a conventional plantation, but the number can reach 10 to 30 tillers in many modern plantations depending on the spaces in between the plants.

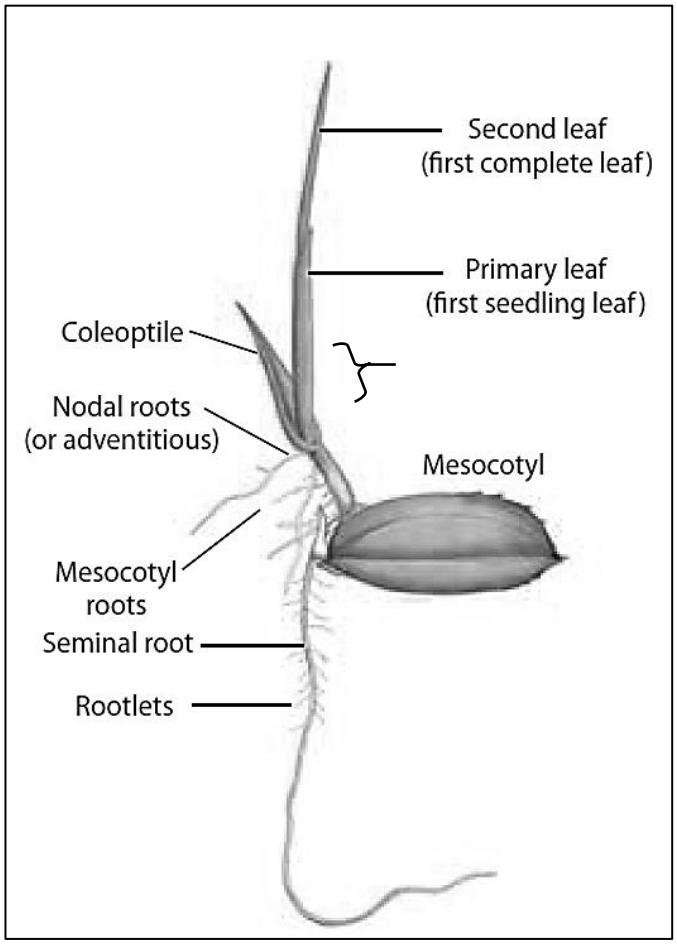


Figure 1 Graphical illustration of a young rice seedling, taken from Maclean and Dawe (2002).

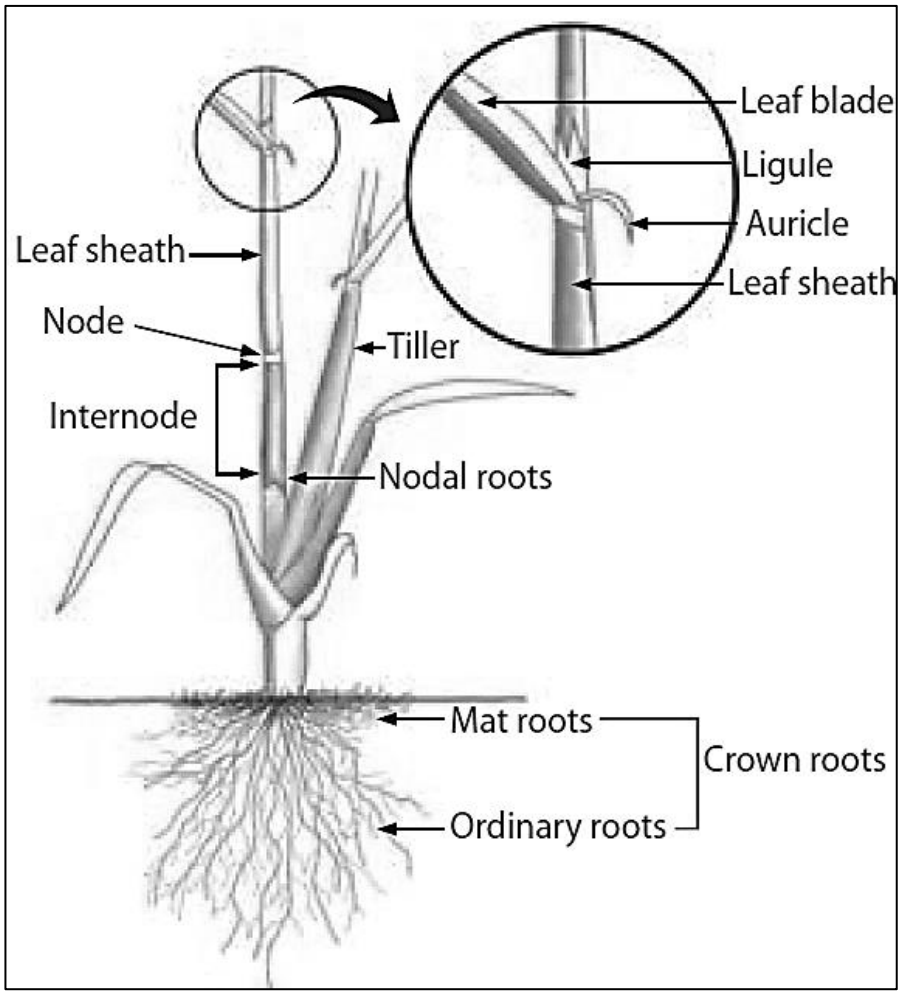


Figure 2 Graphical illustration of rice plant during the vegetative stage, taken from Maclean and Dawe (2002).

2.1.2 Reproductive Stage

The reproductive stage, or internode elongation stage, is approximately 30 days in rice but can vary depending on the weather conditions and can mark by the elongation of culm and the decline in tiller number. The primary processes of the reproductive stage are panicle initiation, internode elongation, panicle differentiation, booting, flag leaf emergences, heading, flowering and anther formation.

A panicle is a cluster of small, wind-pollinated organ at the top of the rice plant and is produced at the end of the vegetative stage. Panicle initiation (PI) usually marks the opening of the reproductive stages. Panicle formation takes place at the "panicle premordia" located at the uppermost node of the culm. The second phase in reproductive stage, also known as internode elongation phase, begins right after panicle initiation and continues until the plant reaches its full height. The top 5 internodes

actively elongate to prepare plants for wind pollination. Next, the panicle differentiation phase is marked by the visible branching of panicles when they are approximately 1 to 2 mm. This phase is often viewed as the most crucial period in the reproductive stage. The booting phase is characterised by the swelling of flag leaf sheath caused by the increased in panicle size as it ripens. This phase is highly sensitive to environmental stress (Moldenhauer & Slaton 2001). The heading phase starts when all panicles are fully visible, which may take over 10 to 14 days depend on the cultivar. In most cases, heading date is calculated as the time when 50% of the panicles are fully visible.

Anthesis is the final phase in the reproductive stage. This phase lasts from the opening of the spikelet to the success fertilisation of ovaries. This usually lasts from 1 to 3 hours. The 6 steps involved in this phase are:

1) Opening of lemma and palea
2) Elongation of filaments
3) Exertion of anthers
4) Further opening of lemma and palea
5) Secondary elongation of filaments
6) Closing of spikelet, leaving anthers exposed for fertilisation (Figure 3)

2.1.3 Ripening Stage

Ripening stage describes the maturation of the grain after fertilisation. In this stage, rice grain increase in mass and volume as the nutrients are translocated from the stem and leaves. The grain transient changes from a green shed to a golden brown colour as it matures. The 4 phases in this stage are the milk phase, soft dough phase, hard dough phase and the maturity phase.

The optimum moisture content in the mature grain is between 12% to 16% (Barber & Benedito de Barber 1978). Any undesirable environmental condition could change moisture content of the grain. Higher moisture content in grain could lead to fungal infection or deterioration of grain while lower moisture content could result in brittle grains that crack or fissure during post processing. The optimum amount of moisture content is dependent on the rice varieties. For milling purposes, the moisture content is kept between 12% to 14% (Gummert & Borlagdan n.d.). The moisture content of the

grain can be affected by insufficient light density, nutrient supply or salinity stress condition.

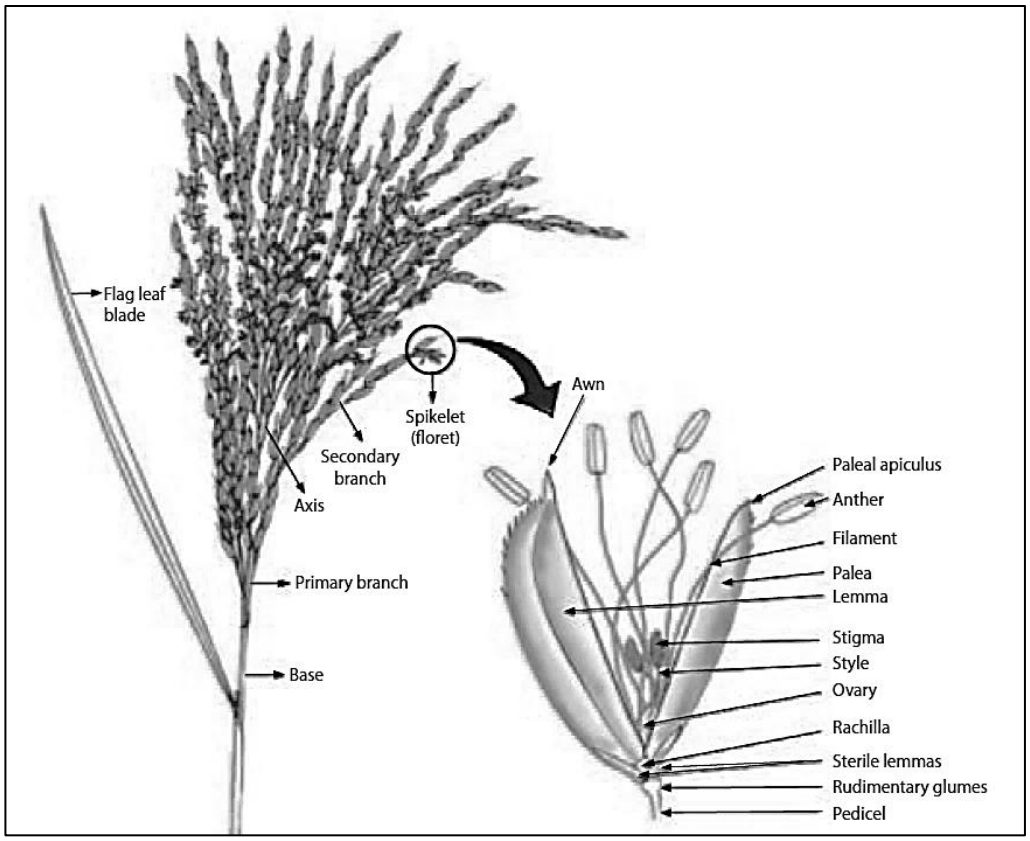


Figure 3 Graphical illustration of rice plant and flower during the reproductive stage, taken from Maclean and Dawe (2002).

2.1.4 Structure and Properties of Rice Grain

Rice grain consists of a husk enclosing an edible rice grain. The husk is not edible and commonly removed before the milling process, but is retain if the grain is needed to grow a new rice crop. A graphical illustration of the detailed structure in the rice grain is shown in Figure 4. The weight of the rice grain is dependent on the variety and the farming condition but is usually around 10 to 45mg at 0% moisture content, with the husk weighing around 20% of the total weight. The husk also served as protective effects against insects infestation or bacterial infection, with the air moisture below 14% relative humidity, rice seed can be stored and remain viable for a few weeks at room temperature (CGIAR n.d.).

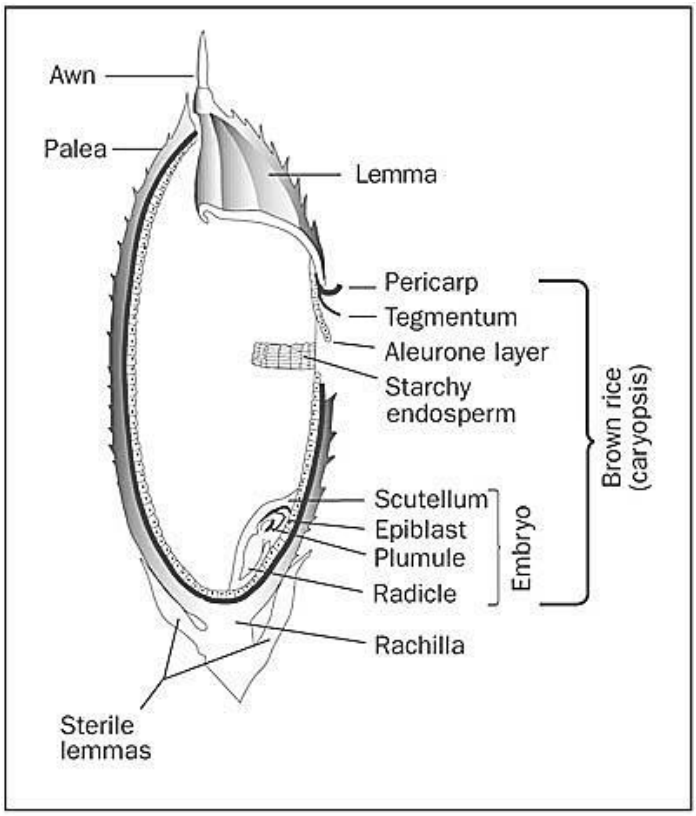


Figure 4 Graphical illustration of a mature rice grain, taken from CGIAR (n.d.)

2.2 Salinity Stress Restricts Normal Growth and Development

The chemical pesticides and fertilisers applied in rice farm usually contain a high amount of salt that will seep into the soil after applications (Atafar et al. 2010). Also, there is a rising environmental concern over the drainage of irrigation water as it is contaminated with pesticide and chemical fertilisers. This awareness has required farmlands to hold water for a longer period, which has allowed the excess salt to seep into the soil. Rice is especially susceptible to salinity at young seedling and reproductive stages (Yeo & Flowers 1984). Many researchers focusing on the genetic inheritances of salt tolerance traits have been done with promising results, but the progress of creating a salt tolerant cultivar is still an on-going struggle (Gao & Lin 2013; Lin et al. 2004).

Salinity affects seedlings growth and development by restricting water uptake and exerting salt-specific damage to the plants (Munns, James & Lauchli 2006). Many commercial crops have developed salt tolerance mechanisms when exposed to salinity stress. Some are capable of responding to a low amount of salinity stress while others require a much higher level of induction for significant responses to occur. Details in

salt tolerance mechanism are complicated and yet to be fully understood. Halophyte displayed extensive biochemical to physiological adaptation toward salinity stress (Flowers & Dalmond 1993; Tester & Davenport 2003). For example, some plants incorporate the capability to excrete excess salt via glandular system (Thomson, Faraday & Oross 1988) while some accumulate high concentration of osmolyte (Meloni et al. 2004). For less salt tolerance crops such as rice, intra-species variation in salt tolerance is usually estimated based on the differences in physiological traits (Foolad & Lin 1997; Yeo et al. 1990). This intra-species variation has been suggested as an excellent source for the discovery of novel salt tolerance mechanism (Flowers & Yeo 1995).

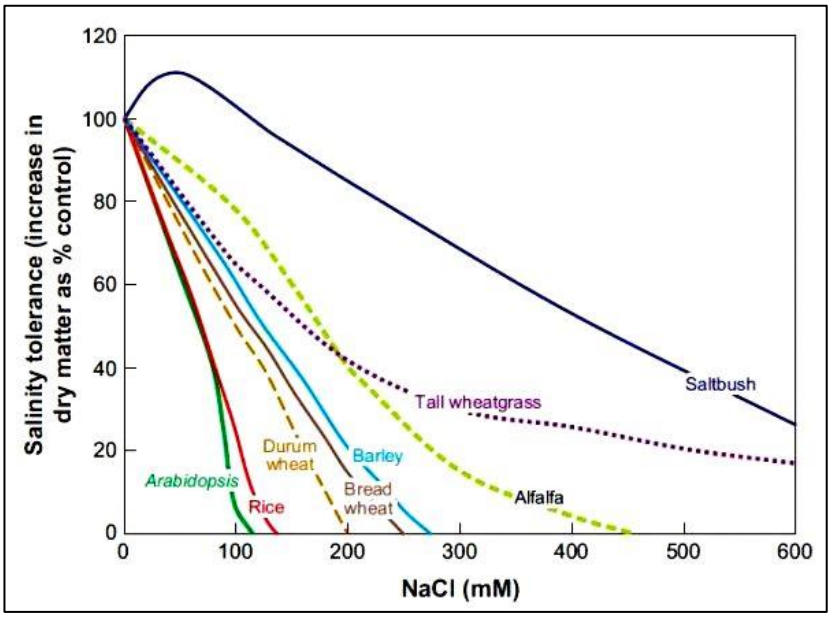


Figure 5 Response of various salt tolerance and salt sensitive plants to varying concentrations of NaCl after 3 weeks of treatment, taken from Munns and Tester (2008).

2.3 Effects of Salinity on Plant Growth and Physiology

Sodium Chloride, also known as common salt, separates into Na^+ and Cl^- ions when dissolved in water. These ions travel into plants and decrease the uptake of other essential ions such as K^+, Ca^{2+} and NO_3^- (Ashraf & Foolad 2007). Salinity stress inhibits the growth of roots (Pujari & Chanda 2002) and weakens the plant's water uptake capability (Tavakkoli, Rengasamy & McDonald 2010). Furthermore, accumulation of high Na^+ and Cl^- ions in leaf negatively affects its capacity in photosynthesis, ion homoeostasis (Karimi et al. 2005), stomatal regulation (Redondo-Gómez et al. 2007),

protein catabolism (Parida & Das 2005) and nitrogen uptake (Evelin, Giri & Kapoor 2012).

High level of Na^+ concentration can affect the ionic ratios of Na^+/Ca^{2+}, Na^+/K^+ and Ca^{2+}/Mg^{2+} (Munns & Tester 2008). Salinity tolerance is complicated and manipulated by The K^+/Na^+ ratio is a key factor of salt tolerance in plants (Gierth & Mäser 2007; Tester & Davenport 2003). Most plant species experience changes in K^+/Na^+ ratio when exposed to salinity stress. This fluctuation disturbs the plant's normal metabolic activities as the antagonistically decreased uptake, translocation and accumulation of K^+, which in turn, affect the proper growth and development of plant seedlings. This phenomenon inhibits metabolic activities by interrupting the operation of osmotic adaptation mechanism. The reduction in total available K^+ in tissue may be due to the direct competition between K^+ and Na^+ at the plasma membrane, inhibition of transport system in the xylem tissues, or specific Na^+ or K^+ efflux from the roots. High Na^+ accumulation has been reported to result in membrane damage, electrolyte leakage and oxidative damage (Mandhania, Madan & Sawhney 2006). Meanwhile, a high concentration of Cl^- damages the chlorophyll production mechanism in the leaf tissue, causing leaves to turn yellow (Pires et al. 2015) and ultimately weakening the plant's photosynthesis mechanism (Slabu et al. 2009). The schematic preview of salinity stress progression in plants has been presented in Figure 6.

Furthermore, the rate and efficiency of photosynthesis are massively reduced upon salinity stress (Chaves, Flexas & Pinheiro 2009). The drop in leaf metabolism decreases the concentration of carbon dioxide in the mesophyll and reduces the efficiency of the carbon-reduction process in the photosynthesis pathway (Flexas et al. 2006). Many photosynthetic enzymes such as rubisco, sucrose phosphate synthase and nitrate reductase can be permanently disabled after experiencing prolong salinity stress (Meyer & Genty 1998).

Symptoms of salinity stresses can first be observed in the change in leaf physiology. Plants close more stomata to reduce transpiration rate upon first exposure to salt. This step allows plants to maintain cell turgor pressure during salinity stress, but it only protects the younger leaves as older leaves tend to accumulate a higher amount of salt (Galmés, Medrano & Flexas 2007). Salinity stress imposed during different development stages in rice at various locations on plants can produce different

responses depending on the intensity, duration and progression rate of salinity stress (Munns 2002a). Additionally, salinity stress reduces the water availability throughout the plant. Since water is used as the final high-energy electron acceptor in photosynthesis (Bolton 1996), the loss of water can also result in the accumulation of high-energy reactive oxidative species molecule in the leaf tissue

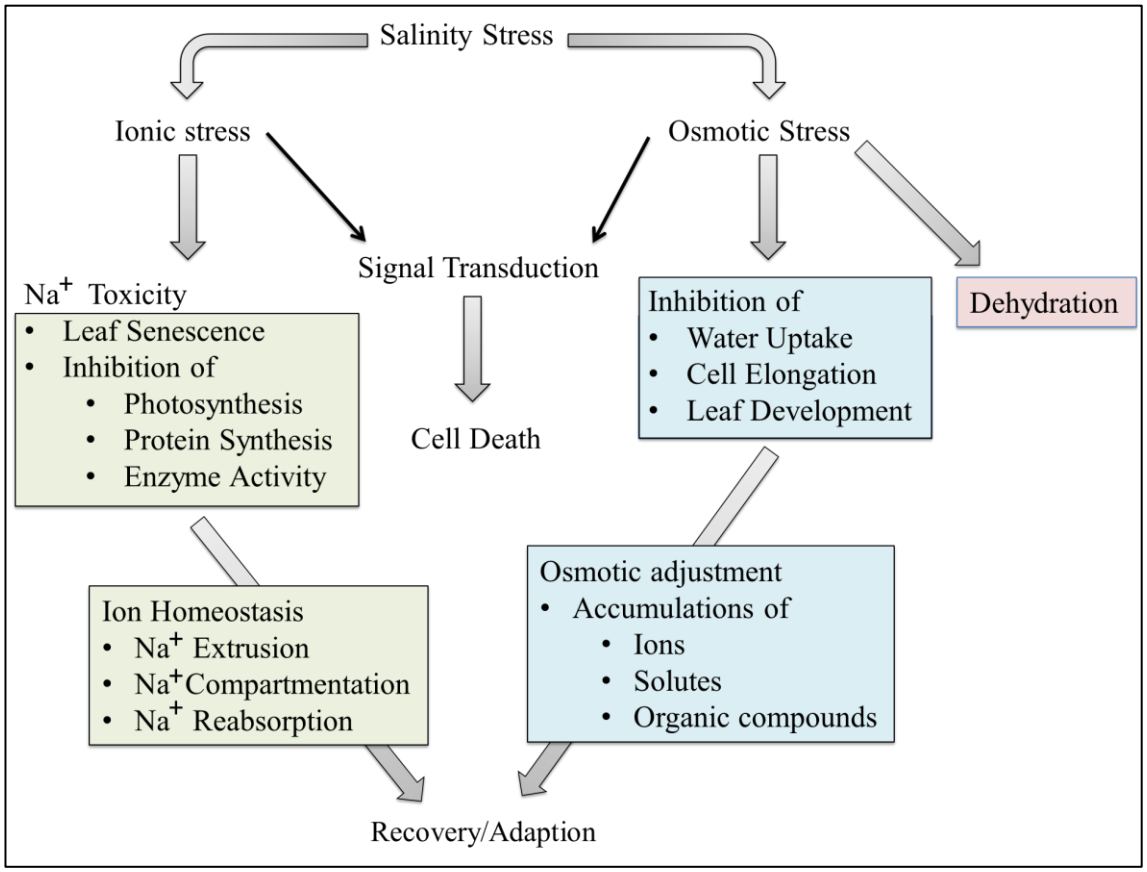


Figure 6 Schematic summary of the stresses that plants suffer from high salinity condition and the subsequent stress responses, adapted from Horie, Kalahari and Katsuhara (2012) with modifications.

2.4 Accumulation of Reactive Oxygen Species (ROS)

Reactive oxygen species such as superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) were widely known as by-products in aerobic respiration and abiotic stress management. Intense light combined with salinity stress resulted in the accumulation of ROS via Mahler Ion Reaction (Møller, Jensen & Hansson 2007). Previous studies have revealed many ROS- related enzymes that are involved in the growth, development, stomatal responses and abiotic and biotic stress responses of the plants. For examples: ascorbate peroxidase (APX), catalase (CAT), mitochondrial alternative oxidase (AOX), thylakoid

APX (tAPX), 2-cysteine peroxiredoxin, Cu/Zn-superoxide dismutase 2 (CSD2) and various NADPH oxidases are all involved in the ROS-mediated pathway (Baier et al. 2000; Miller et al. 2007; Pnueli et al. 2003; Rizhsky, Liang & Mittler 2003; Torres & Dangl 2005; Umbach, Fiorani & Siedow 2005; Vanderauwera et al. 2005).

Since ROS are harmful to the cell's metabolic system, plants have developed complex scavenging networks to counter these hyperosmotic molecules. These elaborate systems have allowed plants to utilised ROS as signal transduction mediators (Bailey-Serres & Mittler 2006). Recent studies have shown that ROS plays an essential role in plants encountering environmental stress, pathogen infection, programmed apoptosis and several developmental stimuli (Mittler et al. 2004; Torres & Dangl 2005). A sudden spike of ROS right after exposure to salinity stress (also known as the "oxidative burst") was found to be a key signal transduction event (Mittler et al. 2004; Torres & Dangl 2005), which leads to the activation of a diverse amount of metabolic responses (Table 1).

2.5 Molecular Adaptation to Salinity Stress

Salinity tolerance is complicated and manipulated by numerous salinity-responsive genes (Parihar et al. 2014). Many physiological processes such as osmotic adjustment, ion homoeostasis, toxic compound scavenging and water regime regulation have been found to contribute to the overall tolerances towards the saline environment. Some long distances responses such as secretion and translocation of hormones, mediators, transcription factors and regulatory molecules have also been studied and characterised. Stress-inducible genes have been classified into main two categories, genes that provide protection directly against external stress and genes that regulated internal metabolic activities to handle hyperosmotic stress exert by the saline environment (Kumar et al. 2013).

Table 1 Major categories of genes and proteins related to salt-stress responses or tolerances in plants, adapted from Sahi et al. (2006).

Type of Responses	Potential roles in salinity tolerance mechanism	References
Signalling molecules	• Gene expression on stress response • Signal transduction in stress response	(Cardinale, Palmer & Collins 2002; Pardo et al. 1998; Saijo et al. 2000; Ulm et al. 2002)
Transcriptional and post-transcriptional mechanism	• Transcriptional manipulation of stress-related gene expression • Maintain transcripts stability, turnover and processing	(Cooper et al. 2003; Lee, Kim & Lee 2001; Park et al. 2001; Sanan-Mishra et al. 2005)
Translational mechanism	• Manipulation of stress-dependent protein translation, transportation and localization	(Wood & Oliver 1999; Wood, Oliver & Cove 2000)
Protein structure scaffolding	• Maintenance of protein structures • Prevention of protein denaturation	(Sun et al. 2001)
Protein Metabolism	• Regulation of protein turnover • Selective protein degradation in stress response	(Khedr et al. 2003; Moon, Parry & Estelle 2004)
Osmolytes production	• Osmotic adaptation • Preservation of cellular structures and macromolecules	(Nomura et al. 1998; Tarczynski, Jensen & Bohnert 1993)
Transport protein channel	• Ion homeostasis • Compartmentalization of solutes and amino acids	(Gisbert et al. 2000; Shi et al. 2000; Zhang & Blumwald 2001)
ROS scavenging and cell death	• Scavenging of ROS • Programmed cell death • Hypersensitive response	(Reddy & Sopory 1999; Roxas et al. 1997)
Photosynthesis	• Regulation of photosynthesis	(Kawasaki et al. 2001; Sahi et al. 2003)
Defence proteins	• Protection against viral, bacterial and fungal infestation	(Cheong et al. 2002; Dombrowski 2003; Reymond et al. 2000)
Hormone-related proteins	• Regulation of hormonal gene expression and metabolism	(Kalifa et al. 2004)
General metabolism	• Housekeeping metabolic pathways • Carbohydrate, fatty acid and protein synthesis • Modifications in membrane fluidity • Nitrogen metabolism	(Jeong, Park & Byun 2001)

2.6 Induction of Salinity Responsive Genes

Mechanism of salt tolerance in plants involves enormous numbers of genetic pathways and feedback loops. Therefore, it is a common practice to study the phenotype of the plant during salinity stress directly. Often, this phenotype needs to be associated with sophisticated analysis technology to comprehend the underlying genetic mechanism. One of the ways we can look into it is by understanding the immediate responses of the plants at its transcriptional level. It can be accomplished by quantifying the amount of mRNA synthesised in plants upon a brief exposure to salinity stress (Tester & Davenport 2003). A large number of salinity-responsive genes from a wide range of crop species have been isolated and characterised in the past decade. These genes originated from crops with very high (*Beta Vulgaris*) (Wakeel et al. 2011) to very low (*Citrus spp*.) salt tolerance (Navarro, Perez-Tornero & Morte 2014). A brief preview of the salt tolerance mechanisms in plants has been summarised in Figure 7 as below. Furthermore, a huge variation of salt tolerance within the same species of plant has also been observed (Kumar et al. 2015). Due to the complexity of such trait, many studies are still trying to understand the mechanism that distinguishes salt sensitive and salt tolerance plants.

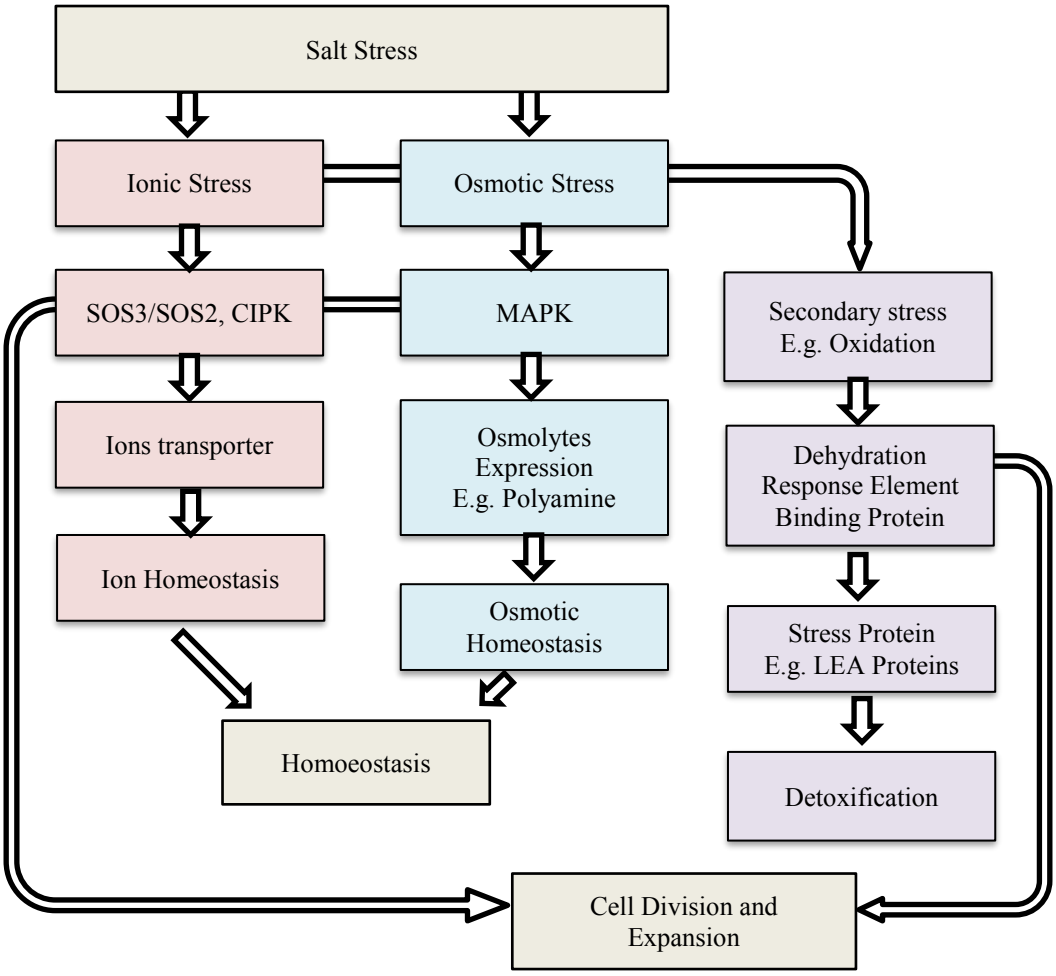


Figure 7 Relations between transcriptional regulatory networks of abiotic stress signals and gene expression, adapted from previous studies (Xiang, Huang & Xiong 2007; Zhu 2001).

2.7 Summary of Salinity Response Genes

2.7.1 Salt Overly Sensitive (SOS) Pathway

The regulation of K^+ and Na^+ is closely linked to the SOS pathway in plants. An excellent review of SOS pathway can be found in a paper published by Ji et al. (2013) and illustration of mechanism in SOS pathway is attached in Figure 8 as follows. In brief, the expression of SOS pathway leads to translation of a trans-membrane ion transporter that directly governs Na^+, K^+ and H^+ concentration. The 3 main components of this pathway are named SOS1, SOS2 and SOS3, which codes for 3 different proteins. They are triggered by the increased intracellular Ca^{2+} concentration during salinity

stress. This increase, also known as the calcium signal, is picked up by a myristoylated calcium binding protein encoded by SOS3 and passed down a serine/threonine kinase encoded by SOS2 that in turn elevates the expression of SOS1, which codes for a protein that actively pump Na^+ out of the cell.

SOS plays an important role in the sodium metabolism of plants under salinity stress. Rice SOS genes (OsSOS1, OsSOS2 and OsSOS3) have been identified and isolated from rice and have been shown to be able to be compatible in the *Arabidopsis* mutant system, indicating the conservation of SOS pathway between many plants (Martinez-Atienza et al. 2007). Differential transcript abundance of SOS pathways genes in wheat has been reported to affect the salinity tolerance of the plant (Sathee et al. 2015) and transgenic *Arabidopsis* expression SOS genes isolated from wheat also shown increased tolerance to salt. Inactivation of SOS1 genes in a halophyte, *thellungiella salsuginea*, has resulted in Na^+ accumulation in the root xylem parenchyma cells and leading to a loss of halophytism (Oh et al. 2009).

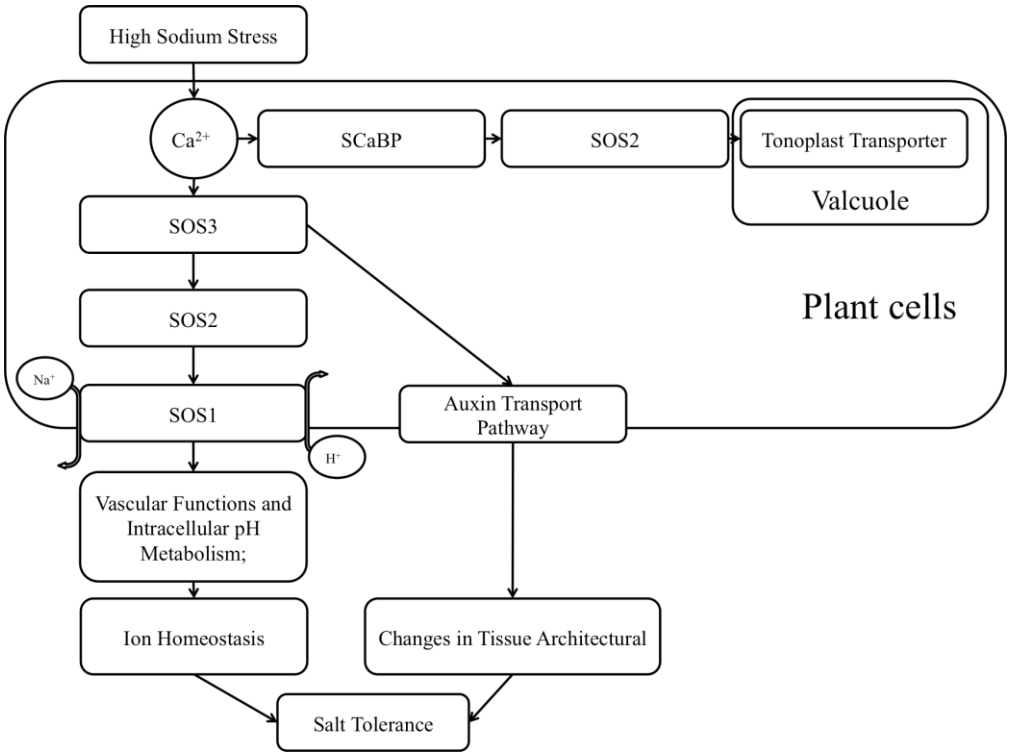


Figure 8 Graphical illustration of SOS pathways, adopted from Ji et al. (2013).

2.7.2 CBL-Interacting Protein Kinase (CIPK)

Bearing similarity to the SOS3 protein, CIPK proteins are a class of protein whose expression is dependent on Ca^{2+} and constitutes as essential relays of the Ca^{2+} signalling pathway in plants. This complex regulates many downstream pathways such as ion channels and transporters such as SOS1 during environmental stress condition (Manik et al. 2015). The detail mechanisms of CIPK in plant's response to abiotic stresses have been summarised in a paper published by Manik et al. (2015).

Unlike an animal, the lack of nervous system in plants has required them to possess a particular regime to response to external stimuli. Calcium is broadly known as a ubiquitous secondary messenger due to the wide range of function in plant's abiotic stress response and the temporary fluctuations of Ca^{2+} concentration in the cytosol, also known as a calcium signal, is often used in plants as a start trigger to various stress-responsive pathways. A brief summary of interactions between CIPK and high Na^{+} stress has been attached as Figure 9 as followed.

Meanwhile, CIPK1 has been known to play a major role in ABA-mediated signalling pathways against osmotic stress, drought and salt responses in *Arabidopsis* by interacting with both CBL1 and CBL9 (D'Angelo et al. 2006). Meanwhile, CIPK11, a SnRK3-type protein kinase, is responsible for the ABA-mediated responses through the phosphorylation of the ABA-INSENSITIVE 5 (AB15) protein and may be activated by many abiotic stress conditions or ABA-induced calcium signal in the cell.

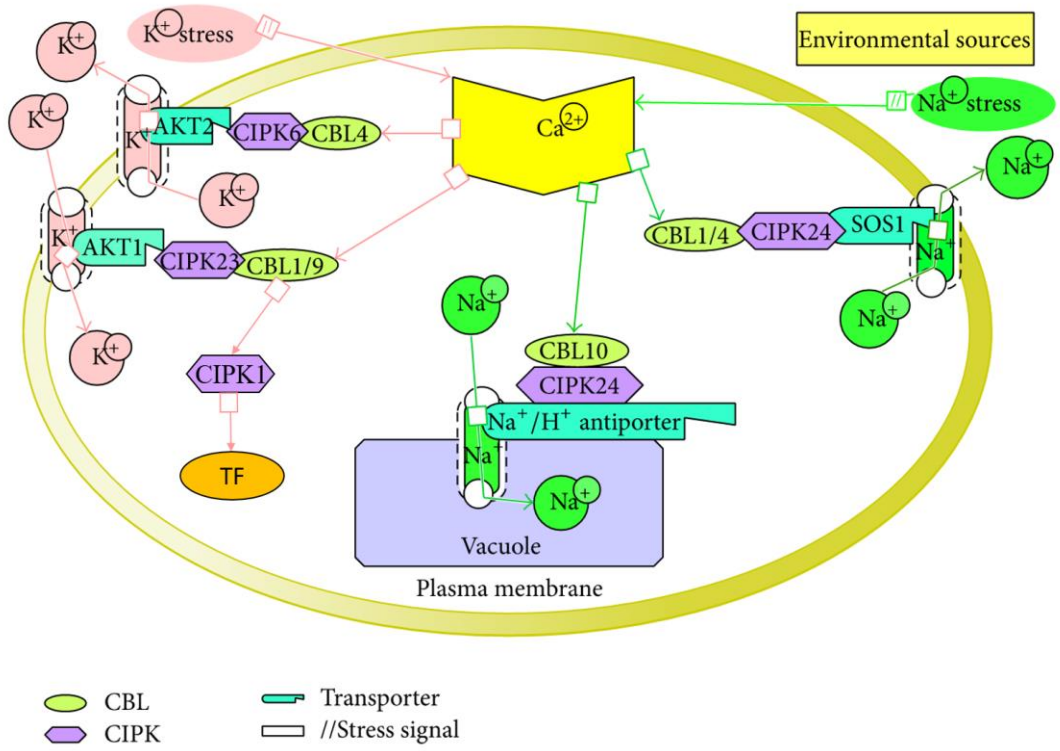


Figure 9 Graphical illustration of interaction between CBLs-CIPK and environmental Na^+ stresses to maintain the homoeostasis in cell, adopted from Manik et al. (2015) and Li et al. (2009b) with slight modification.

KT1: *Arabidopsis* K^+ transporter 1, AKT2: *Arabidopsis* K^+ transporter 2 and SOS1: salt overly sensitive 1, TF: Transcription factors.

2.7.3 Late Embryogenesis Abundant Proteins

Late Embryogenesis Abundant Proteins (LEA) proteins are a collection of many different proteins with a wide range of function. In this study, only one group of LEA protein will be targeted for gene expression studies. Group 1 LEA proteins are a group of highly hydrophilic protein made from a high proportion of charged amino acids. Due to its high polarity, this class of protein can create a micro-aqueous environment to protect essential cellular components from damage during water-deficit stress. It had been found that the expression of LEA1 can be triggered by salinity and water deficit stress (Almoguera & Jordano 1992; Bostock & Quatrano 1992). Previous studies overexpressing LEA1 (isolated from *Brassica napus*) in *Arabidopsis* had shown increased root length and surface area under high salinity stress, which indicates the role of LEA1 in salinity tolerance of the plants.

2.7.4 Synthesis and Accumulation of Polyamine

The role of polyamines such as spermidine, spermine and their biosynthesis precursor putrescine as endogenous growth regulators or intracellular messenger sunder abiotic stress have been well established (Liu et al. 2006). In higher plants such as rice, wheat, or barley, the biosynthesis of putrescine is mainly conducted through the action of ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) (Gemperlova et al. 2006). The biosynthesis of polyamine is briefly summarised in as Figure 10 below.

In previous studies, the biosynthesis of polyamine molecule in rice has been found to increase in response to environmental stress such as chilling (Imai et al. 2004) and salinity stress (Liu et al. 2006; Roy et al. 2005). Transgenic *Arabidopsis* overexpression spermidine synthase has shown increase tolerance to salinity stress and increase intracellular polyamine content after the stress exposure (Kasukabe et al. 2004). Several reports have also demonstrated that transgenic rice overexpressing ADC and ODC has enhanced salinity tolerance (Roy & Wu 2002). Thus, it is speculated that rice variety that displays elevated expression in ADC and ODC genes will have an enhanced salinity tolerance capability.

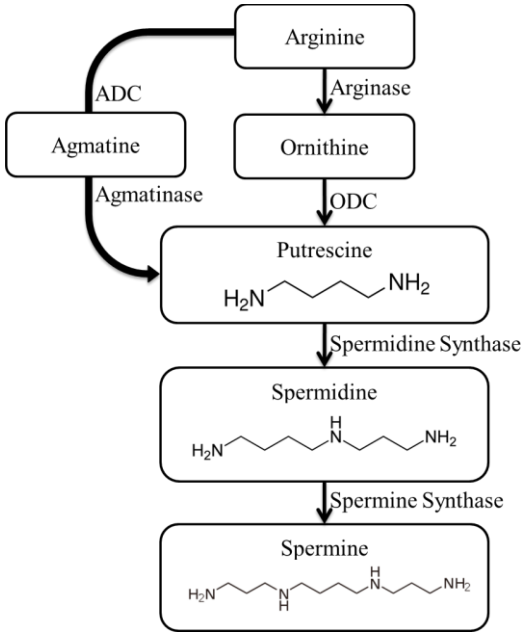


Figure 10 Graphical illustration of putrescine, spermidine and spermine biosynthesis, adopted from Polyamines (n.d.).

2.8 Next Generation Sequencing

In the past decade, the advancement in array based and sequencing-based technologies have identified a significant amount of stress-inducible transcripts from rice (Oono et al. 2016; Venu et al. 2013). Genome-wide identification of saline responsive genes has significant benefits towards the understanding of salinity and drought tolerance in plants. For example, quantitative gene expression data on a wide range of genes can be obtained and annotated. In addition, promoters and cis-elements acting on such gene can be isolated and transformed into plants for basic study and used as a starting point for the creation of salt tolerance cultivar (Garg et al. 2002; Haake et al. 2002; Kasuga et al. 1999; Xu et al. 1996).

The small genome size and salt sensitivity in rice relative to other cereal crops such as wheat or barley have provided a perfect platform for the study of plant stress response. Previous research has identified many transcripts that were upregulated in plants' transportation and defences system, cell tissue recovery and metabolism process via examination of ESTs produced from salinity induced rice plants (Bohnert et al. 2001). Shiozaki, Yamada and Yoshiba (2005) have also isolated 284 different stress related Express Sequence Tags (ESTs) sand roughly 50% of them are in involved in stress response, detoxification and restoration of the plant's tissue. In a separate experiment involving *Arabidopsis*, 53, 194 and 277 genes from 7000 cDNA microarray have been found to be related to cold, salinity and drought stress condition respectively (Seki et al. 2002). Microarray work regarding different abiotic stress on rice plants has also been established all around the world (Ding, Chen & Zhu 2011).

Even though microarray technology has been set up as a model standard for transcriptomic studies, this method is still limited to genes that had been previously identified. Thus, massive multiple parallel sequencing on RNA molecules has emerged as a useful tool for analysing genome-wide transcriptomic expression. Next generation mRNA sequencing provides much higher resolution and sensitivity. Rare transcripts or single nucleotide polymorphism on genes can be revealed to single base resolution (Wang et al. 2009). Furthermore, gene expression levels over a broad dynamic range can be accurately detected, quantified, normalised and compared across different experiments (He et al. 2010; Mizuno et al. 2010). Therefore, RNA-Seq was chosen in

this study to generate a comprehensive overview of rice transcriptomic expression under salinity stress.

2.8.2 Transcriptomic Adaptation of Salinity Responses in Rice

Due to the small genome size, the number of quantitative trait locus (QTLs) in rice is relatively low compared to other crops such as wheat or barley (Leung et al. 2008) and thus making the investigation of saline tolerance in rice fairly straight forward. The understanding of such a system could directly help researchers to identify salt tolerance genotypes using DNA markers. However, the QTLs that controls salt tolerances have low heritability and traits are not easy to study as it demands careful control of environmental parameters (Cuartero et al. 2006) such as cultivation temperature, light intensity and soil pH. Currently, the establishment of salt tolerance rice cultivar is still limited to conventional breeding. A number of salt tolerance rice varieties has been established using such method in India, Bangladesh and Philippines (Ismail et al. 2007) but the progress has not been sufficient for the challenges faced (Flowers 2004; Yamaguchi et al. 2004). Transgenic approaches have shown promising results in the creation of golden rice, but at the same time have raised controversial issues on the use of genetically modified organism.

Transcriptomics studies of rice upon salinity exposure have given many novels insights on the mechanism of salinity tolerance. Garg et al. (2013) have reported the transcriptomic profile of a wild halophyte Rice, *Porteresia coarctata*, during abiotic stress. They have discovered that rigorous transcriptional reprogramming under salinity is responsible for tolerance to these stresses in Porteresia. For example, the elevated expression of transcription factors, suberin and many secondary metabolites such as serotonin amides, hydroxycinnamic acid and phenylpropanoids molecules have been observed in their experiments. Meanwhile, Shankar, Bhattacharjee and Jain (2016) has reported the transcriptomic profile of different rice cultivars under salinity stress and has revealed many significant alternative splicing events during salinity stress.

The mechanisms responsible for salt tolerances are complex, diverse and polygenic (Golldack et al. 2014) and the introduction of one single gene into the system is unlikely to result in the total establishment of a new salt tolerance cultivar. Instead, numerous genes involved in the process of signalling, osmotic adjustment, ion homoeostasis, free radical scavenging, vacuolar compartmentalization of ions, restoration of enzymatic

activity and photorespiration will be necessary to complete the tolerance system (Bohnert et al. 2001). Thus, transcriptomics profile, coupled with extensive bioinformatics analysis, of the local rice varieties could provide vital insight towards the creation of salt tolerance rice cultivar.

2.8.3 Introduction to Bioinformatics Analysis

Bioinformatics is defined as "The science of collecting and analysing complex biological data such as genetic codes", according to the Oxford English Dictionary (Dictionary 2016). It is one of the growing fields of scientific research that applies informatics techniques that is derived from disciplines such as computer science, applied maths and statistics, into biological science to understand and categorise information association with DNA, RNA or proteins sequences.

Many bioinformatics studies are conducted in one or two tactics, by comparing and grouping data in according to any significant biological connections (such as genes to genes interactions), or by organising the information associated with the biological molecules on a large scale (such as transcriptomic changes). Thus, bioinformatics not only provides a different perspective into biological experimentation but also able to quantify many traits that are previously immeasurable.

One of the most common problem in the bioinformatics analysis is that the amount of data generated by next-generation sequencing. At the time of this writing, the data are still relatively large for any common computer to handle and have to be analysed using specific servers or supercomputers. Many of the software are developed on Linux platform and only accessible through a command line interface, which might posed a challenge for personal without a computer science background.

However, many of them are open-source and can be downloaded and utilised by individuals with a small sample size. A typical RNA-Seq workflow is summarised as in Figure 11. The raw reads (usually in FASTA format) obtained from the sequencing platform are first checked through a quality control program such as FastQ Screen (Bioinformatics 2013), FASTX-Toolkit (Gordon & Hannon 2010), NGS QC Toolkit (Patel & Jain 2012), PRINSEQ (Schmieder & Edwards 2011), QC-Chain (Zhou et al. 2013), or QC3 (Guo et al. 2014). The FASTA format is a standard format for text-based representation of the sequence; it consists of the sequence name, single letter coded

nucleotides of amino acids sequence and Phred-Scale base quality scores for each of the data sequence. The Phred scale is usually interpreted as a QV value from 0 to 255. The QV value is a probability score in a negative log configuration, a QV of 10 can be understood as a likelihood of 1 in 10 likelihood of inaccurate base calling while a QV of 20 means 1 in 100 likelihood of inaccurate base calling.

Next, the sequences are trimmed for defects. Low-quality base removed accordingly based on the Phred score as mention above; the usual cutoff point is around a QV value of 10. In addition, the adaptor sequences and unknown bases were removed from the sequencing reads to obtain the "clean reads" required for the next step. The trimming can be done using Cutadapt (Martin 2011) or Trimmomatic (Bolger, Lohse & Usadel 2014) software. The reads are often fed back to the quality control software to validate the quality of the output and can be processed again if needed.

Next, the sequence can be joined using De Novo or mapping assembly. In mapping assembly, the individual reads were mapped onto a reference genome using Burrows–Wheeler Transformation (BWT) compression techniques to search the best alignment match within an acceptable computational time. Many different software had been developed in the past decade, but two of the most commonly used software for mapping assembly are Bowtie2 (Langmead & Salzberg 2012) and BWA (Li & Durbin 2009). In De Novo assembly, individual reads were joined based on the overlapping sequence to form a longer contig. It is often used in an absence of a reference genome, such as in a non-model organism, cancer samples, or in microbiome studies. The commonly used software for this purpose is named Trinity, which consists of 3 individual units named Inchworm, Chrysalis and Butterfly. The detail explanation of the mathematical model behind Trinity can be found in paper by Haas et al. (2013).

In brief, Trinity first extracts all the possible overlapping K-mers from the reads and pass them to the first programme, Inchworm. This programme examines all the unique (k-1) per overlaps and generates a greedy extension. Next, Chrysalis clusters the Inchworm contigs into individual components by generating a de Bruijn graph for each cluster. Finally, Butterfly process each individual graphs in parallel and generate the full-length transcripts based on the individual de Bruijn graph produced by Chrysalis. Many other programmes are also available for de novo assembly. For example, Trans-

ABySS (Robertson et al. 2010), Velvet-Oases (Schulz et al. 2012) and SOAPdenovo-trans (Xie et al. 2014).

Next, the transcripts were compared with several separate online databases for the closest match. The most commonly used tool is BLAST (Basic Local Alignment Search Tool), an algorithm for analysing DNA, RNA or amino acid sequence against an online or local database to identify the sequence that most resemble the query sequence above a certain threshold. Many databases are available for the annotation of the sequences; a table of the database together with a brief description is summarised in Table 2. Some data bank (Nt, Nr) are a collection of sequence submitted by users while some databases (GO, COG, KEGG) provide additional information such as coding region prediction, functional prediction, pathway mapping and co-expression analysis, on top of the sequence annotation. For contigs that cannot be mapped any databases, a separate programme such as ESTSCAN (Iseli, Jongeneel & Bucher 1999) can be used to predict the coding regions of the contigs, so that the expression of that gene can be calculated and compared.

Table 2 Databases used for unigenes annotation.

Name	Website	Description
RAD-DP	http://ricedb.plantenergy.uwa.edu.au/	The Rice Annotation Project (RAP) was form in 2004 upon the completion of the rice genome sequencing with the aim of providing the scientific community with an accurate and timely annotation of the rice genome sequence.
Nt	ftp://ftp.ncbi.nlm.nih.gov/blast/db	Nucleotide sequence database, with entries from all traditional divisions of GenBank, EMBL and DDBJ excluding bulk divisions
Nr	ftp://ftp.ncbi.nlm.nih.gov/blast/db	Non-redundant protein sequence database with entries from GenPept, Swissprot, PIR, PDF, PDB and NCBI RefSeq
GO	http://geneontology.org	The Gene Ontology (GO) project is a major bioinformatics initiative to develop a computational representation of our evolving knowledge of how genes encode biological functions at the molecular, cellular and tissue system levels.
COG	http://www.ncbi.nlm.nih.gov/COG	Cluster of Orthologous Groups of proteins, phylogenetic classification of proteins encoded in complete genomes.
KEGG	http://www.genome.jp/KEGG	KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases dealing with genomes, biological pathways, diseases, drugs and chemical substances.
SwissProt	http://ftp.ebi.ac.uk/pub/databases/swissprot	UniProtKB/Swiss-Prot is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB). It is a high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and scientific conclusions.
Inter Pro	http://www.ebi.ac.uk/interpro	InterPro is a resource that provides a functional analysis of protein sequences by classifying them into families and predicting the presence of domains and relevant sites.
Rfram	http://Rfam.xfam.org/	The Rfam database is a collection of RNA families, each represented by multiple sequence alignments, consensus secondary structures and covariance models (CMs)

The transcript abundance is a major step for many downstream investigations and many separate methods have been developed for the measuring of transcript abundance levels. One of the most popular ways of normalising RNA-Seq data are through the calculation of **R**eads **P**er **K**ilobase of target transcript length per **M**illion reads mapped (**RPKM**) (Mortazavi et al. 2008) for single-end sequences and **F**ragments **P**er **K**ilobase of target transcript length per **M**illion reads mapped (**FPKM**) (Trapnell et al. 2010) for paired-end RNA-Seq data. In brief, the number of fragments from the reads is aligned to a reference genome or the de novo genome from the previous step and RSEM (RNA-Seq by Expectation-Maximization) (Li & Dewey 2011) software is employed to assign reads to each transcript based on probabilities and the positional bias generated by RNA-Seq library. The reads of each unigenes can then be compared between two experiments based on statistical models of expected variation such as under the Poisson or negative binomial distribution. Negative binomial distribution is reported to better accounts for

the increased variation observed between biological replicates and is currently the preferred model for identifying DEGs in many leading software tools such as DSeq or edgeR (Anders & Huber 2010; Lai 2010). A volcano plot or an MA plot can be plotted using the equation below can then be generated using Rstudio (Team 2015) based on the transcript abundance results obtained to check the quality of data normalisation.

$$M = log_2\left(\frac{R}{G}\right);\ A = \frac{1}{2}log_2(RG)$$

Equation for M and A calculation for MA plot; R and G represent two separate colour channels in the experiment

The transcriptome generated is also a useful substrate for assessing any single nucleotide polymorphism mutation in the sample. Software such as SAMtools (Li et al. 2009a), GATK (McKenna et al. 2010) and Atlas2 (Evani et al. 2012) can be used for variant calling within the transcriptome and GATK has been recommended for the general-purpose variant analysis. GATK is an NGS data analysis suite that used "MapReduce framework to parallelise the sequence alignment and implements a simple Bayesian model" to predict the probability of genotype in the sample and have been reported to be more efficient in threads parallelization (McKenna et al. 2010).

Finally, the microsatellite within the transcriptome can be identified using MSA (Microsatellite identification tool) software (Dieringer & Schlötterer 2003), which permits the identification and localisation of perfect microsatellites and compound microsatellites that are separated by a certain number of bases.

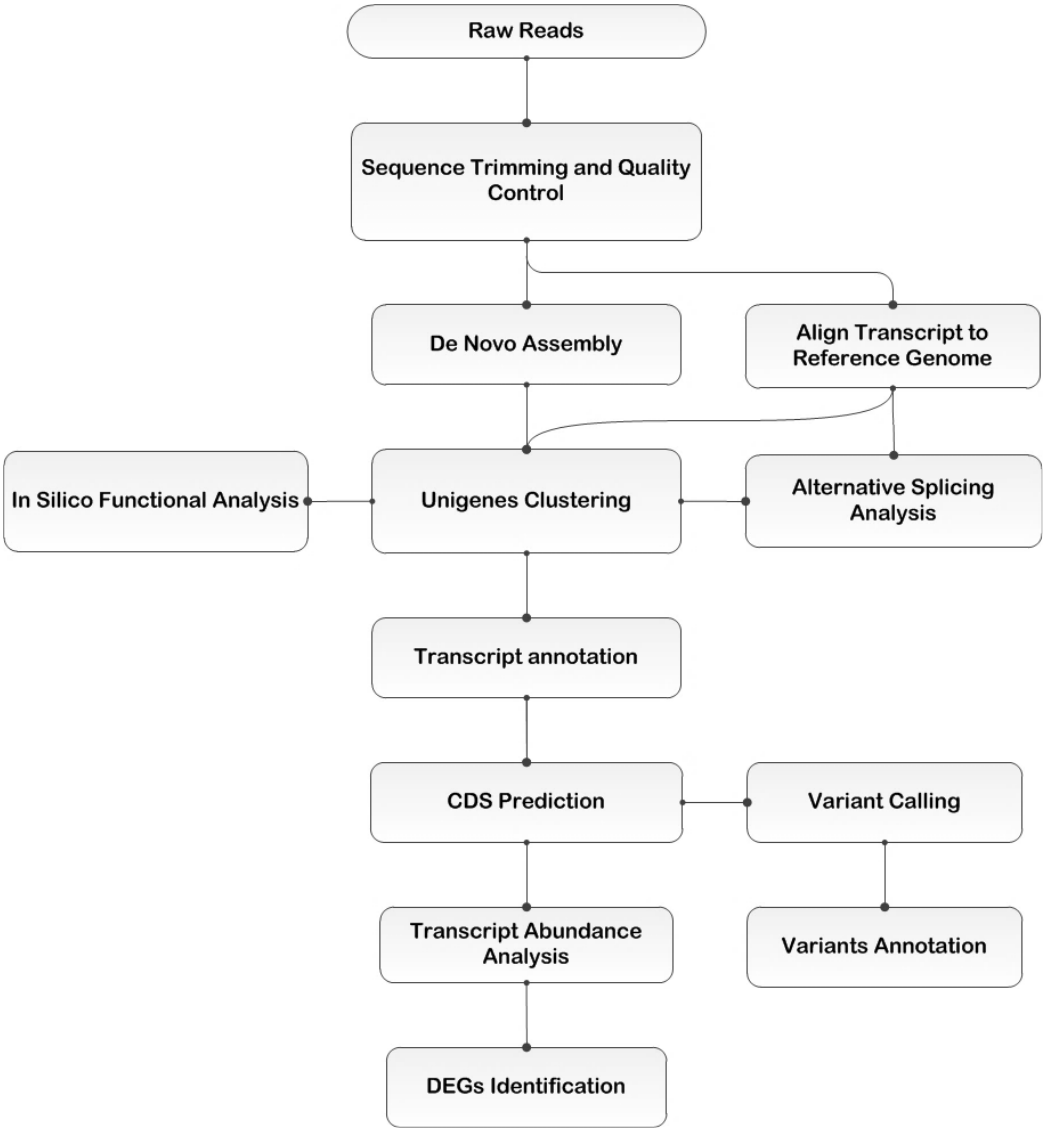


Figure 11 Schematic overview of a typical RNA-Seq analysis pipeline for DEGs identification.

Chapter 3 Physiological and Biochemical Studies of Salinity Stress Responses and Tolerance of Sarawak Rice Varieties

3.1 Executive Summary

Sarawak is blessed with many upland rice varieties that are rain-fed and do not rely on irrigation, which posed a great opportunity for salt tolerance rice to flourish. The quantification of salt tolerance is challenging, as salt tolerance is a complex mechanism controlled by a network of genes. The salt tolerance level is usually estimated by observing the differences between certain physiological and biochemical traits after salinity stress exposure. Salinity stress can impair growth in several manners: the escalation of osmotic pressure in the root system affects the plant's ability to take up more water, resulting in cell level dehydration. Since water molecules play a major role in photosynthesis (Bolton 1996), this cell level dehydration increases the oxidative stress in the leaf tissue. Furthermore, the increase in intracellular Na^+ level results in the shift of essential ions concentration ratio, which impairs many metabolic pathways that require a consistent environment and ultimately affecting many physiological appearances and biochemical content of plants.

In this chapter, the methodologies and results of the performance of the local Sarawak rice varieties after salt induction are presented together with discussions of the outcomes obtained.

3.2 Research Aims and Objectives

The aim of this chapter was to identify the differences in the physiological and biochemical systems of young rice seedlings after salinity stress treatments. With the purpose of completing the aim above, experimental works in this chapter were designed to accomplish the following objectives:

i. Establish an optimum growth condition for rice in controlled environment, avoiding any influences from biotic or abiotic stress
ii. Examine the physiological and biochemical differences in control and stressed samples
iii. Compare the differences in salt tolerances between Sarawak local rice varieties and commercial rice varieties

3.3 Materials and Chemicals

3.3.1 Rice Samples and Chemicals

Three Sarawak local rice varieties, named Bario, Bajong and Biris were used as test samples while one commercial variety known as MR219 was used as a control for salinity stress analyses. All samples in this experiment were provided by the Department of Agriculture Sarawak, Malaysia. Bario was collected from the local farmers located in Bario Highland while Bajong, Biris and MR219 were collected from farmers located in Sri Aman, Sarawak Malaysia. The sources and properties of the chemicals used in this study are summarised in Table 3 as follows.

Table 3 Chemicals used in this study.

Chemicals	Grade	Company	Country of Origin
Sodium Chloride (NaCl)	Analytical grade	Merck	Germany
Concentrated Nitric Acid (HNO_3)	Analytical grade	Fisher Scientific	Malaysia
1000ppm AAS Standards	NA	Fischer Scientific	Malaysia
Absolute Ethanol (EtOH)	Analytical grade	Fisher Scientific	Malaysia
Potassium Nitrate (KNO_3)	Analytical grade	Merck	Germany
Sodium Nitrite ($NaNO_2$)	Analytical grade	Bendosen	Malaysia
Aluminium Trichloride ($AlCl_3$)	Analytical grade	R&M	Malaysia
Potassium Carbonate (K_2CO_3)	Analytical grade	R&M	Malaysia
Sodium Hydroxide (NaOH)	Technical Grade	R&M	Malaysia
Gallic Acid (GA)	Analytical grade	NextGen	Malaysia
Folin-Ciocalteu's Phenol Reagent	Technical Grade	Merck	Germany
Quercetin	Analytical grade	Sigma-Aldrich	USA
Trolox	Analytical grade	EMB Chemicals	USA
2,2-Diphenyl-1-Picrylhydrazyl (DPPH)	Analytical grade	Sigma-Aldrich	USA

3.4 Methodology

3.4.1 Plant Growth Conditions

Seeds were sterilised using 70% (v/v) ethanol for 5 minutes, rinsed twice with distilled water and exposed to UV light for 10 minutes using a biosafety cabinet (1300 Series 2A, Thermo Scientific). Next, they were transferred to 200ml disposable plastic cups containing 80g of 1:1 vermiculite: perlite saturated with distilled water and left for 14 days to grow in a growth chamber (POL-EKO 750, Poland). All seedlings were maintained at 30°C throughout the day and 25°C at night with a constant relative humidity of 83%. Photoperiod was maintained at 12 hours light (300µmol m^{-2} s^{-1}) and

12 hours dark. Each cup was filled twice daily with distilled water to ensure the water level was full throughout the growth period.

3.4.2 Salinity Treatments

Fourteen days seedlings were challenged with 0mM (control), 100mM, 150mM or 200mM sodium chloride (NaCl) by replacing all water in the growth media with salt solutions. The seedlings were maintained in respective salt solution for 5 days before they were harvested for physiological and biochemical studies.

3.4.3 Plant Physiological Analysis

3.4.3.1 Shoot Length

Seedling lengths were measured using a standard ruler after 19 days after sowing (14 days cultivation and 5 days stress period salt). The fresh weights of each seedling shoot were measured directly and dried weights were measured after the samples were left for 24 hours in an 80°C oven (TFAC-136, TUFF).

3.4.3.2 Number of Leaves and Stem Diameters

The total numbers of leaves on the rice seedlings were measured after 19 days of incubation (14 days cultivation and 5 days stress period salt). The stem diameters were measured using a standardised Vernier calliper (Mitutoyo, Japan) on the thickest section of the stem.

3.4.3.3 Relative Water Content

The measurement of relative water content (RWC) of plant tissue can be estimated by using the ratio between its fresh weight, turgid weight and dried weight (Smart 1974). In this study, the RWC of seedlings shoot was analysed using the method described by Smart (1974). The fresh weight of each group was measured directly while the turgid weights were measured after plants were submerged in deionized water for 4 hours. The dried weights were measured after they were oven-dried at 80°C for 24 hours. The RWC of each seedling was calculated using the formula below.

$$\text{RWC (\%)} = \frac{\text{Fresh Weight} - \text{Dried Weight}}{\text{Turgid Weight} - \text{Dried Weight}} \text{ X } 100\%$$

Equation for RWC calculation

3.4.3.4 Germination Analysis

For germination analysis, all seeds from Bario, Bajong, Biris and MR219 varieties were surface-sterilised and transferred to 1:1 vermiculite: perlite as described above. The media were then added with distilled water, 100mM NaCl, 150mM NaCl or 200mM NaCl solution until saturation and left to grow in conditions as described above and observed after 7 days.

3.4.3.5 Growth of Radicle and Plumule

The radicle and plumule length of seedlings were measured using the method by Hakim et al. (2010). Briefly, the radicle and plumule length of One-week-old seedlings grown on 0.6% agarose media containing 0mM (control), 100mM, 150mM or 200mM NaCl were examined. Rice grains were sterilised as described previously and placed on top of 125ml agarose gel in a 250ml cell culture flask. The flasks were positioned in a growth chamber with the condition as described in the section above for 7 days and the radicle and plumule lengths were measured using a standardised Vernier calliper (Mitutoyo, Japan).

3.4.3.6 Ratio of Open Stomata

To assess the number of open stomata after salinity stress, seedlings growth and stress treatment were conducted as described in the section above. Seeds were incubated in a growth chamber for 14 days and subjected to 100mM of salinity stress for 5 consecutive days. Stomatal counting was conducted using the method as described by Zelitch (1961). Briefly, the undersides of the second leaf of the living plants were coated with a thin layer of cellulose acetate solution (commercial nail varnish) and left to dry. It was then peeled off with a scapula and observed directly under a microscope at x400 magnification. Pictures of open stomata were captured and measured using NIS-Elements D3.0 imaging software (Nikon, Japan) using a haemocytometer as size standard. Stomata were considered as open if the width of individual stoma exceeds 20.0µm.

3.4.4 Biochemical Analyses

3.4.4.1 Ion Concentration Analysis

Ion concentration analysis was conducted on dried shoot sample of salt-stressed and control plants. Fresh shoot tissue of seedlings was dried in an oven at 105ºC for 48

hours and placed into a 15mL centrifuge tube. Each sample group was individually digested in 0.5M nitric acid with 2000-ppm caesium chloride (80°C, 24 hours digestion). Concentration of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions in each sample were analysed using an atomic absorption analyser (XplorAA, GBC Scientific Equipment). The details of the analyses and standard curve are summarised in Table 4 and Figure 12 as followed. The Relative Standard Deviation (RSD) of each measurement was set at a maximum threshold of 1.5%; e.g., when exceeded, the entire experiment was optimised and repeated.

Table 4 Parameters used in ions determination.

Parameter	**Analyses**		
	Na^+	**K^+**	**Ca^{2+}**
Concentration of Standard solution	0.5ppm to 50ppm	25ppm to 400ppm	0.5ppm to 50ppm
R^2 Value	0.9993	0.9982	0.9951
Number of Calibration Points	9	7	7
Wavelength	589.00 nm	766.50 nm	422.70nm
Lamp Current	5.0mA	6.0mA	10.0mA
Matrices	0.5M nitric acid with 2000-ppm caesium chloride	0.5M nitric acid with 2000-ppm caesium chloride	0.5M nitric acid
Type of Flame	Air-Acetylene (Oxidizing)		
Sample Feeds	3 feeds for each sample, 3 seconds each		
Method of Measurement	Integration		

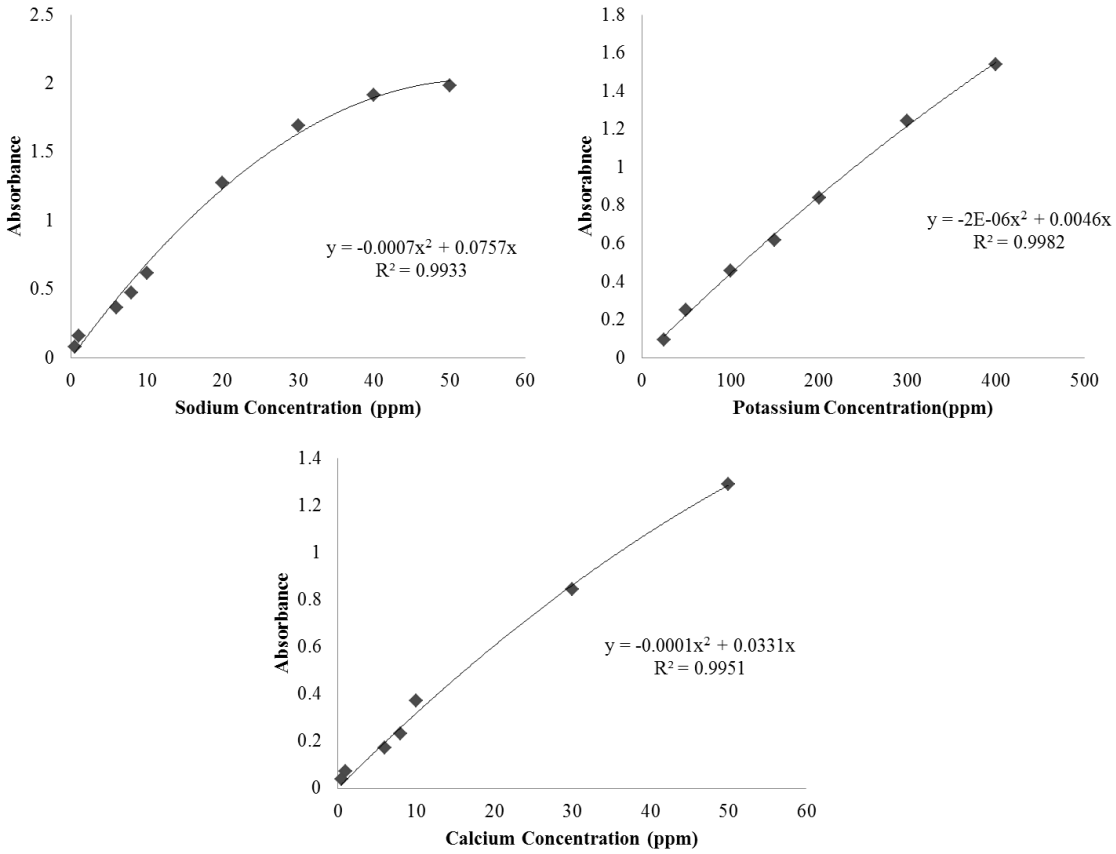


Figure 12 Standard curves for Na^+, K^+ and Ca^{2+} analyses.

3.4.4.2 Antioxidant Assays

Each sample group was weighted and added with absolute ethanol at a sample mass to solvent ratio of 1:10 (gramme to millilitres ratio). The mixture was placed in an ultrasonic generator (Model B5510, Branson) and sonicated for 60 minutes at room temperature. Then, the mixture was centrifuged (Centrifuge 5702, Eppendorf) for 15 minutes at 6000rpm at 4°C. The supernatants were collected as crude extracts and keep immediately at -22°C until further use.

Total flavonoid content of seedlings was measured using aluminium trichloride complexation method by Zhishen, Mengcheng and Jianming (1999) and Herald, Gadgil and Tilley (2012) with slight modification. Briefly, 250µL of crude extract was mixed with 1000µL of ultrapure water (Millipore) and 75µL of 5% (w/v) of sodium nitrite ($NaNO_2$). The mixture was incubated for 5 minutes at room temperature. Then, the mixture was added with 150µL of 10% (w/v) aluminium trichloride ($AlCl_3$), vortexed and incubated at room temperature for another 6 minutes. Later, the mixture was added

with 500µL of 1M sodium hydroxide (NaOH) and centrifuge at 3000rpm for 5 minutes. Finally, the absorbance of the collected supernatant was measured using a spectrometer (Genesys 20, Thermo Scientific) at 510 nm. Different concentrations of quercetin (0.008-1.000mg/mL) diluted in ethanol were used to prepare standard curve and the total flavonoid contents of seedlings were expressed in the unit of "mmol of quercetin equivalents (QE)/100gram of fresh rice grass".

Total phenolic contents of seedlings were measured using the method by with minor modification. Briefly, 100µL of crude extract was aliquoted into a cuvette followed by 500µL of Folin-Ciocalteu reagent (diluted 10-folds) and 400µL of 7.5% (w/v) sodium carbonate (Na_2CO_3). The mixture was allowed to stand in the dark and at room temperature (25ºC) for 60 minutes. Then, the absorbance of the solutions was measured using a spectrometer (Genesys 20, thermo-scientific) at 765nm. Different concentrations of Gallic acid (10-100mg/mL) diluted in ethanol were used to prepare a standard curve and the total phenolic contents of extracts were expressed in "mmol of Gallic acid equivalent (GAE) per 100gram of fresh rice grass".

The antioxidant-scavenging assay was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radicle molecule. Antioxidant capacity in compounds is assessed as the capability of the sample to convert DPPH molecules into a stable non-free radicle form. DPPH free radical scavenging assay was performed following the method from Herald, Gadgil and Tilley (2012) with slight amendments. Briefly, 200mM of DPPH solution was prepared using absolute ethanol. A serially diluted concentration of crude extracts (0.1g/ml – 0.7mg/ml) and positive controls, Trolox (0.02 mg/ml – 2.0 mg/ml, diluted in ethanol), were prepared directly in individual wells of a 96 wells microtiter plate to a total volume of 100µL. Next, 100µL of 0.4mM DPPH solution was then added to each well and the solution is mixed with a microplate reader (Synergy HT, Biotek) for 10 seconds. The plate was held in the dark 30 minutes at room temperature and the absorbance was measured at 517nm via a microplate reader (Synergy HT, Biotek). DPPH free radical scavenging capacity of was estimated via the following formula as expressed as Trolox equivalent antioxidant capacity (TEAC)/100g of rice grass.

$$\text{DPPH free radical scavenging capacity} = \frac{(A0-A)}{A0} \times 100\%$$

Equation for DPPH free radical scavenging capacity Calculation

**where A0 = absorbance of control sample; A = absorbance of test sample*

3.4.5 Statistical Analysis

Data were tabulated using Microsoft Excel and GraphPad (www.graphpad.com). Means and standard deviation of the sample group were calculated using Microsoft Excel and manually imported into GraphPad Prism software. The significant differences between samples were calculated using two-way ANOVA with Dunnett's post-test ($P<0.05$). Effective concentration for 50% DPPH inhibition (EC_{50}) was calculated using the dose-response analysis [log (agonist) versus response (three parameters)] method in GraphPad Prism.

3.4.6 Biological Replicates

The number of biological replicates used in each analysis is summarised in Table 5.

Table 5 Number of biological replicates used in each analysis.

Parameter	Conditions			
	Control	**100mM**	**150mM**	**200mM**
Shoot Length	30 plants	30 plants	30 plants	30 plants
Fresh Weight	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Dried Weight	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Turgid Weight	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Relative Water Content	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Number of Leaves	30 plants	30 plants	30 plants	30 plants
Stem Diameter	30 plants	30 plants	30 plants	30 plants
Germination Rate	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Open Stomata	3 groups x 3 plants	3 groups x 3 plants	3 groups x 3 plants	3 groups x 3 plants
K^+ Concentration	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Na^+ Concentration	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Ca^{2+} Concentration	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Total Phenolic Content	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
Total Flavonoid Content	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants
DPPH scavenging assay	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants	3 groups x 10 plants

3.5 Results

3.5.1 Physiological Studies

3.5.1.1 Seedling Length, Fresh Weight, Dried Weight and RWC

The results obtained are summarised in Table 6. Bario showed 18% reduction in seedling growth when exposed to 200mM NaCl. Seedlings from Bario, Bajong and MR219 showed no significant reduction in length under the same condition. Average seedling lengths of Bario treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 19.5cm, 18.8 m, 18.7cm and 16.0cm respectively. Bario also displayed the longest seedling length at 19.5cm among all tested varieties. The average seedling lengths of Bajong, Biris and MR219 were 16.2 cm, 15.9 cm and 15.1cm respectively. However, Bario also displayed a significant reduction in shoot length ($P<0.05$) when exposed to 200mM of NaCl solution while Bajong, Biris and MR219 displayed no significant reduction after the stress treatment.

All varieties exhibited reductions in fresh weight upon exposure to equal and more than 150mM of NaCl (Table 6). Statistical analysis has unveiled that many sample groups displayed a significant reduction ($P<0.05$) in fresh weight relative to the control. Upon exposure to 200mM of NaCl, Bario displayed the highest reduction at 28% while MR219 displayed the least reduction at 5% relative to the control. Average fresh weights of Bario treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 44.4mg, 43.2mg, 42.2mg and 32.0mg respectively. Meanwhile, average fresh weights of MR219 treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 45.6mg, 51.0mg, 42.2mg and 44.2mg respectively. In this study, MR219 had significant increased ($P<0.05$) fresh weight when exposed to 100mM of NaCl, while Bario and Bajong had no significant changes in fresh weight when exposed to 200mM of NaCl. No significant reduction ($P<0.05$) was observed in Biris under all level of stress treatments.

Seedlings exposed to high salinity (200mM NaCl) decreased significantly ($P<0.05$) in dried weight in Bario and MR219 (Table 6). Dried weight of all other plant samples was not affected by low-level salinity stress imposed by 100mM and 150mM NaCl solution. Bario displayed the highest reduction at 16% ($P<0.05$) while MR219 displayed the second highest reduction at 12% relative to the controls ($P<0.05$). Dried weight in each

group of Bario rice sample treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 89.3mg, 90.9mg, 88.2mg and 75.1mg respectively. Meanwhile, dried weight in each group of plants of MR219 rice sample treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 89.1mg, 94.7mg, 86.5mg and 78.8mg respectively. In summary, the dried weight of Bario and MR219 had significantly decreased ($P<0.05$) after high (200mM) stress induction while Bajong and Biris displayed no significant changes after the treatment.

The RWC estimates the plant's dehydration status. In this study, the RWC in Bario had suffered the highest reduction after salinity treatment (Table 6). Average RWC of Bario treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 88.89%, 91.25%, 85.07% and 83.13% relative to the control respectively. RWC in Bajong sample only showed a significant decrease ($P<0.05$) after being subject to 200mM of NaCl. Biris had not shown any significant decrease in RWC regardless of the salinity stress imposed. All varieties also displayed a slight increase in RWC when exposed to 100mM NaCl for 5 consecutive days, but the increase was not statistically significant ($P<0.05$). Bario, Bajong, Biris and MR219 displayed an increase of 3.0%, 4.0%, 2.0% and 2.0% relative to the control. In this analysis, all variety displayed a significant reduction in RWC expect Biris. The ability of rice varieties to retain water can be ranked in ascending order as Bario, MR219, Bajong and Biris.

Table 6 Shoot length, dried weight and RWC of test samples.

Varieties	Condition	Shoot Length (cm)	Fresh Weight (mg)	Dried Weight (mg)	RWC (%)
Bario	Control	19.48 ± 1.6	44.37 ± 6.9	8.93 ± 0.6	88.89 ± 0.1
	100mM	18.82 ± 1.7	43.21 ± 8.1	9.09 ± 0.4	91.25 ± 0.1*
	150mM	18.72 ± 1.3	42.24 ± 7.0	8.82 ± 0.5	85.07 ± 0.2*
	200mM	15.96 ± 1.6*	31.96 ± 6.9	7.51 ± 0.4*	83.13 ± 0.5*
Bajong	Control	16.2 ± 1.2	37.54 ± 6.2	8.65 ± 0.2	90.14 ± 0.3
	100mM	16.04 ± 1.9	37.18 ± 6.7	8.61 ± 0.5	94.32 ± 0.5
	150mM	14.97 ± 1.5	34.65 ± 5.8	8.49 ± 0.8	91.22 ± 0.8
	200mM	15.15 ± 1.3	33.74 ± 4.2	8.06 ± 1.3	81.90 ± 0.5*
Biris	Control	15.89 ± 1.4	42.39 ± 5.6	9.17 ± 0.7	91.02 ± 0.3
	100mM	16.21 ± 1.4	40.92 ± 6.0	9.13 ± 0.6	92.38 ± 0.9
	150mM	15.64 ± 1.1	42.79 ± 5.5	9.07 ± 0.4	91.29 ± 0.0
	200mM	16.20 ± 1.7	38.43 ± 6.5	9.06 ± 0.6	90.45 ± 0.1
MR219	Control	15.06 ± 1.5	46.55 ± 11.0	8.91 ± 0.3	90.81 ± 2.0
	100mM	14.68 ± 1.3	51.02 ± 7.5	9.47 ± 0.2	92.70 ± 1.9
	150mM	13.66 ± 1.9	42.20 ± 9.8	8.65 ± 0.1	88.78 ± 2.7*
	200mM	14.33 ± 1.5	44.23 ± 10.0	7.88 ± 0.3*	85.07 ± 2.0*

*Data were taken 5 days after stress treatment on 14 days old seedlings. Asterisks denote significant reduction to control at $P<0.05$ (Dunnett's test).

3.5.1.2 Germination Rate of Seed in Saline Solution

All rice varieties showed a sharp decline in seed germination rate with increasing concentration of environmental stress (Table 7). The germination rate of Biris was found to be higher than that of Bario, Bajong and MR219 ($P<0.05$) in all condition. Germination rates of Biris treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 97.8%, 86.7%, 73.3% and 26.7% relative to the controls. Germination rate in Bario was found to be lower than Bajong, Biris and MR219. Germination rate of Bario treated with 0mM (control), 100mM, 150mM and 200mM of NaCl solution were 65.6%, 51.1%, 15.6% and 1.1% relative to the controls. The germination capability of these varieties in saline condition can be arranged in ascending order as Bario, Bajong, MR219 and Biris.

3.5.1.3 Radicle and Plumule Length, Stomatal Opening, Stem Diameter and Leaves Number

The growth of radicle and plumule of Bario, Bajong, Biris and MR219 in saline solution were assessed (Table 7). Biris had consistently shown relatively better growth ($P<0.05$) while experiencing salinity stress. Bario displayed the reduced early seedlings growth under normal and salinity stress compared to Bajong, Biris and MR219. Bajong and MR219 exhibited roughly equal performance in term of plumule growth.

All varieties displayed decreases in the total stomatal opening after being subjected to 100mM of NaCl (Table 8). The total stomatal opening of Bario, Biris and MR219 had demonstrated 19.7%, 17.8% and 24.6% decrease relative to the controls while the reduction in the total open stomatal of Bajong was relatively higher at 42.6% relative to the control. The reduction in the open stomata was statistically significant ($P<0.05$) in Bajong but not in Bario, Biris and MR219.

The average stem diameter of all varieties had displayed slight reduction after being subjected to 100mM of NaCl (Table 8). However, no significant reduction can be detected between control and stressed sample. Inter-varieties comparison displayed that MR219 had the thickest stem while Bajong had the thinnest stem. No other observation can be made in this analysis.

The average number of leaves of all varieties had displayed no noticeable reduction after being subjected to 100mM of NaCl for 5 consecutive days (Table 8). The average numbers of leaves in Bario, Bajong, Biris and MR219 in the control group were 2.87, 2.87, 2.73 and 2.87 respectively. The average numbers of leaves in Bario, Bajong, Biris and MR219 in the stressed samples are 2.83, 2.87, 2.76 and 2.73 respectively. No other observation can be made in this analysis.

Table 7 Germination rates, plumule and radicle length of test samples expressed in percentage relative to control in each variety.

Varieties	Condition	Germination Rate (%)	Plumule Length (%)	Radicle Length (%)
Bario	Control	65.6 ± 1.9	100 ± 30	100 ± 28
	100mM	51.1 ± 3.8	105 ± 16	109 ± 99
	150mM	15.6 ± 5.1	28 ± 29	52 ± 15
	200mM	1.1 ± 1.9	12 ± 67	12 ± 33
Bajong	Control	92.2 ± 1.9	100 ± 15	100 ± 27
	100mM	80.0 ± 3.3	85 ± 55	73 ± 50
	150mM	35.6 ± 3.8	75 ± 62	69 ± 0
	200mM	11.1 ± 1.9	46 ± 17	36 ± 25
Biris	Control	97.8 ± 1.9*	100 ± 27	100 ± 29
	100mM	86.7 ± 3.3	61 ± 26	273 ± 27
	150mM	73.3 ± 3.3*	47 ± 47	91 ± 40
	200mM	16.7 ± 3.3	32 ± 30	73 ± 25
MR219	Control	78.9 ± 5.1	100 ± 30	100 ± 28
	100mM	43.3 ± 3.3	105 ± 16	109 ± 99
	150mM	27.8 ± 3.8	28 ± 29	52 ± 15
	200mM	13.8 ± 3.3	12 ± 67	12 ± 33

*Data were taken 7 days after incubation. Asterisks denote significant differences to other varieties in the same condition at $P<0.05$ (Dunnett's test).

Table 8 Stem diameters and number of leaves of test samples in saline solution.

Varieties	Conditions	Ration of Open Stomata (%)	Stem Diameters (cm)	Number of Leaves
Bario	Control	70.5 ± 15.1	0.118 ± 0.004	2.87 ± 0.3
	Stressed	56.7 ± 10.5	0.103 ± 0.009	2.83 ± 0.4
Bajong	Control	80.2 ± 4.0	0.098 ± 0.013	2.87 ± 0.3
	Stressed	46.0 ± 10.9*	0.092 ± 0.015	2.87 ± 0.3
Biris	Control	71.8 ± 5.5	0.121 ± 0.008	2.83 ± 0.4
	Stressed	59.0 ± 7.1	0.108 ± 0.015	2.77 ± 0.4
MR219	Control	79.8 ± 8.7	0.134 ± 0.017	2.87 ± 0.3
	Stressed	60.2 ± 5.7	0.120 ± 0.016	2.73 ± 0.4

*Data were taken 7 days after grains were immersed in saline solution. Asterisks denote significant differences to control at $P<0.05$ (Dunnett's test).

3.5.2 Biochemical Analysis

3.5.2.1 Ion Concentration Analysis

In this study, the concentration of Na^+ was around 0.040% to 0.052% of the plant's dried weight (Table 9). Biris seems to be more vigorous in Na^+ accumulation compared to Bario, Bajong and MR219. Bario was found to have accumulated the least amount of Na^+ after being exposed to 200mM of NaCl, about 34.61% lower than Biris.

Total K^+ concentration was around 2.89% to 4.25% of the plant's dried weight (Table 9). Bario contained the highest concentration of K^+ and was able to maintain a constant amount of K^+ regardless the strength of stress applied. Bajong, Biris and MR219 showed the slight increase in K^+ concentration after the treatment. The increased in K^+ concentration after treatment with 200mM of NaCl in Bajong, Biris and MR219 were 18.70%, 13.90% and 17.90% respectively.

All varieties showed noticeably declined in K^+/Na^+ ratio after exposed to 100mM of salinity stress (Table 9). The K^+/Na^+ ratios for Bario, Bajong, Biris and MR219 after 200mM salinity stress treatment suffered a reduction of 85.30%, 88.30%, 90.50% and 89.10% relative to the controls. After treated with 200mM of NaCl, Biris exhibited the lowest K^+/Na^+ ratio while Bario had the highest.

The concentration of Ca^{2+} ranged approximately 0.15% to 0.40% of the plant's dry weight (Table 9). Bario and MR219 contain the least amount of Ca^{2+} while Biris contain the highest amount of Ca^{2+} in the plant tissue. The Ca^{2+} concentration in Bajong was inversely proportional to the amount of salinity stress imposed. Biris have the highest Ca^{2+} concentration among all varieties regardless of the stress applied.

In summary, Biris accumulated the highest amount of Na^+ after stress treatment. Even though an increase accumulation of K^+ was observed, Biris still exhibited the lowest K^+/Na^+ ratio when compared to the Bario, Bajong and MR219. On the other hand, Bajong displayed an increased level of Ca^{2+} accumulation after stress treatment while Bario displayed a reduction in Ca^{2+} concentration. However, no statistical significances ($P<0.05$) can be found in the comparison of all the data due to the small amount of biological replicates involved.

Table 9 Na^+, K^+ and Ca^{2+} concentration and the K^+/Na^+ ratio of the test sample.

Varieties	Condition	Na^+ Concentration %Dried weight	K^+ Concentration %Dried weight	Ca^{2+} Concentration %Dried weight	K^+/Na^+ Ratio	Decrease Over Control (%)
Bario	Control	0.052 ± 0.004	4.196 ± 0.16*	0.334 ± 0.04	80.5	N/A
	100mM	0.191 ± 0.02	4.086 ± 0.10	0.322 ± 0.02	21.4	73.41
	150mM	0.239 ± 0.04	4.245 ± 0.16	0.319 ± 0.02	17.8	77.88
	200mM	0.345 ± 0.06	4.069 ± 0.07	0.331 ± 0.02	11.8	85.34
Bajong	Control	0.041 ± 0.00	2.891 ± 0.03	0.286 ± 0.01	70.8	N/A
	100mM	0.264 ± 0.03	3.082 ± 0.10	0.212 ± 0.01	11.7	83.47
	150mM	0.286 ± 0.01	3.460 ± 0.04	0.256 ± 0.01	12.1	82.90
	200mM	0.377 ± 0.08	3.561 ± 0.08	0.248 ± 0.01	9.4	86.72
Biris	Control	0.051 ± 0.01	3.435 ± 0.10	0.372 ± 0.01	67.0	N/A
	100mM	0.207 ± 0.02	3.738 ± 0.07	0.336 ± 0.04	18.0	73.13
	150mM	0.411 ± 0.05	4.040 ± 0.24	0.345 ± 0.02	9.8	85.37
	200mM	0.523 ± 0.05	3.994 ± 0.06	0.367 ± 0.04	7.6	88.65
MR219	Control	0.053 ± 0.05	3.091 ± 0.11	0.203 ± 0.01	60.2	N/A
	100mM	0.148 ± 0.03	3.269 ± 0.12	0.172 ± 0.04	22.6	62.40
	150mM	0.256 ± 0.01	3.294 ± 0.08	0.174 ± 0.03	13.1	78.10
	200mM	0.432 ± 0.02	3.761 ± 0.07	0.203 ± 0.05	8.7	85.47

Data were taken 5 days after stress treatment on 14 days old seedlings.

3.5.2.2 Antioxidant Concentration in Plant Tissue

In this study, the Total Phenolic Content (TPC) of rice seedling ranged from 0.04 to 0.10mmol/GAE for every 100g of fresh rice grass (Table 10). Bario exhibited a reduction in total phenolic content after they were subjected to 5 days of salinity stress treatment. Meanwhile, Bajong, Biris and MR219 exhibited an increase in TPC after stress treatment. In the control condition, Bario had exhibited the highest concentration of TPC at 0.08mmol GAE/100g fresh rice grass, but MR219 displayed higher TPC at 0.09mmol GAE per 100g of fresh rice grass in the stressed condition.

The Total Flavonoid Content (TFC) was found to be between 0.09 to 0.26mmol QE for every 100g of fresh rice (Table 10). Bario and MR219 exhibited a reduction in TFC after the treatment. Meanwhile, Bajong and Biris displayed a slight increase in TFC after the stress treatment. Bario contained the highest amount of TFC at 0.26mmol QE/100g fresh rice in the control condition and 0.23mmol QE/100g fresh rice in the stress condition. However, Biris had significantly lower ($P<0.01$) TFC relative to Bario, Bajong and MR219.

The scavenging activity of various extracts was evaluated using DPPH assay. The total radical scavenging power was represented as EC50, (Table 10) the concentration of extracts to scavenge half (50%) of the starting DPPH concentration. Extracts from rice seedlings between 1.6 to 100.0mg/ml displayed DPPH radical scavenging activity in a dose-dependent manner. The highest scavenging activity was observed in Biris sample after stress treatment, for which the TEAC value increase from 0.17 to 0.26mmol /100g fresh seedlings weight.

Table 10 TPC, TFC and DPPH scavenging activity of the samples.

Varieties	Conditions	TPC mmol GAE/100g Ricegrass	TFC mmol QE/100g Ricegrass	DPPH Scavenging Activity mmol TEAC/100g Ricegrass
Bario	Control	0.0816 ± 0.0050	0.2585 ± 0.0051	0.15
	Stressed	0.0623 ± 0.0017*	0.2307 ± 0.0101*	0.03*
Bajong	Control	0.0551 ± 0.0061	0.2129 ± 0.0097	0.17
	Stressed	0.0735 ± 0.0045*	0.2176 ± 0.0160	0.12
Biris	Control	0.0750 ± 0.0066	0.0925 ± 0.0041	0.17
	Stressed	0.0843 ± 0.0031	0.0997 ± 0.0149	0.26*
MR219	Control	0.0692 ± 0.0012	0.1896 ± 0.0208	0.25
	Stressed	0.0949 ± 0.0041*	0.1688 ± 0.0034*	0.18*

*Data were taken 5 days after stress treatment on 14 days old seedlings. Asterisks denote significant differences to control at $P<0.05$ (Dunnett's test).

3.6 Discussion

3.6.1 General Physiological Studies

The phylogenetic relationship between all the Sarawak local rice varieties is not well understood. Lee et al. (2011) have reported the genetic diversity of Sarawak local rice varieties using microsatellites markers. It was found that Biris and Bajong were closely related, showing high similarity in their SSR patterns. Microsatellite patterns in Bario were significantly different from Bajong, Biris and MR219. Meanwhile, MR219 was shown to belong to its clusters, showing equal distances in dissimilarity to Bajong, Biris or Bario. The pattern in genetic diversity is similar to the differences in salinity tolerance found in this study. Biris was more resistance to salinity stress, which is followed closely by Bajong. MR219 had a lowered salinity tolerance than Biris and Bajong but was still significantly better than Bario.

Brondani et al. (2006) have proposed that genetic variation within the same species could result from many generations of successive cultivation in different geographical settings. Thus, it was speculated the salt tolerance capability of Biris and salt sensitivity of Bario could be an unintended selection of natural mutants that could better adapt to the respective cultivating environment

Results from this study have also indicated that short period of NaCl treatment did not significantly affect stem diameter and the number of leaves in the seedlings (Table 8). Thus, screening for changes in these parameters above might not be a good method to quantify the short-term salt sensitivity in rice. Meanwhile, an increase in RWC can be observed in all samples under 100mM NaCl stress. Since the measurement of relative water content is based on the ratio between fresh weights, turgid weights and dried weights of the samples. It was speculated in this study, the number of osmolytes within the plant cells has deviated the measurements of turgid weights when plants accumulate osmoprotective molecules (Hare, Cress & Van Staden 1998; Khan, Mazid & Mohammad 2011). Biochemical studies have revealed that all rice varieties displayed elevated TFC and TPC after low stress (100mM) treatment (Section 3.5.2.2). The presence of such molecules could reduce the overall osmotic pressure in the cells, thus resulting in the drop in overall turgid weight and increases the relative water content. Thus, future work should consider this factor when measuring RWC on plants directly.

To counter this problem, an electroconductivity meter can be used to crosscheck the RWC obtained.

In this study, we can briefly conclude that Biris is more salt tolerance than Bario, Bajong and MR219.

3.6.2 Germination Rate and Ratio of Open Stomata

High seed germination rate and vigorous seedling growth could indicate the better growth and productivity of the plants under salinity stress (Carpýcý, Celýk & Bayram 2009). Therefore, screening for salt tolerance in different growth stages could result in a change in perspective in the conventional idea of salt tolerance in the plants. The germination rate of Biris in 100mM NaCl was approximately 87%, which is higher than all rice varieties tested in previous studies (Table 11). Thus, we should see a better performance in Biris when cultivated in the saline environment. The results from plumule and radicle lengths analyses were in accordance with the hypothesis proposed above. However, reasons for such outcome are unknown and require further investigation.

Meanwhile, guard cells or stomatal apparatus cells regulate the transpiration rate and CO_2 levels in the leaves. Reactive Oxidative Species (ROS) have also been proposed as a secondary messenger in the guard cells (Schroeder, Kwak & Allen 2001). Therefore, the percentage of the stomatal opening could serve as an indication of the oxidative stress level in the leaf tissue (Yan et al. 2007). In this study, Bajong displayed the highest reduction in the stomatal opening when compared to Bario, Biris and MR219. However, the reasons for such outcome are unknown and will need further investigation.

Table 11 Germination rates of rice varieties in previous studies.

Name of Variety	Germination Rate (%)	Stress Condition	References
NJ11	80.00	100mM NaCl*	(Jiang et al. 2013)
Balilla	60.00		
HN2026	55.00		
Nipponbare	75.00		
IR50	28.33	12dS/m (Approximately 102mM NaCl)**	(Anbumalarmathi & Mehta 2013)
MDU5	23.33		
ADT43	28.33		
ADT47	23.33		
CO49	23.33		
PMK3	28.33		
Jeeraga Samba	21.67		
TKM11	16.67		

* Germination rate of Nj11, Balila, HN2026 and Nipponbare were estimated from a bar chart provided.
** Conductance of NaCl in water was obtained from Haynes (2014)

3.6.4 Accumulation of Ions

Potassium is one of the most abundant elements in plants and could accumulate up to 6% of the plant's dry weight (Raven, Evert & Eichhorn 2005). Plants often accumulate K^+ during high Na^+ stress, which could help to lower the amount of ROS present in the cytoplasm (Cakmak 2005). In general, the concentration and accumulation of Na^+ and K^+ upon salinity exposure is similar to a previous study conducted by Walia et al. (2005b), showing increase in Na^+ accumulation and decrease in K^+ concentration after the induction. The Na^+ concentration was also higher when compared to salt-sensitive species such as *Arabidopsis* (Mason et al. 2010) and lower than more salt tolerance species such as Maize (Estrada et al. 2013).

In this study, a high Na^+ accumulation in Biris under salinity stress had been observed. Blumwald (2000) has suggested that plant could actively transport any excess Na^+ into the vacuole of the cell to prevent ion-excess damage to the cell. It is speculated that Biris contain high levels of translocation activities while experiencing salinity stress. Future work can employ Biris as model plants for plant's Na^+ translocation studies. Meanwhile, Bario had the highest ratio of K^+/Na^+ among all the tested varieties regardless of the treatment strength. However, the K^+ concentration had not shown any

increase with the increased in treatment strength. Also, Bario exhibited relatively weaker physiological performance when experiencing salinity stress. It had the highest reduction in RWC and had the lowest germination rate when subjected to salinity stress. Therefore, the high K^+/Na^+ ratio is speculated to be because of lowered water absorption and not a direct consequence of the salt tolerance system.

Ca^{2+} is another vital plant macronutrient that takes part in numerous structural and signalling roles (White & Broadley 2003) as tight regulation of transportation in Ca^{2+} is essential for the function and responses of plants towards environmental stress (Dodd, Kudla & Sanders 2010; McAinsh & Pittman 2009). The range of calcium concentration found in this study is similar to one previous study (Rahman et al. 2016), which is around 36 - 57μmol/g (approximately 0.156% to 0.228% of the plant's dried weight), with a slight decrease in calcium concentration after the stress induction. Little data can be found targeting the change in calcium accumulation upon salinity stress despite the importance of Ca^{2+} in the plant stress signalling pathway. The data generated from this study might serve as a baseline for any future studies looking into this aspect. Furthermore, results from the current study could also suggested that Bario, Bajong and Biris have a more efficient uptake mechanism compared to MR219. However, the direct relationship between Ca^{2+} accumulation and salt tolerance was not fully understood and will need further investigation.

3.6.5 Antioxidant Concentration

Plants increase the accumulation of osmolytes when exposed to high salinity (Feng et al. 2002; Hare, Cress & Van Staden 1998). Salinity stress stimulates the synthesis of superoxide by sub-mitochondrial particles and increases the overall oxidative stress in plants (Hernández et al. 1993), which called for the plant's natural defence system to synthesise and accumulate anti-oxidant molecules. Results from the antioxidant assay suggested that Bajong tended to be more responsive towards salinity stress and was capable in the reduction of the oxidative stress. Radical scavenging activity in Bario, Bajong and MR219 had shown a slight decrease in value, which could be due to the depletion of the antioxidant molecules. Since little has touched on this topic, more studies will be needed to validate such hypothesis.

In the meantime, the TPC and TFC in Sarawak local rice extracts were found to be comparable to extracts from wheatgrass seedlings (Kulkarni et al. 2006). Aqueous and

ethanol extracts from common wheat (*Triticum aestivum*) has been widely used as health-promoting drinks (Falcioni et al. 2002; Gruenwald 2009). They have been investigated in numerous studies for radical-scavenging (Kulkarni et al. 2006), immuno-stimulating (Hemalatha et al. 2012) and anti-carcinogenic effects *in vivo* and *in-vitro* (Ben-Arye et al. 2002). In this study, TPC and TFC from rice grass were approximately half and one-third the value when compared to the extract from wheatgrass seedlings (Table 10, Table 12) while the radical scavenging capacity was approximately one-third of wheatgrass seedlings (Kulkarni et al. 2006).

Little data have reported the antioxidant levels of seedlings tissue. One recent study has reported antioxidant level in rice seedlings by analysing the antioxidant in juice extracted from jointing space rice plants (Table 12). However, due to the differences in sample preparation, the values could not be compared with the current study. Nevertheless, they have shown that extracts from rice grass possess significant antioxidant and DNA protection properties and indicates the potential of using rice grass as a raw material for health drinks.

In the current study, direct relations between fresh weight and antioxidant level have been reported. Even though it was lower when compared to extracts from wheatgrass of similar age in the same condition, further optimisation of extraction method and controlled environmental stresses could increase the amount of antioxidant in the plants, resulting in a higher efficacy of the product. The use of rice seedlings as health drinks would benefit the local community as rice can be grown in Malaysia but wheatgrass cannot be cultivated easily due to the tropical weather condition.

Table 12 Antioxidants content from wheatgrass and rice grass juice.

Sample	**TPC (mmol GAE/100g)**	**TFC (mmol GAE/100g)**	**DPPH (mmol TEAC/100g)**	**References**
Wheatgrass	0.7	0.55	1.4	(Kulkarni et al. 2006)
Rice grass juice Extracts	2.6	N/A	8.8	(Khanthapoka, Muangpromb & Sukronga 2015)

*Units are expressed as 100g of fresh wheatgrass and 100g of dried extracts from rice grass juice.

3.7 Conclusion

In this study, 4 rice varieties had been tested for salt tolerance properties. No direct changes can be found in the plant's shoot length, stem diameter or number of leaves on young seedlings after salinity stress induction. Biris was found to be more tolerant to saline stress compare to Bajong, MR219 and Bario with higher RWC, germination rate, radicle and plumule length under salinity stress. However, Biris also contain a higher level of Na^+ concentration and had the lowest K^+/Na^+ ratio. Meanwhile, Bajong was found to be more responsive towards salinity stress with the lowered open stomata and increased antioxidant under salinity stress. The antioxidant assay had also revealed that the antioxidant levels in rice seedlings were comparable to wheatgrass extracts. In summary, it appears that Biris is most adapted to salinity stress while Bajong is most responsive to salinity stress. Both samples should be further investigated for the molecular mechanism of salinity tolerance.

Chapter 4 Molecular Studies of Salinity Stress in Sarawak Rice Targeting Specific Salt-inducible Pathway

4.1 Executive Summary

Salinity tolerance is a complex trait manipulated by numerous salinity-responsive genes that control many different physiological and biochemical responses. Upon exposure to environmental salinity stress, a massive signal cascade is triggered. Plants can response to salinity stress by using several strategies. For instances, abscisic acid (ABA) signalling pathway and calcineurin B-like (CBL) proteins kinases control many of the immediate stress responses. Meanwhile, Late embryogenesis abundant proteins (LEA) pathway and salt overly sensitive (SOS) pathway are involved in the later part of the tolerance mechanism. Some plants also accumulated polyamines molecule to encounter the rising intercellular osmotic pressure.

In this chapter, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the changes in expression levels in several salinity-responsive pathways after stress treatment. Several genes were chosen to examine the changes in gene expression that took part in various different mechanisms. For examples, SOS1 and SOS2 were chosen to reflect the initiation of ion homoeostasis, CIPK1 to reflect the initiation of transcription factors, CIPK11 to reflect the initiation of the ABA-mediated pathway, LEA1 and LEA2 to reflect the changes in expression of stress proteins and finally, ADC and ODC to reflect the changes in the plant's polyamine biosynthesis activity. In the meantime, a moderate level of salinity stress (100mM) was chosen in this study to avoid extreme stress level to the plants and to investigate whether these varieties can response without an extreme environmental stimulation. Finally, the results are presented together with discussions of the outcomes obtained.

4.2 Research Aims and Objectives

The aim of this chapter of research work was to identify the differences in expression of several previously identified salt-inducible genes. Research works in this chapter were designed to accomplish the following objectives:

i. Optimisation of RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)
ii. Study of expression levels on several salt-inducible genes in SOS, CIPK, LEA pathways and two putrescence production genes after salinity stress exposure
iii. Narrow down target variety for transcriptome sequencing

4.3 Materials and Methodology

4.3.1 RNA Extraction

Samples from 30 seedlings from each variety (15 controls, 15 stressed) were included for expression profiling exactly 6 hours after stress treatment using 100mM of NaCl. The seedlings were cultivated as described in Section 3.4.1 and induced with salinity stress as outlined in Section 3.4.2. All control plants were kept at identical conditions alongside stressed sample. Upon harvesting, 5 whole shoots tissue were pooled, pulverised in liquid nitrogen and stored immediately at -80ºC until further use. In each variety, 3 biological replicates (3 groups x 5 plants per group) were included in both control and stressed environment.

Total RNA was isolated from the pool of shoot tissue using QIAGEN RNeasy kit (Qiagen, Valencia, CA) and purified using the RNase-Free DNase Set (Qiagen, Valencia, CA) following manufacturer instructions. The quality and integrity of each RNA extracts were checked using a Synergy HT Multi-Mode Reader (Bio-Tek Instruments, Inc., USA). The integrity of RNA extracts was double-checked using gel electrophoresis technique; employing 1.5% agarose gel, 100V and 60 minutes separation time. All steps were performed following manufacturer's instructions. A total of 50-100ng was used for each qRT-PCR reaction. The dilution was made directly in the preparation of master mix, so all reactions contain the exact amount of RNA except for non-template control. All steps were performed following manufacturer's instruction.

4.3.2 Quantitative Reverse Transcription Real-Time PCR (qRT-PCR)

Several genes involved in stress signalling or accumulation of stress response-related metabolites were selected for gene expression analysis. One housekeeping gene (Rice Actin 11) was used as an internal expression reference. Primer sets were designed using the Primer-3-plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al. 2012). The expression profiling was conducted using QIAGEN OneStep RT-PCR Kit (Qiagen, Valencia, CA). The name, accession number and sequences of forward and reverse primers are summarised in Table 13. The PCR cycles were as follows: 50°C for 10 minutes for reverse transcription; 95°C for 5 minutes for template denaturation; 40 cycles of template amplification at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Relative quantification method was used in this study using comparative CT method (Schmittgen & Livak 2008) based on the equation as attached assuming equal PCR efficiency among primer sets. The results were expressed as fold changes between control and treated sample, with each fold representing a 100% increase in expression level. The measurement of CT, normalisation of expressions and comparison of expression levels between samples were conducted directly on Rotorgene Q software v2.3.1.49 using the included function on default parameters.

$$2^{-\Delta\Delta\, Ct} = \left[(Ct\ Gene\ of\ interest - Ct\ Internal\ Control) Sample\ A - \left((Ct\ Gene\ of\ interest - Ct\ Internal\ Control) Sample\ B\right)\right]$$

4.4 Results

In this study, the expressions of several salt-inducible genes in 3 Sarawak local rice varieties (Bario, Bajong and Biris) and one commercial variety (MR219) were investigated using Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The gel Image of RNA extracts from each sample is enclosed in Figure 13 while the quantity and quality of RNA extracts are attached as follows.

The schematic preview of the changes in expression is enclosed in Figure 14. The expression levels of SOS1 and SOS2 in Bario and MR219 did not appear to change significantly. The expression level of SOS1 was upregulated in Bajong by eight-fold, but it was downregulated by two-fold in Biris. The expression levels of SOS2 were elevated by approximately two-folds in Biris and Bajong (Figure 14). Meanwhile, the expression level of CIPK1 and CIPK11 had shown similar trend relative to SOS pathway. The expression of CIPK1 and CIPK11 was upregulated in Bajong by approximately two-folds but were downregulated in all Bario, Biris and MR219 (Figure 14).

The expression of LEA1 was upregulated in Bajong but was repressed in Bario, Biris and MR219. Results targeting the expression levels of LEA2 were disregarded, as the Ct value was higher than 30 cycles in all samples. In Bajong, the expression of ADC was slightly upregulated. However, expression of ADC was downregulated by 2 to 8 folds in Bario, Biris and MR219 respectively (Figure 14). Results targeting the expression levels of ODC were disregarded, as the Ct value was higher than 30 cycles in the control sample (Table A).

Table 13 List of primer pairs used in gene expression studies.

Name of Gene	Accession Number	Expected Size (bp)	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	References
Rice Expression Reference – Actin 11 Production Gene					(Chern et al. 2013)
Actin 11	KC140129.1	239	TACACGTACGCCTCGTCAAG	CAGGATACCCCT TTTTGCCT	
Rice Salt Overly Sensitive (SOS) Pathway Production Genes					(Soni et al. 2013)
SOS1	AK065608	76	GAGCAATTGCGTCAATCAGA	AAAGCCTGGCAACGACTAGA	
SOS2	AK102270.1	81	GCCTGAAAACCTGCTTCTTG	ACTCCTTTCTGGGCCAAAGT	
Rice Calcineurin B-Like Protein-Interacting Protein Kinases (CIPK) Genes					(Xiang, Huang & Xiong 2007)
CIPK1	AK065588.1	90	GTCCTTAAGCCTCCCAAACC	TGACCATGCGTATCCTCAAA	
CIPK11	AK103032.1	93	GTCCTTAAGCCTCCCAAACC	TGACCATGCGTATCCTCAAA	
Rice Late Embryogenesis Abundant Proteins (LEA) Production Genes					(Wang et al. 2007)
LEA1	AK063682	58	TAAAAACACGTCGCAAGTCG	GCCTGTGGATTGAGATTCGT	
LEA2	AK107973	93	AGCACAGGCTCCATAAGCAT	TAGCTAGCAGGTGGGAGGTG	
Rice Polyamine Production and Accumulation Genes					(Quinet et al. 2010)
ADC	AY604047	69	AACCTGTCCGTGTTCACCTC	GATTGGGATGATCGGGAAC	
ODC	NM_001070362.2	62	AACCTGTCCGTGTTCACCTC	GATTGGGATGATCGGGAAC	

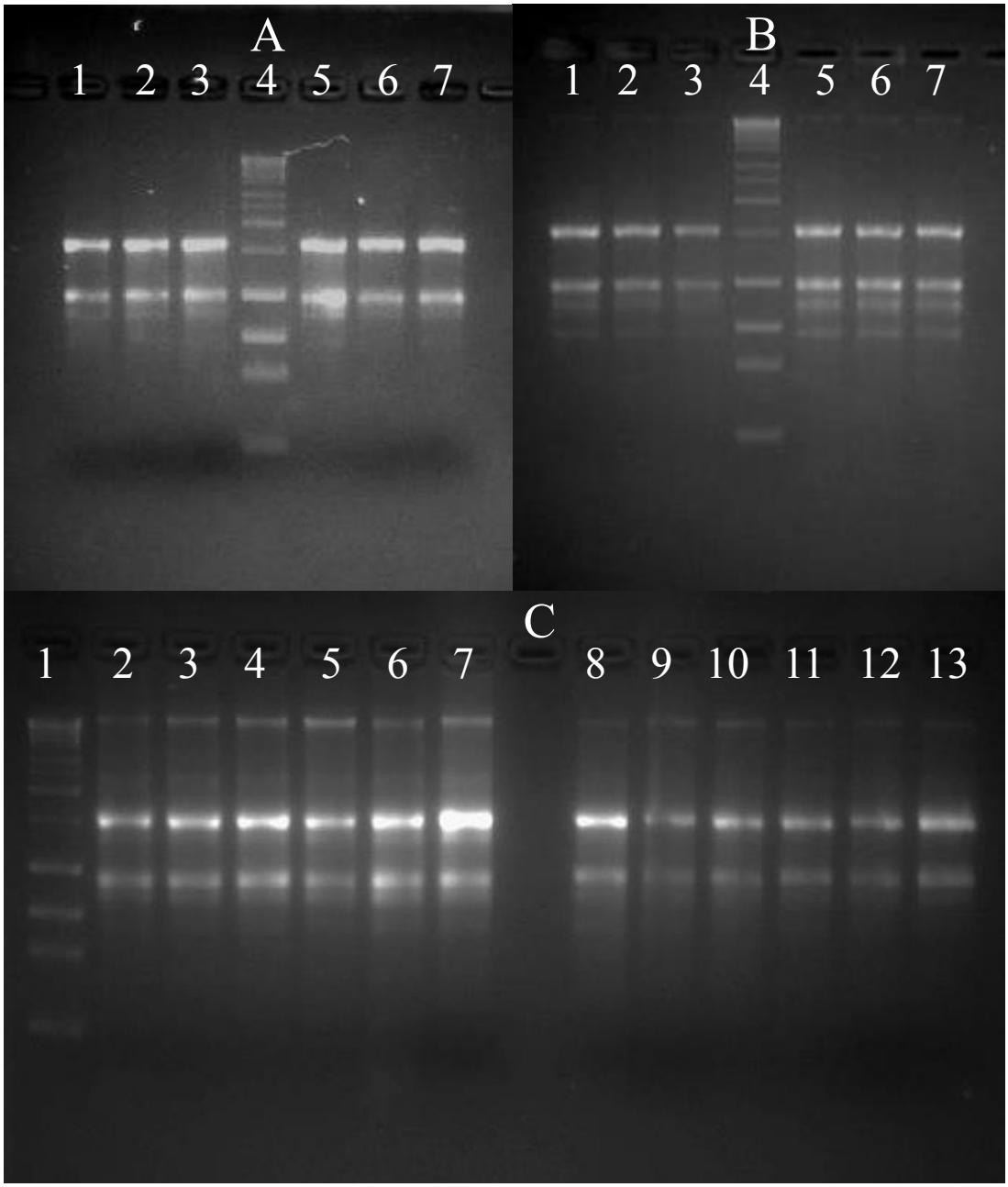


Figure 13 Gel image of RNA extracts.

Gel A: Lane 1 to 3: Bario Control; Lane 5 to 7: Bario Treated; Lane 4: DNA Ladder*

Gel B: Lane 1 to 3: Bajong Control; Lane 5 to 7: Bajong Treated; Lane 4: DNA Ladder*

Gel C: Lane 2 to 4: Biris Control; Lane 5 to 7: Biris Treated: Lane 1 DNA Ladder*

Gel C: Lane 8 to 10: MR219 Control; Lane 11 to 13: MR219 Treated

*DNA Ladder= Bioline HyperLadder™ 1kb

Table 14 Quality and quantity of RNA extracts.

Varieties	Conditions	Elution Volume(µL)	Concentration(ng/µL)	260/280	260/230
Bario	Control	35	607.30 ± 60.5	2.10	4.79
	Treated	35	684.61 ± 104.6	2.12	2.82
Bajong	Control	35	400.77 ± 185.1	2.06	1.86
	Treated	35	482.41 ± 34.3	2.08	3.04
Biris	Control	35	154.07 ± 41.3	1.98	1.62
	Treated	35	178.65 ± 29.2	2.06	1.43
MR219	Control	35	375.10 ± 35.1	2.21	1.91
	Treated	35	181.40 ± 18.0	2.06	2.39

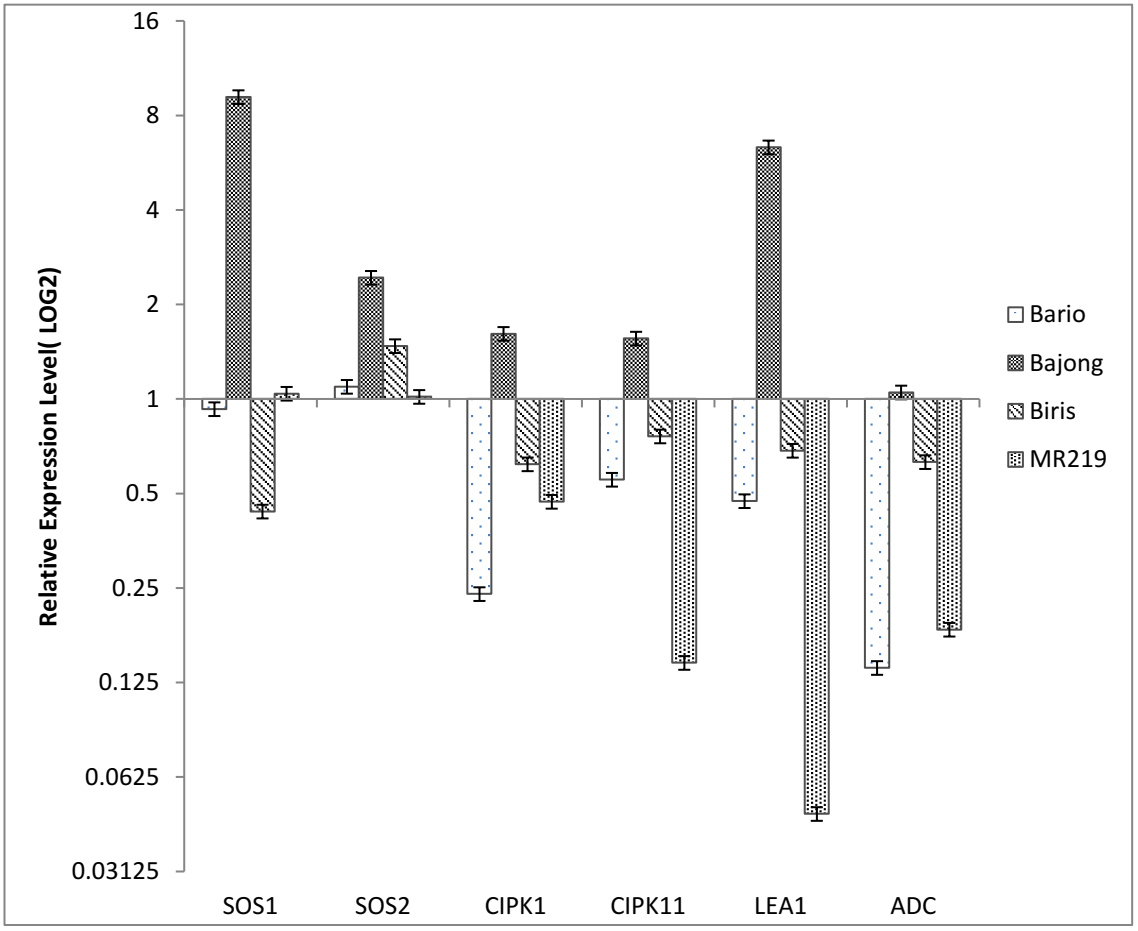


Figure 14 Relative gene expression levels of several salinity-responsive pathway genes. Samples were treated with 100mM of NaCl for 6 hours. Data represent the mean ± standard error of 5%.

4.5 Discussion

Salinity tolerance is a multi-factorial phenomenon, which involved complex physiological and biochemical traits such as salt exclusion, ions compartmentation, early triggering of transcription factors and the active accumulation of competitive metabolite (Sahi et al. 2006). Organisms have developed many different methods of maintaining their ion concentrations within the cytoplasm. In a normal growing condition, the concentration of intracellular Na^+ cannot be higher than 1mM (Sharma et al. 2012) and any excess of Na^+ needs to be excluded or sequestered into the vacuolar compartment of the cell. The overexpression of a vacuolar Na^+/H^+ antiporter protein (SOS1) has shown to increase the salt tolerances of *Arabidopsis* (Shi et al. 2000).

In this study, the expression of SOS1 and SOS2 were examined. Among all the varieties, Bajong had increased expression levels of both SOS1 and SOS2, while Biris had increased expression of SOS2 only. This elevation could indicate that these varieties are responsive towards the sudden increase in environmental salinity and could be a potential candidate for further investigation.

Meanwhile, many plants respond to hostile environments by intensifying the expression of Calcineurin B-Like Protein-Interacting Protein Kinases (CIPKs) pathway (Mallikarjuna et al. 2011). In this study, we have observed elevated expressions of CIPK1 and CIPK11 in Bajong samples and repressions of CIPK1 and CIPK11 in Bario, Biris and MR219. Concerning results obtained from Section 3.5.2.1, Bajong had shown a decrease in Ca^{2+} concentration when exposed to increasing levels of salinity stress, while Bario had shown an increase in Ca^{2+} concentration. The reason for such correlation is unclear and in need further investigation.

LEA proteins are known to act as the “hydration buffer agent” in the cell (Hand et al. 2011). Thus, LEA proteins can retard water loss in times of dehydration and the over-production of LEA proteins might help plants to encounter salinity stress (Duan & Cai 2012; Xiao et al. 2007). It is speculated that the overexpression of LEA1 in Bajong might indicate the accumulation of LEA protein molecules in the plants, which is in accordance with the results we obtained in Section 3.4.1. Bajong had shown the highest increase in RWC after low (100mM) salinity stress induction when compared to Bario, Biris and MR219. After medium (250mM) salinity stress treatment, Bajong was able to maintain a comparable level of RWC, while Bario and MR219 had suffered a

significant decrease in RWC value. Biris had shown a slight decrease in expression levels of LEA1, which was followed by Bario and MR219. Interestingly, Biris had also shown better water retention capability when compared to Bario and MR219. The reason for this phenomenon is unknown and will need further investigation.

Several studies have demonstrated that genetic manipulations of plants that overproduce osmolytes have increased salinity tolerance capability (Ghosh et al. 2012; Groppa & Benavides 2008). Plants that possess the ability of early sensing of stress followed by adequate reactions are also generally more tolerances (Jakab et al. 2005). Thus, early accumulation of polyamine in plants could serve as an indication of salt tolerance properties. Therefore, it was speculated that Bajong would perform better when experiencing salinity stress based on the result obtained. The exact causation of this phenomenon is unclear and in need of further investigation.

4.6 Conclusion

Bajong displayed elevated expression in SOS, CIPK, LEA and putrescence production genes. Having elevated expression of SOS genes might indicate that Bajong comprises mechanism activated for Na^+ translocation. The elevated expression in CIPK might indicate the activation of calcium signal cascade and many other counteractions against osmotic stress. The elevated expression of LEA and polyamine might be due to the synthesis of osmolytes in the plants. Meanwhile, Bario, Biris and MR219 have repressed expression in SOS, CIPK, LEA and putrescence production genes, but the reason for such phenomenon is unknown and need further investigation. However, the result indicates Bajong is more responsive to salinity stress and thus is the most suitable candidate for transcriptomic studies.

Chapter 5 Transcriptomic Studies of Salinity Stress in Sarawak Rice Using RNA-Seq

5.1 Executive Summary

In the past decade, the advancement in sequencing technology has allowed researchers to identify a broad range of stress-inducible genes in a single experiment. This breakthrough has made a significant impact on the understanding of salinity tolerance in several crop varieties such as *Arabidopsis*, rice, wheat and barley. Rice is one of the most salt sensitive cereal crops in the world. The small genome size (480Mb) in rice relatively to wheat (17 GB) or barley (5.1 GB) has made it the model plant for salinity stress study. Currently, it has one of the richest genomic backgrounds available in the stated area. Incorporating advanced technology such as massive multiple parallel sequencing can have many benefits. Most importantly, it can generate extremely detailed information on the transcriptome and allowed the identification of many salinity-responsive pathways at a reasonable cost. Thus, the aim of this chapter was to examine the changes in the transcriptomic profile of Bajong rice after the salinity stress treatment. Bajong is chosen in this study as the candidate variety due to the elevated expression of salt-inducible genes upon salinity exposure as stated in Chapter 4. The data obtained from transcriptomic sequencing are used to identify any potential genes that could contribute to the salinity stress tolerance in Bajong.

In this chapter, the transcriptomic sequence of Bajong together with a list of salinity-responsive genes is reported together with the discussion of the outcome obtained.

5.2 Research Aims and Objectives

The aim of this chapter was to identify the differences in the transcriptomic profile of Bajong rice after salinity stress exposure. With the purpose of completing the objectives above, research works in this chapter were designed to accomplish the following objectives:

i. Examine the changes in transcriptome during salinity stress with the use of RNA-Seq technology.
ii. Examine and identify a board spectrum of salinity-responsive genes in Sarawak local rice variety, Bajong.

iii. Identify any potential pathways that were most responsive towards salinity stress.

5.3 Methods and Material

5.3.1 Sample Preparation and RNA Extraction

Rice seedlings were cultivated in condition as described in Section 3.4.1 for 14 days and were exposed to 100mM of NaCl for exactly 6 hours. The plants were harvested and submerged immediately in liquid nitrogen. In order to reduce matrix effects, 5 individual plants were used for each extraction. RNA was extracted using the method described as in Section 4.3.1. Briefly, total RNA was isolated from seedling tissue using QIAGEN RNeasy kit (Qiagen, Valencia, CA) and purified using the RNase-Free DNase Set (Qiagen, Valencia, CA) following manufacturer instructions. The quality and integrity of each RNA extracts were checked using a Synergy 2 Multi-Mode Reader (Bio-Tek Instruments, Inc., USA). The integrity of the RNA was checked using Bioanalyzer 2100 (Agilent Technologies Inc., USA) and Agilent RNA 6000 Nano Kit (Agilent Technologies Inc., USA). All steps were performed according to manufacturer's instruction. All RNA extracts were then double-checked using gel electrophoresis technique; employing 1.5%, agarose gel, 100V running voltage and 60 minutes separation time. Then, the extracts were preserved in dry ice and shipped to BGI Co. Ltd. (Hong Kong) for library preparation, DNA sequencing and bioinformatics analysis. BGI Co. Ltd. was chosen in this study as the sole service provider as they are one of the world's leading genetics research centre and genomics, proteomics and bioinformatics analyses in the field. They have published more than 1500 research papers with an average of 61 citations per articles, many of papers are also in top-tier journals such as Nature and Science (BGI 2016a). Their services for RNA-Seq and bioinformatics analysis have also been certified by both Agilent and Illumina (BGI 2016b).

5.3.2 Preparation of cDNA library and Massive Multiple Parallel Sequencing

Steps below were all conducted by BGI Co. Ltd (http://www.genomics.cn/index). The total RNA from the previous step was first treated with DNase-I and the mRNA was isolated using Oligo-dT beads. Random hexamers were then utilised for the synthesis of cDNA fragments from the mRNA templates obtained from the previous step and fragmented into short sequences (100bp) suitable for sequencing. Short fragments were

resolved with EB buffer for end repairing and ligated with adapter suitable for PCR amplification. Finally, their quality and quantity of the library were checked with Agilent 2100 Bio-analyser and ABI Step One Plus Real-Time PCR System before proceeding to the sequencing cycle. The sequencing was conducted using Illumina HiSeq 4000 platform. The schematic overview of the bioinformatics pipeline is summarised in Figure 15 and all software utilised in this study are summarised in Table 15. All parameters are set as default unless stated.

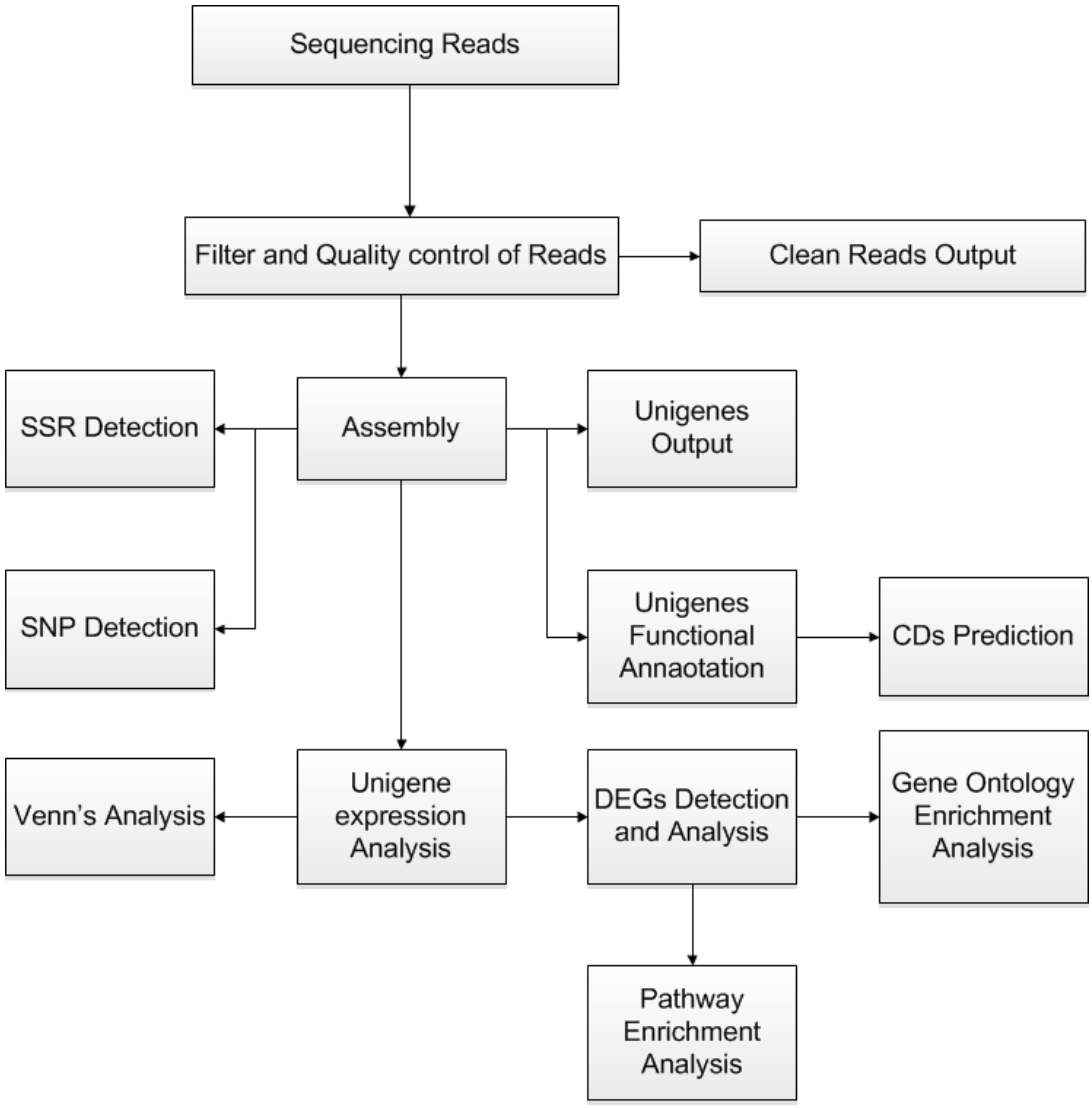


Figure 15 Bioinformatics analysis pipeline for RNA-Seq.

5.3.3 Quality Control and Filtering of Sequencing Reads

Reads containing low quality (Phred score <10), adaptor sequences, or high content of unknown base (N) were removed using the steps below. First, all adaptors sequences were eliminated from the data, then reads that contains more than 5% of unknown bases (N) were removed. Next, Trinity software (Grabherr et al. 2011) was used to assemble the clean reads into individual contigs and "TIGR Gene Indices clustering tools" (TGICL) software, version 2.0.6, (Pertea et al. 2003) was used for the clustering of transcripts into individual unigenes. Next, the contigs sequences were annotated with several online databases. The sequence of the databases used, in descending order, was RAP-DB, Nt, Nr, GO, COG, KEGG, SwissProt, InterPro and Rfam databases. Unigenes that doesn't align to any of the databases above were predicted using ESTSCAN software with Blast-predicted CDS. The minimum contig length was set as 150bp in Trinity while the rest of the parameters were set as default throughout the analysis. Micro Satellite identification tool (MSA) was utilised for the detection of SSR markers while Pimer3Plus software was then used for the design of SSR marker. Next, the SNP within the reads were called using Genome Analysis Toolkit (GATK) software and recorded in the variant call format (VCF) format after filtering.

After mapping the clean reads to unigenes using Bowtie2 software, the expression levels of unigenes were then calculated using "RNA-Seq by Expectation-Maximization" (RSEM) software. Finally, individual DEGs have been computed using Poisson-Dis software based on the methodology as described by Audic and Claverie (1997). The fold change was kept at a minimum of 1.2 while the FDR was maintained at a maximum of 0.001. MA plot and Volcano Plot of the DEGs were generated using RStudio, using default parameters. KEGG database was used for pathway analysis using Blast2GO software. The MA plot and Volcano plot was constructed in RStudio using the method by Ritchie et al. (2015), utilising *limma* package from Bioconductor.

Table 15 Software employed for bioinformatics analyses in this study.

Software	**Version**	**Website**
Trinity	v2.0.6	https://trinityRNAseq.github.io/
TGICL	v1.0	http://compbio.dfci.harvard.edu/tgi/
Blast	v2.2.23	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Blast2GO	v2.5.0	https://www.blast2go.com
InterProScan5	v5.11	https://code.google.com/p/interproscan/wiki/Introduction
ESTScan	v3.0.2	http://sourceforge.net/projects/estscan
MISA	v1.0	http://pgrc.ipk-gatersleben.de/misa
Primer3	v2.2.2	website: http://bioinfo.ut.ee/primer3
GATK	v3.4.0	https://www.broadinstitute.org/gatk
Bowtie	v2.1.0	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
RSEM	v1.2.12	http://deweylab.biostat.wisc.edu/rsem
RStudio	V3.2.4	https://www.rstudio.com/
		https://cran.r-project.org/bin/windows/base/

5.4 Results

5.4.1 Quality of RNA Extracts

The RNA Gel is attached as Figure 16 as below. The amount of RNA in the control sample is 1.66µg and while the amount of RNA in the treated sample is 1.25µg. Both RNA had obtained a Class A rating, which indicates the samples were in good integrity when it reaches the service provider.

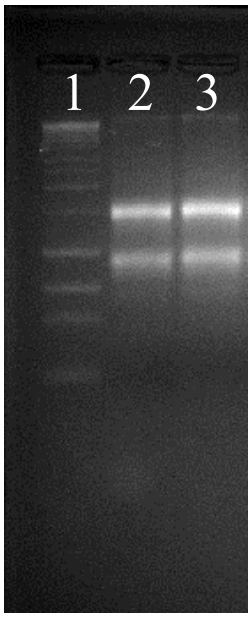


Figure 16 Electrophoresis results for RNA extracts.

Lane 1: Bioline 1Kb Hyperladder, Lane 2: Bajong Control, Lane 3: Bajong Stressed

Table 16 Quality control results for RNA extracts.

Name of Variety	Bajong Control	Bajong Stressed
Concentration (ng/µL)	64.00	50.00
Volume (µL)	26.00	25.00
Total Mass(µg)	1.66	1.25
RIN Number	8.50	8.70
28S/18S	1.40	1.50
OD260/280	1.99	1.90
OD260/230	1.73	1.30
Test Results	Level A*	Level A*

* Level A indicates the sample is qualified and the amount of RNA satisfies at least two library construction

**Results obtained from service provider

5.4.2 Filtering and Analysis of Sequencing Reads

The size of the raw output from the sequencing run was 13.21GB for both samples. The sequencing reads were the filtered and had defects removed. The control sample contained 66.99MB of reads, occupying 6.66GB, while the stressed sample contained 65.86MB of reads, occupying 6.55GB. The reads quality and metrics were shown in Table 17 while the distribution of base content and quality are summarised in the appendices (Figure A-1, Figure A-2, Figure A-3 and Figure A-4).

Table 17 Quality of reads from control and stressed sample.

Parameters	Control Sample	Stressed Sample
Number of Total Raw Reads (Mb)	66.99	65.86
Number of Total Clean Reads (Mb)	66.63	65.52
Size of Total Clean Bases (Gb)	6.66	6.55
Clean Reads Q20 (%)	97.40	98.09
Clean Reads Q30 (%)	93.23	94.99
Clean Reads Ratio (%)	99.46	99.48

5.4.3 De Novo Assembly of Clean Reads

After filtering and quality checking, the reads were fed into Trinity software to be assembled. The quality metrics of the transcripts are summarised in Table 18. The control sample had produced 71,121 transcripts, with a total length of 68,193,430nt. The average transcripts length was 945nt with a GC content of 48.8%. The stressed sample produced 70,926 transcripts, with a total length of 67,924,611nt. The average transcripts length was 957nt with a GC content of 48.8%. Next, all sequences were load into TGICL software for the clustering analysis. The quality metrics of the unigenes had been summarised in Table 19. The control sample contained 55,009 transcripts with a total length of 59,127,769nt. The average size of the unigenes was 1,074nt, with a GC content of 48.83%. The stressed sample contained 54.346 transcripts with a total length of 59,105,296nt. The average size of the unigenes was 1087nt, with a GC content of 48.83%. Combining both samples, the total numbers of unigenes were 61,316, with a total non-overlapping length of 73,588,661nt. A schematic previous of the distribution of Contig length in the stressed and control sample have been summarised in Figure A-5 and Figure A-6 in the appendices as attached.

Table 18 Quality metrics of transcripts.

Parameters	Control Sample	Stressed Sample
Total Number of Contigs	72,121	70,926
Total Length of Contigs (nt)	68,193,430	67,924,611
Mean Length of Contigs (nt)	945	957
Q50	1,683	1,703
Q70	1,038	1,065
Q90	363	369
GC Content (%)	48.8	48.8

Q50 represented a weighted median statistic that at least 50% of the total length contained in transcripts was great than or equal to this value. GC (%) was calculated based on the percentage of G and C bases in all transcripts.

Table 19 Quality metrics of unigenes.

Parameters	Control Sample	Stressed Sample	Combined
Total Number of Unigenes	55,009	54,346	61,316
Total Length (nt)	59,127,769	59,105,296	73,588,661
Mean Length (nt)	1,074	1,087	1,200
Q50	1,780	1,796	1,931
Q70	1,168	1,195	1,326
Q90	441	450	537
GC Content (%)	48.83	48.83	48.61

Q50 represented a weighted median statistic that at least 50% of the total length contained in transcripts was great than or equal to this value. GC (%) was calculated based on the percentage of G and C bases in all transcripts.

5.4.4 Annotation and Identification of Transcripts

The transcripts were then mapped to several databases to find matches. In this study, 7 databases were used in this study (Nt, Nr, GO, COG, KEGG, Swissprot and Interpro). The annotation summary was shown in Table 20. Around 99.75% of the total discovered unigenes had been mapped. A total of 61,302 unigenes had been mapped using Nt databases, representing 99.54% of the total unigenes discovered (Table 21). The annotated results using various databases are summarised in Figure 17, Figure 18, Figure 19, and Figure 20 as attached. The number of overlapping unigenes between Nr, COG, KEGG, swissplot and InterPro databases had also been arranged into a Venn's diagram (Figure A-8).

Table 20 Summary of functional annotation.

Name of Databases	**Number of Annotated Unigenes**	**Percentage**
Nr-Annotated	46,657	76.09%
Nt-Annotated	61.302	99.54%
SwissProt-Annotated	32.206	52.52%
KEGG-Annotated	30.118	49.12%
COG-Annotated	21,016	34.27%
Interpro-Annotated	27,808	45.35%
GO-Annotated	33,288	54.28%
Total annotated unigenes	61.164	99.75%
Total Unigenes	**63,316**	**100.00%**

Table 21 Summary of predicted CDs using the ESTSCAN software.

Parameters	**BLAST**	**ESTSCAN**	**Overall**
Total number of unmapped unigenes	5,543	657	46,200
Total length of unigenes (nt)	39,527,487	189,795	39,717,282
Mean length (nt)	867	288	859
GC (%)	52.41	57.47	52.44

Data from COG annotation databases had revealed that a significant amount of unigenes is responsible for the general function of the cell, with 8,433 genes in this category. There were 7,684 genes that had unknown functions. A large number of genes were also involved in the translation, ribosomal and biogenesis of the cells, with 6,317 genes in the stated category. Furthermore, it had revealed that a significant number of the genes are responsible for the biogenesis of cell wall/membrane/envelope, with 5,358 genes taking part in the above-mention activity. Investigation of functional distribution in unigenes using GO annotation database had revealed a similar pattern. Most unigenes belongs to the "cellular component" category of the GO database, with more than 23,399 genes in the "cell part" and "cell" categories. A large number of genes also belong to the "organelle" category of the database. Furthermore, 16,969 and 15,688 genes were found to take part in the metabolic and cellular process of the cell.

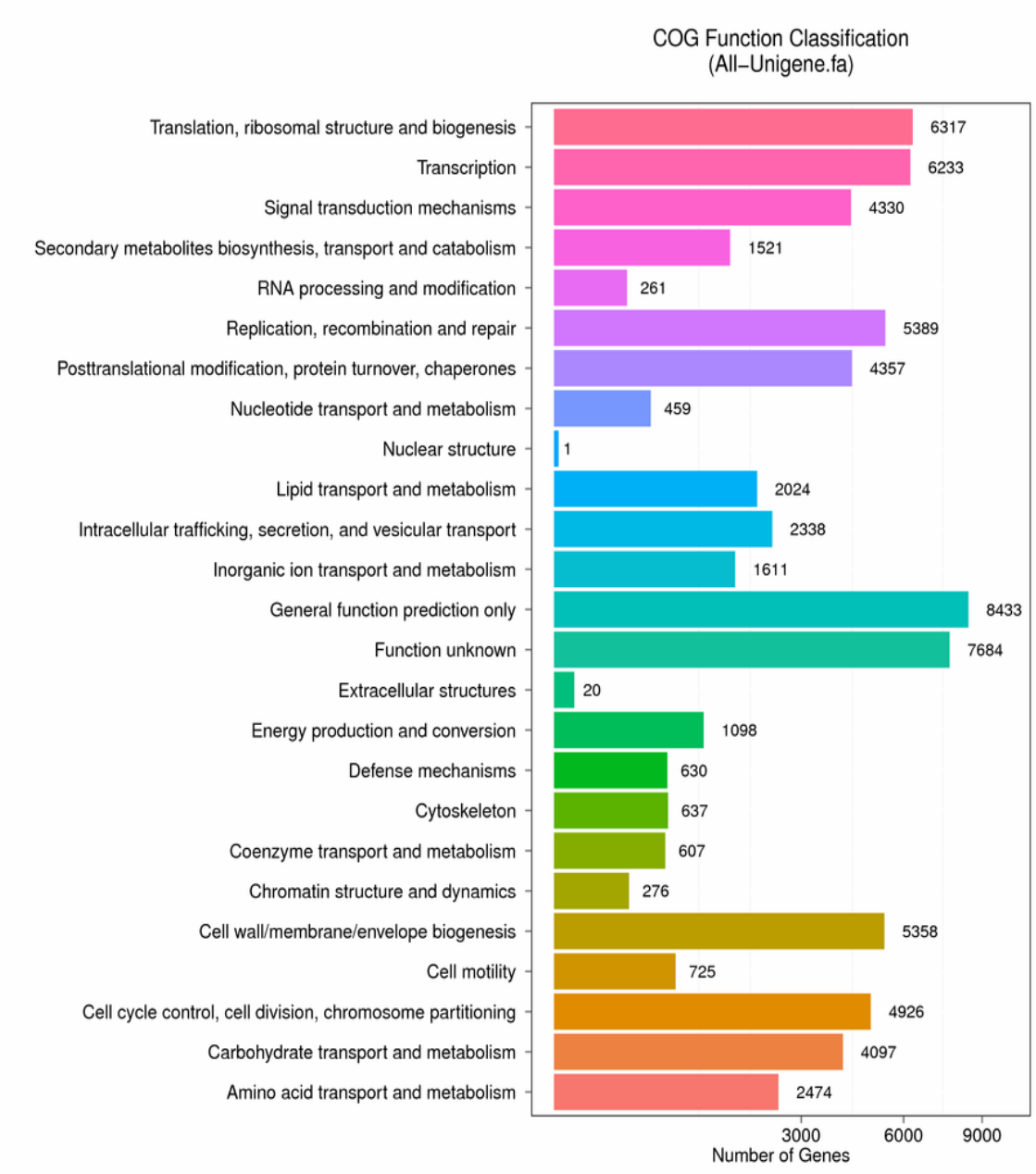


Figure 17 Functional distribution of COG annotation of all mapped unigenes.

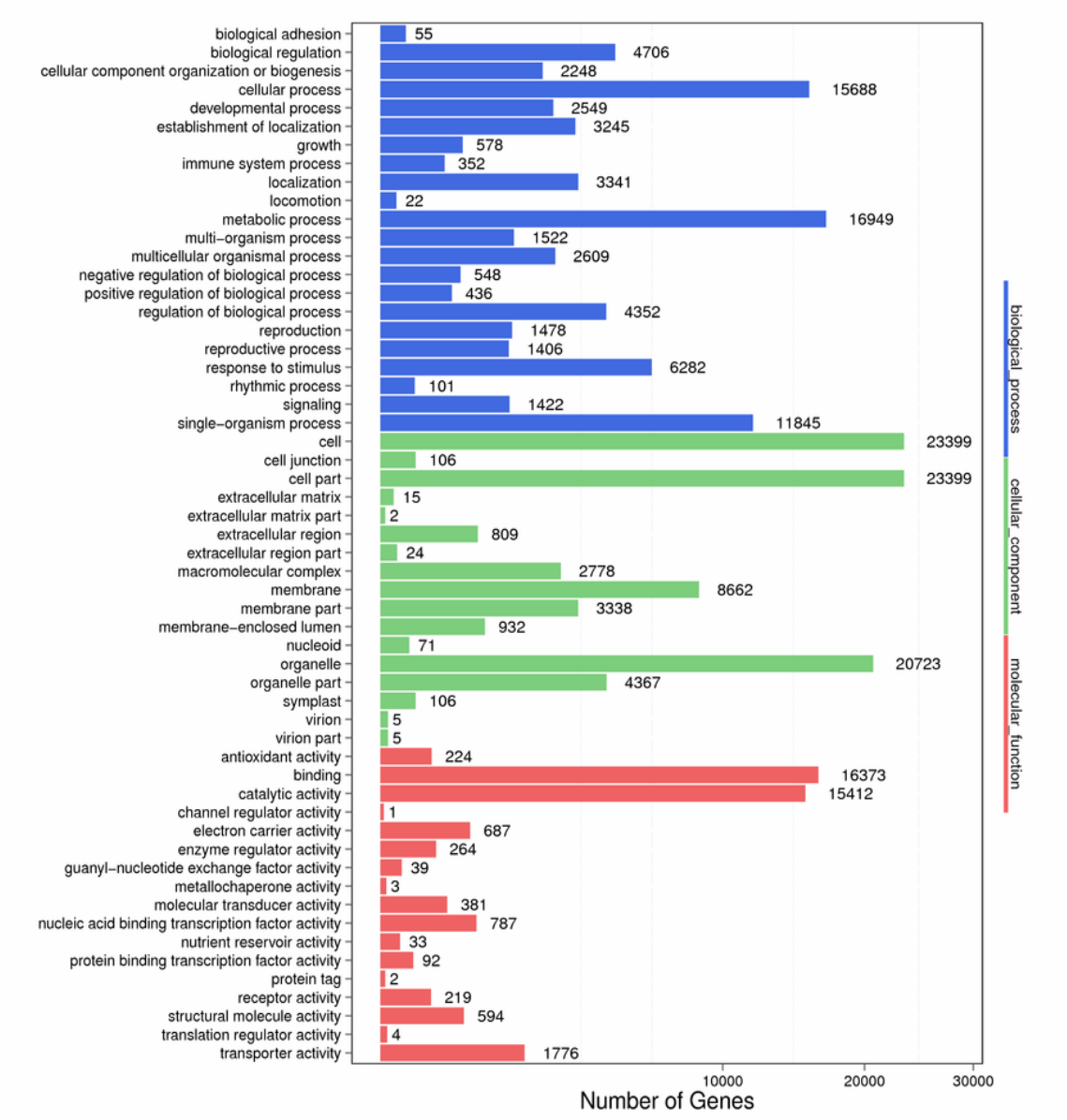


Figure 18 Functional distribution of GO annotation of the mapped unigenes.

Investigation of functional distribution of DEGs using KEGG database had revealed that a large number of unigenes were responsible for the environmental adaptation of the plants. We had also found that 3915, 4253 and 2143 unigenes were responsible for the transport and catabolism activity, lipid metabolism and environmental adaptation metabolism of the cell.

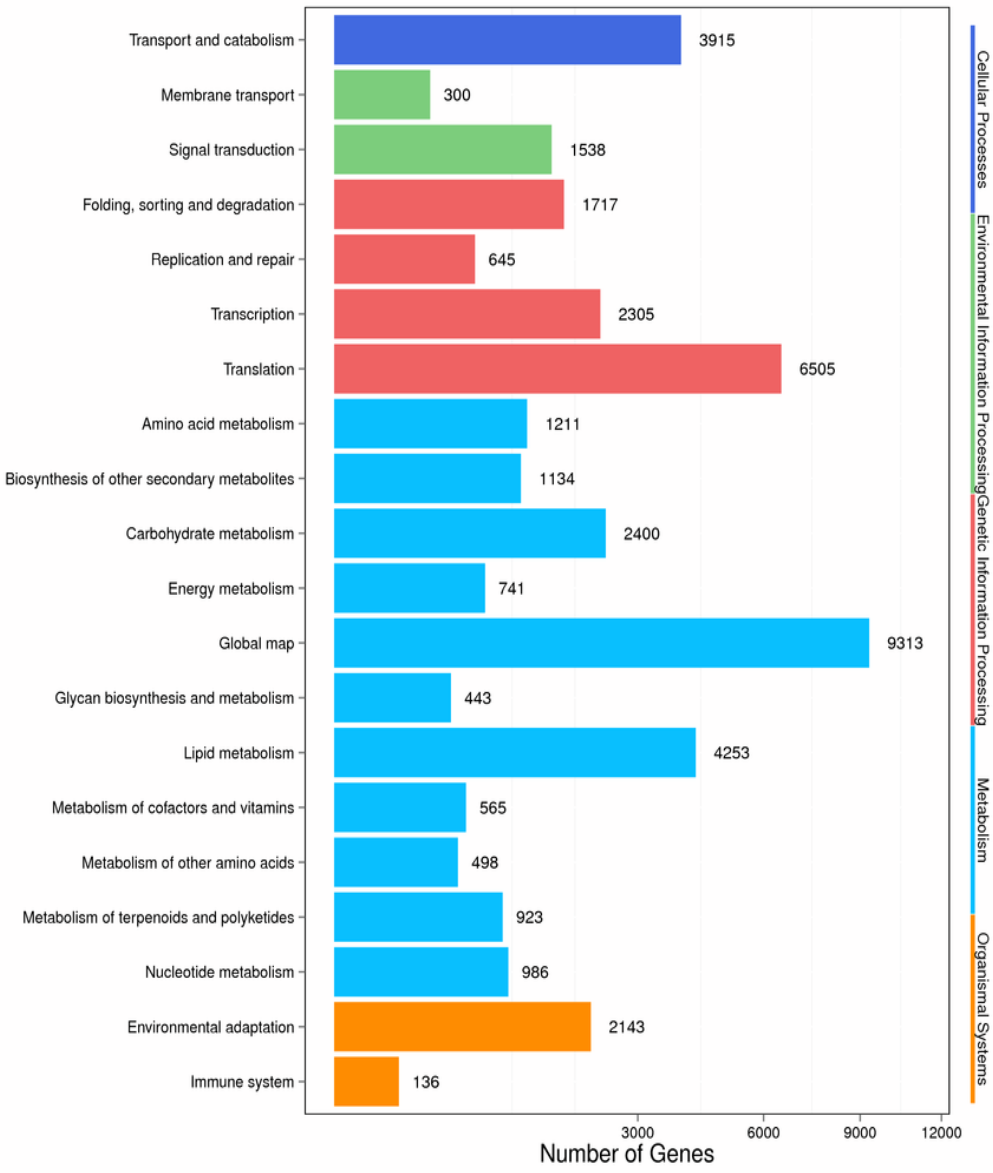


Figure 19 Functional distribution of KEGG annotation of all mapped unigenes.

The data also revealed that more than 75% of the unigenes were best mapped onto genes originated from *Oryza sativa japonica* while 19% of the unigenes were best mapped onto genes originated from *Oryza sativa indica*. A small portion of the unigenes was found to be similar to genes originated from *Oryza brachyantha* while 4.51% of the unigenes did not match any rice species present in the databases.

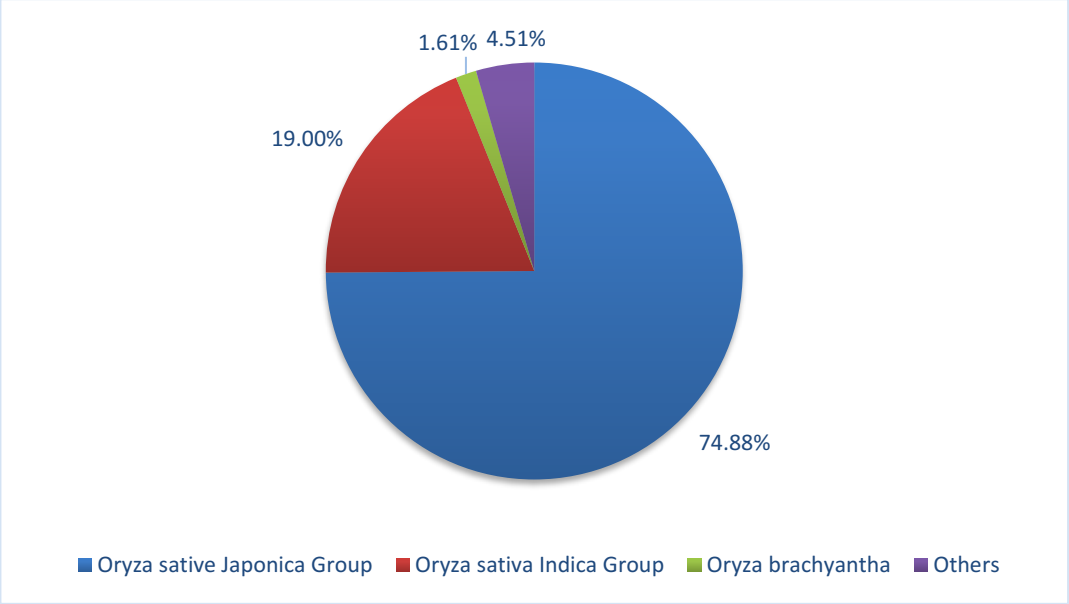


Figure 20 Distribution of annotated species from all unigenes based on Blastn results obtained.

After the assembly, the SSR within the samples were detected and suitable primers were designed. The size summary and the size distributions of SSR are summarised in Figure 21 and Table A-2 in the appendices attached. The SNP variant was called using GATK software and the results are summarised in Figure 22 as follows.

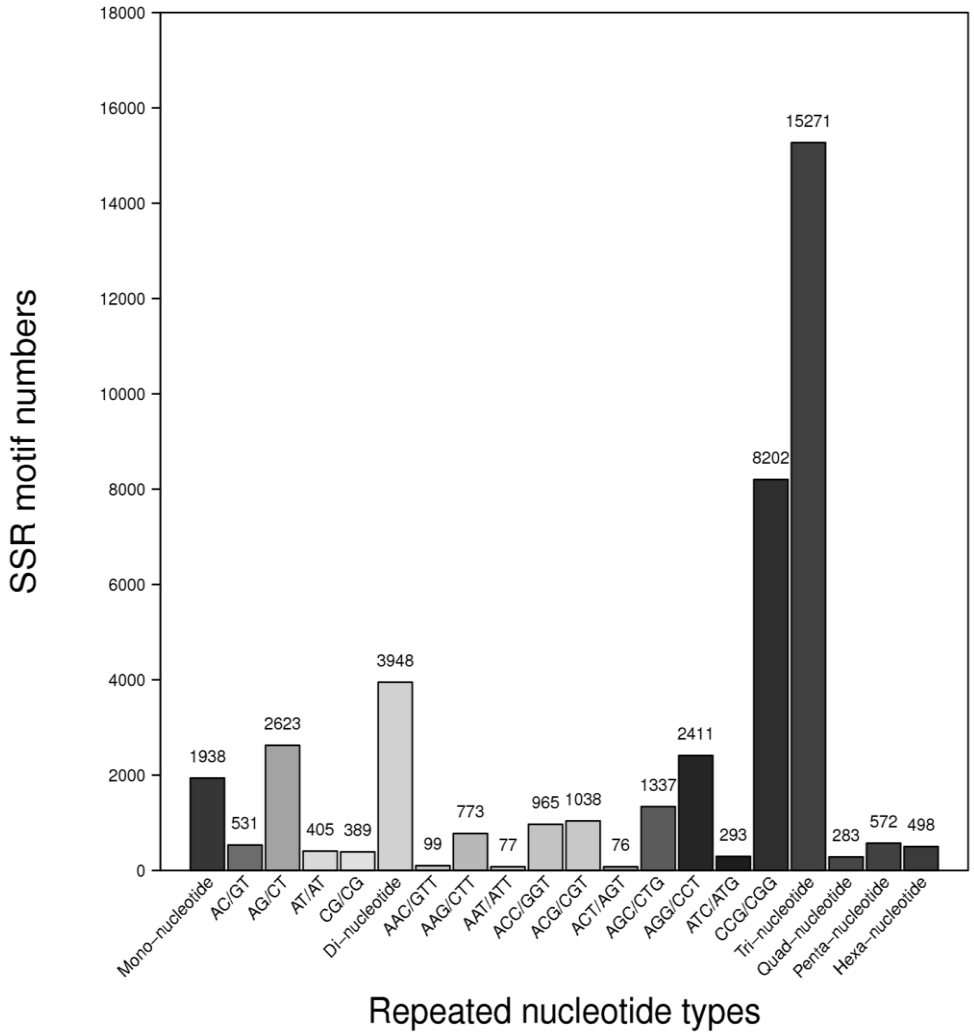


Figure 21 Type and size distribution of SSR.

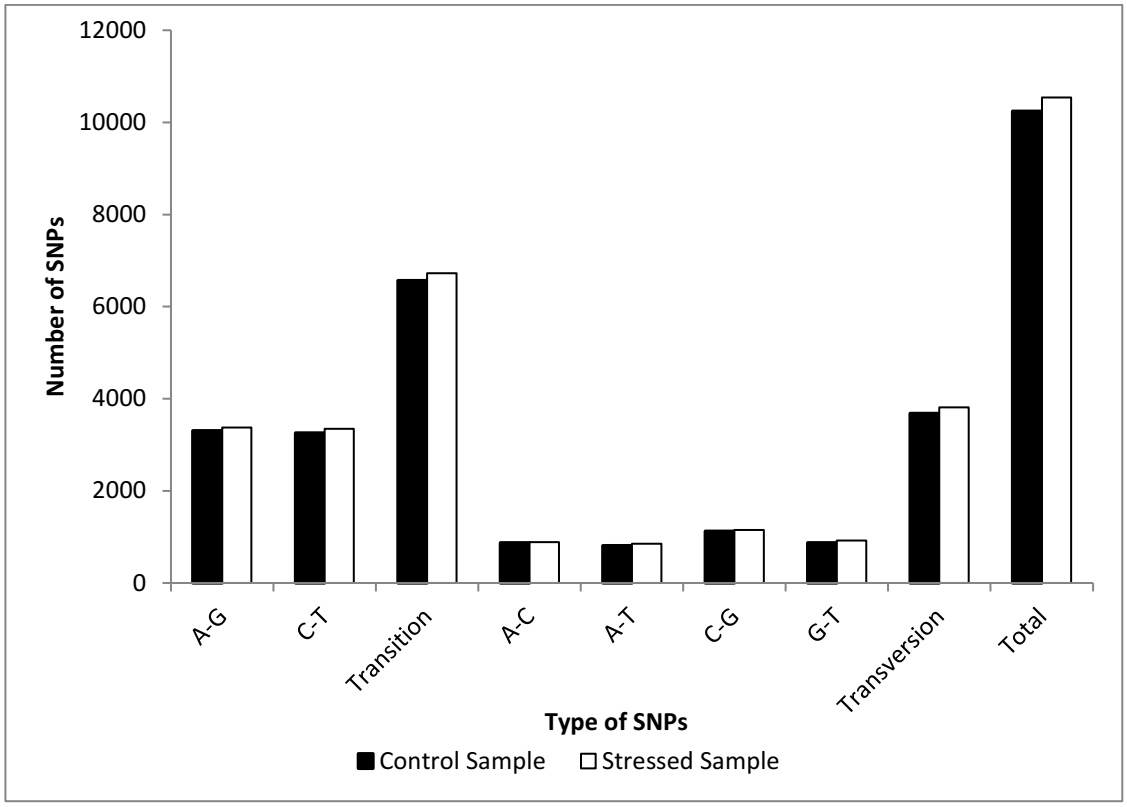


Figure 22 Distribution of SNP variants.

5.4.5 DEGs Analysis

In this study, we have discovered 1,976 unigenes that had been upregulated and 2,048 unigenes that had been downregulated in the stressed sample. The expressed level versus fold changes is summarised in a volcano plot (Figure 23) while significances of transcripts versus fold changes had been summed up in an MA plot the (Figure 24). The patterns of the DEGs on MA plot and Volcano plot indicates accurate normalisation of the data

A list of 20th most upregulated and downregulated DEGs has also been attached (Table 22). No obvious trend can be observed from all the upregulated and downregulated genes. The closest hit for the first, second, third and fifth most upregulated genes (CL2272.Contig7_All, CL4527.Contig1_All, CL2272.Contig4_All and Unigene22754_All) were best mapped towards genes from varieties' other than rice. Meanwhile, all down regulated genes but one (CL7571.Contig1_All) were found to be originated from *Oryza Sativa* species. Interestingly, all of the unknown DEGs mentioned above were found to be similar to RF02543 in the Rfam database, which

codes for the large subunit ribosomal RNA of the plants. Another DEGs with the ID CL7640.Contig8_All had been found to code for MIR1846, which is a micro-RNA found to be involved in the stress regulation of rice seedlings during arsenic (Pandey et al. 2015) and low nitrogen (Nischal et al. 2012) stresses.

One DEG named CL6647.Contig2_All was found to be similar to a "Ubiquitin Ligase AtAIRP3" from *Arabidopsis* thaliana. This protein was previously identified to be involved in the active regulation of high salt and drought stress in the plants (Kim & Kim 2013). By using a yeast hybrid assay, they had identified that this protein is a "positive regulator of the ABA-mediated drought and salt stress tolerance mechanism via the ubiquitination of RD21", which plays an important role in the "drought stress response and amino acid transport in *Arabidopsis*". This indicates that this transcript can be utilised as a potential marker for any marker selection studies in the future.

Next, the DEGs were filtered for their involvement in salt tolerance based on the locus description. Using the keyword "salt", we had discovered 15 DEGs that could contribute towards the plant's salt tolerance properties. The unigenes ID and locus description of these DEGs have been summarised in Table 23. The descriptions include "putative low temperature and salt responsive protein", "salt tolerance protein", "salt stress root protein RS1" and "membrane-associated salt inducible protein" while the fold change (presented in log2) expression of these DEGs ranged from 2.17 (upregulation) to -2.54 (downregulation). In addition, 6 unigenes were found to be part of the OsRCI2 gene family, homologs of a protein family responsible for the low temperature and salt response of the *Arabidopsis* plant (Medina, Ballesteros & Salinas 2007) .

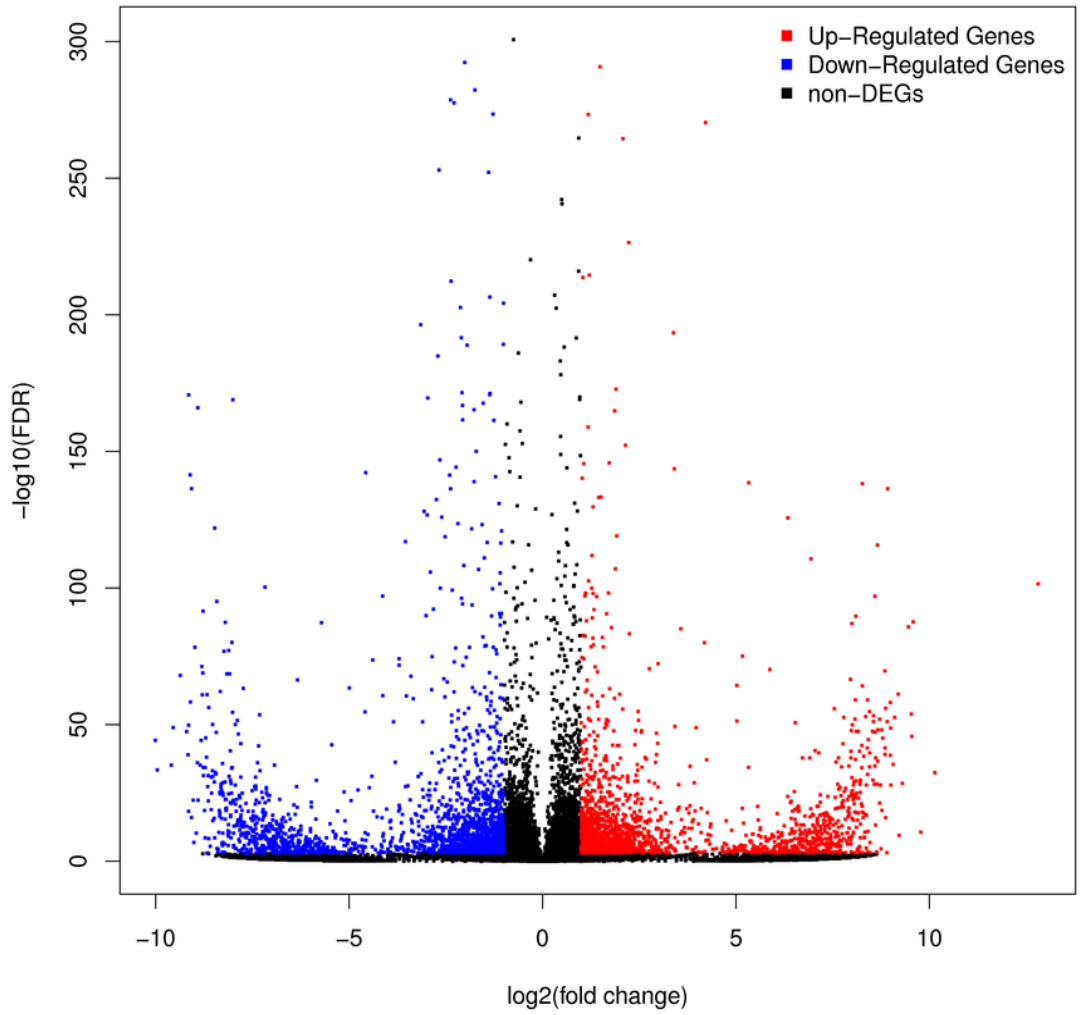


Figure 23 Volcano plot of DEGs.

Y-axis represents the significance of data after $-LOG_{10}$ transformation while X-axis represents fold changes of DEGs after LOG_2 transformation. Red points represent upregulated DEGs. Blue points represent downregulated DEGs. Black points represent DEGs without statistically significance changes in expression.

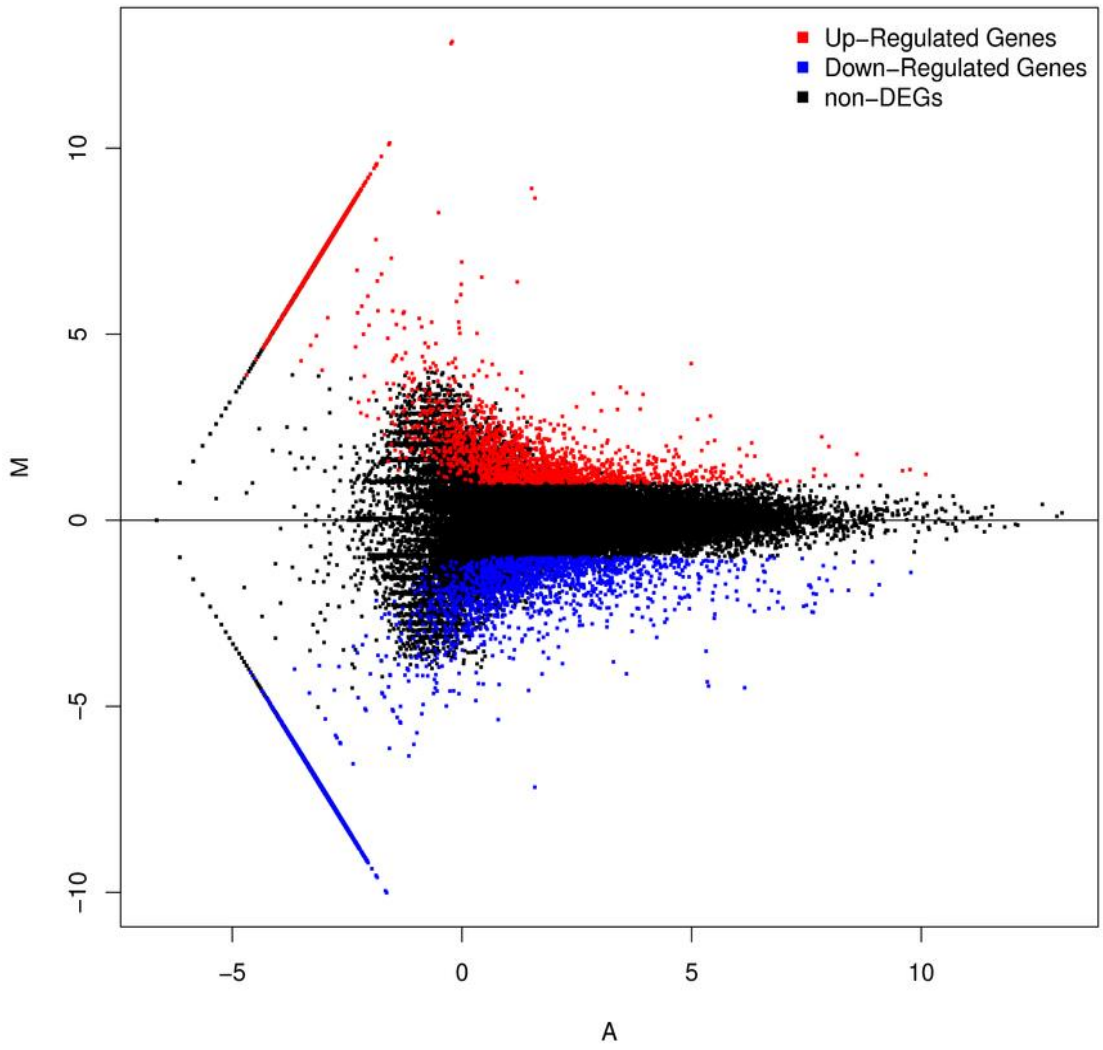


Figure 24 MA plot of DEGs.

The Y-axis represents value A (LOG2 transformed mean expression level) while X-axis represents value M (LOG2 transformed fold change). Red points represent upregulated DEGs. Blue points represent downregulated DEGs. Black points represent DEGs without statistically significance changes in expression.

Table 22 Summary of 20 most up regulated and down regulated DEGs.

					Name of Databases																
						Nr				Nt				Swissprot	KEGG		COG	Interpro	GO	Rfam	
Sequence ID	Length	Regulation	Fold Change (LOG2)	Locus	Locus Description	Identifier Tag	Accession Number	E-Value	Description	Identifier Tag	Accession Number	E-Value	Description			Enzyme Code			Decription	ID	Description
CL2272.Contig7_All	1044	Up	12.86	N/A	N/A	gb	EXC34899.1	3.59E-20	Metal transporter Nramp5 [Morus notabilis]	gb	JQ020079.1	5.00E-71	Pinus taeda isolate 4724 anonymous locus 0_9696_01 genomic sequence	NA	NA	NA	NA	NA	NA	RF02543	LSU_rRNA_eukarya
CL4527.Contig1_All	343	Up	12.81	N/A	N/A	ref	XP_007161040.1	8.12E-13	hypothetical protein PHAVU_001G037800g [Phaseolus vulgaris]	emb	HG737342.1	3.00E-36	Pyrus spinosa chloroplast, isolate PYR002, complete sequence	NA	NA	NA	NA	NA	biological_process:GO:0006810//transport;molecular_function:GO:0016787//hydrolase activity;	RF02543	LSU_rRNA_eukarya
CL2272.Contig4_All	667	Up	10.14	N/A	N/A	dbj	BAJ11784.1	3.36E-20	dehydration responsive protein [Corchorus olitorius]	gb	DQ115326.1	1.00E-178	Gossypium hirsutum strain CNH123 genomic sequence	NA	NA	NA	NA	NA	NA	RF02543	LSU_rRNA_eukarya
CL6647.Contig2_All	11580	Up	10.10	LOC_Os12g24080genomic	HECT-domain domain containing protein, expressed	gb	EEC69178.1	0	hypothetical protein OsI_38149 [Oryza sativa Indica Group]	ref	XM_006663934.1	0	PREDICTED: Oryza brachyantha E3 ubiquitin-protein ligase UPL2-like (LOC102712681), mRNA	sp|Q8H0T4|UPL2_ARATH/0.0/E3 ubiquitin-protein ligase UPL2 OS=*Arabidopsis* thaliana GN=UPL2 PE=1 SV=3	bdi:100821130/0.0/E3 ubiquitin-protein ligase UPL1-like; K10592 E3 ubiquitin-protein ligase HUWE1	[EC:6.3.2.19]	SPAC19D5.04/1e-138/---	PF14377/1.9E-30/Domain of unknown function (DUF4414)	biological_process:GO:0016567//protein ubiquitination;cellular_component:GO:0005622//intracellular;molecular_function:GO:0004842//ubiquitin-protein transferase activity;	N/A	N/A
Unigene22754_All	325	Up	9.78	N/A	N/A	ref	XP_007161040.1	4.85E-10	hypothetical protein PHAVU_001G037800g [Phaseolus vulgaris]	gb	GQ252834.1	1.00E-44	Phyllostachys edulis clone 00077 genomic sequence	NA	NA	NA	NA	NA	biological_process:GO:0006810//transport;molecular_function:GO:0016787//hydrolase activity;	RF02543	LSU_rRNA_eukarya

CL3038.Contig5_All	2447	Up	9.58	LOC_Os01g04900genomic	peptidase M50 family protein, putative, expressed	sp	B8AD72.1	0	RecName: Full=Probable zinc metalloprotease EGY2, chloroplastic; AltName: Full=Protein ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN 2; Flags: Precursor [Oryza sativa Indica Group]	ref	NM_001048524.1	0	Oryza sativa Japonica Group Os01g0142100 (Os01g0142100) mRNA, complete cds	sp|B8AD72|EGY2_ORYSI/0.0/Probable zinc metalloprotease EGY2, chloroplastic OS=Oryza sativa subsp. indica GN=EGY2 PE=3 SV=1	NA	NA	alr2114/1e-63/---	PTHR31412/0.0/--	biological_process:GO:0006508//proteolysis;cellular_component:GO:0009535//chloroplast thylakoid membrane;molecular_function:GO:0004222//metalloendopeptidase activity;	N/A	N/A
CL184.Contig3_All	1355	Up	9.55	LOC_Os01g28989genomic	expressed protein	gb	EEC70696.1	3.35E-43	hypothetical protein Osl_02051 [Oryza sativa Indica Group]	ref	NM_001185426.1	0	Oryza sativa Japonica Group Os01g0386700 (Os01g0386700) mRNA, complete cds	sp|Q944A7|Y4523_ARATH/7e-12/Probable serine/threonine-protein kinase At4g35230 OS=*Arabidopsis* thaliana GN=At4g35230 PE=1 SV=1	mtr:MTR_5g098970/5e-12/Receptor like protein kinase; K14500 BR-signaling kinase	[EC:2.7.11.1]	NA	NA	biological_process:GO:0006468//protein phosphorylation;molecular_function:GO:0005524//ATP binding;GO:0004672//protein kinase activity;	N/A	N/A
Unigene22568_All	1589	Up	9.53	LOC_Os02g45490genomic	expressed protein	gb	EAY87063.1	2.39E-65	hypothetical protein Osl_08460 [Oryza sativa Indica Group]	ref	NM_001054265.1	0	Oryza sativa Japonica Group Os02g0677800 (Os02g0677800) mRNA, partial cds	sp|Q69UI2|RS131_ORYSJ/2e-64/40S ribosomal protein S13-1 OS=Oryza sativa subsp. japonica GN=Os08g0117200 PE=3 SV=2	osa:4344523/5e-64/Os08g0117200; K02953 small subunit ribosomal protein S13e	NA	SPAC6F6.07c/2e-45/---	PF08069/5.3E-30/Ribosomal S13/S15 N-terminal domain	biological_process:GO:0010090//trichome morphogenesis;GO:0009965//leaf morphogenesis;GO:0000911//cytokinesis by cell plate formation;GO:0006412//translation;cellular_component:GO:0016020//membrane;GO:0005763//mitochondrial small ribosomal subunit;GO:0005618//cell wall;GO:0005730//nucleolus;GO:0009507//chloroplast;GO:0022626//cytosolic ribosome;molecular_function:GO:0003735//structural constituent of ribosome;GO:0019843//rRNA binding;	N/A	N/A
CL5412.Contig2_All	2601	Up	9.46	LOC_Os04g54790genomic	ELMO/CED-12 family protein, putative, expressed	ref	NP_001054036.1	0	Os04g0640500 [Oryza sativa Japonica Group]	ref	NM_001060571.1	0	Oryza sativa Japonica Group Os04g0640500 (Os04g0640500) mRNA, complete cds	sp|Q94BU1|Y1181_ARATH/0.0/Uncharacterized aarF domain-containing protein kinase At1g71810, chloroplastic OS=*Arabidopsis* thaliana GN=At1g71810 PE=2 SV=1	osa:4337164/0.0/Os04g0640500; K08869 aarF domain-containing kinase	NA	sll0005/2e-121/---	PTHR10566/4.0E-212/--	biological_process:GO:0006468//protein phosphorylation;cellular_component:GO:0010287//plastoglobule;molecular_function:GO:0005524//ATP binding;GO:0004672//protein kinase activity;	N/A	N/A
CL4199.Contig1_All	1026	Up	9.30	LOC_Os08g36450genomic	transcription regulator, putative, expressed	dbj	BAD09553.1	0	putative storekeeper protein [Oryza sativa Japonica Group]	dbj	AP006461.3	0	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0104B02	sp|Q9FPQ6|GP1_CHLRE/5e-28/Vegetative cell wall protein gp1 OS=Chlamydomonas reinhardtii GN=GP1 PE=1 SV=1	gmx:100776781/2e-16/probable pectinesterase/pectinesterase inhibitor 25-like; K01051 pectinesterase	[EC:3.1.1.11]	Rv3876/5e-16/---	PTHR31662/2.8E-49/--	cellular_component:GO:0005739//mitochondrion;	N/A	N/A

CL2272.Contig1_All	415	Up	9.22	---NA---		ref	YP_001152214.1	1.50E-08	ORF124 [Pinus koraiensis]	gb	BT132544.1	1.00E-26	Oryza sativa clone RRIBB00557 mRNA sequence	NA	NA	NA	NA	NA	NA	RF0254 3	LSU_rRNA_eukarya
CL5680.Contig2_All	2255	Up	9.19	LOC_Os01g04870genomic	WD domain, G-beta repeat domain containing protein, expressed	dbj	BAB16450.1	0	UV-damaged DNA binding protein 2 [Oryza sativa Japonica Group]	dbj	AK112097.1	0	Oryza sativa Japonica Group cDNA clone:002-108-A04, full insert sequence	sp|Q6NQ88|DDB2_ARATH/0.0/Protein DAMAGED DNA-BINDING 2 OS=*Arabidopsis* thaliana GN=DDB2 PE=1 SV=1	osa:4325482/0.0/Os01g0141700; K10140 DNA damage-binding protein 2	NA	NA	PTHR15169:SF0/1.1E-187/--	biological_process:GO:0006281//DNA repair;GO:0010224//response to UV-B;cellular_component:GO:0005634//nucleus; molecular_function:GO:0008270//zinc ion binding;GO:0003676//nucleic acid binding;	N/A	N/A
CL1416.Contig7_All	2086	Up	9.10	LOC_Os10g02220genomic	peptide transporter PTR2, putative, expressed	gb	ABB46608.2	0	Major Facilitator SuperRfamily protein, expressed [Oryza sativa Japonica Group]	ref	NM_001189077.1	0	Oryza sativa Japonica Group Os10g0110800 (Os10g0110800) mRNA, complete cds	sp|P46032|PTR2_ARATH/2e-150/Protein NRT1/PTR FAMILY 8.3 OS=*Arabidopsis* thaliana GN=NPF8.3 PE=1 SV=1	mtr:MTR_4g015080/3e-133/Peptide transporter PTR1; K14638 solute carrier family 15 (peptide/histidine transporter), member 3/4	NA	ECU11g1050/1e-24/---	PF00854/2.7E-79/POT family	biological_process:GO:0006857//oligopeptide transport;cellular_component:GO:0016021//integral component of membrane;molecular_function:GO:0005215//transporter activity;	N/A	N/A
CL121.Contig12_All	1479	Up	9.06	LOC_Os01g50050genomic	polyprenyl synthetase, putative, expressed	gb	EEC71327.1	2.31E-166	hypothetical protein OsI_03373 [Oryza sativa Indica Group]	ref	NM_001050494.1	0	Oryza sativa Japonica Group Os01g0695300 (Os01g0695300) mRNA, complete cds	sp|P49353|FPPS_MAIZE/4e-126/Farnesyl pyrophosphate synthase OS=Zea mays GN=FPS PE=2 SV=1	zma:100273420/1e-128/uncharacterized LOC100273420; K00787 farnesyl diphosphate synthase	[EC:2.5.1.10]	YJL167w/5e-74/---	PF00348/3.3E-57/Polyprenyl synthetase	biological_process:GO:0045337//farnesyl diphosphate biosynthetic process;GO:0033384//geranyl diphosphate biosynthetic process;GO:0006695//cholesterol biosynthetic process;cellular_component:GO:0005737//cytoplasm;molecular_function:GO:0004337//geranyltranstransferase activity;GO:0046872//metal ion binding;GO:0004161//dimethylallyltranstransferase activity;	N/A	N/A
CL1286.Contig12_All	1233	Up	9.01	LOC_Os02g43090genomic	myristoyl-acyl carrier protein thioesterase, chloroplast precursor, putative, expressed	ref	NP_001068400.1	1.80E-93	Os11g0659500 [Oryza sativa Japonica Group]	dbj	AP005291.3	0	Oryza sativa Japonica Group genomic DNA, chromosome 2, BAC clone:OJ1282_H11	sp|Q9SQI3|FATB_GOSHI/7e-47/Palmitoyl-acyl carrier protein thioesterase, chloroplastic OS=Gossypium hirsutum GN=FATB1 PE=1 SV=1	osa:4351056/9e-95/Os11g0659500; K10781 fatty acyl-ACP thioesterase B	[EC:3.1.2.14 3.1.2.-]	NA	PTHR31727/7.8E-86/--	biological_process:GO:0006633//fatty acid biosynthetic process;cellular_component:GO:0009536//plastid;molecular_function:GO:0016297//acyl-[acyl-carrier-protein] hydrolase activity;	N/A	N/A
Unigene20082_All	2083	Up	9.00	LOC_Os03g08360genomic	3-ketoacyl-CoA synthase 10, putative, expressed	gb	EEE58439.1	0	hypothetical protein OsJ_09660 [Oryza sativa Japonica Group]	gb	AC126223.2	0	Oryza sativa Japonica Group chromosome 3 clone OSJNBb0076N15, complete sequence	sp|Q570B4|KCS10_ARATH/1e-179/3-ketoacyl-CoA synthase 10 OS=*Arabidopsis* thaliana GN=FDH PE=1 SV=2	osa:4332236/0.0/Os03g0245700; K15397 3-ketoacyl-CoA synthase	[EC:2.3.1.-]	DRA0326/6e-13/---	PTHR31561:SF2/0.0/--	biological_process:GO:0009913//epidermal cell differentiation;GO:0000038//very long-chain fatty acid metabolic process;GO:0042335//cuticle development;GO:0009409//response to cold;GO:0009416//response to light stimulus;GO:0010025//wax biosynthetic process;GO:0030497//fatty acid elongation;GO:0080167//response to karrikin;cellular_component:GO:0016020//membrane;GO:0005783//endoplasmic reticulum;GO:0022626//cytosolic ribosome;molecular_function:GO:0009922//fatty acid elongase activity;	N/A	N/A

CL6935.Contig2_All	1678	Up	8.99	LOC_Os12g06490genomic	STE_PAK_Ste20_Slob_Wnk.6 - STE kinases include homologs to sterile 7, sterile 11 and sterile 20 from yeast, expressed	ref	NP_001066222.1	0	Os12g0162100 [Oryza sativa Japonica Group]	ref	NM_001072754.1	0	Oryza sativa Japonica Group Os12g0162100 (Os12g0162100) mRNA, complete cds	sp|Q2QXC6|WNK9_ORYSJ/0.0/Probable serine/threonine-protein kinase WNK9 OS=Oryza sativa subsp. japonica GN=WNK9 PE=2 SV=1	gmx:100301899/2e-91/WNK3; with no lysine kinase; K08867 WNK lysine deficient protein kinase	[EC:2.7.11.1]	YAR019c/3e-21/---	PF00069/2.0E-49/Protein kinase domain	biological_process:GO:0006468//protein phosphorylation;molecular_function:GO:0004674//protein serine/threonine kinase activity;GO:0005524//ATP binding;	N/A	N/A
CL4755.Contig3_All	2470	Up	8.99	LOC_Os02g32530genomic	SAM domain family protein, expressed	ref	NP_001061807.1	2.14E-100	Os08g0416000 [Oryza sativa Japonica Group]	dbj	AP004777.3	0	Oryza sativa Japonica Group genomic DNA, chromosome 2, PAC clone:P0458B05	sp|Q6ZA74|HOX5_ORYSJ/4e-102/Homeobox-leucine zipper protein HOX5 OS=Oryza sativa subsp. japonica GN=HOX5 PE=1 SV=1	gmx:100783566/8e-11/uncharacterized LOC100783566; K14325 RNA-binding protein with serine-rich domain 1	NA	DR2133/1e-07/---	PTHR24326/1.6E-19/--	biological_process:GO:0006355//regulation of transcription, DNA-templated;cellular_component:GO:0005634//nucleus;GO:0005773//vacuole;GO:0016021//integral component of membrane;GO:0005739//mitochondrion;GO:0009941//chloroplast envelope;GO:0005886//plasma membrane;molecular_function:GO:0000976//transcription regulatory region sequence-specific DNA binding;GO:0003700//sequence-specific DNA binding transcription factor activity;	N/A	N/A
CL7475.Contig3_All	506	Up	8.92	LOC_Os04g16722genomic	uncharacterized protein ycf68, putative, expressed	gb	EPS74505.1	8.87E-39	hypothetical protein M569_00222, partial [Genlisea aurea]	gb	EU600086.1	1.00E-146	Pueraria montana var. lobata 16S ribosomal RNA gene, partial sequence	NA	NA	NA	NA	NA	biological_process:GO:0015986//ATP synthesis coupled proton transport;GO:0015991//ATP hydrolysis coupled proton transport;GO:0022900//electron transport chain;cellular_component:GO:0045261//proton-transporting ATP synthase complex, catalytic core F(1);GO:0016021//integral component of membrane;GO:0009507//chloroplast;molecular_function:GO:0005524//ATP binding;GO:0046961//proton-transporting ATPase activity, rotational mechanism;GO:0046933//proton-transporting ATP synthase activity, rotational mechanism;	RF01960	SSU_rRNA_eukarya
Unigene4162_All	2310	Up	8.91	LOC_Os11g19790genomic	O-methyltransferase, putative, expressed	ref	NP_001067738.1	1.94E-71	Os11g0303200 [Oryza sativa Japonica Group]	gb	AC112208.3	0	Oryza sativa Japonica Group chromosome 11 clone OSJNBa0015P05 map C53961S, complete sequence	NA	NA	NA	NA	NA	NA	N/A	N/A
CL4283.Contig2_All	969	Down	-10.01	LOC_Os04g28180genomic	ribosomal protein, putative, expressed	ref	NP_001052526.1	9.96E-123	Os04g0349500 [Oryza sativa Japonica Group]	dbj	AK288317.1	0	Oryza sativa Japonica Group cDNA, clone: J090021K08, full insert sequence	sp|P49199|RS8_ORYSJ/6e-120/40S ribosomal protein S8 OS=Oryza sativa subsp. japonica GN=RPS8 PE=2 SV=2	osa:4335547/5e-124/Os04g0349500; K02995 small subunit ribosomal protein S8e	NA	SPAC521.05/2e-63/---	PS01193/--/Ribosomal protein S8e signature.	biological_process:GO:0000462//maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA);GO:0006414//translational elongation;cellular_component:GO:0016020//membrane;GO:0022627//cytosolic small ribosomal subunit;GO:0009507//chloroplast;molecular_function:GO:0003735//structural constituent of ribosome;GO:0019843//rRNA binding;	N/A	N/A
CL2678.Contig3_All	779	Down	-9.96	LOC_Os01g70460genomic	expressed protein	ref	NP_001045285.1	2.34E-78	Os01g0930000 [Oryza sativa Japonica Group]	ref	NM_001051820.1	0	Oryza sativa Japonica Group Os01g0930000 (Os01g0930000) mRNA, complete cds	sp|Q9FPQ6|GP1_CHLRE/2e-13/Vegetative cell wall protein gp1 OS=Chlamydomonas reinhardtii GN=GP1 PE=1 SV=1	gmx:100787779/6e-09/uncharacterized LOC100787779; K03126 transcription initiation factor TFIID subunit 12	NA	Rv3876/3e-09/---	NA	cellular_component:GO:0009536//plastid;	N/A	N/A

CL317.Contig5_All	1033	Down	-9.60	LOC_Os12g02960genomic	glutathione S-transferase, putative, expressed	gb	ABA95696.2	7.63E-116	Glutathione S-transferase, C-terminal domain containing protein, expressed [Oryza sativa Japonica Group]	dbj	AK240983.1	0	Oryza sativa Japonica Group cDNA, clone: J065050H09, full insert sequence	sp|Q9FQA3|GST23_MAIZE/3e-55/Glutathione transferase GST 23 OS=Zea mays PE=2 SV=1	bdi:100844756/2e-79/glutathione transferase GST 23-like; K00799 glutathione S-transferase	[EC:2.5.1.18]	NA	PF00043/1.7E-7/Glutathione S-transferase, C-terminal domain	NA	N/A	N/A
CL184.Contig1_All	1450	Down	-9.55	LOC_Os01g28989genomic	expressed protein	gb	EEC70696.1	3.69E-43	hypothetical protein Osl_02051 [Oryza sativa Indica Group]	ref	NM_001185426.1	0	Oryza sativa Japonica Group Os01g0386700 (Os01g0386700) mRNA, complete cds	sp|Q944A7|Y4523_ARATH/3e-12/Probable serine/threonine-protein kinase At4g35230 OS=*Arabidopsis* thaliana GN=At4g35230 PE=1 SV=1	mtr:MTR_5g098970/4e-12/Receptor like protein kinase; K14500 BR-signaling kinase	[EC:2.7.11.1]	NA	NA	molecular_function:GO:0016772//transferase activity, transferring phosphorus-containing groups;	N/A	N/A
CL5680.Contig1_All	2240	Down	-9.36	LOC_Os01g04870genomic	WD domain, G-beta repeat domain containing protein, expressed	dbj	BAB16450.1	0	UV-damaged DNA binding protein 2 [Oryza sativa Japonica Group]	dbj	AK112097.1	0	Oryza sativa Japonica Group cDNA clone:002-108-A04, full insert sequence	sp|Q6NQ88|DDB2_ARATH/0.0/Protein DAMAGED DNA-BINDING 2 OS=*Arabidopsis* thaliana GN=DDB2 PE=1 SV=1	osa:4325482/0.0/Os01g0141700; K10140 DNA damage-binding protein 2	NA	NA	PTHR15169/1.1E-187/--	biological_process:GO:0006281//DNA repair;GO:0010224//response to UV-B;cellular_component:GO:0005634//nucleus; molecular_function:GO:0008270//zinc ion binding;GO:0003676//nucleic acid binding;	N/A	N/A
CL7640.Contig8_All	1777	Down	-9.20	LOC_Os03g18270genomic	expressed protein	dbj	BAG93073.1	1.68E-40	unnamed protein product [Oryza sativa Japonica Group]	ref	NM_001056348.1	0	Oryza sativa Japonica Group Os03g0293900 (Os03g0293900) mRNA, partial cds	sp|P08823|RUBA_WHEAT/1e-21/RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment) OS=Triticum aestivum PE=1 SV=1	smo:SELMODRAFT_165043/3e-06/hypothetical protein; K04077 chaperonin GroEL	NA	sll0416/2e-11/---	NA	biological_process:GO:0009658//chloroplast organization;GO:0009790//embryo development;GO:0042026//protein refolding;cellular_component:GO:0016020//membrane;GO:0048046//apoplast;GO:0009579//thylakoid;GO:0005739//mitochondrion;GO:0009941//chloroplast envelope;GO:0009570//chloroplast stroma;GO:0022626//cytosolic ribosome;molecular_function:GO:0005524//ATP binding;	RF02000	MIR1846
CL14.Contig2_All	1522	Down	-9.16	LOC_Os01g09370genomic	ankyrin repeat domain-containing protein 28, putative, expressed	gb	EAY72742.1	1.26E-115	hypothetical protein Osl_00609 [Oryza sativa Indica Group]	ref	NM_001185285.1	0	Oryza sativa Japonica Group Os01g0188900 (Os01g0188900) mRNA, complete cds	sp|Q9M8S6|SKOR_ARATH/1e-08/Potassium channel SKOR OS=*Arabidopsis* thaliana GN=SKOR PE=1 SV=1	vvi:100257739/8e-08/serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C-like; K06694 26S proteasome non-ATPase regulatory subunit 10	NA	NA	NA	NA	N/A	N/A
Unigene29535_All	772	Down	-9.15	LOC_Os09g18230genomic	expressed protein	gb	EEC84422.1	3.22E-80	hypothetical protein Osl_31015 [Oryza sativa Indica Group]	ref	NM_001069491.1	1.00E-134	Oryza sativa Japonica Group Os09g0351700 (Os09g0351700) mRNA, complete cds	sp|C0LGR6|Y4291_ARATH/3e-29/Probable LRR receptor-like serine/threonine-protein kinase At4g29180 OS=*Arabidopsis* thaliana GN=At4g29180 PE=2 SV=2	NA	NA	NA	NA	PTHR11795:SF358/1.9E-44/--	biological_process:GO:0006468//protein phosphorylation;cellular_component:GO:0016023//cytoplasmic membrane-bounded vesicle;molecular_function:GO:0004674//protein serine/threonine kinase activity;GO:0005524//ATP binding;	N/A	N/A

CL4499.Contig3_All	1931	Down	-9.15	LOC_Os06g21330genomic	ABB1 - Ankyrin repeat region with 2 Bric-a-Brac, Tramtrack, Broad Complex BTB domains, expressed	ref	NP_001057501.1	0	Os06g0318200 [Oryza sativa Japonica Group]	ref	NM_001064036.1	0	Oryza sativa Japonica Group Os06g0318200 (Os06g0318200) mRNA, complete cds	sp|Q9SJ85|Y2474_ARATH/0.0/BTB/POZ domain-containing protein At2g04740 OS=*Arabidopsis* thaliana GN=At2g04740 PE=2 SV=2	mtr:MTR_2g069490/1e-09/RNA-binding protein with serine-rich domain-containing protein; K14325 RNA-binding protein with serine-rich domain 1	NA	SPAC13D6.04c/7e-13/---	SM00225/9.5E-17/Broad-Complex, Tramtrack and Bric a brac	biological_process:GO:0006414//translational elongation;molecular_function:GO:0003746//translation elongation factor activity;	N/A	N/A
Unigene29264_All	6337	Down	-9.15	LOC_Os01g37280genomic	expressed protein	dbj	BAH00660.1	3.53E-76	unnamed protein product [Oryza sativa Japonica Group]	ref	NM_001187852.1	0	Oryza sativa Japonica Group Os06g0361500 (Os06g0361500) mRNA, complete cds	NA	NA	NA	NA	NA	cellular_component:GO:0016023//cytoplasmic membrane-bounded vesicle;molecular_function:GO:0003676//nucleic acid binding;	N/A	N/A
CL1431.Contig3_All	5426	Down	-9.11	LOC_Os03g38740genomic	Dicer, putative, expressed	ref	NP_001050564.1	0	Os03g0583900 [Oryza sativa Japonica Group]	ref	NM_001057099.1	0	Oryza sativa Japonica Group Os03g0583900 (Os03g0583900) mRNA, complete cds	sp|Q10HL3|DCL2A_ORYSJ/0.0/Endoribonuclease Dicer homolog 2a OS=Oryza sativa subsp. japonica GN=DCL2A PE=2 SV=1	osa:4333337/0.0/Os03g0583900; K11592 endoribonuclease Dicer	[EC:3.1.26.-]	SPCC188.13c/2e-29/---	PS50142/21.586/Ribonuclease III family domain profile.	biological_process:GO:0006396//RNA processing;GO:0040029//regulation of gene expression, epigenetic;GO:0031047//gene silencing by RNA;GO:0050896//response to stimulus;GO:0090305//nucleic acid phosphodiester bond hydrolysis;cellular_component:GO:0005634//nucleus;molecular_function:GO:0005524//ATP binding;GO:0004525//ribonuclease III activity;GO:0003725//double-stranded RNA binding;GO:0008026//ATP-dependent helicase activity;GO:0046872//metal ion binding;	N/A	N/A
CL4552.Contig3_All	2320	Down	-9.10	LOC_Os01g71000genomic	protein kinase APK1B, chloroplast precursor, putative, expressed	ref	NP_001045326.1	0	Os01g0936100 [Oryza sativa Japonica Group]	ref	NM_001051861.1	0	Oryza sativa Japonica Group Os01g0936100 (Os01g0936100) mRNA, complete cds	sp|Q9FE20|PBS1_ARATH/3e-165/Serine/threonine-protein kinase PBS1 OS=*Arabidopsis* thaliana GN=PBS1 PE=1 SV=1	vvi:100254113/2e-174/serine/threonine-protein kinase PBS1-like; K13430 serine/threonine-protein kinase PBS1	[EC:2.7.11.1]	Cgl2127_1/4e-17/---	PF00069/5.8E-45/Protein kinase domain	biological_process:GO:0006468//protein phosphorylation;cellular_component:GO:0005886//plasma membrane;molecular_function:GO:0004674//protein serine/threonine kinase activity;GO:0005524//ATP binding;	N/A	N/A
CL634.Contig1_All	714	Down	-9.07	LOC_Os11g29290genomic	cytochrome P450, putative, expressed	ref	NP_001067905.2	2.88E-85	Os11g0483000 [Oryza sativa Japonica Group]	gb	AC131343.4	0	Oryza sativa Japonica Group chromosome 11 clone OSJNBa0057E15, complete sequence	sp|Q9SMP5|C94B3_ARATH/4e-40/Cytochrome P450 94B3 OS=*Arabidopsis* thaliana GN=CYP94B3 PE=2 SV=1	aly:ARALYDRAFT_324175/1e-27/CYP94D2; K00517	[EC:1.14.-.-]	NA	PTHR24296/2.5E-88/--	biological_process:GO:0055114//oxidation-reduction process;cellular_component:GO:0016023//cytoplasmic membrane-bounded vesicle;molecular_function:GO:0009055//electron carrier activity;GO:0020037//heme binding;GO:0070330//aromatase activity;GO:0005506//iron ion binding;	N/A	N/A
CL7571.Contig1_All	5367	Down	-9.07	LOC_Os01g56590genomic	guanine nucleotide exchange family protein, putative, expressed	ref	XP_004970129.1	0	PREDICTED: LOW QUALITY PROTEIN: protein MON2 homolog [Setaria italica]	ref	XM_006644720.1	0	PREDICTED: Oryza brachyantha protein MON2 homolog (LOC102715156), mRNA	sp|F4IXW2|BIG5_ARATH/1e-23/Brefeldin A-inhibited guanine nucleotide-exchange protein 5 OS=*Arabidopsis* thaliana GN=BIG5 PE=1 SV=2	ath:AT3G43300/3e-23/ATMIN7; guanine nucleotide-exchange factor; K13462 guanine nucleotide-exchange factor	NA	NA	PTHR10663:SF114/4.5E-269/--	NA	N/A	N/A

CL4199.Contig2_All	990	Down	-9.03	LOC_Os08g36450genomic	transcription regulator, putative, expressed	dbj	BAD09553.1	4.56E-179	putative storekeeper protein [Oryza sativa Japonica Group]	dbj	AP006461.3	0	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0104B02	sp|Q9FPQ6|GP1_CHLRE/4e-28/Vegetative cell wall protein gp1 OS=Chlamydomonas reinhardtii GN=GP1 PE=1 SV=1	gmx:100776781/2e-16/probable pectinesterase/pectinesterase inhibitor 25-like; K01051 pectinesterase	[EC:3.1.1.11]	Rv3876/5e-16/---	PF04504/3.2E-29/Protein of unknown function, DUF573	cellular_component:GO:0005739//mitochondrion;	N/A	N/A
CL6231.Contig2_All	366	Down	-9.01	LOC_Os02g37290genomic	heavy metal associated domain containing protein, expressed	ref	NP_001173045.1	3.01E-12	Os02g0584700 [Oryza sativa Japonica Group]	dbj	AP005800.3	0	Oryza sativa Japonica Group genomic DNA, chromosome 2, BAC clone:OSJNBa0016D04	NA	NA	NA	NA	NA	NA	biological_process:GO:0006952//defense response;GO:0030001//metal ion transport;molecular_function:GO:0043531//ADP binding;GO:0046872//metal ion binding;GO:0005524//ATP binding;GO:0017111//nucleoside-triphosphatase activity;	N/A	N/A
CL1096.Contig6_All	3316	Down	-8.99	LOC_Os12g18650genomic	Regulator of chromosome condensation domain containing protein, expressed	ref	NP_001066582.1	0	Os12g0284000 [Oryza sativa Japonica Group]	dbj	AK065992.1	0	Oryza sativa Japonica Group cDNA clone:J013049P07, full insert sequence	sp|Q9FN03|UVR8_ARATH/6e-34/Ultraviolet-B receptor UVR8 OS=*Arabidopsis* thaliana GN=UVR8 PE=1 SV=1	cme:CMB070C/8e-14/ubiquitin protein ligase E3A; K10615 E3 ubiquitin-protein ligase HERC4	[EC:6.3.2.19]	SPBC557.03c/1e-15/---	PS50012/12.997/Regulator of chromosome condensation (RCC1) repeat profile.	NA	N/A	N/A
Unigene29245_All	538	Down	-8.95	LOC_Os05g01500genomic	tubulin-specific chaperone E, putative, expressed	gb	EEE62030.1	3.91E-41	hypothetical protein OsJ_16812 [Oryza sativa Japonica Group]	ref	NM_001060945.1	0	Oryza sativa Japonica Group Os05g0105300 (Os05g0105300) mRNA, complete cds	sp|Q8GRL7|TBCE_ARATH/5e-25/Tubulin-folding cofactor E OS=*Arabidopsis* thaliana GN=TFCE PE=2 SV=1	NA	NA	NA	NA	NA	biological_process:GO:0000910//cytokinesis; GO:0009793//embryo development ending in seed dormancy;cellular_component:GO:0009507//chloroplast;	N/A	N/A
CL3097.Contig1_All	1661	Down	-8.92	LOC_Os04g57700genomic	expressed protein	emb	CAH67770.1	4.77E-174	H0322F07.7 [Oryza sativa Indica Group]	dbj	AK068390.1	0	Oryza sativa Japonica Group cDNA clone:J013147K07, full insert sequence	NA	NA	NA	NA	PTHR14000/1.6E-48/--	NA	N/A	N/A
CL1597.Contig2_All	7268	Down	-8.91	LOC_Os02g01740genomic	U5 small nuclear ribonucleoprotein 200 kDa helicase, putative, expressed	gb	EAY84119.1	0	hypothetical protein OsI_05501 [Oryza sativa Indica Group]	ref	XM_006648151.1	0	PREDICTED: Oryza brachyantha U5 small nuclear ribonucleoprotein 200 kDa helicase-like (LOC102699844), mRNA	sp|Q9FFW5|PERK8_ARATH/1e-12/Proline-rich receptor-like protein kinase PERK8 OS=*Arabidopsis* thaliana GN=PERK8 PE=1 SV=1	bdi:100827281/0.0/SNRNP200; small nuclear ribonucleoprotein 200kDa (U5); K12854 pre-mRNA-splicing helicase BRR2	[EC:3.6.4.13]	SPAC9.03c/0.0/---	SM00973/1.3E-104/Sec63 Brl domain	cellular_component:GO:0016020//membrane; GO:0005730//nucleolus;molecular_function:GO:0005524//ATP binding;GO:0003676//nucleic acid binding;GO:0008026//ATP-dependent helicase activity;	N/A	N/A

Table 23 List of DEGs involved in plant salinity response pathway found using locus mapping.

Name	Length	Log2 Fold Change	Locus	Description
Unigene25044_All	574	2.170	LOC_Os05g03130genomic	OsRCI2-7 - Putative low temperature and salt responsive protein, expressed
Unigene26426_All	247	1.035	LOC_Os03g25460genomic	OsRCI2-4 - Putative low temperature and salt responsive protein, expressed
Unigene14210_All	565	0.453	LOC_Os03g17790genomic	OsRCI2-5 - Putative low temperature and salt responsive protein, expressed
Unigene8708_All	806	0.329	LOC_Os06g08564genomic	OsRCI2-8 - Putative low temperature and salt responsive protein, expressed
Unigene5725_All	545	0.327	LOC_Os01g18390genomic	OsRCI2-1 - Putative low temperature and salt responsive protein, expressed
Unigene8512_All	1453	0.321	LOC_Os01g58080genomic	Membrane-associated salt-inducible protein, putative, expressed
Unigene21941_All	720	0.207	LOC_Os02g35880genomic	Salt tolerant protein, putative, expressed
CL3163.Contig1_All	342	0.053	LOC_Os08g36550genomic	Salt tolerant protein, putative, expressed
Unigene132_All	1567	-0.020	LOC_Os02g42210genomic	Membrane-associated salt-inducible protein like, putative, expressed
Unigene390_All	1143	-0.175	LOC_Os02g18410genomic	Salt stress root protein RS1, putative, expressed
Unigene2646_All	945	-0.340	LOC_Os01g13210genomic	Salt stress root protein RS1, putative, expressed
CL6845.Contig2_All	1267	-0.452	LOC_Os01g13210genomic	Salt stress root protein RS1, putative, expressed
Unigene34759_All	450	-1.294	LOC_Os03g25460genomic	OsRCI2-4 - Putative low temperature and salt responsive protein, expressed
CL6845.Contig1_All	271	-2.541	LOC_Os01g13210genomic	Salt stress root protein RS1, putative, expressed

Finally, the DEGs were examined for the involvement in metabolic and biosynthesis pathways. The DEGs classification results were shown in Figure 25 while the pathway functional enrichment results using KEGG database were presented in Figure 26. The data from pathway classification had revealed that many DEGs belong to the "genetic information pathway" and "global map" categories of the database. Extensive activities in the plant's "translation" metabolism had also been observed, with 497 DEGs in the stated category. The third largest group of DEGs was responsible for the plants' "transport and catabolism" activity, with 362 DEGs in the stated category. Pathway functional enrichment of DEGs had revealed that more than 12% (344 DEGs) of the total DEGs are responsible for the biosynthesis of secondary metabolites. The second largest group of DEGs was in charge of the starch and sucrose metabolism of in the plants, representing 4.69% (127 DEGs) of the total DEGs.

The summary of pathway functional enrichment is summarised in the section below (Figure 27) revealing an extensive transcriptional reprogramming in the secondary metabolite biosynthesis pathway upon salinity stress exposure. More details in the functional enrichment of DEGs against all annotated unigenes are included in Table A-3 in the appendices.

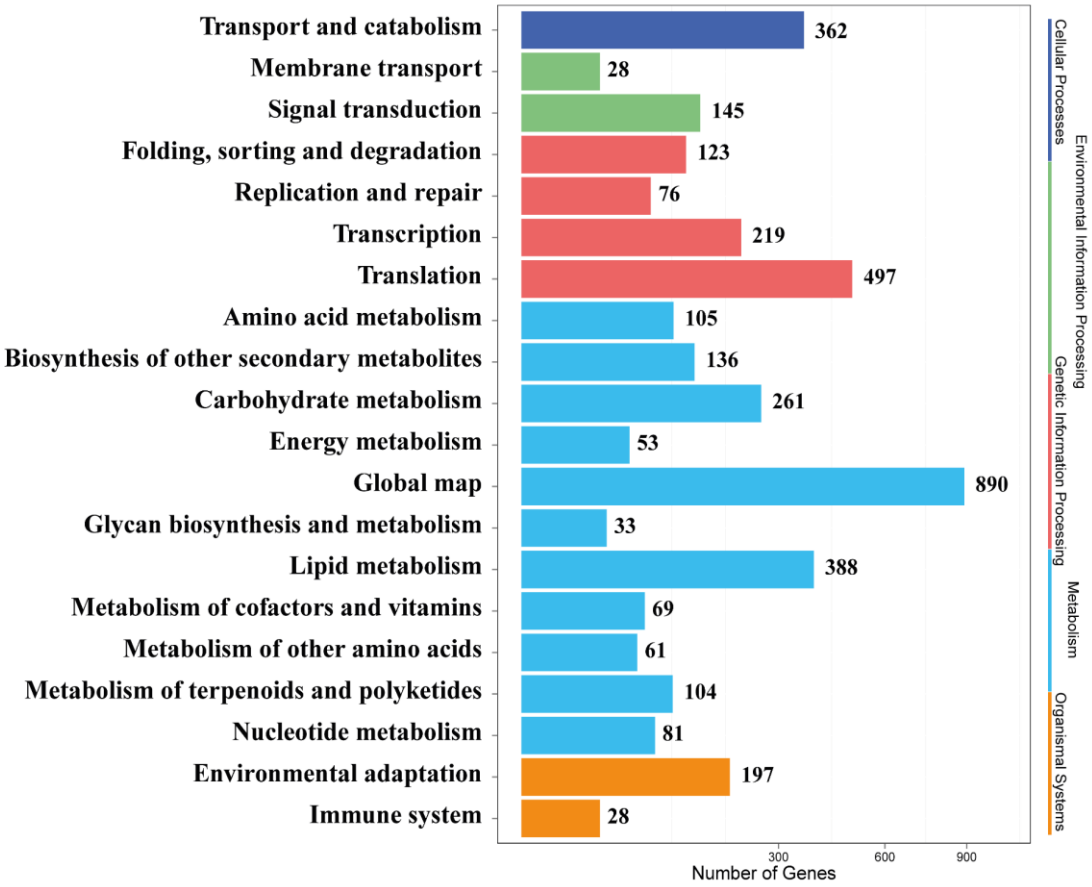


Figure 25 Pathway classification of DEGs based on KEGG database.

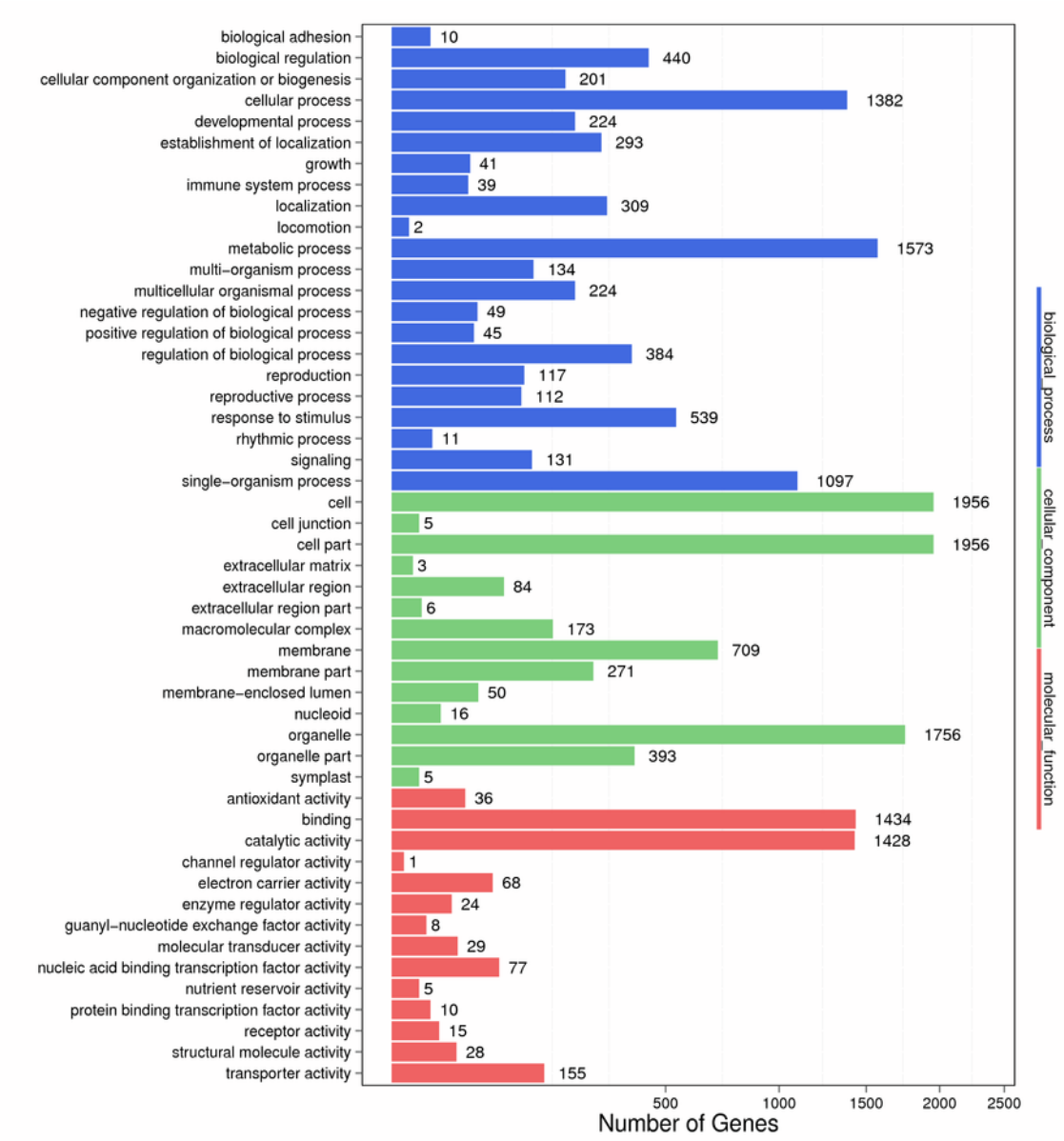


Figure 26 Functional analysis of DEGs based on GO database.

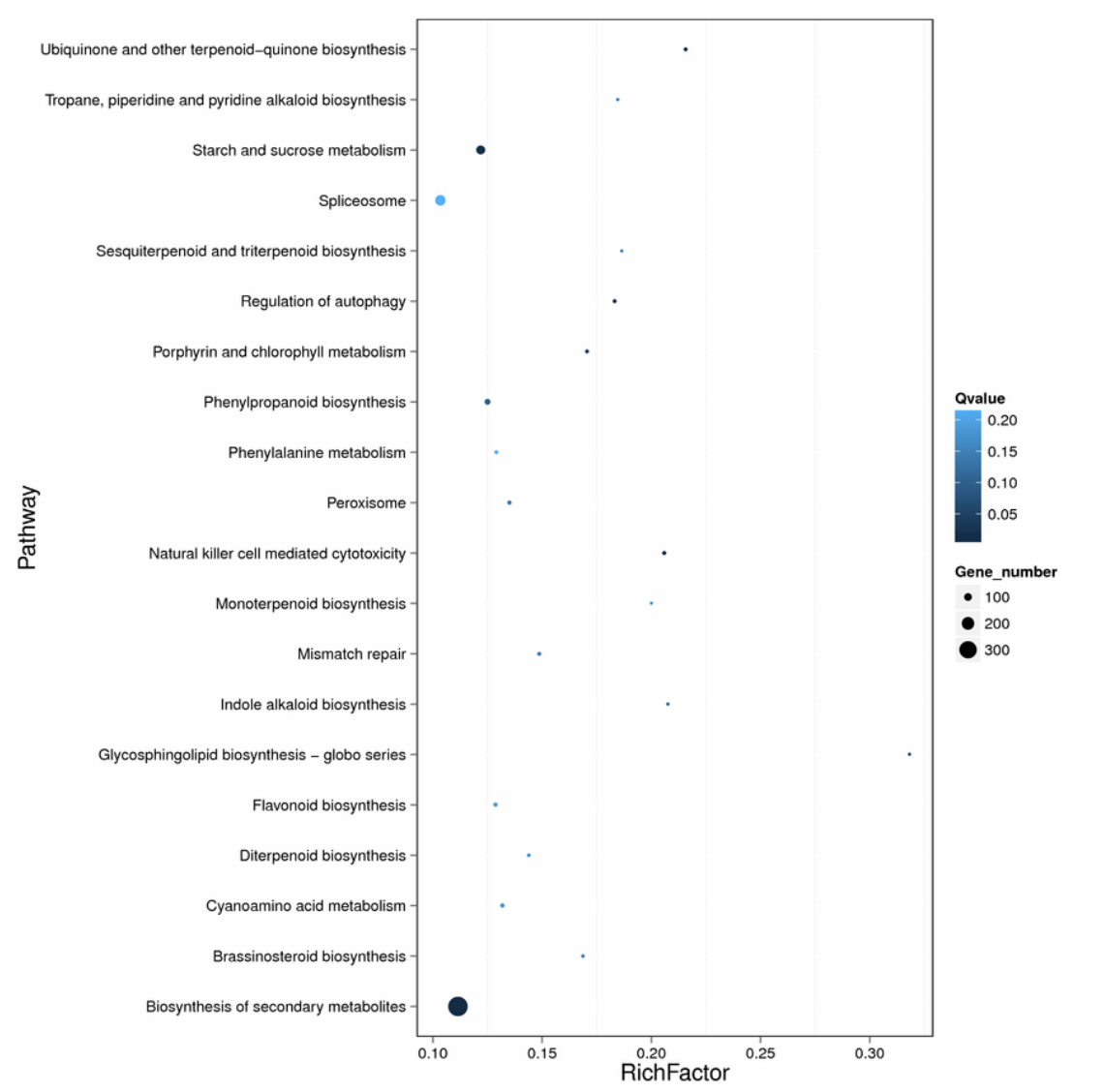


Figure 27 Pathway functional enrichment analysis on DEGs.

Colouring indicates Q value; A lower Q value indicates a more significant enrichment. Point size shows the number of DEG present in a group. Details in enrichment data have been attached in Table A-3 in the appendices.

4.5.10 Pathway analysis of DEGs

Significant regulation of gene expressions can be observed in the conversion of phenylpyruvate to phenylalanine (Figure 28). There was also up and down-regulations in the production of 2-phenylacetaminde molecules. Next, there were repressed gene expressions for the conversion of trans-4-hydroxyl cinnamate to 4-coumaroyl-CoA and an elevated gene expression for the transformation from caffeoyl-CoA to feruloyl-CoA. The phenylpropanoids synthesis pathway had significantly more complicated regulatory mechanisms (Figure 29). Many regulation activities can be found in the biosynthesis of syringyl lignin, guaiacyl lignin and p-hydroxyphenyl lignin. Additionally, there is also much regulation activity in the production of a coumarinate molecule. Furthermore, pathway leading to biosynthesis of cinnamaldehyde had shown an increased activity in gene expression.

The flavonoid biosynthesis pathway in Bajong was significantly disturbed under salinity stress (Figure 30). There had been an elevated expression in the production and accumulation of 5-deoxyleucopelargonidin and 5-deoxylecocyanidin. Many DEGs were also involved in the manufacture in precursors of these two molecules. Furthermore, the elevated expression of 3 enzymes labelled as 2.1.1.104 1.1.1.234 and 1.17.13 could lead to the accumulation of feruloyl-CoA, apiforol and ($^{+}$) gallocatechin molecules within the plant. In the isoflavonoid biosynthesis pathway, salt-treated Bajong has altered regulatory activities in the enzymes 2-hydroxyisoflavanone synthase and CYP93C (Figure 31). Both enzymes are responsible for biosynthesis of apigenin, liquiritigenin, 7-4-dihydroflavone, 2,6,7,4-tetrahydroxyl isoflavone and naringenine molecules. Additionally, elevated enzyme activity related to the production of genistein and daizein can be observed. As shown in Figure 32, relatively small changes in the photosynthesis pathway can be seen in this study. We have detected elevated gene expression related to the metabolism of Psb P (OEC), Psb Q, Pet F and the delta component in the F-type ATPase molecule. Meanwhile, we have observed that there was significantly high response in the antioxidant system of the plants (Figure 33), especially in the SOD and CAT metabolic system. There was also up-regulation of the genes responsible for ROS metabolism and fatty acid oxidation such as MPV17 and MVYCD. Furthermore, up-regulation can be observed in the MVK gene responsible for sterol precursor biosynthesis.

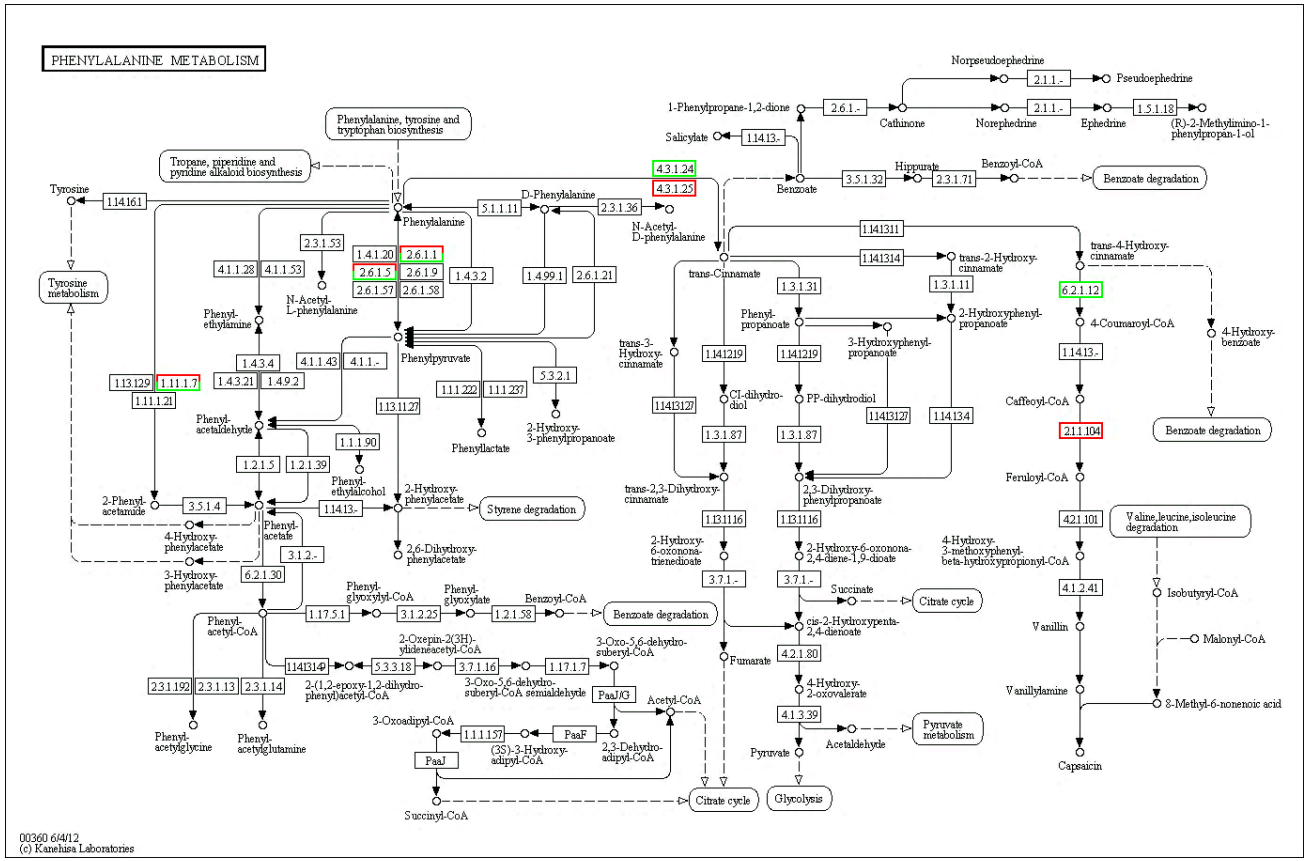


Figure 28 Phenylalanine metabolism pathway analysis of DEGs in controls versus stressed sample based on KEGG database.

Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

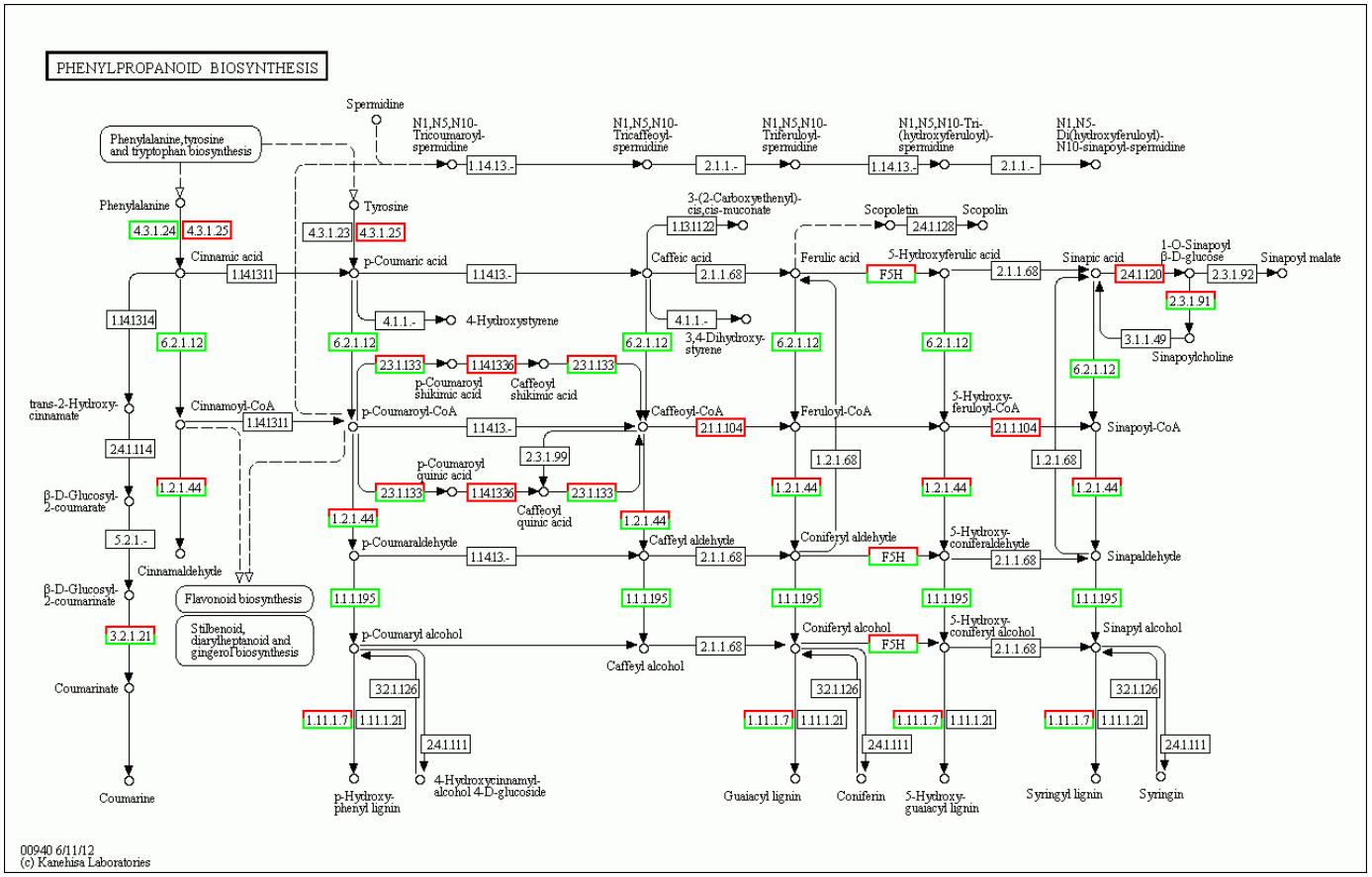


Figure 29 Phenylpropanoids biosynthesis pathway analysis of DEGs in controls versus stressed sample based on KEGG database. Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

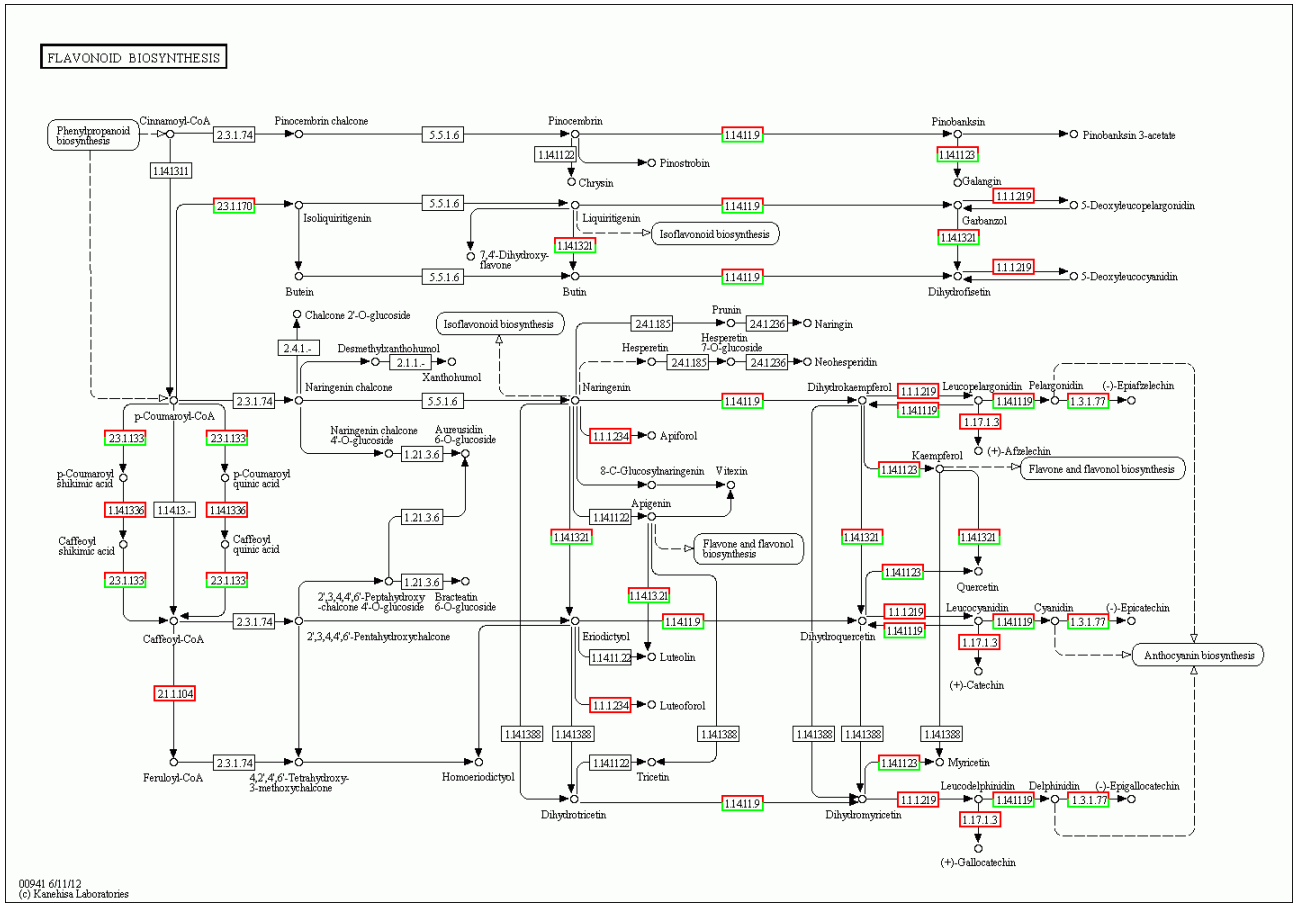


Figure 30 Flavonoid biosynthesis pathway analysis of DEGs in controls versus stressed sample based on KEGG database.

Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

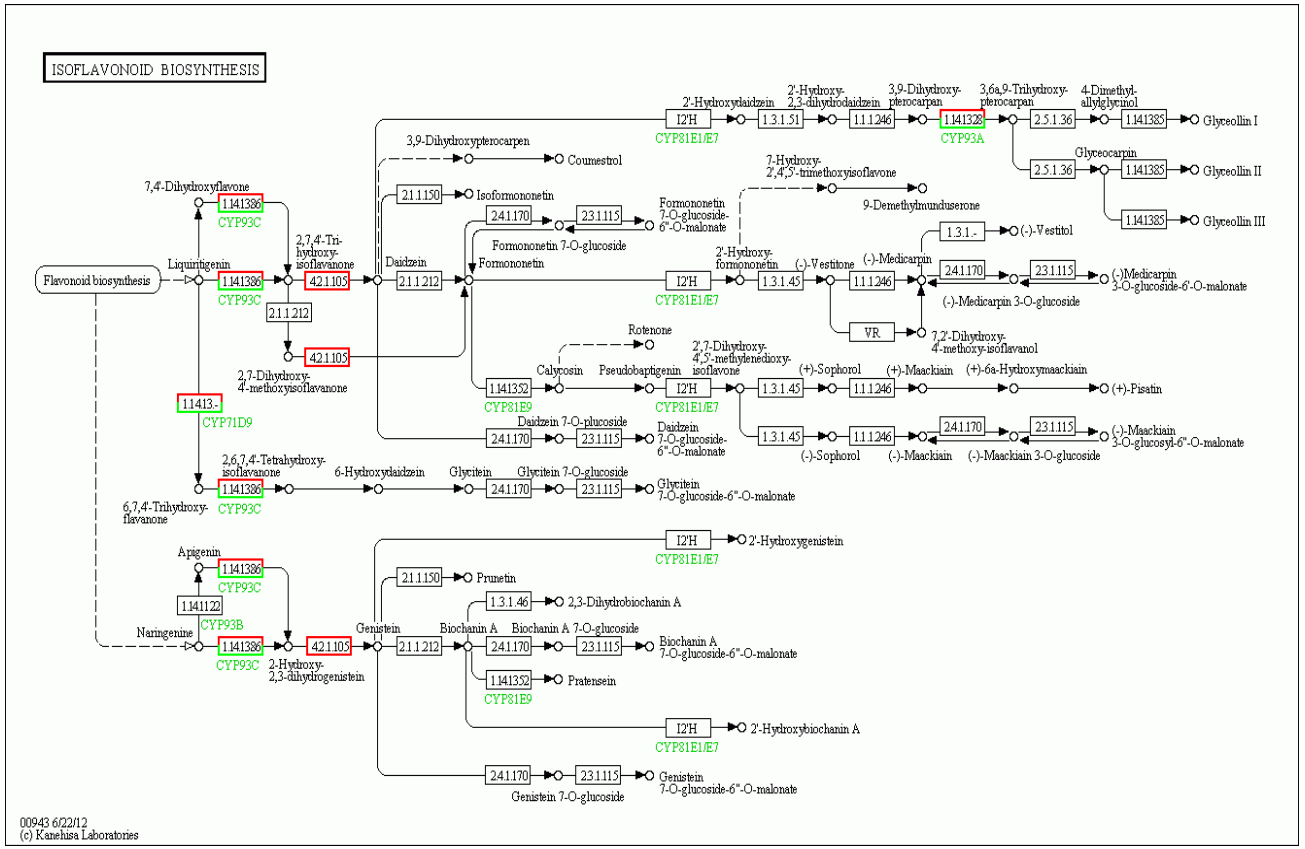


Figure 31 Isoflavonoid biosynthesis pathway analysis of DEGs in controls versus stressed sample based on KEGG database.

Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

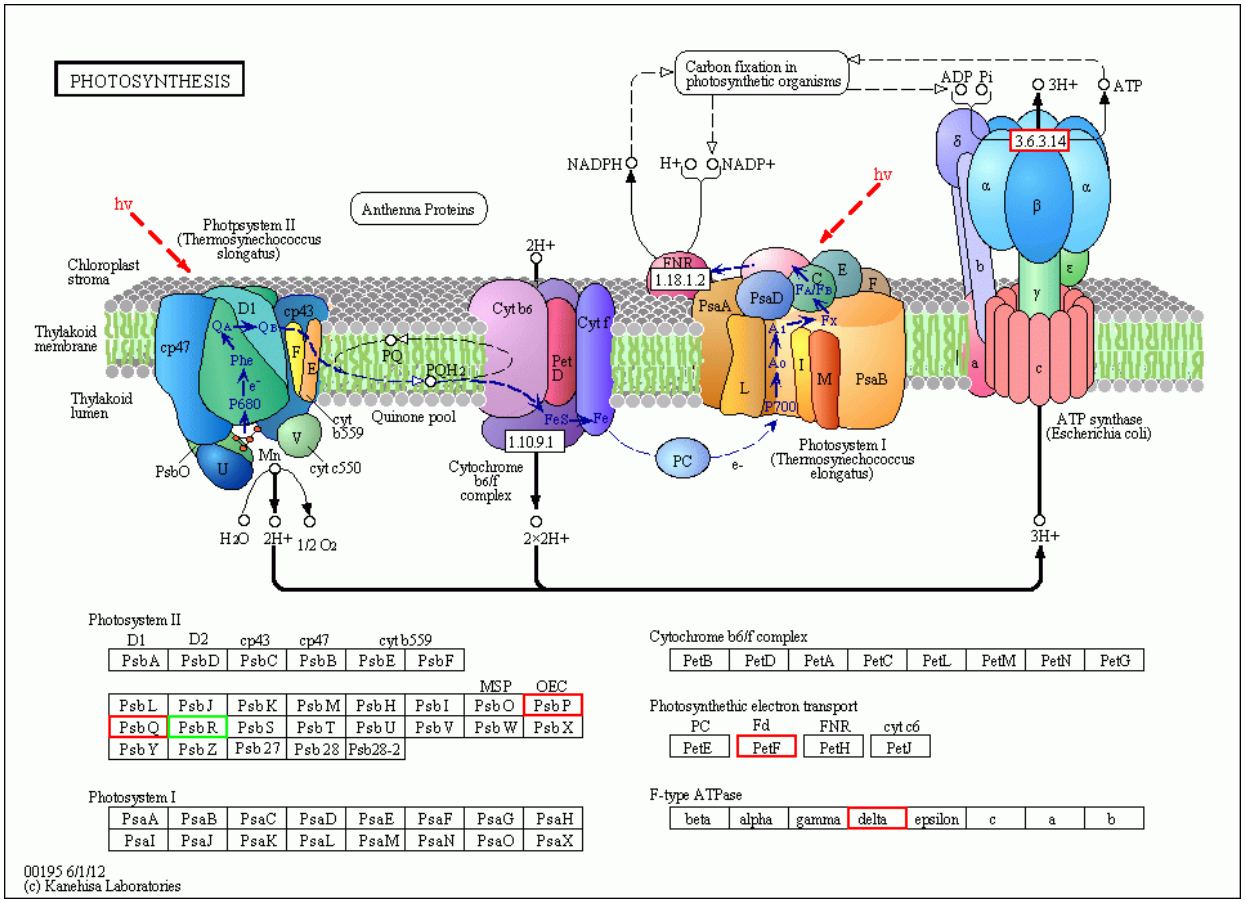


Figure 32 Photosynthesis pathway analysis of DEGs in controls versus stressed sample based on KEGG database.

Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

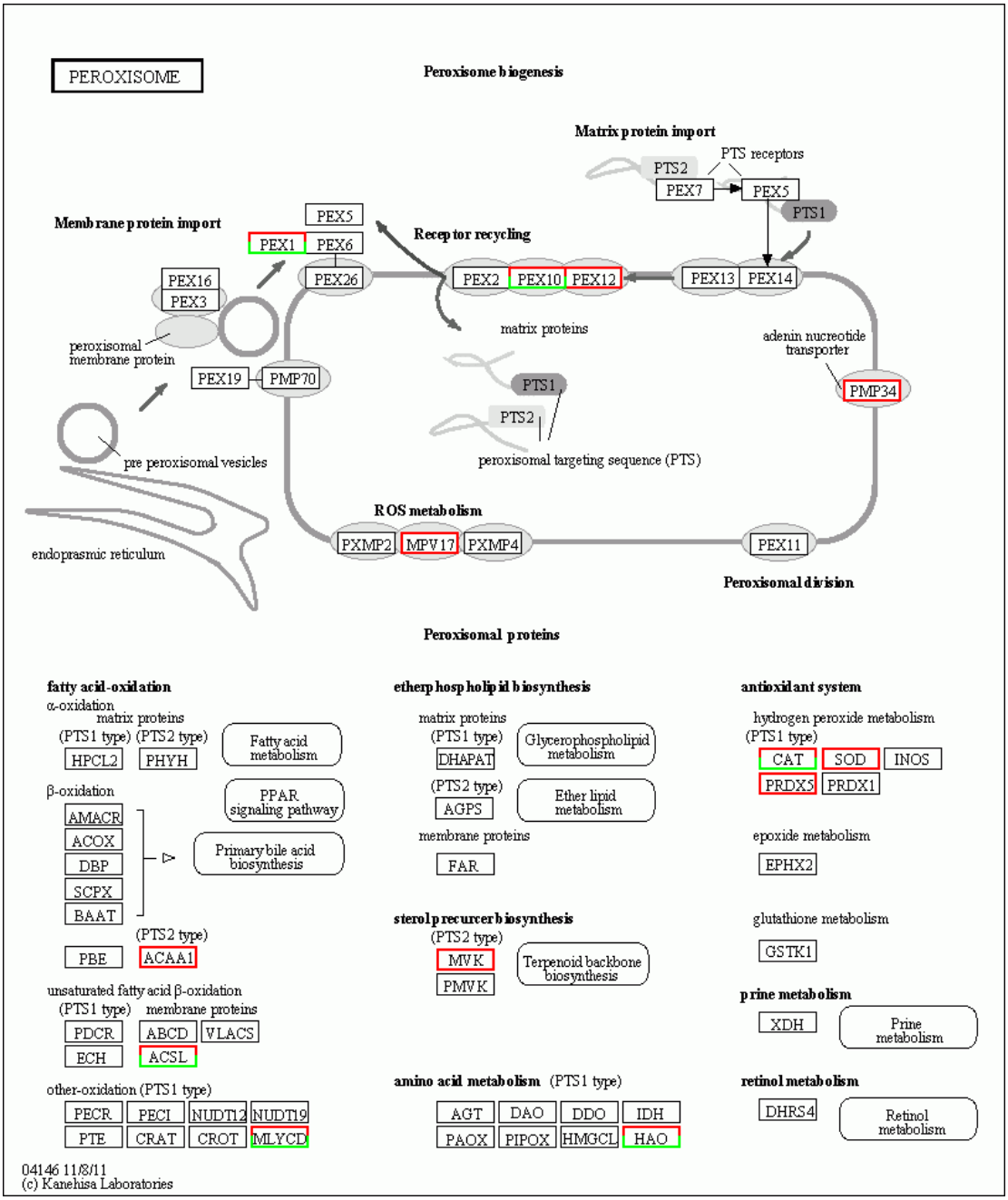


Figure 33 Peroxisome metabolism pathway analysis of DEGs in controls versus stressed sample based on KEGG database.

Upregulated genes are labelled with red borders while downregulated genes are labelled with green borders. Non-DEGs are labelled with black borders.

5.5 Discussion

5.5.1 Sequencing Quality and Transcriptome Assembly

The aim of this work is to identify a broad spectrum of salinity-responsive genes and pathways in local Sarawak rice varieties to predict the influence of salinity stress towards the physiological, metabolic and cellular processes in the plants. In this study, the transcriptome of Sarawak local rice varieties, Bajong, after salinity stress treatment (100mM, 6 hours) was sequenced using Illumina HiSeq 4000 platform.

More than 99.7% of the transcripts had been successfully annotated to various online databases, which account for 61,164 transcripts. In addition, 657 new sequences that cannot be annotated to any databases had also been discovered, which could be further processed for novel proteins or functional peptides. The high similarity of transcriptomic sequences towards *Oryza Japonica* indicates that Bajong could be originated from this variety, but has accumulated significant mutation across the many generations of traditional farming. Some crossbreeding between *Oryza indica* could also have taken place for the high similarity of genes towards this variety. The reason for transcript similarity of towards a wild rice (*Oryza brachyantha*) was unknown as this variety was native from Africa (Joshi et al. 2000) and no previous history of such introduction has been recorded. Thus, it is speculated that the similarities are due to natural convergent mutation. Since the mutation rates in transcripts are high, future study should use genomics sequences for the diversity mapping process.

Meanwhile, the functional annotation using GO database of all unigenes showed a similar trend towards previous studies (Huang et al. 2014; Yang et al. 2015; Zhai et al. 2013) with most of the transcripts mapped onto the cellular component of the cells such as cell, cell part, organelle and organelle ontologies.

5.5.2 Functional Analysis of Unigenes

In this study, 4024 DEGs has been identified and annotated. They were involved in metabolic, stimulant, single organism processes and cell binding activity, environmental adaptation and catalytic activity of the plant. The environmental adaptation categories comprise of 197 DEGs, which accounts for 5 % of the DEGs number. This value was relatively higher when compared to the data from the raw reads, which is about 3.5% (Figure 25).

The most elevated gene was found to be most similar to a metal transporter in *Morus notabilis* while the third most elevated genes were found to be most similar to a dehydration-responsive protein from *Corchorus olitorius* (Table 22). It has been widely accepted that ions transportation is important towards the development of salt tolerance of the plant (Munns & Tester 2008), this has suggested that local Sarawak rice varieties could have developed a slightly different method of ions -transportation using different proteins compared to common rice varieties. However, this funding is very preliminary and needs further investigation.

Locus mapping had revealed that 6 DEGs belong to the OsRCI2 gene family. Specifically, Unigene14210_All was found to be highly similar to OsRCI2-5, which had been previously identified and proven to improve drought resistance in rice when cloned into a mutant (Li et al. 2014). A total of 12 different OsRCI2 has been described (Medina, Ballesteros & Salinas 2007) based on the sequenced genome of the rice. However, by scanning the available cDNA collection available as performed by Fu et al. (2012), only OsRCI2-3, OsRCI2-5, OsRCI2-6 (Oslti6B), OsRCI2-8, OsRCI2-9, OsRCI2-10 (Oslti6A) and OsRCI2-11 have their corresponding cDNA in the database examined (http://rice.plantbiology.msu.edu/). This study had potentially identified the full sequences of OsRCI2-1, OsRCI2-4 and OsRCI2-7 within the cDNA collection of the rice transcriptome and proven their involvement in salinity response. These genes could be further investigated for the capabilities to improve salt tolerance in rice.

5.5.3 Pathway Classification and Functional Enrichment

In this study, the transcriptome of Bajong in control and stressed condition are reported. Among the DEGs discovered, many of them had been found to be involved in the osmo-protection pathways of the plants. Six different pathways showing the most regulation activities upon exposure to salinity are reported here.

A large number of regulation activities in the phenolic biosynthesis pathway was observed. Phenolic compounds are widely known to protect the plant from excessive light and UV radiation, (Winkel-Shirley 2002). Significant changes in phenylalanine metabolism pathways could be an indication of the accumulation of phenolic compound. This is in accordance with the increased with TPC in Bajong as observed in the Section 3.5.2.2. Meanwhile, intracellular accumulation of phenylpropanoids such as caffeoylquinic acid and phenylalanine was found to be an effective defence mechanism

against salinity stress in plants (Lugan et al. 2009) and has been shown to improve the stress acclimation capability of the plant. Phenylpropanoids also serve as a precursor for the biosynthesis of lignin, which is a crucial molecule in the stress defences mechanism in the plant as it modulates cell wall composition and thus affects the stiffness of the plants (D'Auria & Gershenzon 2005; Van Poecke, Posthumus & Dicke 2001). The effect of lignin is especially important for the maintenance of plant's structure during salinity stress and drought stress as the plants lose its turgor pressure. Thus, it is speculated that Bajong contains effective salt acclimation abilities due to the upregulation of all the genes mentioned above.

An increase in TFC content can also be observed in Bajong after the stress treatment as shown in the section above, which is in accordance with the upregulation of many genes observed in the flavonoid biosynthesis (Figure 30) and isoflavonoid biosynthesis pathways (Figure 31). Meanwhile, the increase in Psb P, Psb Q and PetF protein could be due to the repairing of the damaged protein in photosynthetic machinery caused by the high concentration of chlorine ions (Figure 32). Also, significant upregulation can be observed in the synthesis of 4-coumaroyl-CoA (Figure 33). It has been previously reported that 4-coumaroyl-CoA plays an important role in the synthesis of naringenin chalcone, a flavonoid involved in salinity stress response (Walia et al. 2005a). Meanwhile, feruloyl-CoA plays an important in the synthesis of suberin, a polyester polymer that is responsible for the protection of the plants from environmental stresses (Gou, Yu & Liu 2009). The upregulation of suberin synthesis genes could indicate the importance of this molecule in the salt acclimation capability of Bajong.

Previous studies have covered the role of ROS in signalling pathways involved in plant growth, development, gravitropism, hormonal action and many other physiological phenomena (Foyer & Noctor 2005; Mittler et al. 2004), which indicates the important roles of ROS molecules as secondary messengers in signal transduction pathway (Foyer and Noctor, 2005). In this study, a significant increase in the MPV, SOD, CAT and PRDX5 genes can also be observed, indicating the changes in ROS metabolism system of the plants in response to salinity stress (Figure 33). The previous study reported the possibility of the halo-tolerance plant to possess a unique ROS system that provides protection towards the plant's photosynthesis system during salinity stress exposure (Sengupta & Majumder 2010). Thus, it is speculated that Bajong could have different

ROS metabolism system due to the high activity of gene regulation observed. However, such mechanism is complex and required further investigation.

Based on the pathway results obtain, a list of potential genes and respective expression data had also been included in the appendices (Table A-4). These data can be used as potential targets for marker design breeding or gene expression studies in any future studies. All unigenes had also been attached with forward and reverse primers for them to be used in future gene expression studies (Table A-5). However, all primer sequence should be tested before any analysis.

5.6 Conclusion

In conclusion, the changes in transcriptome sequence of Sarawak Rice, Bajong, after salinity stress was reported. It was found that this variety displayed high similarity towards *Oryza Sativa Japonica* species. Most of the transcriptomic sequences are responsible for the general activity of the cells. We have also unveiled 4096 DEGs upon salinity stress exposure, with 5% of the total DEGs involved in the environmental adaptation of the cell. The results suggested that extensive transcriptional reprogramming under salinity stress is necessary for the salt acclimation capability of the variety. The transcriptome analysis present in the current study provides several candidate pathways such as phenylalanine and flavonoid synthesis pathway that are involved upon salinity stress exposure. These candidate genes can be used as potential targets for genetic engineering and open new possibilities for future research.

Chapter 6 General Discussion and Future Directions

6.1 General Discussion

This study was set out to examine different varieties of Sarawak local rice varieties towards their salt tolerance capability. Due to the space constraint, all samples in this study were planted inside the growth chamber without any field test. Even though this significantly restricts many external influences during the growth, it might not properly represent the real world. Furthermore, the parameters chosen were based on the weather reports for the average temperature during daytime and nightfall. No actual trial runs have been done on the rice plants on various temperature and lighting conditions. This might result in the overestimation of growth rate about field studies. Furthermore, no nutrient has been added to the growing media and that might have the slight variation in the initial growth and salt tolerant capacity of the rice. Further optimisation of the growing condition could be done in future studies targeting on the progress of growing. This would allow us to track the growth rate on a day-to-day basis instead of a snapshot of the physiological and biochemical traits.

The genetic diversity in the Sarawak rice varieties have not been classified and studied thoroughly. Furthermore, Sarawak rice varieties are not limited to those 3 varieties. There are more rice varieties locally known as Padi Pandan, Padi Bali, Padi Bubuk, Wangi Mumut, Bajong Lubok Nibong, Padi Chelum, Padi Kurau, Padi Pasur and Padi Sangau. Lastly, the name of the variety is not entirely tied to the genetic diversity of the plants as some varieties are named accordingly depending on the planting location.

Also, due to the time and budget constraint, the sample size for each group was set at a maximum of 30 plants in each analysis. The study period for stress induction was also limited to 19 days due to the size of the growth chamber and space available for plant cultivation. Future studies could be done targeting a much larger sample size across each variety and include a longer period of stress induction. More sophisticated equipment can also be used to obtain an in-depth view of the physiological changes after stress induction. This could be done by incorporating automated phenomics recording equipment together with more specific analysis such as isotope tagging, leaf conductivity measurement and scanning electron microscopy on the microdissected root tissue. This could provide more insight towards the progression of salinity stress in rice. More antioxidant test such as oxygen radical absorbance capacity (ORAC) and ferric

reducing antioxidant power (FRAP) can be incorporated into the antioxidant assay. For the rice grass to be developed into a health drink, cytotoxicity test such as brine shrimp bioassay (BSB) and cell culture assay can be utilised to test for any toxicity assay of the extracts.

More extensive quantitation should also be included in quantitative PCR experiments. According to the MIQE standards (Minimum Information for Publication of Quantitative) (Bustin et al. 2009), a minimum of 2 reference genes should be required for each quantitation. This has not been done in this study as the current equipment only allows 36 reactions per run and incorporating more standards could result in a significant increase in cost and time. Future research that focuses on the specific genes should include more standards with more biological replicates.

The biological replicates in this transcriptomic study were only limited to one per condition due to the budget constraint. This has been considered and thus, RNA extract from 5 individual plants were mixed to overcome any samplings effect that could have been present. Furthermore, total RNA from the whole seedlings was incorporated to visualise the overall changes of gene expression. This could have diluted any differential expression genes that were elevated in certain regions but was repressed in another. Future work could incorporate more individual samples with more specific tissue to get a clearer view of gene expression within the organism during salinity stress.

All the genes discovered in the qRT-PCR experiment cannot be found in the list of DEGs resulted from transcriptomic sequencing. It is speculated that the resolution of the sequencing was insufficient to significantly identified the changes in expression between the two samples and a deeper sequencing run will be required to study these salt responsive genes. Alternatively, targeted sequencing technology such as CHIP-Seq or Microarray can be used to study the expression levels of these genes in future.

Finally, all DEGs found should also be compared to other studies to find any potential salt responsive genes that had not been validated. However, comparison of reads data requires an extensive computational power and therefore was not included in this study.

6.2 Future Directions

Results from this study are expected to increase the exposure of Sarawak rice varieties in the research community and enhance the international knowledge base of salt

tolerance properties on Sarawak local rice varieties by the discovery of salt tolerance variety, Biris and salinity-responsive variety, Bajong. Meanwhile, it also hopes to raise awareness on the protection of traditional farming methods in the rural areas as it cultivates many indigenous species that could possess better abiotic stress tolerance.

The high germination rate of Biris and high tolerance towards salinity stress in the early seedlings stage is of particular importance in seedlings cultivation industries. The extreme weather pattern had made it increasingly challenging to maintain a stable supply of fresh water towards rice cultivation industries. Thus, seedlings that could tolerate an increased salinity during germination stage could result in the direct increase in final seedlings output. Biris can be used as a model system for the study of salinity tolerance system in young seedlings and could be included as one of the breeds for any cross-breeding programs.

The high enrichment of secondary metabolite pathways present in Bajong could also be exploited for the investigation of salt tolerance mechanism in rice. The gene expression of a large number of genes involved in phenolic and flavonoids production had been identified in this study. They can be further investigated for their role in salt tolerance properties of rice. These potential salt responsive genes can also be incorporated into CHIP-Seq or microarray chips to study the changes in expression upon exposure to various environmental stresses. This could reveal any wide spectrum stress-signalling gene that is capable of triggering all possible stress responses mechanism in the plant.

Meanwhile, the presences of high antioxidant in rice seedlings also could encourage any further investigation to discover any pharmaceutical or nutraceutical potential of these rice varieties. Previous research by Tan et al. (2016) has indicated the rice bran extracts from Sarawak local rice varieties contained significant cytoprotective effects. Thus, it is speculated that extracts from these seedlings could also possess similar cytoprotective properties and should be further investigated.

Appendices

Table A-1 Gene expression data from real-time PCR (CT value)

Name of	Bario		Bajong		Biris		MR219	
Genes	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
ACTIN	25.77	24.12	23.67	23.66	23.44	23.38	24.45	21.94
ACTIN	24.20	23.80	23.31	24.60	23.41	22.28	25.10	22.19
ACTIN	25.32	23.43	24.73	24.81	25.07	22.13	25.35	21.83
SOS1	27.13	25.31	27.30	24.91	24.70	23.77	26.67	23.16
SOS1	26.23	25.38	28.07	25.02	24.98	24.13	26.10	23.39
SOS1	26.06	25.11	27.75	24.97	22.23	23.45	25.86	22.97
SOS2	22.37	21.40	22.43	23.18	22.38	20.47	24.45	21.01
SOS2	23.15	21.36	25.01	23.38	21.86	21.15	24.01	21.23
SOS2	22.68	21.11	24.13	22.52	24.06	20.87	24.10	21.31
CIPK1	24.82	24.75	25.09	25.03	24.47	23.85	23.86	21.85
CIPK1	24.33	24.70	24.69	24.20	23.53	23.51	24.60	21.92
CIPK1	24.55	26.50	24.97	24.81	24.38	22.96	22.67	21.68
CIPK11	25.62	24.52	25.77	26.50	24.24	23.17	23.31	22.95
CIPK11	24.73	24.79	24.72	24.62	24.23	23.28	22.18	22.51
CIPK11	24.86	24.52	25.48	24.29	24.22	23.31	22.20	21.80
LEA1	29.19	28.65	28.94	26.48	26.14	25.94	24.07	25.72
LEA1	27.35	27.66	27.50	27.11	27.57	25.63	24.20	25.60
LEA1	28.21	27.74	30.35	26.57	26.65	26.30	24.49	25.67
LEA2	32.7	34.24	32.6	32.39	27.00	30.94	26.5	30.33
LEA2	30.08	N/A	33.13	33.26	28.33	30.90	29.25	30.85
LEA2	29.53	33.10	31.64	33.55	28.51	30.55	26.83	31.42
ADC	25.11	26.74	30.31	30.07	25.7	25.72	27.77	25.3
ADC	26.32	27.21	29.78	31.47	26.21	26.05	27.93	29.1
ADC	24.9	26.97	29.25	28.95	27.38	25.39	29.16	28.84
ODC	N/A	N/A	N/A	30.03	N/A	30.41	N/A	N/A
ODC	N/A	N/A	N/A	31.03	N/A	27.16	N/A	N/A
ODC	N/A	N/A	N/A	29.96	N/A	28.61	N/A	N/A

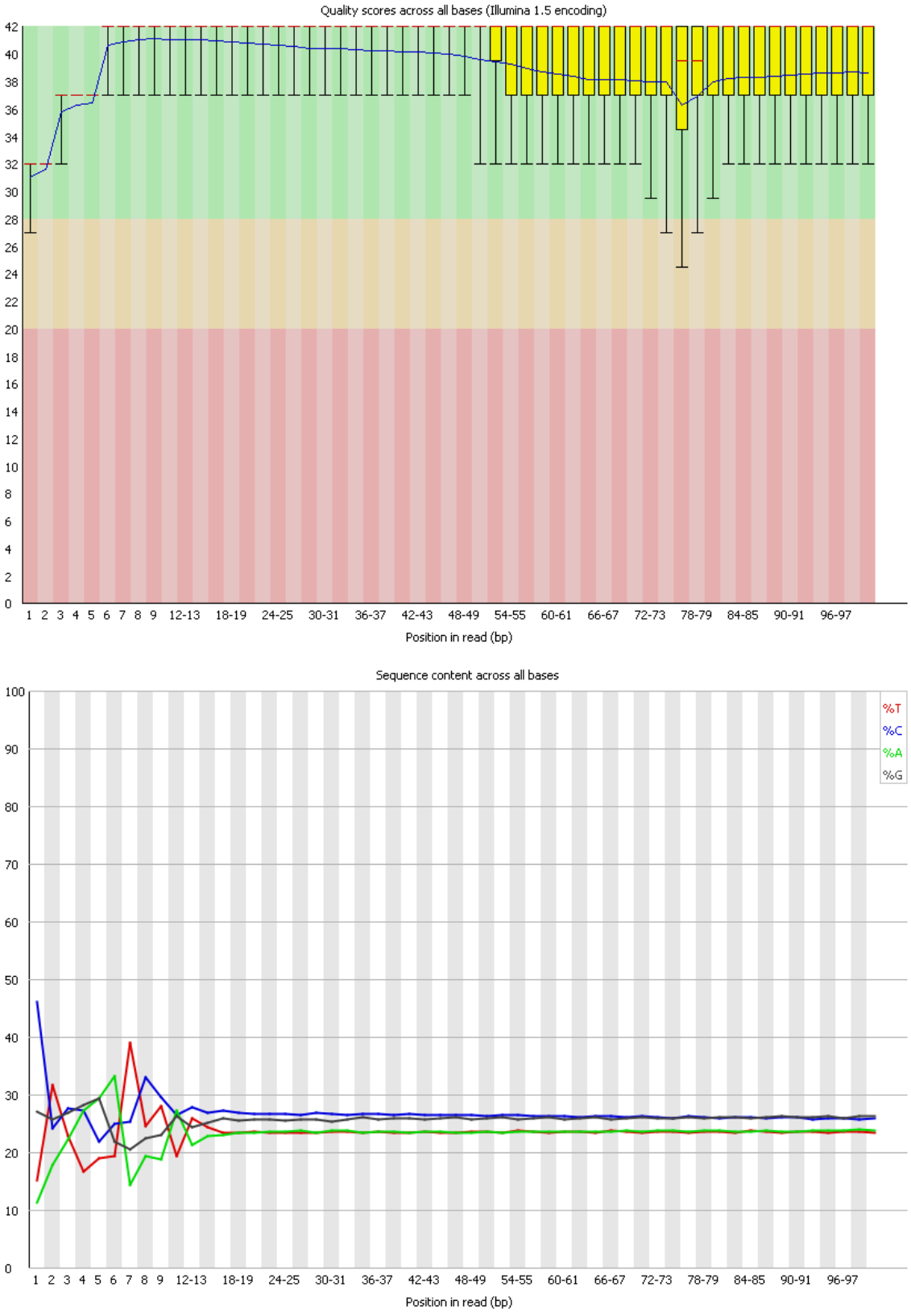
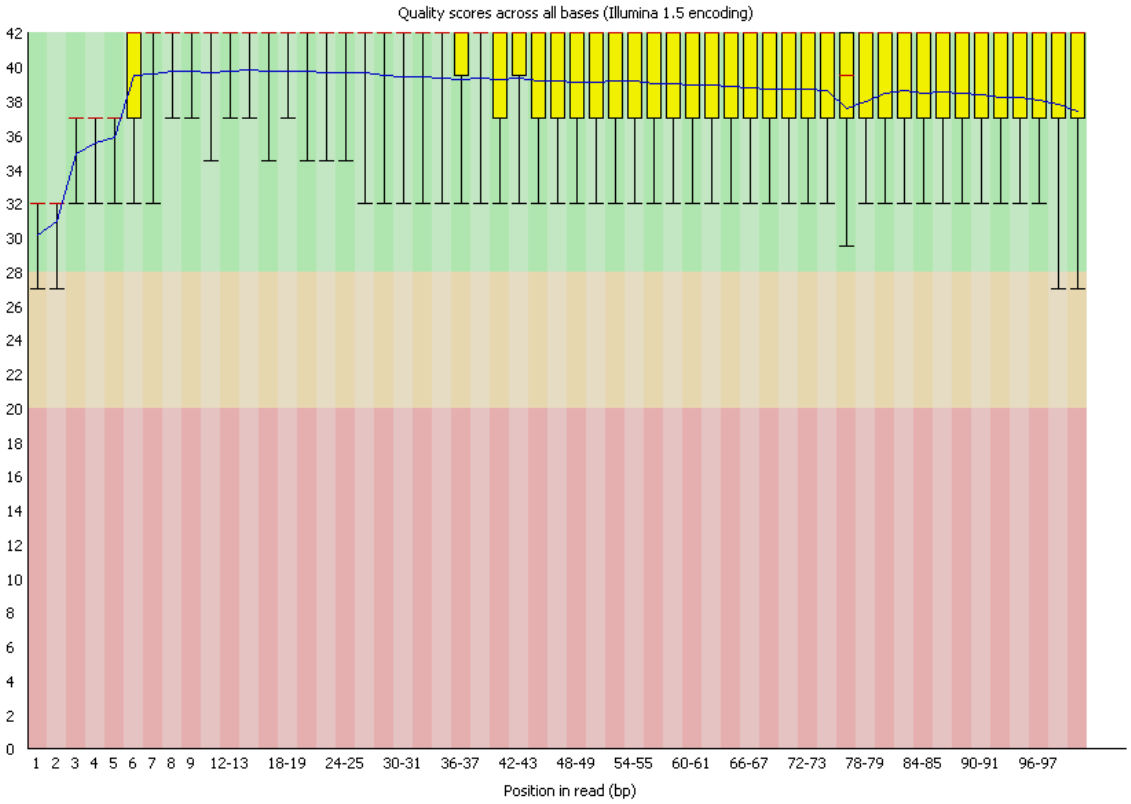


Figure A-1 Schematic overview of quality values across all bases at in the control sample (forward) at each position.



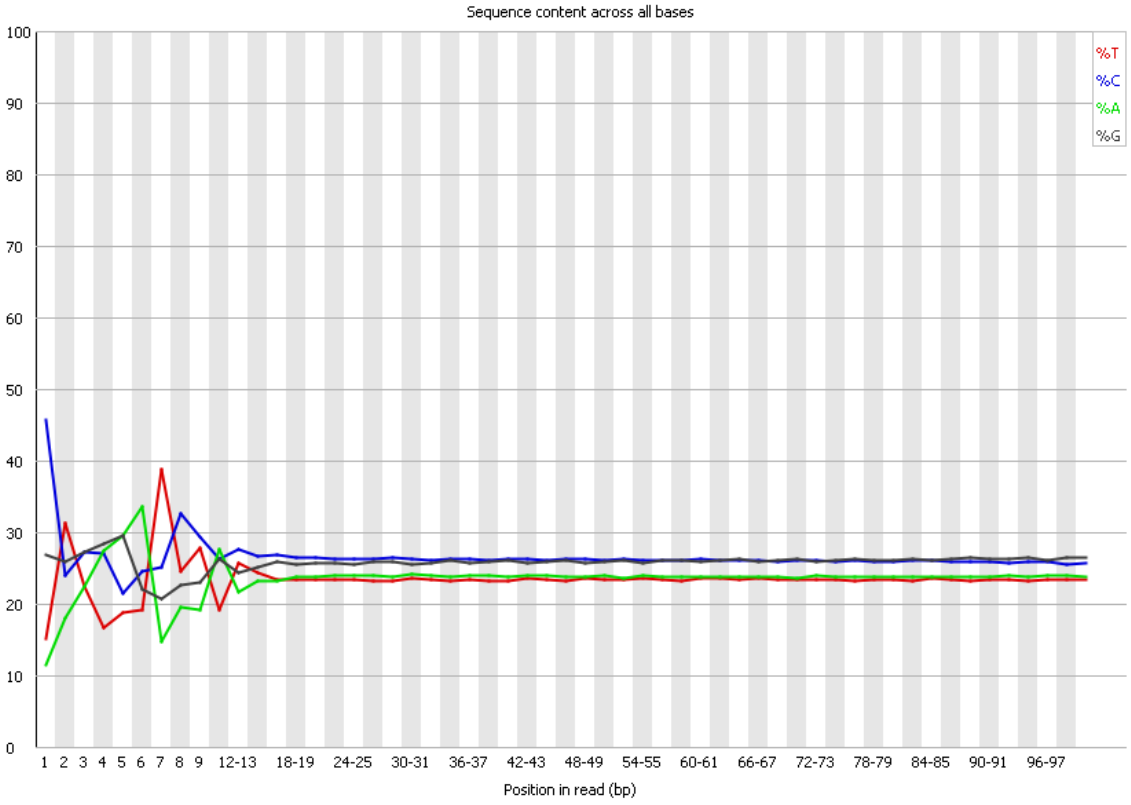
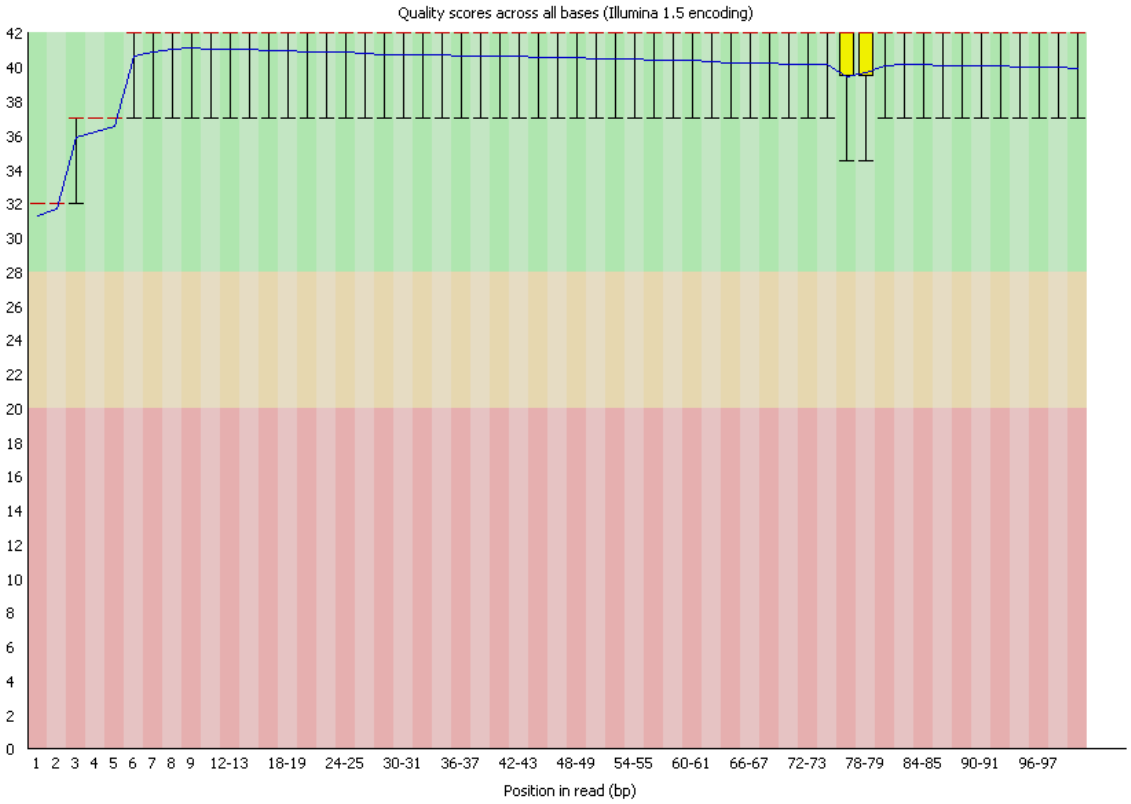


Figure A-2 Schematic overview of quality values across all bases at in the control sample (reverse) at each position.



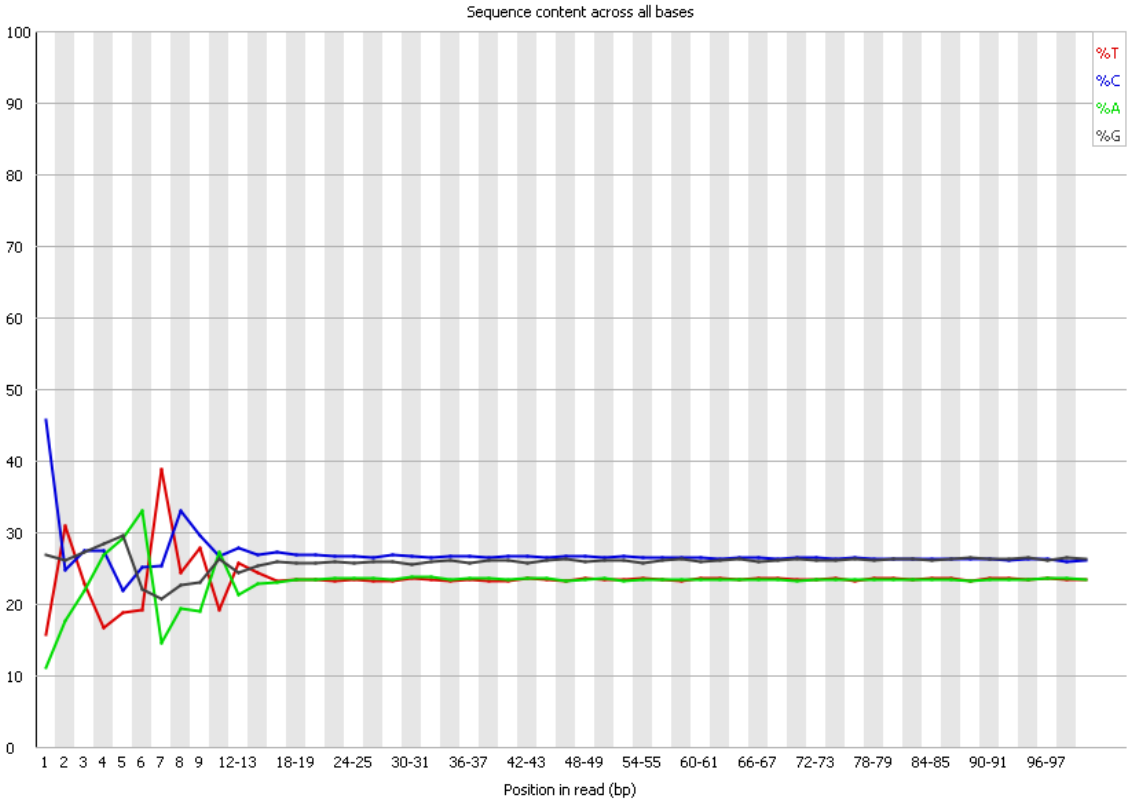


Figure A-3 Schematic overview of quality values across all bases at in the stressed sample (forward) at each position.

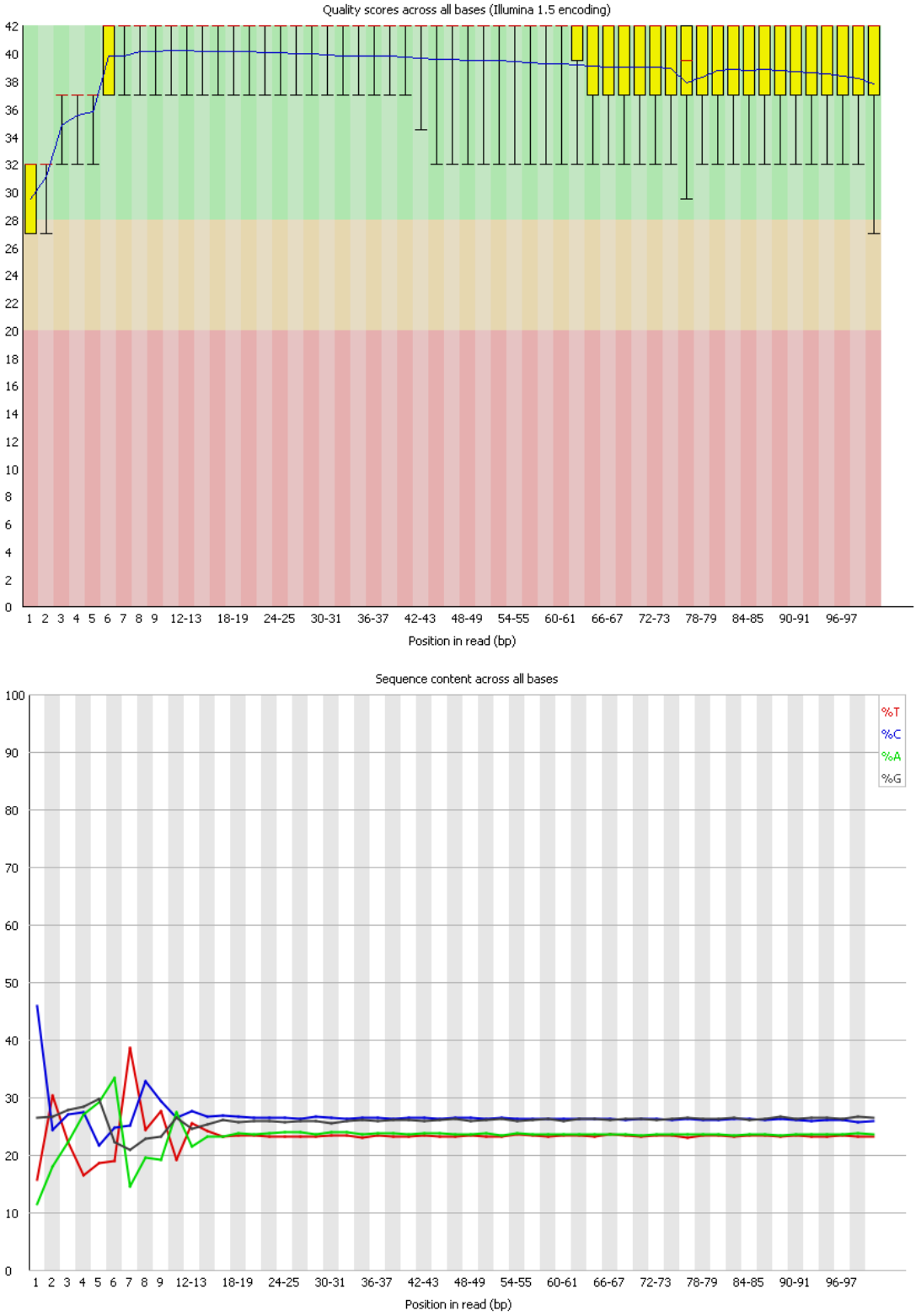


Figure A-4 Schematic overview of the quality values across all bases at in the stressed sample (reverse) at each position.

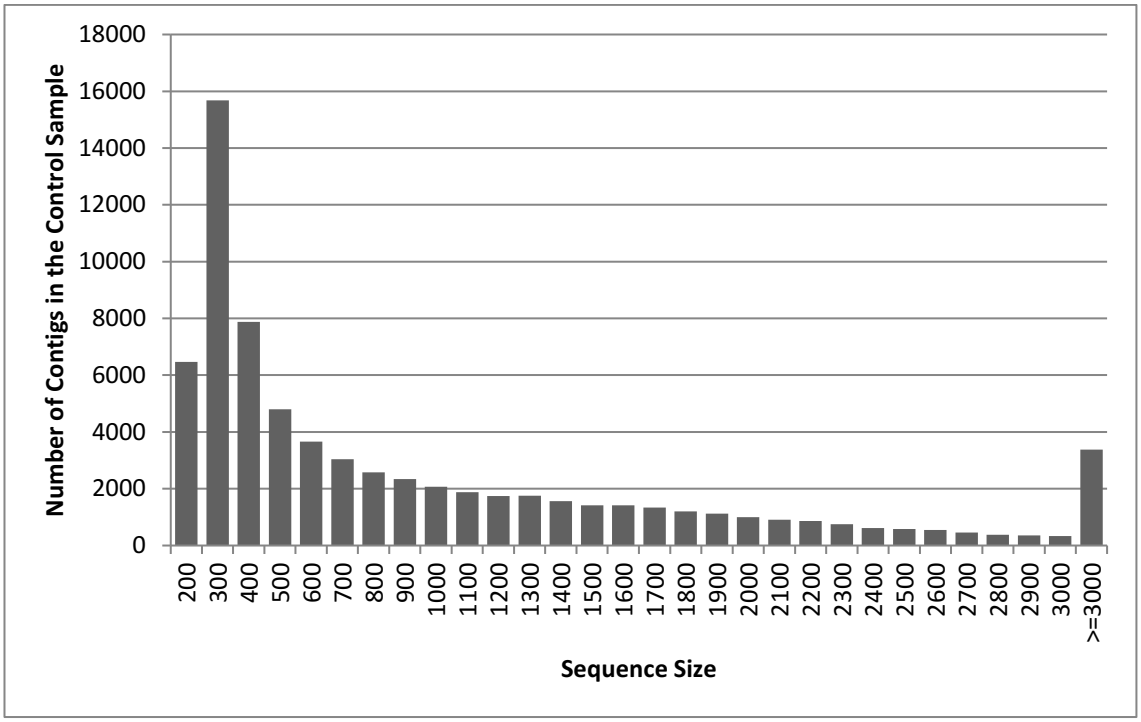


Figure A-5 Contigs length distribution in the control sample.

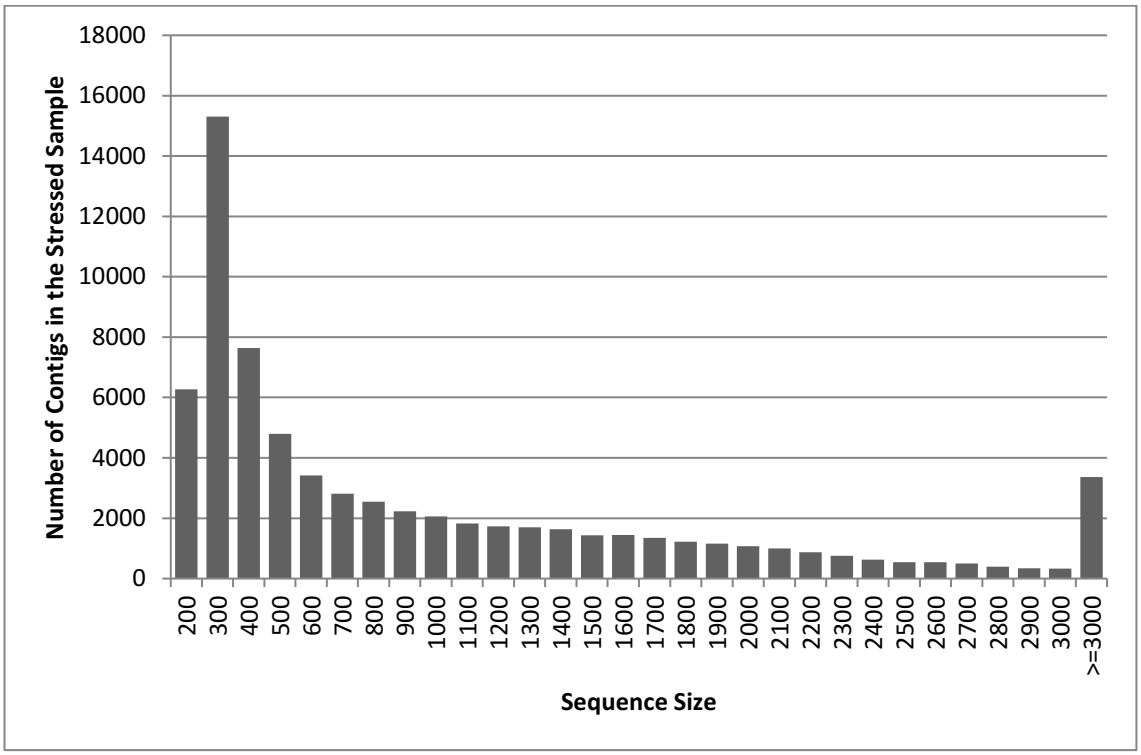


Figure A-6 Contigs length distribution in the stressed sample.

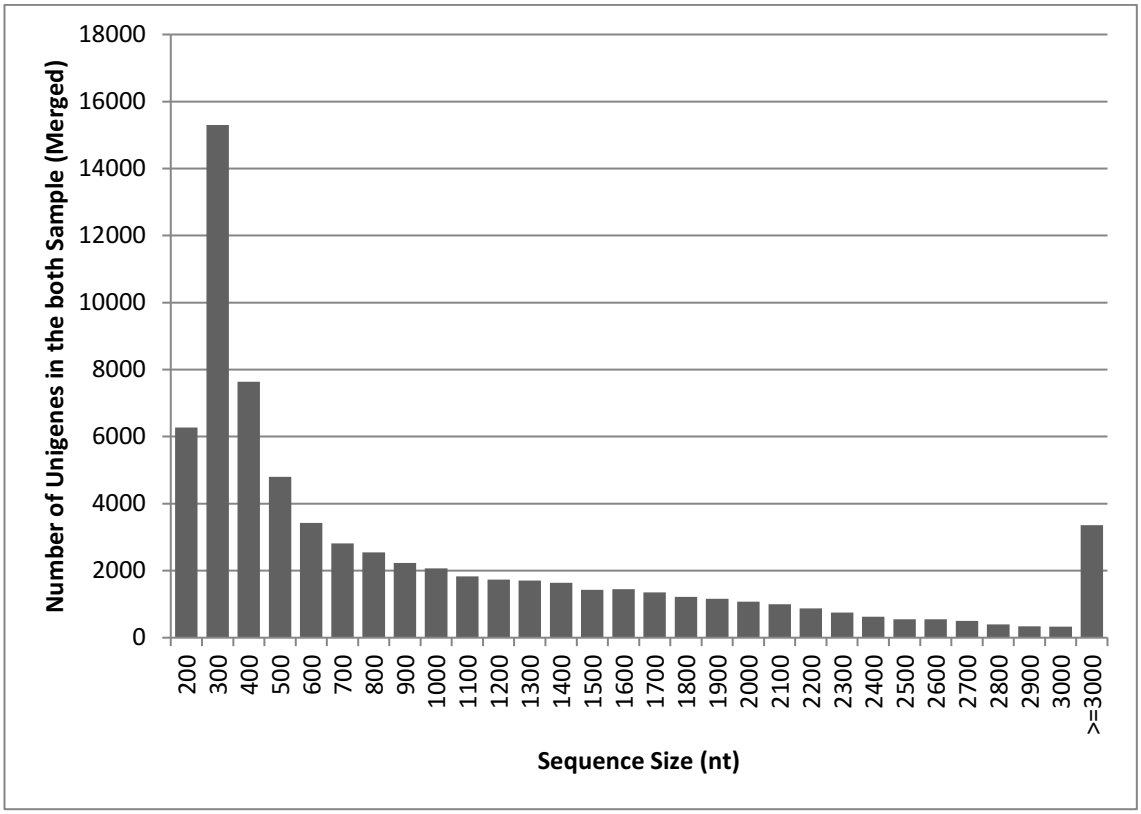


Figure A-7 Length distributions of all mapped unigenes.

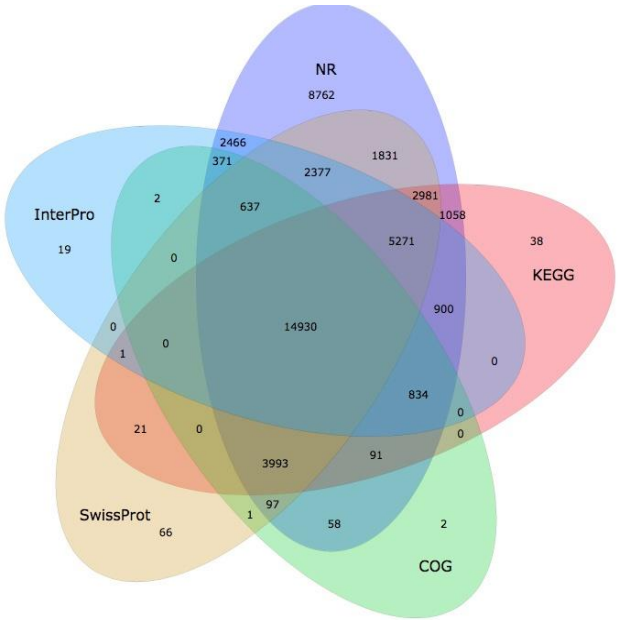


Figure A-8 Venn diagram showing the overlapping annotation of unigenes between Nr, COG, KEGG, Swissprot and Interpro databases.

Table A-2 Summary size of single sequence repeat (SSR).

Number of SSR	Mono-nucleotide	Di-nucleotide	Tri-nucleotide	Quad-nucleotide	Penta-nucleotide	Hexa-nucleotide
4	0	0	0	0	474	440
5	0	0	8,474	207	91	30
6	0	1,365	3,979	61	5	14
7	0	674	1,702	5	0	10
8	0	593	727	5	2	1
9	0	314	127	0	0	1
10	0	217	112	0	0	0
11	0	212	53	0	0	1
12	593	152	22	0	0	1
13	313	30	20	1	0	0
14	236	67	11	2	0	0
15	155	47	4	2	0	0
16	79	39	5	0	0	0
17	58	34	2	0	0	0
18	57	41	5	0	0	0
19	33	29	8	0	0	0
20	32	15	6	0	0	0
21	22	27	4	0	0	0
22	21	13	5	0	0	0
23	211	17	2	0	0	0
24	7	14	0	0	0	0
25	2	15	0	0	0	0
26	3	5	1	0	0	0
27	5	5	0	0	0	0
28	4	12	1	0	0	0
29	14	5	0	0	0	0
30	5	2	0	0	0	0
31	6	1	0	0	0	0
32	4	1	0	0	0	0
33	9	0	1	0	0	0
34	6	1	0	0	0	0
35	5	0	0	0	0	0
Total	1,880	3,947	15,271	283	572	498

Table A-3 List of DEGs enrichment relative to full-annotated unigenes set.

No	Pathway	DEGs with pathway annotation (2706)	All genes with pathway annotation (30118)	P-value	Pathway ID	Level 1	Level 2
1	Biosynthesis of secondary metabolites	344 (12.71%)	3086 (10.25%)	9.30E-06	ko01110	Metabolism	Global map
2	Natural killer cell mediated cytotoxicity	28 (1.03%)	136 (0.45%)	2.58E-05	ko04650	Organismal Systems	Immune system
3	Ubiquinone and other terpenoid-quinone biosynthesis	22 (0.81%)	102 (0.34%)	8.84E-05	ko00130	Metabolism	Metabolism of cofactors and vitamins
4	Starch and sucrose metabolism	127 (4.69%)	1042 (3.46%)	0.000253031	ko00500	Metabolism	Carbohydrate metabolism
5	Regulation of autophagy	24 (0.89%)	131 (0.43%)	0.000591464	ko04140	Cellular Processes	Transport and catabolism
6	Glycosphingolipid biosynthesis - globo series	7 (0.26%)	22 (0.07%)	0.002380507	ko00603	Metabolism	Glycan biosynthesis and metabolism
7	Porphyrin and chlorophyll metabolism	22 (0.81%)	129 (0.43%)	0.002535756	ko00860	Metabolism	Metabolism of cofactors and vitamins
8	Phenylpropanoid biosynthesis	60 (2.22%)	480 (1.59%)	0.005757089	ko00940	Metabolism	Biosynthesis of other secondary metabolites
9	Indole alkaloid biosynthesis	11 (0.41%)	53 (0.18%)	0.006675348	ko00901	Metabolism	Biosynthesis of other secondary metabolites
10	Tropane, piperidine and pyridine alkaloid biosynthesis	12 (0.44%)	65 (0.22%)	0.01221046	ko00960	Metabolism	Biosynthesis of other secondary metabolites
11	Mismatch repair	22 (0.81%)	148 (0.49%)	0.01305656	ko03430	Genetic Information Processing	Replication and repair
12	Peroxisome	32 (1.18%)	237 (0.79%)	0.01331591	ko04146	Cellular Processes	Transport and catabolism
13	Sesquiterpenoid and triterpenoid biosynthesis	11 (0.41%)	59 (0.2%)	0.01494241	ko00909	Metabolism	Metabolism of terpenoids and polyketides
14	Brassinosteroid biosynthesis	14 (0.52%)	83 (0.28%)	0.01555559	ko00905	Metabolism	Metabolism of terpenoids and polyketides
15	Cyanoamino acid metabolism	29 (1.07%)	220 (0.73%)	0.02379624	ko00460	Metabolism	Metabolism of other amino acids
16	Diterpenoid biosynthesis	20 (0.74%)	139 (0.46%)	0.02392558	ko00904	Metabolism	Metabolism of terpenoids and polyketides
17	Monoterpenoid biosynthesis	8 (0.3%)	40 (0.13%)	0.02393686	ko00902	Metabolism	Metabolism of terpenoids and polyketides
18	Flavonoid biosynthesis	31 (1.15%)	241 (0.8%)	0.0271162	ko00941	Metabolism	Biosynthesis of other secondary metabolites
19	Phenylalanine metabolism	28 (1.03%)	217 (0.72%)	0.03311569	ko00360	Metabolism	Amino acid metabolism
20	Spliceosome	154 (5.69%)	1489 (4.94%)	0.03532108	ko03040	Genetic Information Processing	Transcription

Table A-4 List of potential salt tolerant genes and their expression profiles in several pathways.

						Fold Change (Expressed in Log_2)	
Reference Pathway Code	**Name of Pathways**	**Name of Enzyme**	**Enzyme Code**	**Gene Orthology Entry**	**Name of Unigene Involved**	**Downregulated**	**Upregulated**
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL2013.Contig3_All	6.0	
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL2013.Contig5_All		7.2
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL2013.Contig6_All	3.7	
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL3180.Contig3_All	1.9	
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL6846.Contig1_All		1.1
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL7410.Contig1_All	2.4	
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	CL7410.Contig2_All	1.4	
ko00905	Brassinosteroid biosynthesis	PHYB activation tagged suppressor 1	1.14.-.-	K15639	Unigene4484_All	2.3	
ko00905	Brassinosteroid biosynthesis	steroid 22-alpha-hydroxylase	1.14.13. -	K09587	CL3646.Contig1_All		2.1
ko00905	Brassinosteroid biosynthesis	steroid 22-alpha-hydroxylase	1.14.13. -	K12639	CL982.Contig2_All	6.7	
ko00905	Brassinosteroid biosynthesis	steroid 22-alpha-hydroxylase	1.14.13. -	K12639	CL982.Contig7_All		2.9
ko00905	Brassinosteroid biosynthesis	steroid 22-alpha-hydroxylase	1.14.13. -	K12639	Unigene29494_All	1.8	
ko00905	Brassinosteroid biosynthesis	steroid 22-alpha-hydroxylase	1.14.13. -	K09587	Unigene29494_All	1.8	
ec00906	Carotenoid biosynthesis	lycopene cyclase CruA	5.5.1.19	K14606	CL4937.Contig7_All	6.9	
ec00906	Carotenoid biosynthesis	lycopene cyclase CruA	5.5.1.19	K14606	CL4937.Contig8_All		2.9
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	CL35.Contig6_All	1.5	
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	CL3853.Contig2_All	1.4	
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	CL3895.Contig3_All	2.0	
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	CL5898.Contig3_All		1.6
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	CL6866.Contig2_All		1.9
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	Unigene10573_All		1.5
ec00906	Carotenoid biosynthesis	zeta-carotene desaturase	1.3.5.6	K00514	Unigene6153_All		1.0
ec00941	Flavonoid biosynthesis	anthocyanidin reductase	1.3.1.77	K08695	CL2123.Contig3_All		1.6
ec00941	Flavonoid biosynthesis	anthocyanidin reductase	1.3.1.77	K08695	CL2123.Contig7_All	7.5	

ec00941	Flavonoid biosynthesis	anthocyanidin reductase	1.3.1.77	K08695	Unigene16115_All	1.4	
ec00941	Flavonoid biosynthesis	flavonoid 3'-monooxygenase	1.14.13.21	K05280	CL2131.Contig5_All	1.5	
ec00941	Flavonoid biosynthesis	flavonoid 3'-monooxygenase	1.14.13.21	K05280	CL4397.Contig1_All		1.2
ec00941	Flavonoid biosynthesis	flavonoid 3'-monooxygenase	1.14.13.21	K05280	CL4397.Contig3_All	4.4	
ec00941	Flavonoid biosynthesis	flavonoid 3'-monooxygenase	1.14.13.21	K05280	CL684.Contig3_All		6.9
ec00941	Flavonoid biosynthesis	flavonoid 3'-monooxygenase	1.14.13.21	K05280	Unigene6237_All	1.3	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	CL1650.Contig2_All	1.3	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	CL4431.Contig2_All	1.9	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	CL5668.Contig2_All		2.8
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	CL7260.Contig1_All	1.2	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	CL7260.Contig2_All	1.9	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	Unigene22569_All		8.2
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	Unigene22571_All	8.3	
ec00941	Flavonoid biosynthesis	flavonol synthase	1.14.11.23	K05278	Unigene29035_All	1.1	
ec00941	Flavonoid biosynthesis	leucoanthocyanidin dioxygenase	1.14.11.19	K05277	CL7260.Contig1_All	1.2	
ec00941	Flavonoid biosynthesis	leucoanthocyanidin dioxygenase	1.14.11.19	K05277	Unigene22569_All		8.2
ec00941	Flavonoid biosynthesis	leucoanthocyanidin dioxygenase	1.14.11.19	K05277	Unigene22571_All	8.3	
ec00941	Flavonoid biosynthesis	naringenin 3-dioxygenase	1.4.11.9	K00475	CL5668.Contig2_All		2.8
ec00941	Flavonoid biosynthesis	naringenin 3-dioxygenase	1.4.11.9	K00475	Unigene18822_All	1.1	
ec00941	Flavonoid biosynthesis	polyketide reductase	2.3.1.170	K08243	CL2262.Contig3_All		1.2
ec00941	Flavonoid biosynthesis	polyketide reductase	2.3.1.170	K08243	Unigene17442_All	2.1	
ec00941	Flavonoid biosynthesis	polyketide reductase	2.3.1.170	K08243	Unigene18098_All		1.9
ec00943	Isoflavonoid biosynthesis	2-hydroxyisoflavanone dehydratase	4.2.1.105	K13258	Unigene11370_All		1.5
ec00943	Isoflavonoid biosynthesis	2-hydroxyisoflavanone dehydratase	4.2.1.105	K13258	Unigene22586_All		1.8
ec00943	Isoflavonoid biosynthesis	2-hydroxyisoflavanone dehydratase	4.2.1.105	K13258	Unigene22950_All		3.1
ec00943	Isoflavonoid biosynthesis	2-hydroxyisoflavanone synthase	1.14.13.136	K13257	CL4397.Contig1_All		1.2
ec00943	Isoflavonoid biosynthesis	2-hydroxyisoflavanone synthase	1.14.13.136	K13257	CL4397.Contig3_All	4.4	
ko00360	Phenylalanine metabolism	aspartate aminotransferase, cytoplasmic	2.6.1.1	K15849	CL3360.Contig2_All	1.5	
ko00360	Phenylalanine metabolism	aspartate aminotransferase, cytoplasmic	2.6.1.1	K15849	CL794.Contig7_All		2.2

ko00360	Phenylalanine metabolism	tyrosine aminotransferase	2.6.1.5	K00815	CL4762.Contig2_All		3.8
ko00360	Phenylalanine metabolism	tyrosine aminotransferase	2.6.1.5	K00815	CL6408.Contig2_All	1.9	
ko00360	Phenylalanine metabolism	tyrosine aminotransferase	2.6.1.5	K00815	Unigene30369_All		2.1
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	CL1840.Contig4_All	1.2	
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	CL2201.Contig1_All	1.5	
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	CL2201.Contig3_All	8.5	
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	CL2201.Contig4_All		7.6
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	CL7045.Contig2_All		1.6
ec00940	Phenylpropanoid biosynthesis	cinnamoyl-CoA reductase	1.1.2.44	K09753	Unigene12817_All		2.5
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL2993.Contig7_All		2.0
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL2993.Contig8_All		2.0
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL3048.Contig1_All	1.4	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL5081.Contig1_All	8.2	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL5665.Contig2_All		1.2
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL6872.Contig1_All		6.1
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL6896.Contig2_All	1.2	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	CL7588.Contig2_All	7.8	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene11777_All	2.0	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene15871_All	1.7	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene20083_All	2.0	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene22612_All		1.5
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene29255_All	2.1	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene3003_All	1.2	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene32135_All	2.4	
ko00940	Phenylpropanoid biosynthesis	peroxidase	1.11.1.7	K00430	Unigene3945_All	1.9	
ec00940	Phenylpropanoid biosynthesis	shikimate O-hydroxycinnamoyltransferase	2.3.1.133	K13065	CL3006.Contig3_All		1.7
ec00940	Phenylpropanoid biosynthesis	shikimate O-hydroxycinnamoyltransferase	2.3.1.133	K13065	CL4556.Contig3_All	6.6	
ec00940	Phenylpropanoid biosynthesis	shikimate O-hydroxycinnamoyltransferase	2.3.1.133	K13065	CL61.Contig4_All		1.0

ec00940	Phenylpropanoid biosynthesis	shikimate O-hydroxycinnamoyltransferase	2.3.1.133	K13065	CL6653.Contig1_All	8.3	
ec00940	Phenylpropanoid biosynthesis	shikimate O-hydroxycinnamoyltransferase	2.3.1.133	K13065	Unigene2710_All	1.8	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL1900.Contig3_All		1.8
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL1904.Contig2_All	1.2	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL1904.Contig2_All	1.2	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL2160.Contig1_All	1.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL2160.Contig1_All	1.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL4317.Contig2_All		1.4
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL4317.Contig3_All		1.4
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL5565.Contig1_All	1.7	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL5565.Contig1_All	1.7	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL762.Contig1_All		1.8
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig11_All		1.2
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig13_All		7.7
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL80.Contig13_All		7.7
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig14_All	4.8	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL80.Contig14_All	4.8	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig18_All	5.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL80.Contig18_All	5.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig2_All	1.7	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig26_All	1.4	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig4_All	4.2	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	CL80.Contig7_All	5.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	CL80.Contig7_All	5.6	
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K01188	Unigene22405_All		1.4
ko00500	Starch and sucrose metabolism	beta-glucosidase	3.2.1.21	K05350	Unigene22405_All		1.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1114.Contig2_All	1.7	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1158.Contig11_All		1.8

ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1407.Contig8_All	1.3	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1499.Contig3_All		7.7
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL165.Contig1_All	1.3	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1704.Contig4_All	2.9	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL1821.Contig1_All		1.2
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2000.Contig7_All	2.5	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2139.Contig2_All	1.3	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2139.Contig5_All		1.7
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2139.Contig7_All		1.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig1_All	8.2	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig13_All		5.3
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig16_All		2.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig18_All	1.9	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig20_All		7.6
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig31_All		1.2
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig34_All	7.8	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2361.Contig9_All		1.5
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL2480.Contig1_All	1.2	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL260.Contig1_All		1.2
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL260.Contig2_All		8.1
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL288.Contig4_All		1.3
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3275.Contig1_All	6.7	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3483.Contig2_All		1.3
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3718.Contig1_All	1.1	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3737.Contig1_All	2.1	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3737.Contig4_All	2.4	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL3737.Contig5_All	3.0	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL413.Contig6_All		6.1
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL413.Contig7_All		2.2

ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL4193.Contig2_All		2.1
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL4368.Contig2_All	1.8	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL4498.Contig2_All		5.6
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL5487.Contig1_All	1.3	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL563.Contig16_All	1.8	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL5726.Contig1_All		6.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL5736.Contig4_All		1.9
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL5811.Contig2_All	1.5	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL616.Contig3_All		2.6
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL6324.Contig2_All		6.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL6350.Contig3_All	1.3	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL6446.Contig2_All	2.1	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL666.Contig1_All	1.2	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL666.Contig2_All		2.5
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL666.Contig4_All		1.2
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL666.Contig5_All	1.8	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL666.Contig6_All		2.7
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL6741.Contig3_All		2.1
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL7433.Contig1_All	2.6	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	CL7433.Contig3_All		1.8
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene14222_All	1.7	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene1531_All		1.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene1842_All	1.2	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene1904_All	1.1	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene22514_All	2.8	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene22647_All		2.0
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene22815_All	1.2	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene24798_All		6.4
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene29569_All		1.8

ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene29691_All		1.5
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene30410_All	4.1	
ko00500	Starch and sucrose metabolism	pectinesterase	3.1.1.11	K01051	Unigene5407_All		3.9
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	tocopherol cyclase	5.5.1.24	K09834	CL1718.Contig3_All	5.3	
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	tyrosine aminotransferase	2.6.1.5	K00815	CL4762.Contig2_All		3.8
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	tyrosine aminotransferase	2.6.1.5	K00815	Unigene30369_All		2.1
map00195	Photosynthesis	Psb P	-	K02717	CL6488.Contig2_All		7.7
map00195	Photosynthesis	Psb P	-	K02717	CL4346.Contig2_All		1.4
map00195	Photosynthesis	Psb Q	-	K08901	Unigene17657_All		1.3
map00195	Photosynthesis	Psb F	-	K02639	Unigene22260_All		1.5
map00195	Photosynthesis	Psb F	-	K02639	CL5724.Contig2_All		1.1
map4146	Peroxisome	MVP 17	-	K13348	CL107.Contig1_All		8.1
map4146	Peroxisome	MVP 17	-	K13348	Unigene25002_All		1.1
map4146	Peroxisome	MLYCD	-	K01058	CL1858.Contig1_Al		5.6
map4146	Peroxisome	MLYCD	-	K01058	CL1858.Contig4_All		1.5
map4146	Peroxisome	MLYCD	-	K01058	CL1858.Contig2_All	2.5	
map4146	Peroxisome	MLYCD	-	K01058	CL1858.Contig3_All	2.4	
map4146	Peroxisome	MVK	-	K00869	CL202.Contig2_All		7.8
map4146	Peroxisome	CAT	-	K03781	CL2719.Contig3_All		1.4
map4146	Peroxisome	CAT	-	K03781	CL4183.Contig2_All	2.2	2.3
map4146	Peroxisome	SOD1	-	K04565	CL4466.Contig3_All		2.3
map4146	Peroxisome	SOD1	-	K04565	CL4599.Contig6_All		2.2
map4146	Peroxisome	SOD1		K04565	CL4599.Contig3_All		2.0
map4146	Peroxisome	SOD1		K04565	CL4599.Contig2_All		1.3
map4146	Peroxisome	SOD1		K04565	CL4599.Contig4_All		1.2

Table A-5 List of Primer sequences designed from newly discovered salinity-responsive genes

No	Seq ID	Count	Orientation	Start	Primer Length	Melting Temperature	GC%	Primer Sequence	Product Size	Sequence Length	Included Length
1	CL107.Contig1_All	1	FORWARD	1573	20	60.08	45.00	TTGTTCGAACCTGGAAAAGG	201	2432	2432
	CL107.Contig1_All	1	REVERSE	1773	20	60.2	60.00	GCCAACCTCTCCTCTTAGGG			
2	CL1114.Contig2_All	1	FORWARD	497	20	59.8	55.00	CGAAGATCTCGGTGAAGGAC	251	751	751
	CL1114.Contig2_All	1	REVERSE	747	21	60.35	47.62	TGAGGTAAACGCAGTGACACA			
3	CL1158.Contig11_All	1	FORWARD	525	20	59.82	50.00	CATGGCCAAGAGTCAGCATA	251	1471	1471
	CL1158.Contig11_All	1	REVERSE	775	20	59.7	55.00	GGCTGTCTTGGTGAAGTTCC			
4	CL1407.Contig8_All	1	FORWARD	893	20	60.61	45.00	TTATGCCGCTGCCAGTTAAT	248	1545	1545
	CL1407.Contig8_All	1	REVERSE	1140	20	59.87	50.00	GGAATTCCCTCAAGGAAAGG			
5	CL1467.Contig2_All	1	FORWARD	934	20	60.13	50.00	AGCAGGCTTGGAAACAGAGA	256	1527	1527
	CL1467.Contig2_All	1	REVERSE	1189	20	59.89	55.00	CAGCAGAGCAGCAAGATCAC			
6	CL1499.Contig3_All	1	FORWARD	92	20	60.74	50.00	CATATTCATCTGGCGGTGGT	242	556	556
	CL1499.Contig3_All	1	REVERSE	333	19	61.12	57.89	GGTCGCCGGATCTAGCATA			
7	CL165.Contig1_All	1	FORWARD	68	20	59.38	40.00	TTACATCCGGGGAAGAAAAA	238	1185	1185
	CL165.Contig1_All	1	REVERSE	305	20	59.97	50.00	GGGCACTAAACACCCAGAAA			
8	CL1650.Contig2_All	1	FORWARD	512	20	59.89	50.00	TCTCTATTCCATCCCCAACG	252	1636	1636
	CL1650.Contig2_All	1	REVERSE	763	20	59.98	55.00	GCTTGAGCGAGGGATACTTG			
9	CL1704.Contig4_All	1	FORWARD	761	20	60.05	45.00	TTCTGAATCCATCCGTGTCA	247	1067	1067
	CL1704.Contig4_All	1	REVERSE	1007	20	59.73	60.00	GCTCCGACTACAGGACCAAC			
10	CL1718.Contig3_All	1	FORWARD	989	20	60.01	50.00	GTGCCTGGTTCTTTTGTGGT	247	2805	2805
	CL1718.Contig3_All	1	REVERSE	1235	20	59.98	50.00	ATGTCTTCTCAGGCGCATCT			
11	CL1821.Contig1_All	1	FORWARD	205	20	60.17	50.00	GGGTTGGGGCTATATTTGCT	252	1428	1428
	CL1821.Contig1_All	1	REVERSE	456	20	60.36	55.00	GGATAGCAGGCAGTCCAAGA			
12	CL1840.Contig4_All	1	FORWARD	1025	20	59.83	55.00	CTTCTAGATGGGCGACCTTG	250	1308	1308
	CL1840.Contig4_All	1	REVERSE	1274	20	59.81	45.00	GACATGGCTGCATGAAAGAA			
13	CL1858.Contig1_All	1	FORWARD	450	20	60.11	50.00	AAGGGCATTCAACAGAGTGG	205	1115	1115
	CL1858.Contig1_All	1	REVERSE	654	20	60.13	45.00	AGCGAGAAAACAAAGGCAGA			
14	CL1858.Contig2_All	1	FORWARD	214	20	60.09	40.00	TTGAACATGCTGGTGGAAAA	198	1025	1025
	CL1858.Contig2_All	1	REVERSE	411	20	60.38	40.00	TTGCTCCATTTTGCAAGTGA			

15	CL1858.Contig3_All	1	FORWARD	970	20	60.05	55.00	GATGACCCTCCAACTCCTGA	197	2199	2199
	CL1858.Contig3_All	1	REVERSE	1166	20	60.08	45.00	AGAAGCCATTGCATGAAACC			
16	CL1858.Contig4_All	1	FORWARD	970	20	60.05	55.00	GATGACCCTCCAACTCCTGA	197	2109	2109
	CL1858.Contig4_All	1	REVERSE	1166	20	60.08	45.00	AGAAGCCATTGCATGAAACC			
17	CL1900.Contig3_All	1	FORWARD	1484	20	60.35	55.00	TCCTTCAGGAGGCTCACATC	253	1794	1794
	CL1900.Contig3_All	1	REVERSE	1736	20	59.98	55.00	CCTTGTTCTTGGGAGCTCTG			
18	CL1904.Contig2_All	1	FORWARD	1578	20	59.93	50.00	GGAGCCAATGTGAAGGGTTA	254	2057	2057
	CL1904.Contig2_All	1	REVERSE	1831	20	60.07	50.00	ATCATGCACTCCCGATAAGC			
19	CL2000.Contig7_All	1	FORWARD	384	20	59.76	50.00	AGCCACAACACAGCAATCAC	248	959	959
	CL2000.Contig7_All	1	REVERSE	631	20	59.68	50.00	TGACAGAACCGACATCAAGG			
20	CL202.Contig2_All	1	FORWARD	374	20	60	55.00	CCTGTCCACCAACCTCAACT	207	2102	2102
	CL202.Contig2_All	1	REVERSE	580	20	59.96	50.00	GCATGGGTGTTAGCCATTCT			
21	CL2123.Contig3_All	1	FORWARD	711	20	59.75	50.00	GGAGATATCCCGTTGGTTGA	245	1335	1335
	CL2123.Contig3_All	1	REVERSE	955	20	59.81	55.00	GCTCCGATGAACCTCAAGTC			
22	CL2123.Contig7_All	1	FORWARD	26	20	59.64	50.00	GCGTTGCAAAGAGTGTTGAG	251	1455	1455
	CL2123.Contig7_All	1	REVERSE	276	20	59.89	50.00	TGAGCTAGAAGCACCAGCAA			
23	CL2131.Contig5_All	1	FORWARD	114	20	60.52	55.00	TCTCGTCGACCAAGAAGAGG	238	1438	1438
	CL2131.Contig5_All	1	REVERSE	351	20	59.8	60.00	GTCCTCATCACCTCCTCAGC			
24	CL2139.Contig2_All	1	FORWARD	811	20	59.98	45.00	AAACACCCAAATCGCTCAAC	256	2448	2448
	CL2139.Contig2_All	1	REVERSE	1066	20	60.02	50.00	AGCACGCCACTCTTCAATCT			
25	CL2139.Contig5_All	1	FORWARD	524	20	60.11	55.00	CCACAGGTCAATCGACTCCT	254	807	807
	CL2139.Contig5_All	1	REVERSE	777	20	60.42	55.00	CAAGTCTGGCTGGCGACTAT			
26	CL2139.Contig7_All	1	FORWARD	325	20	59.65	45.00	CTCAATCACCAAGCCATCAA	248	2088	2088
	CL2139.Contig7_All	1	REVERSE	572	20	60.08	40.00	ATCACAAGCATTGGCATCAA			
27	CL2160.Contig1_All	1	FORWARD	1081	20	60.32	55.00	CTGCATGAGTGAACGTGTCC	248	1366	1366
	CL2160.Contig1_All	1	REVERSE	1328	20	60.01	50.00	AGTTCGAACCAACCAACCAG			
28	CL2201.Contig1_All	1	FORWARD	1251	20	59.8	45.00	ATGTCCATCCCAGTTTTTGC	251	1908	1908
	CL2201.Contig1_All	1	REVERSE	1501	20	59.65	55.00	ACGGCTGTAACTGGGACACT			
29	CL2201.Contig3_All	1	FORWARD	679	20	59.8	45.00	ATGTCCATCCCAGTTTTTGC	251	1035	1035
	CL2201.Contig3_All	1	REVERSE	929	20	59.65	55.00	ACGGCTGTAACTGGGACACT			
30	CL2201.Contig4_All	1	FORWARD	797	20	59.8	45.00	ATGTCCATCCCAGTTTTTGC	251	1153	1153

	CL2201.Contig4_All	1	REVERSE	1047	20	59.65	55.00	ACGGCTGTAACTGGGACACT			
31	CL2262.Contig3_All	1	FORWARD	71	20	59.99	55.00	AGGCCACCCTTAAGACCTGT	249	1908	1908
	CL2262.Contig3_All	1	REVERSE	319	20	60.01	45.00	TCGAAATTCCTCCCTGAATG			
32	CL2361.Contig1_All	1	FORWARD	1445	20	60.66	55.00	GGCAATGGAGGAGTGAGTTG	253	1751	1751
	CL2361.Contig1_All	1	REVERSE	1697	20	59.96	50.00	AATCTCGGTGCCCTTACCTT			
33	CL2361.Contig13_All	1	FORWARD	1395	20	59.89	40.00	TTTTTGCTTTTGTGGTGCAG	250	2302	2302
	CL2361.Contig13_All	1	REVERSE	1644	20	60.19	45.00	TCGCCCACAAATTCTTCTTC			
34	CL2361.Contig16_All	1	FORWARD	1358	21	60.08	47.62	CAAGACCTGGATGATTGCCTA	248	1816	1816
	CL2361.Contig16_All	1	REVERSE	1605	20	60.08	45.00	GAACTGCATTTGCCTCCATT			
35	CL2361.Contig18_All	1	FORWARD	1016	20	59.66	55.00	CTACCTTCAACGTGCTGCTG	250	2473	2473
	CL2361.Contig18_All	1	REVERSE	1265	20	59.84	50.00	GAGTGGCATTCAGTCGAACA			
36	CL2361.Contig20_All	1	FORWARD	1356	20	59.84	50.00	TGAGTGGCATTCAGTCGAAC	251	2286	2286
	CL2361.Contig20_All	1	REVERSE	1606	20	59.21	50.00	GGAGCCGATAGCGTAATGTT			
37	CL2361.Contig31_All	1	FORWARD	796	20	59.89	40.00	CCTGTGCACAAAAGCAAAAA	246	2120	2120
	CL2361.Contig31_All	1	REVERSE	1041	20	59.85	45.00	TTGCTGCTTGTTCACCATTC			
38	CL2361.Contig34_All	1	FORWARD	646	20	59.89	40.00	CTGCACCACAAAAGCAAAAA	252	1970	1970
	CL2361.Contig34_All	1	REVERSE	897	20	59.79	45.00	AACAGCTTGCTGCTTGTTCA			
39	CL2361.Contig9_All	1	FORWARD	791	20	59.66	55.00	CTACCTTCAACGTGCTGCTG	250	2116	2116
	CL2361.Contig9_All	1	REVERSE	1040	20	59.84	50.00	GAGTGGCATTCAGTCGAACA			
40	CL260.Contig1_All	1	FORWARD	460	20	59.1	55.00	CTGTGGTTGAGGAGGAGGAT	249	1453	1453
	CL260.Contig1_All	1	REVERSE	708	20	60.05	55.00	TGTCAGCTGCCCTGTTGTAG			
41	CL260.Contig2_All	1	FORWARD	460	20	59.1	55.00	CTGTGGTTGAGGAGGAGGAT	249	1469	1469
	CL260.Contig2_All	1	REVERSE	708	20	60.05	55.00	TGTCAGCTGCCCTGTTGTAG			
42	CL2719.Contig3_All	1	FORWARD	49	19	59.1	52.63	TCTCCGCGAAGAAGTTGTC	199	281	281
	CL2719.Contig3_All	1	REVERSE	247	20	60.91	55.00	AAGGACCTCACCGACTCCAT			
43	CL288.Contig4_All	1	FORWARD	512	20	59.83	50.00	GCCCCTTCGTCTTTCTTCTT	248	2304	2304
	CL288.Contig4_All	1	REVERSE	759	20	59.93	50.00	ATCCACCGAATCGACTCAAC			
44	CL2993.Contig7_All	1	FORWARD	235	20	60.19	55.00	AGCTGTGCGAGGCTATCTGT	244	800	800
	CL2993.Contig7_All	1	REVERSE	478	20	59.71	50.00	CAGCCCCAAACTTTTCTGTC			
45	CL2993.Contig8_All	1	FORWARD	608	20	59.76	60.00	CTCTCTCAGGAGCACACACG	251	1280	1280
	CL2993.Contig8_All	1	REVERSE	858	20	60.45	55.00	GTTGCTGAACAGCTCCTGGT			

46	CL3006.Contig3_All	1	FORWARD	621	20	60.31	55.00	AGTTGCCGAGGTACTTGTCG	257	1603	1603
	CL3006.Contig3_All	1	REVERSE	877	20	59.99	60.00	CTTCACCCTGTCCAGAGAGC			
47	CL3048.Contig1_All	1	FORWARD	637	20	59.99	55.00	GCTTCTTGGAGAAGGTGTCG	245	1284	1284
	CL3048.Contig1_All	1	REVERSE	881	20	60.33	60.00	ACCCTCGTACGCAGTGTCTC			
48	CL3275.Contig1_All	1	FORWARD	1104	20	59.78	50.00	GATCAAGGCAAGCAGATTCC	249	1546	1546
	CL3275.Contig1_All	1	REVERSE	1352	20	59.98	45.00	TTGCTTGCTATCTTGCATGG			
49	CL3360.Contig2_All	1	FORWARD	134	20	59.96	55.00	CCACAGGGTGGATGAGTCTT	250	2981	2981
	CL3360.Contig2_All	1	REVERSE	383	20	60.11	50.00	TACAGTGACAGCCCAATGGA			
50	CL3483.Contig2_All	1	FORWARD	1985	20	59.79	50.00	TCATCAGCTCATCACGAACC	247	2527	2527
	CL3483.Contig2_All	1	REVERSE	2231	20	60.12	50.00	CAAATCGCAGATGACCACAC			
51	CL35.Contig6_All	1	FORWARD	517	20	60.1	50.00	AATCAGGCTGTATGGCAAGG	252	1957	1957
	CL35.Contig6_All	1	REVERSE	768	20	59.87	55.00	TTGCAGAGGTAGACCCTCGT			
52	CL3718.Contig1_All	1	FORWARD	877	20	60.13	55.00	CCGAGAAGAGCTCAAAGGTG	249	3165	3165
	CL3718.Contig1_All	1	REVERSE	1125	20	59.96	55.00	GCACTCCGTATCCCATGTCT			
53	CL3737.Contig1_All	1	FORWARD	2093	20	60.11	50.00	TGTGTGTGGGAAGCCTGATA	249	3147	3147
	CL3737.Contig1_All	1	REVERSE	2341	20	60.17	55.00	AGGCCGGTCTATCCATTACC			
54	CL3737.Contig4_All	1	FORWARD	2147	20	60.17	55.00	AGGCCGGTCTATCCATTACC	249	4518	4518
	CL3737.Contig4_All	1	REVERSE	2395	20	60.11	50.00	TGTGTGTGGGAAGCCTGATA			
55	CL3737.Contig5_All	1	FORWARD	2318	20	60.11	50.00	TGTGTGTGGGAAGCCTGATA	249	4712	4712
	CL3737.Contig5_All	1	REVERSE	2566	20	60.17	55.00	AGGCCGGTCTATCCATTACC			
56	CL3853.Contig2_All	1	FORWARD	1094	20	59.65	50.00	AGTCTCCGATGGTTCTTCCA	250	1394	1394
	CL3853.Contig2_All	1	REVERSE	1343	20	60.16	50.00	ACAATGGCAGCTCAAGCTCT			
57	CL3895.Contig3_All	1	FORWARD	366	20	60.07	50.00	CCGGAGTTTCATCTCGTCAT	252	1421	1421
	CL3895.Contig3_All	1	REVERSE	617	20	60.67	55.00	CCAGCGAGCTTCTCCTTGTA			
58	CL413.Contig6_All	1	FORWARD	140	20	60.14	55.00	ACAGCCTATGGAGCACAACC	248	2095	2095
	CL413.Contig6_All	1	REVERSE	387	20	59.93	45.00	TGAGGAAGGCTTGGAAAGAA			
59	CL413.Contig7_All	1	FORWARD	140	20	60.14	55.00	ACAGCCTATGGAGCACAACC	248	1571	1571
	CL413.Contig7_All	1	REVERSE	387	20	59.93	45.00	TGAGGAAGGCTTGGAAAGAA			
60	CL4183.Contig2_All	1	FORWARD	1330	20	59.98	45.00	GTGAATGCACCAAAATGTGC	203	2086	2086
	CL4183.Contig2_All	1	REVERSE	1532	20	59.97	45.00	CCAGCCTGTTGGAAATTGTT			
61	CL4193.Contig2_All	1	FORWARD	62	19	61.23	57.89	GATTTGGGGGAGCCGTACT	107	242	242

	CL4193.Contig2_All	1	REVERSE	168	18	61.21	61.11	AACGACGTGCTCGTCTCG			
62	CL4317.Contig2_All	1	FORWARD	660	20	59.98	50.00	CTGATCAACGCACTCCTTCA	247	2127	2127
	CL4317.Contig2_All	1	REVERSE	906	20	60.11	55.00	AGTGGTTCGGAGGGAACTCT			
63	CL4317.Contig3_All	1	FORWARD	590	20	59.98	50.00	CTGATCAACGCACTCCTTCA	247	2057	2057
	CL4317.Contig3_All	1	REVERSE	836	20	60.11	55.00	AGTGGTTCGGAGGGAACTCT			
64	CL4346.Contig2_All	1	FORWARD	63	20	59.93	45.00	TCCTTTTTCCTGGAAGCTGA	198	996	996
	CL4346.Contig2_All	1	REVERSE	260	20	59.99	55.00	GAGGAACAGGCTCAAAGTCG			
65	CL4368.Contig2_All	1	FORWARD	564	20	59.11	55.00	CTGTCTCAACGCGTCGTACT	236	880	880
	CL4368.Contig2_All	1	REVERSE	799	20	60.25	50.00	CGCTTGAGGTGAATCAAGGT			
66	CL4397.Contig1_All	1	FORWARD	2206	20	60.09	50.00	TAAAGCAGCCCCACCAATAG	260	2808	2808
	CL4397.Contig1_All	1	REVERSE	2465	20	60.32	40.00	CATTGCATGTGGATTTTGGA			
67	CL4397.Contig3_All	1	FORWARD	3009	20	60.03	55.00	CGGCTTAGAAGCGTTGGTAG	250	3419	3419
	CL4397.Contig3_All	1	REVERSE	3258	20	60.79	45.00	ACCCAACATTTGCTTTGTGG			
68	CL4431.Contig2_All	1	FORWARD	670	20	59.99	55.00	TCTGACGCTGATCTGGTGAC	249	1323	1323
	CL4431.Contig2_All	1	REVERSE	918	20	60	45.00	ACCTACAATGGCGTTTTTGC			
69	CL4466.Contig3_All	1	FORWARD	490	20	59.72	50.00	CACTATTTCACCGTGCAGGA	200	887	887
	CL4466.Contig3_All	1	REVERSE	689	20	59.71	50.00	GAGGCTTTTGTGAACCTTGG			
70	CL4498.Contig2_All	1	FORWARD	279	20	59.87	60.00	GCCACCTCCTACAACTGCTC	249	1122	1122
	CL4498.Contig2_All	1	REVERSE	527	20	59.6	55.00	GCAGGTGTACTGGTTGGTCA			
71	CL4556.Contig3_All	1	FORWARD	1203	20	60.69	60.00	GAGATGCCGTCGGTGTAGTC	242	1527	1527
	CL4556.Contig3_All	1	REVERSE	1444	20	60.91	60.00	AGCTACTTCGACGGGGAGAG			
72	CL4599.Contig2_All	1	FORWARD	1060	20	59.8	45.00	TAACAAAGCCAAACCCATCC	199	1854	1854
	CL4599.Contig2_All	1	REVERSE	1258	20	60.04	50.00	ATCCCCTCTTCTTCCATGCT			
73	CL4599.Contig3_All	1	FORWARD	289	20	60.06	55.00	TGTGAAGCTGCCTGTACGAC	191	792	792
	CL4599.Contig3_All	1	REVERSE	479	20	59.88	55.00	GACAACGGAGCCAAGTAAGC			
74	CL4599.Contig4_All	1	FORWARD	887	20	59.8	45.00	TAACAAAGCCAAACCCATCC	199	1681	1681
	CL4599.Contig4_All	1	REVERSE	1085	20	60.04	50.00	ATCCCCTCTTCTTCCATGCT			
75	CL4599.Contig6_All	1	FORWARD	527	20	60.04	50.00	ATCCCCTCTTCTTCCATGCT	199	1601	1601
	CL4599.Contig6_All	1	REVERSE	725	20	59.8	45.00	TAACAAAGCCAAACCCATCC			
76	CL4762.Contig2_All	1	FORWARD	1153	20	59.8	55.00	GATCATAGAGCCGCACTTCC	255	1676	1676
	CL4762.Contig2_All	1	REVERSE	1407	20	59.9	60.00	GCGACTACAACGGCTACTCC			

77	CL4937.Contig7_All	1	FORWARD	1321	20	60.13	55.00	CCTGCTTACGAGGATGTGGT	252	2266	2266
	CL4937.Contig7_All	1	REVERSE	1572	20	60.08	50.00	CAGCTGCAAACTACGTGCAT			
78	CL4937.Contig8_All	1	FORWARD	1274	20	60.13	55.00	CCTGCTTACGAGGATGTGGT	252	2219	2219
	CL4937.Contig8_All	1	REVERSE	1525	20	60.08	50.00	CAGCTGCAAACTACGTGCAT			
79	CL5081.Contig1_All	1	FORWARD	289	20	59.83	50.00	CAGATCAGGGTCAACTGCAA	250	566	566
	CL5081.Contig1_All	1	REVERSE	538	20	59.88	45.00	GCAAATGCACGTACGAAAGA			
80	CL5487.Contig1_All	1	FORWARD	1190	20	60.08	50.00	CCCTTTGACCAACCTCTTCA	251	1917	1917
	CL5487.Contig1_All	1	REVERSE	1440	20	60.22	40.00	TCGATGCTTTGCATTACCAA			
81	CL5565.Contig1_All	1	FORWARD	854	20	60.01	50.00	TAGCAATGTCGGCAGTCAAG	250	1575	1575
	CL5565.Contig1_All	1	REVERSE	1103	20	60.26	50.00	ATTGGTGCCACTGAAAGAGC			
82	CL563.Contig16_All	1	FORWARD	1449	20	60.14	50.00	TGTGCTTGGAGCATCTCTTG	252	2263	2263
	CL563.Contig16_All	1	REVERSE	1700	20	59.93	50.00	CTTGGGTATCCGGAGTTCAA			
83	CL5665.Contig2_All	1	FORWARD	641	20	59.09	50.00	AGGCTCAACTGCAGGAAAGT	248	889	889
	CL5665.Contig2_All	1	REVERSE	888	20	59.06	50.00	CCGTCGCAATAGTTACATGG			
84	CL5668.Contig2_All	1	FORWARD	936	20	60.02	50.00	AACGGAGTGCTGAAGAGCAT	246	1391	1391
	CL5668.Contig2_All	1	REVERSE	1181	20	60.22	50.00	CCGCTGTCAGGATCAGATTT			
85	CL5724.Contig2_All	1	FORWARD	257	20	60.27	55.00	GACAACATGAGCTCCCGACT	205	1015	1015
	CL5724.Contig2_All	1	REVERSE	461	20	60	50.00	GGGCTATGCATTGTTGTGTG			
86	CL5726.Contig1_All	1	FORWARD	162	20	60.32	45.00	CGGCCATTTCTGAAGAAGAA	250	1169	1169
	CL5726.Contig1_All	1	REVERSE	411	20	60.27	60.00	CTGGGGAGGCACTACTACGA			
87	CL5736.Contig4_All	1	FORWARD	173	20	59.99	40.00	TGCAAGCATTGAAAACGAAG	253	1907	1907
	CL5736.Contig4_All	1	REVERSE	425	20	59.97	40.00	TGTTTTGGCATCGTCAATGT			
88	CL5811.Contig2_All	1	FORWARD	354	20	59.93	50.00	ATCCCGAGACCTTTGTTCCT	249	726	726
	CL5811.Contig2_All	1	REVERSE	602	20	60.09	50.00	GCTCTTCGCGAATGCTAATC			
89	CL5898.Contig3_All	1	FORWARD	363	20	59.9	40.00	CGAATTCGGATTCAACGATT	256	1247	1247
	CL5898.Contig3_All	1	REVERSE	618	20	59.74	50.00	TAGCTCGTTCTGCACCTTCA			
90	CL61.Contig4_All	1	FORWARD	1541	20	58.78	50.00	GCGTGTCCAAACTCAACAGT	256	2653	2653
	CL61.Contig4_All	1	REVERSE	1796	20	59.93	50.00	AGTCGAAGTGGGATGGATTG			
91	CL616.Contig3_All	1	FORWARD	405	20	59.96	55.00	CCACACCAACCTCTCCATCT	249	719	719
	CL616.Contig3_All	1	REVERSE	653	20	60.02	60.00	GTCCAAGCTGGTCGAGCTAC			
92	CL6324.Contig2_All	1	FORWARD	23	20	60.12	45.00	CGCTTGAGCATGCATAGAAA	248	1128	1128

	CL6324.Contig2_All	1	REVERSE	270	20	59.9	55.00	GGAAGCTCTGCAGCTTGTCT			
93	CL6350.Contig3_All	1	FORWARD	37	20	60.03	50.00	TAGAAATCCCTTGCCCCTCT	257	1328	1328
	CL6350.Contig3_All	1	REVERSE	293	20	60.46	55.00	GGTATACCCGTGGACCATGA			
94	CL6408.Contig2_All	1	FORWARD	824	20	59.62	40.00	CGCATGGATTATTGGGATTT	254	1623	1623
	CL6408.Contig2_All	1	REVERSE	1077	20	59.83	55.00	CATCTTCCTAACCGCTGGAG			
95	CL6446.Contig2_All	1	FORWARD	562	20	59.84	55.00	CGGTATCGAGGAACAGAAGC	260	914	914
	CL6446.Contig2_All	1	REVERSE	821	20	60.14	55.00	TCGATCCTCTGTCACTGCTG			
96	CL6488.Contig2_All	1	FORWARD	1925	20	59.95	50.00	AGAGAGAAAGCGGATCACCA	201	2878	2878
	CL6488.Contig2_All	1	REVERSE	2125	s	59.92	50.00	TGGCCGAATTACTAGGGATG			
97	CL6653.Contig1_All	1	FORWARD	91	20	59.98	55.00	CTAAACTCCGGCATGCTCTC	222	321	321
	CL6653.Contig1_All	1	REVERSE	312	18	60.35	61.11	CATGAACCTGCCGAGGAG			
98	CL666.Contig1_All	1	FORWARD	585	20	60.29	50.00	CAGACAACGGAACCTTGCTT	250	2577	2577
	CL666.Contig1_All	1	REVERSE	834	20	59.99	45.00	TTGGACTCAGCTGCCTTTTT			
99	CL666.Contig2_All	1	FORWARD	610	20	60.29	50.00	CAGACAACGGAACCTTGCTT	250	2742	2742
	CL666.Contig2_All	1	REVERSE	859	20	59.99	45.00	TTGGACTCAGCTGCCTTTTT			
100	CL666.Contig4_All	1	FORWARD	611	20	60.29	50.00	CAGACAACGGAACCTTGCTT	250	2620	2620
	CL666.Contig4_All	1	REVERSE	860	20	59.99	45.00	TTGGACTCAGCTGCCTTTTT			
101	CL666.Contig5_All	1	FORWARD	610	20	60.29	50.00	CAGACAACGGAACCTTGCTT	250	1975	1975
	CL666.Contig5_All	1	REVERSE	859	20	59.99	45.00	TTGGACTCAGCTGCCTTTTT			
102	CL666.Contig6_All	1	FORWARD	610	20	60.29	50.00	CAGACAACGGAACCTTGCTT	250	2108	2108
	CL666.Contig6_All	1	REVERSE	859	20	59.99	45.00	TTGGACTCAGCTGCCTTTTT			
103	CL6741.Contig3_All	1	FORWARD	94	20	59.87	60.00	CCGCAACTCTCTCCTACCAC	248	918	918
	CL6741.Contig3_All	1	REVERSE	341	20	60.07	50.00	CGTAACTCTTCCCCGATCAA			
104	CL684.Contig3_All	1	FORWARD	1320	20	60.25	50.00	CAAGCGGTGATCAAAGAGGT	244	2263	2263
	CL684.Contig3_All	1	REVERSE	1563	20	60.02	50.00	ACCCCATTATCGGAAACTCC			
105	CL6866.Contig2_All	1	FORWARD	724	20	60.33	50.00	CCTTGATCAGCCTCTGGAAA	258	1609	1609
	CL6866.Contig2_All	1	REVERSE	981	20	59.72	45.00	TGCAAGGAGTTCTTCAGCAA			
106	CL6872.Contig1_All	1	FORWARD	305	20	59.97	50.00	AACTTGATCGGGTTGGTGAG	254	1275	1275
	CL6872.Contig1_All	1	REVERSE	558	20	60.28	60.00	CTGTACCAGGGGAACACCAC			
107	CL6896.Contig2_All	1	FORWARD	57	20	60.04	50.00	AATGTCGCAGTGCAGCTATG	250	1243	1243
	CL6896.Contig2_All	1	REVERSE	306	20	60.2	55.00	ACAACGCCTACTACGCCAAC			

108	CL7045.Contig2_All	1	FORWARD	1159	20	60.24	45.00	TATCTTTGCGCACCGTATCA	260	1652	1652
	CL7045.Contig2_All	1	REVERSE	1418	20	60.02	50.00	TAAGGCAGAACGCAAGGTCT			
109	CL7260.Contig1_All	1	FORWARD	330	20	59.52	50.00	TTGTGAACCATGGAGTGGAG	246	1362	1362
	CL7260.Contig1_All	1	REVERSE	575	20	60.11	50.00	TAAAGTTGGCCGGAGTTGTC			
110	CL7260.Contig2_All	1	FORWARD	221	20	59.52	50.00	TTGTGAACCATGGAGTGGAG	246	1253	1253
	CL7260.Contig2_All	1	REVERSE	466	20	60.11	50.00	TAAAGTTGGCCGGAGTTGTC			
111	CL7433.Contig1_All	1	FORWARD	224	20	60.03	50.00	AAACGGCACAAGTAGGTTGG	252	1825	1825
	CL7433.Contig1_All	1	REVERSE	475	20	60.04	50.00	GAAGATTGGACGGAGGCATA			
112	CL7433.Contig3_All	1	FORWARD	224	20	60.03	50.00	AAACGGCACAAGTAGGTTGG	247	1425	1425
	CL7433.Contig3_All	1	REVERSE	470	20	59.78	50.00	TTGGACGGAGGCATATAACC			
113	CL7588.Contig2_All	1	FORWARD	732	20	59.71	50.00	GCGGCAAGGACTAAAATGTC	251	1383	1383
	CL7588.Contig2_All	1	REVERSE	982	20	60.54	60.00	GTGAGGGATGTCGTCAGGTC			
114	CL762.Contig1_All	1	FORWARD	241	20	60.02	55.00	TGTGTCAGCCTCAGTCCTTG	251	2779	2779
	CL762.Contig1_All	1	REVERSE	491	20	59.92	40.00	GCCAAAGAAATTTTGGGACA			
115	CL794.Contig7_All	1	FORWARD	160	20	60.18	55.00	AACGGTGTTGACGAGTAGCC	248	1810	1810
	CL794.Contig7_All	1	REVERSE	407	20	60.18	55.00	TCGTACACCAGCCTACACCA			
116	CL80.Contig11_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	4619	4619
	CL80.Contig11_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
117	CL80.Contig13_All	1	FORWARD	253	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	3575	3575
	CL80.Contig13_All	1	REVERSE	502	20	59.79	50.00	TAAACCCCACGACCAGATTC			
118	CL80.Contig14_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	4265	4265
	CL80.Contig14_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
119	CL80.Contig18_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	3232	3232
	CL80.Contig18_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
120	CL80.Contig2_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	4553	4553
	CL80.Contig2_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
121	CL80.Contig26_All	1	FORWARD	253	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	2784	2784
	CL80.Contig26_All	1	REVERSE	502	20	59.79	50.00	TAAACCCCACGACCAGATTC			
122	CL80.Contig4_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	4475	4475
	CL80.Contig4_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
123	CL80.Contig7_All	1	FORWARD	1123	20	59.99	45.00	CGAAAAGCAAGAACGAAAGG	250	4367	4367

	CL80.Contig7_All	1	REVERSE	1372	20	59.79	50.00	TAAACCCCACGACCAGATTC			
124	Unigene10573_All	1	FORWARD	803	20	59.25	55.00	AGGAGGTCTCGTTGAGGATG	250	1194	1194
	Unigene10573_All	1	REVERSE	1052	20	60.04	55.00	GTGGGCAACAACGACTACCT			
125	Unigene11370_All	1	FORWARD	769	20	60.2	50.00	TGCTTCTCCACCCATTCTTC	252	1335	1335
	Unigene11370_All	1	REVERSE	1020	20	59.71	55.00	TCAGCACCTCCACGTACATC			
126	Unigene11777_All	1	FORWARD	228	20	59.11	55.00	TCGTACTACGCCGATAGCTG	243	548	548
	Unigene11777_All	1	REVERSE	470	20	60.08	55.00	GATCTGGTCGATGACCTCGT			
127	Unigene11842_All	1	FORWARD	113	20	59.03	50.00	TTTACTGATCAGGCGAGTGC	186	375	375
	Unigene11842_All	1	REVERSE	298	21	59.84	38.10	TCTTTTCGTATTTGCCTCGAA			
128	Unigene12817_All	1	FORWARD	459	20	59.81	45.00	ACATCCCGCACATCAACATA	250	1200	1200
	Unigene12817_All	1	REVERSE	708	20	60	55.00	GGAGCAACCTTGACTTCTGC			
129	Unigene14222_All	1	FORWARD	151	20	60.74	55.00	ATCCTCCTCATCCACCACCT	236	568	568
	Unigene14222_All	1	REVERSE	386	20	60.02	55.00	TCGACCGAGTGACACAAGAG			
130	Unigene1531_All	1	FORWARD	373	20	59.72	60.00	CTACGACGACCTCCAGAACC	250	1470	1470
	Unigene1531_All	1	REVERSE	622	20	59.95	50.00	CTTGATACCCGCACCCTTTA			
131	Unigene15871_All	1	FORWARD	181	20	59.4	45.00	CCTCCATGCCACAATACAAA	256	1065	1065
	Unigene15871_All	1	REVERSE	436	20	59.73	50.00	ATCTTGCAGGGTCGTCAACT			
132	Unigene16115_All	1	FORWARD	308	20	59.99	50.00	GATGAGGCGCAGCTAAAAAC	262	790	790
	Unigene16115_All	1	REVERSE	569	20	59.98	55.00	GCTTCTCCGACGAATAGCAC			
133	Unigene17442_All	1	FORWARD	877	20	59.98	60.00	GTCCAGATCTGCGCCTACTC	246	1599	1599
	Unigene17442_All	1	REVERSE	1122	20	59.91	60.00	GATCCTCTGCCTCTCCTCCT			
134	Unigene17657_All	1	FORWARD	255	20	60.26	60.00	GTACCTCGACCTCGACCTCA	195	608	608
	Unigene17657_All	1	REVERSE	449	21	59.17	52.38	AGGAACTCTGTCACGTCATCC			
135	Unigene18098_All	1	FORWARD	653	20	60.07	40.00	CTTGGCCTTGCAAAAATGAT	251	1310	1310
	Unigene18098_All	1	REVERSE	903	20	59.96	50.00	GCGGCAATCTCTTTCAGAAC			
136	Unigene18822_All	1	FORWARD	95	20	60.11	50.00	AAGGGGTGAGGCTTTGAACT	250	1516	1516
	Unigene18822_All	1	REVERSE	344	20	59.56	40.00	CATAAACAGGCCATGCAAAA			
137	Unigene1904_All	1	FORWARD	59	20	59.55	45.00	GAAAATTGCCCGAGTCAAAC	244	841	841
	Unigene1904_All	1	REVERSE	302	20	59.95	60.00	GTCCTGCTCCTCCTCTCCTT			
138	Unigene20083_All	1	FORWARD	199	20	60.18	50.00	ATCTTGAAGGAGCCGAGGAT	255	1179	1179
	Unigene20083_All	1	REVERSE	453	20	59.96	45.00	GATTGAATCACGAGCAGCAA			

139	Unigene22260_All	1	FORWARD	638	20	60.41	50.00	TGCTCGATCGTCACCTTGTA	201	859	859
	Unigene22260_All	1	REVERSE	838	20	60.11	50.00	TCCTAAGCACACACCATCCA			
140	Unigene22405_All	1	FORWARD	420	20	59.6	50.00	CACGAATGACCACACGAAGT	260	911	911
	Unigene22405_All	1	REVERSE	679	20	59.96	55.00	CCAAGACAGATCCACCACCT			
141	Unigene22514_All	1	FORWARD	842	20	60.13	50.00	TTTGCCTCTTGGCTCACTCT	266	1273	1273
	Unigene22514_All	1	REVERSE	1107	20	59.91	45.00	TTGAACCCTTTCGGTGAATC			
142	Unigene22568_All	1	FORWARD	312	20	59.93	50.00	CGTCCAATTGATCTCCCAGT	250	1589	1589
	Unigene22568_All	1	REVERSE	561	20	60.01	60.00	GCGCTGAGGTACTGCCTATC			
143	Unigene22569_All	1	FORWARD	196	20	60.15	50.00	TAAAAGCCTGGCACTCTGCT	252	515	515
	Unigene22569_All	1	REVERSE	447	20	60.07	55.00	GAGAGGTAGGCCATCCAACA			
144	Unigene22571_All	1	FORWARD	61	20	59.84	55.00	CTCCGAGGACCAGAAACTTG	250	594	594
	Unigene22571_All	1	REVERSE	310	20	59.85	45.00	GCCATGAACTCAAACAAGCA			
145	Unigene22612_All	1	FORWARD	427	20	60.48	60.00	ACTGTCGACTGGGCTACTGC	262	1060	1060
	Unigene22612_All	1	REVERSE	688	20	59.72	45.00	TTCGACGTGAGCTTCTTCAA			
146	Unigene22647_All	1	FORWARD	1269	20	59.93	50.00	GCATAGTTGGGGGTGAAGAA	253	3768	3768
	Unigene22647_All	1	REVERSE	1521	20	59.91	50.00	GCAAACTCGTGCATCACTGT			
147	Unigene22815_All	1	FORWARD	376	20	60.11	45.00	TACACCGATTGCAACCAGAA	253	1029	1029
	Unigene22815_All	1	REVERSE	628	20	59.31	55.00	GTGAGCTGGTAGCCCAATCT			
148	Unigene22950_All	1	FORWARD	290	19	60.94	57.89	CCTCCAAGGACGTGGTCAT	235	626	626
	Unigene22950_All	1	REVERSE	524	20	59.88	60.00	AGGTGGTACTCCACGGACAC			
149	Unigene24798_All	1	FORWARD	1	18	59.29	61.11	GGTGATCAGGGGTGTCGT	238	286	286
	Unigene24798_All	1	REVERSE	238	18	59.98	61.11	CGTGCTGGACAGCATGAC			
150	Unigene25002_All	1	FORWARD	1119	20	60.2	60.00	GCCAACCTCTCCTCTTAGGG	201	2891	2891
	Unigene25002_All	1	REVERSE	1319	20	60.08	45.00	TTGTTCGAACCTGGAAAAGG			
151	Unigene2710_All	1	FORWARD	404	20	59.95	55.00	CCGGAGGTATGGTCGTTCTA	245	1718	1718
	Unigene2710_All	1	REVERSE	648	20	59.36	55.00	GGCGTGGAGATCTCTCTGAT			
152	Unigene29035_All	1	FORWARD	1149	20	60.31	55.00	ACCTCGACCAAGAACACTGC	251	1503	1503
	Unigene29035_All	1	REVERSE	1399	20	59.96	55.00	CCTCTCCCAAACACCACCTA			
153	Unigene29255_All	1	FORWARD	632	20	59.1	55.00	ACCTCGACATCAAGGACCTC	244	1220	1220
	Unigene29255_All	1	REVERSE	875	20	59.64	55.00	ACATGGCGGTAGTAGCTGGT			
154	Unigene29569_All	1	FORWARD	1389	20	59.93	50.00	GCATAGTTGGGGGTGAAGAA	253	2841	2841

	Unigene29569_All	1	REVERSE	1641	20	59.91	50.00	GCAAACTCGTGCATCACTGT			
155	Unigene29691_All	1	FORWARD	42	20	59.41	40.00	AAAGGCGTTTCCCATATTGA	259	777	777
	Unigene29691_All	1	REVERSE	300	20	60.35	55.00	GGAGGAACACAAACCCTCCT			
156	Unigene3003_All	1	FORWARD	326	20	59.97	50.00	AACTTGATCGGGTTGGTGAG	254	1504	1504
	Unigene3003_All	1	REVERSE	579	20	60.28	60.00	CTGTACCAGGGGAACACCAC			
157	Unigene30369_All	1	FORWARD	766	20	60.03	50.00	GTCATCAACCCCAACAATCC	249	1680	1680
	Unigene30369_All	1	REVERSE	1014	20	60.55	55.00	GTCACAGAAGGCCAACCAAC			
158	Unigene30410_All	1	FORWARD	134	20	60.19	40.00	TAAAACCATCGGTGGCAAAT	251	1141	1141
	Unigene30410_All	1	REVERSE	384	20	59.86	50.00	TGATGCTAGGCACACAAAGG			
159	Unigene32135_All	1	FORWARD	186	20	60.03	50.00	TCCCTCCTAAGGCTCCATTT	244	980	980
	Unigene32135_All	1	REVERSE	429	20	60.09	55.00	CGTCATAGTAAGGGCCTCCA			
160	Unigene3945_All	1	FORWARD	136	20	59.65	45.00	CAGAGAATGCATGGAAACCA	247	1458	1458
	Unigene3945_All	1	REVERSE	382	20	59.79	50.00	CATGGTCAAGATGAGCCAGA			
161	Unigene5407_All	1	FORWARD	121	20	60.28	50.00	GCGCAGTAACCAAGACGAAT	244	844	844
	Unigene5407_All	1	REVERSE	364	20	60.03	45.00	TCTTGTGTTCGCAGTTTTGC			
162	Unigene6153_All	1	FORWARD	195	20	60.12	50.00	ACGTTGATGGGAGAAGCAAC	252	1687	1687
	Unigene6153_All	1	REVERSE	446	20	60.14	55.00	CACGTACGAGATCGTGATGG			
163	Unigene6237_All	1	FORWARD	823	20	60.14	60.00	CACTCGCTCTCCTCCTTGTC	244	1439	1439
	Unigene6237_All	1	REVERSE	1066	20	59.78	50.00	CCATGTTCAGGGACCTCATT			

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