Investigation of the Molecular Response of Grapevine to Salt Stress Through Transcriptomics and Proteomics Approaches

Thesis Submitted to AcSIR for the
Award of the Degree of

DOCTOR OF PHILOSOPHY

In

BIOLOGICAL SCIENCES

By

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July 2019

Dedicated to…

Aai, Baba

& all the teachers who made me eligible to undertake this research

Certificate

This is to certify that the work incorporated in this Ph.D. thesis entitled "**Investigation of the molecular response of grapevine to salt stress through transcriptomics and proteomics approaches**" submitted by **Ms. Sucheta Sanjay Patil** to **Academy of Scientific and Innovative Research (AcSIR)** in fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Biological Sciences**, embodies original research work under my guidance. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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Declaration by the candidate

I hereby declare that the thesis entitled **"Investigation of the molecular response of grapevine to salt stress through transcriptomics and proteomics approaches"** submitted for the degree of Doctor of Philosophy to the **Academy of Scientific and Innovative Research (AcSIR)** has been carried out by me at the **Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune – 411008**, India, under the supervision of **Dr. Vidya Gupta** and co-supervision of **Dr. Narendra Kadoo**. Any material that has been obtained by other sources has been duly acknowledged in this thesis. I declare that the present work or any part thereof has not been submitted to any other University for the award of any other degree or diploma.

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Acknowledgements

This dissertation is built on the foundation of blessings and contributions from everyone who accompanied me throughout this journey of doctoral research.

First and foremost, I would like to express my sincere gratitude to my advisor Dr. Vidya Gupta for believing in me and for giving me the opportunity to work under her fabulous guidance. I am extremely thankful to her for her continuous support and meticulous attention throughout my Ph.D. research and for her tremendous patience and motivation during the writing of this thesis. The freedom that she gave me to think and work throughout my doctoral studies has taught me to become an independent researcher. Every discussion with her was a tremendous boost full of enthusiasm, precise directions and encouragement. I feel extremely fortunate for having her as my Ph.D. mentor.

I would also like to convey my sincere gratitude to my co-advisor, Dr. Narendra Kadoo. I am very much thankful to him for his constant support from the first day of this journey. His immense support during academic activities and the completion of this thesis has made this journey a completely effortless process. His enthusiasm for teaching as well as learning new computational tools has greatly influenced me and has instilled in me the interest for bioinformatic studies. His timely instructions and meticulous attention towards the details of this research has remarkably polished all the results from this thesis.

I am extremely grateful to Dr. Anuradha Upadhyay (National Research Center for Grapes (NRCG), Pune) for her help during the salinity stress experiments and for providing all the facilities for sample collection and storage at her institute. Her expert advice and knowledge of viticulture and salinity stress problems in the fields were of great help. I also thank her for providing the results of sodium and chloride content analysis to be used in this thesis. I would also like to thank Dr. Ramya Prashant for introducing me to the research on gene family studies and for guiding me in designing the experiments for in-silico analyses. Her cheerful nature and wisdom has always motivated me during the tough times in this journey.

I want to thank my DAC members Dr. H.V. Thulasiram, Dr. Ramchandra Gadre, Dr. Dhanasekaran Shanmugam, and Dr. Ashok Giri, for their timely feedback and assessment of the progress of my doctoral research. I am also grateful to Dr. Mahesh Kulkarni and Dr. Santhakumari for providing enough access to central mass spectrometric facilities for proteomics. I wish to thank the Director, CSIR-NCL, and Head of Department, Biochemical Sciences Divison, Dr. Ashok Giri, and former heads for providing the infrastructure and lab facilities. I would like to thank the Council for Scientific and Industrial Research (CSIR), New Delhi for the financial support in the form of Junior and Senior Research fellowships.

Special thanks to Dr. Manisha Shinde and the plant-nursery department (NRCG, Pune) for raising the grapevines and for conducting the salinity stress experiments at NRCG Pune. I am very grateful to my colleagues, Dr. Manisha Shinde, Ms. Uma Jadhav, and Ms. Smita Mhaske, for helping me during measurement of morphological and physiological parameters and sample collection.

I would like to express immense gratitude to Tejas Chirmade and Dr. Sheon Mary for their invaluable help in troubleshooting every minute step in proteomics experiments and data acquisition on LC-MS. I am also thankful to Dr. Arati Deshmukh, Dr. Jagdeeshprasad, and Swami for helping me throughout my data acquisition on LC-MS. Special thanks to Deepika Somani for teaching me the accurate way of qRT-PCR analysis in a very comprehensive manner. I extend my gratitude to Pooja Sethiya and Gaurav Dube for teaching me the means to explore various bioinformatic tools. I am also thankful to my project trainees Manali Zamindar, Deepak Tiwari, Sonal More, and Ruchi Kumari whose assistance in my research activities has been a great help.

It gives me immense pleasure to express my affectionate gratitude to all my lab members for their assistance during my tenure at NCL It was a blessing to have the splendid company of Deepika Somani, Mhd. Saleem Dar, Smrati Sanghi, Tejas Chirmade and Dr. Nidhi Saikhedkar throughout the academic struggle. It was an amazing experience to share the bench with Deepika and to indulge into fruitful discussions with her on diverse topics. I feel extremely fortunate for having found true friends like Vaishnavi Salunkhe, Pranjali Oak and Gayatri Salunke who have transformed me into a better person. Their constant care and motivation are priceless. Special mention to Vaishnavi for her enthusiasm and love that has always added more

fun during all the celebrations in this vibrant journey. I also feel thankful for the wonderful company of friends like Amol Kasodekar, Sonal Channale, Amol Jadhav, Uma Jadhav, Gauri Pathak, Monika Mathur, Sagar Yadav, Divya Karade, Santosh Lavhale, Gopal Kallure, Ranjeet Barbole, Veena Dewangan and Bhakti Dayama whose co-ordination in daily lab activities has helped me enormously in carrying out my research smoothly in a healthy and co-operative atmosphere. I am also grateful to my seniors Drs. Yashwant Kumar, Rakesh Joshi, Medha Upasani, Ashish Deshpande, Rahul Tanpure, Neha Khandelwal, Ameya Bhide, Yojana Chikate, and Priyanka Singh whose teachings and achievements have always inspired me throughout my research.

My sincere thanks also go to my teachers from college, Dr. Shampa Chakraborti, Dr. Dhanashri Godbole, Dr. Sangita Sathey, Dr. Girish Pathade, and many other teachers who instilled in me the interest for research in biotechnology and motivated me to undertake the doctoral research.

Last, but not the least, I would like to thank my parents for their incessant support and constant encouragement that gave me the strength and ability to undertake this research work with perseverance to complete it satisfactorily. Without their blessings, this achievement would not have been possible.

Besides this, I would like to thank all those people who have knowingly and unknowingly helped me in the successful completion of this project.

Sucheta Patil

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Abbreviations

Abbreviation	Description
110R	110R Richter (hybrid grapevine rootstock of *Vitis ruperstris* and *Vitis berlanderie*)
2D-GE	Two-Dimensional Polyacrylamide Gel Electrophoresis
ABA	Abscisic acid
ABC	ATP-Binding Cassette
ABRE	ABA-Responsive Element Binding
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BEH	Bridged-Ethyl Hybrid
bHLH	basic Helix-Loop-Helix
BLAST	Basic local Alignment Search Tool
BP	Biological Process
bZIP	basic-leucine zipper
CC	Cellular Component
cDNA	Complementary Deoxyribonucleic Acid
DEGs	Differentially Expressed Genes
DEPs	Differentially Expressed Proteins
DTT	Dithiothretol
EC	Electrical Conductivity
ECe	Electrical Conductivity of saturated soil solution
ECw	Electrial Conductivity of water
EDTA	Ethylenediaminetetraacetic acid
EREB	Ethylene Responsive Element Binding
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organization of the United Nations
FBP	Fructose Bisphosphate
FDR	False Discovery Rate
fmol	Femtomole
FPKM	Fragments per kilobase of transcript per million mapped reads

Abbreviation	Description
FWHM	Full Width Half Maximum
FYM	Farm Yard Manure
GA	Gibberellin
GDH	Glutamate dehydrogenase
GEO	Gene Expression Omnibus
GO	Gene Ontology
GS	Glutamine Synthase
GTT	Genome Guided Trinity
HDMS	High Definition Mass Spectroscopy
HKT	High-Affinity Potassium Transporters
IRGA	Infrared Gas Analysis system
JA	Jasmonate
JAZ	Jasmonate-ZIM domain
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LEA	Late Embryogenesis Abundant
MF	Molecular Function
MFS	Major Facilitator Superfamily
MFSS	MFS Superfamily
MFSST	MFS Superfamily transporter
mRNA	messenger RNA
MS	Mass Spectrometry
NGS	Next Generation Sequencing
NPF	NRT/ POT Family transporter
NRT	Nitrate transporter
PCR	Polymerase Chain Reaction
PIP	Plasma membrane intrinsic protein
POT	Proton dependent Oligopeptide Transporter
ppm	Parts per million
PPO	Polyphenol oxidase
PPP	Pentose Phosphate Pathway

Abbreviation	Description
PR	Pathogenesis related
PSII	Photosystem II
qRT-PCR	quantitative Reverse Transcriptase PCR
QST	glycine, serine, and threonine
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RNAseq	RNA sequencing / transcriptomics
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
RSEM	RNA-Seq by Expectation-Maximization
SA	Salicylic Acid
SAGE	Serial Analysis of Gene Expression
SAR	Systemic Acquired Resistance
SOS	Salt Overly Sensitive
SP	Sugar Porter
SSH	Suppression Subtractive Hybridization
TCA	Trichloroacetic acid
TCDB	Transporter Classification Database
TCID	Transporter Classification Identifier
TF	Transcription Factor
TMH	Transmembrane Helix
TPI	Triosephosphate Isomerase
TS	Thomson Seedless grapevine
TS110R	Thompson Seedless grafted on 110R rootstock
TSOR	Thompson Seedless grown on own roots
UMF	Unknown MFS
UPLC	Ultra-Pressure Liquid Chromatography
WUE	Water Use Efficiency

Synopsis

Chapter 1: Introduction and Review of Literature

Grape (*Vitis vinifera* L.) is a perennial woody vine cultivated across the world mainly for wine production and for consumption of fresh grapes or processed raisins. In India, grapes top the fruit exports of the country however, the country ranks 7th in grape production across the world with an annual production of three million tonnes while, China tops with thirteen million tonnes (FAO 2017). Viticulture has been facing various environmental threats like climate change, salinity stress, drought stress, and pathogen attack. Soil salinity is becoming a major shortcoming to the viticulture industry (Jogaiah, 2016). Therefore, farmers have now switched to grafting of grapevines on wild rootstocks for drought and salt stress tolerance. A hybrid grapevine (*V. ruperstris x V. berlanderie*) rootstock, 110-Richter (110R), has been identified as the most potent salt stress tolerant rootstock (Upadhyay et al., 2013). Salt stress in plants primarily affects water conductance and photosynthesis leading to osmotic imbalance, ionic toxicity and production of reactive oxygen species (ROS), which ultimately results into poor growth and severe foliar damage. These conditions induce changes in expression of several genes and proteins necessary for alleviating the stress. In the current study we have proposed to understand these gene expression changes in response to salt stress in grapevine using transcriptomics and proteomics approaches. The grapevines used in the study include – salinity sensitive Thomson seedless on its own roots (TSOR) and salinity tolerant Thompson seedless grafted on 110R rootstock (TS110R). Additionally, we have studied a transporter protein superfamily from gene annotation to in-silico functional characterization to identify salt stress responsive novel transporters.

Chapter 2: Comparative proteomics unravels the differences in salt stress response of own rooted and 110R grafted Thompson Seedless grapevines

The proteomic makeup of a tissue gives a more direct and closer glance of the molecular mechanisms underlying the physiological changes. The first proteomic study on the grapevine investigated the differences between *V. vinifera* cv. Chardonnay and cv. Cabernet Sauvignon and their responses to water deficit and salinity using Two-

dimentional gel electophoresis (2D-GE) based proteomics approach (Vincent et al., 2007). It revealed downregulation of proteins involved in photosynthesis, protein synthesis, and protein destination in grapevines subjected to water and salinity stress for 8 and 16 days. Another proteomic study on salt tolerant Tunisian grapevine cultivar Razegui identified over-expression of a pathogenesis-related protein 10 in response to salt stress on 15th day (Jellouli et al., 2008). However, these studies are restricted to late stages of salt stress and cover a small number of proteins due to their limited separation on 2D-GE. In the present study we have used untargeted shotgun proteomics approach to compare the effect of salt stress in salt sensitive TSOR grapevine and salt tolerant TS110R grapevine.

We profiled the salinity induced protein expression patterns across 6 hours (early stage), 48 hours (mid stage) and 7 days (late stage) of salt treatment in TSOR and TS110R grapevines. The TSOR grapevines displayed modulation in expression patterns of more number of proteins relative to those in the salinity tolerant TS110R vines. The early phase of salt stress displayed more differential protein expression in both the grapevines however; the processes affected were not similar. Photosynthesis was highly upregulated in TSOR vines while translation and amino acid metabolism was upregulated in TS110R in early phase. Moreover, chlorophyll biosynthesis was upregulated in both the vines; the chlorophyll-ab binding proteins were highly upregulated in TS110R vines, which implies more efficient trapping of light energy in TS110R than in TSOR vines. A few proteins upregulated in late stress in TS110R were involved in desiccation tolerance, chlorophyll biosynthesis and histone methylation. Hence, the decrease in shoot growth of TSOR vines despite increased CO_2 assimilation rate and photosynthesis could be due to partitioning of generated energy towards osmotic adjustments and stress tolerance whereas, the TS110R grapevines displayed early adaptation to prevent oxidative stress leading to efficient utilization of energy for continuing shoot growth.

Chapter 3: Transcriptomic regulation of salt stress response in 110R grafted Thompson Seedless grapevines

Since the availability of *V. vinifera* genome in 2007 (Jaillon et al., 2007) there have been many transcriptomic studies on grapevines unraveling the genes involved in salinity stress response (Cramer et al., 2007; Daldoul et al., 2010). Cramer et al. (2007)

used microarray- based transcript profiling to identify genes and metabolic pathways in wine variety Cabernet Sauvignon under water-deficit stress and equivalent salinity stress. Daldoul et al. (2010) identified salt tolerance related genes through a comparative study between salt tolerant and sensitive grapevine cultivars Razegui and Syrah, respectively using suppression subtractive hybridization (SSH) and microarray approach (Daldoul et al., 2010). These studies have focused on salinity stress in the wine varieties of grapevines. In our study we focus on early and late stages of salt stress in the table-grape variety - Thompson Seedless.

The effect of salt stress on grapevines was studied across three phases of stress, early (6 hours), mid (24 hours and 48 hours) and late (7 days). The sixteen months old potted grapevines were subjected to salt stress by irrigating them with 150 mM NaCl salt solution. The control vines were irrigated with potable water (EC<0.7). The salt sensitive TSOR vines displayed immediate uptake of sodium and chloride ions while, TS110R vines suppressed the uptake of ions till 15 days of salt treatment. Sixteen months old potted TS110R grapevines were used for the study. The RNASeq data was generated for young TS110R leaves sampled at 6 hours (early stage), 24 hours (mid stage) and 7 days (late stage) of salt stress. The high quality RNA samples were sequenced using Illumina paired end sequencing technique. The transcriptome analysis revealed differential regulation of several genes related to transcription factor activity, abiotic stress and photosynthesis.

Chapter 4: Global study of Major Facilitator Superfamily transporters in Arabidopsis and grape reveals their salt stress specific response

The Major Facilitator Superfamily (MFS) is the largest superfamily of secondary transporters that transport diverse molecules like sugars, vitamins, amino-acids, hormones, etc. across cell membrane. Most of these transporters use proton gradient for transport of molecules. Its presence in all organisms from prokaryotes to higher eukaryotes marks it an evolutionarily conserved superfamily. Later the superfamily was expanded to MFS Superfamily (MFSS) to integrate MFS with nine more families. There are more than 80 subfamilies in MFSS; however, only a subset of them is present in individual organisms. It is widely studied in fungi and other microbes for their importance in drug extrusion. In plants, some transporters form this superfamily, such

as, zinc induced facilitator like and phosphate transporters were identified to be involved in regulation of stomatal opening and salinity tolerance in *Arabidopsis* (Remy et al., 2013; Cubero et al., 2009) which, indicated the possibility of more such salt stress responsive transporters to be present in this superfamily. We therefore performed identification and *in-silico* functional analysis of this superfamily in *Arabidopsis* and grapes.

We identified 213 and 203 MFSS transporters in *Arabidopsis thaliana* and *Vitis vinifera* (grapes) respectively and classified them into six families and 20 subfamilies. The phylogenetic tree constructed using the 416 MFSS proteins revealed expansion of sugar porter and proton dependent oligopeptide transporter families suggesting their importance in regulating sugar and nitrogen flux in plant shoots and roots. Subcellular localization prediction showed that majority of these transporter proteins were plasma membrane localized. The microarray datasets from Gene Expression Omnibus revealed that these transporters were expressed in different plant tissues indicating their multi-tissue-specificity. Some transporters from sugar porter, oligopeptide transporter and phosphate transporter families were identified to be differentially expressed in salt stress studies which suggest their role in regulation of transport of sugars, nitrates, peptides and phosphates during the stress condition. The plasma membrane localization of these transporters and use of protons for transport of molecules indicates the role of stress induced localized proton gradients in regulating the function of these transporters. Moreover, transporters from Unknown MFS 23 (UMF23) family were also recognized as salinity stress responsive transporters through qRT-PCR analysis in salt stressed TSOR and TS110R grapevines.

Chapter 5: Summary and future prospects

Summary

The TSOR grapevines displayed diversion of photo assimilates towards osmotic adjustments and tolerance of oxidative stress while, TS110R grapevines displayed specific upregulation of abiotic stress related proteins responsible for prevention of oxidative stress. Several transporter proteins were also affected by salt stress in both the vines. The MFS Superfamily study revealed modulation of sugar, nitrate and peptide transporters along with some transporters with unknown function.

Future prospects

- The salinity tolerance phenotype displayed by upregulation of proteins in TS110R vines needs to be validated through their overexpression in Thompson seedless grapevines
- Transcriptomic analysis of salt tolerant 110R rootstock under saline conditions
- Studies on xylem sap of grafted grapevines to identify the molecules responsible for modulation of gene expression in the scion
- Prediction of substrates transported by UMF23 transporter

Chapter 1

Introduction and Review of Literature

Chapter 1 Introduction and Review of Literature

1.1 Origin and spread of grape cultivation

1.1.1 Fossil records of grape cultivation

Grape (*Vitis vinifera* L.) is an ancient fruit crop consumed by humans since prehistoric times. It a perennial woody vine cultivated across the world mainly for wine production and consumption of fresh grapes or processed raisins. Grape cultivation and winemaking are depicted in several ancient Egyptian paintings and murals dating back to 1500 BC (**Figure 1.1**) (Janick, 2000). Grape domestication has been linked to winemaking with the earliest evidence from Iran of around 7000 years ago (McGovern, 2013). The majority of archaeological records of fossil leaves and seeds of wild grape varieties come from the Neolithic Era and Bronze Age (Winkler, 1974). However, the latest grape seed fossils discovered from India pushes back the existence of the Vitaceae family to the Cretaceous period (Manchester et al., 2013).

1.1.2 The spread of grape cultivation in India

A large number of fossil records from the Neolithic period from Europe suggests the cultivation of grapes to have originated in Armenia near the Caspian Sea from where it was spread to Europe, Iran, and Afghanistan (Papademetriou and Dent, 2001). Grape cultivation reached China in the second century and to Japan around 3200 years ago (Royer, 1988). The cultivated grape varieties were introduced into north India by the Persian invaders in 1300 AD and were spread southwards to Daulatabad, Maharashtra by King Mohammed-bin-Tughlak. Grapes were also introduced by the Christian missionaries into Salem and Madurai districts of Tamil Nadu around 1832, and by the Nizam of Hyderabad into Hyderabad province in the early 20th century (Shikhamany, 2001). Apart from the newly introduced grape cultivation in India, some indigenous varieties of northwestern Himalayan foothills were also cultivated in Himachal Pradesh (Shikhamany, 2001).

Figure 1.1 Egyptian mural paiPlatenting of winemaking by workers

From a tomb at Thebes, Egypt, ca. 1500 BCE (Picture taken from Google images)

1.2 Current status of grape cultivation

1.2.1 Grape producing countries

Grapevines are cultivated through clonal propagation of hardwood cuttings of a vine with desirable qualities. Currently, grapevines are cultivated across different countries in the world. China tops the world grape production with thirteen million tonnes in the year 2017 (**Figure 1.2**). In the year 2000, Italy was the leading country in grape production, but within a decade, China overtook the production by systematically increasing the area under grape cultivation (**Figure 1.3**). The average national yields vary from country to country (**Figure 1.3B**) depending on the varieties cultivated and the climatic conditions. India has the highest national average yield with 20 to 30 tonnes per hectare (Shikhamany, 2001, **Figure 1.3B**).

1.2.2 Grape cultivation in India

The grape production in India has almost tripled from 1.13 million tonnes to 2.9 million tonnes from the year 2000 to 2017 (**Figure 1.2**). The total area under grape production in India is 88,160 hectares as of year 2017. In India, grapes are cultivated under three distinct climatic zones, namely, sub-tropical, hot tropical and mild tropical. The sub-tropical region covers the northwestern plains including Delhi, Uttar Pradesh, Haryana, and Punjab.

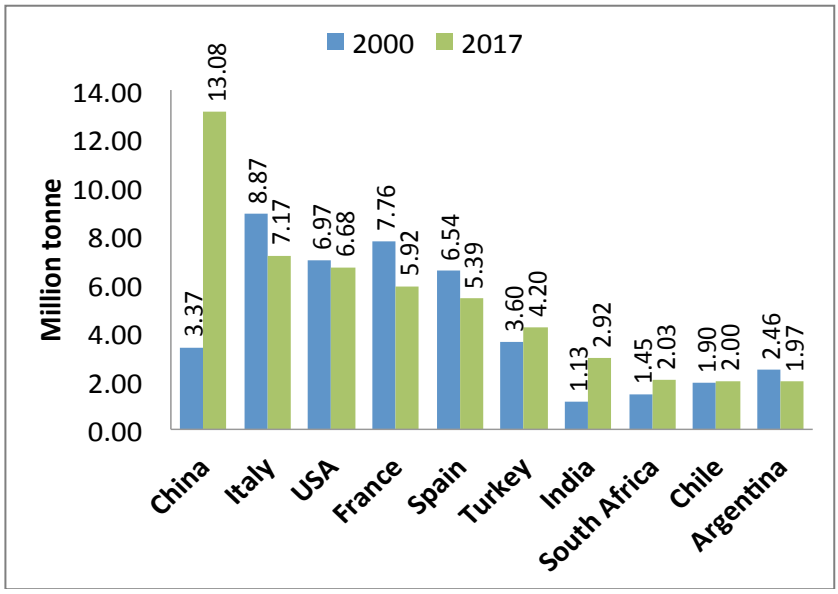


Figure 1.2 Annual grape production in the years 2000 and 2017 (FAOSTAT, 2018)

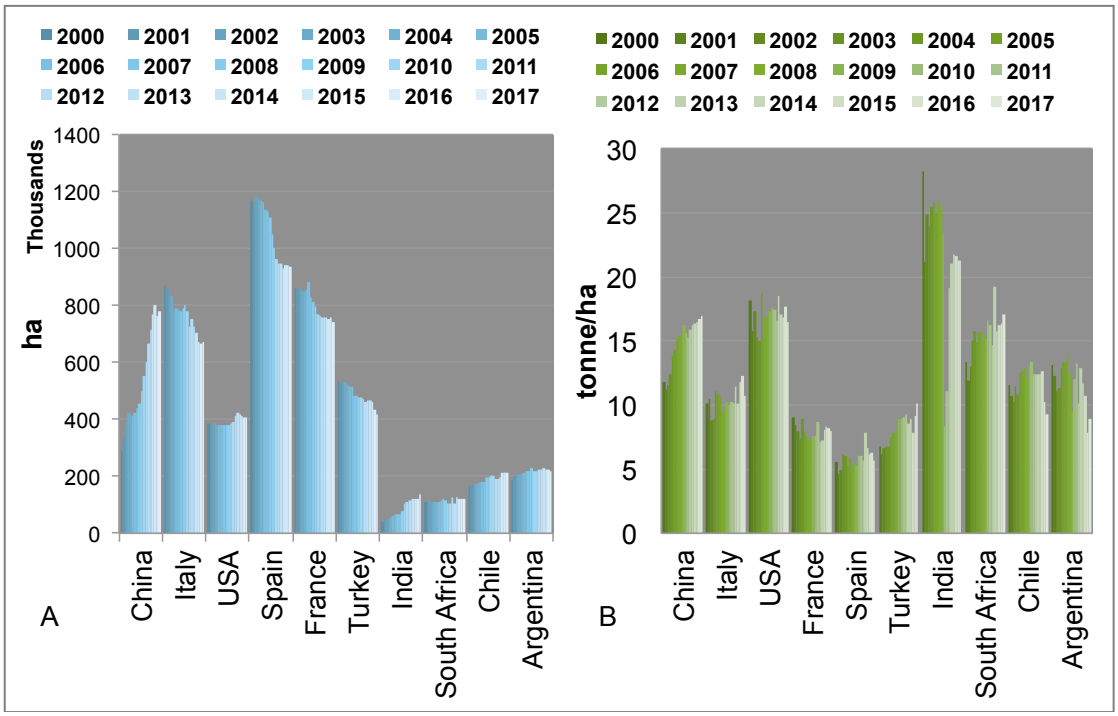


Figure 1.3 Grape production trend over the years 2000 to 2017

A – Changes in the area under grape cultivation in the top ten countries in the years 2000 to 2017. B – Grape yield of top ten countries in the years 2000 to 2017. (FAOSTAT, 2018)

Due to the dormancy of vines grown in this region, only 90-95 days are available for growth of berries from bud burst to harvest, i.e., from March to June; and

therefore, only an early ripening vine variety, Perlette, is grown in this region (Shikhamany, 2001). The vines grown in hot tropical regions such as Maharashtra, Andhra Pradesh, and northern Karnataka do not show dormancy and therefore undergo pruning twice a year. This region accounts for 70% of the area under grape cultivation in the country. A small share of grape production comes from the mild tropical region, which includes southern Karnataka, Andhra Pradesh, and Tamil Nadu.

1.2.3 Challenges in grape cultivation

In India, the area under grape cultivation has not shown a significant increase across the country (**Figure 1.3A**) owing to various constraints. The average yield has significantly dropped to 21 tonnes per hectare in the last decade (**Figure 1.3B**). The establishment of vineyards requires high investments, plus the cost of maintenance, nutrition, and disease control adds to the cost of production. Grapes are grown in both temperate and tropical climate; in the latter, climate vines remain evergreen. Usually, dry weather during flowering and ripening is preferred for high-quality grape production. Regions having heavy rainfall or hailstorms are not suited for grape cultivation. For instance, a sudden drop in grape yield from India observed in the year 2011 was a result of unseasonal heavy rainfall and hailstorms during the harvest season in the Nashik district of Maharashtra, which contributes to more than 70% of the grapes exported from the state (**Figure 1.3B**, https://www.business-standard.com/article/markets/nashik-grape-exports-dip-58-111042100008_1.html).

Viticulture has been facing various environmental threats like salinity stress, drought, and high temperature while the risk of these abiotic stresses to the industry is increasing with the increasing climate change (Carvalho and Amâncio, 2018). The narrow genetic base of commercial grape varieties has also led to their vulnerability to diseases and pests. Among diseases, anthracnose, downy mildew, and powdery mildew are the most serious ones (Shikhamany, 2001). Among the abiotic stresses, drought, soil salinity, nutrient deficiencies, high temperature, and untimely rains are the major limiting factors. Soil salinity is becoming a major problem to the viticulture industry resulting in poor vine growth and severe foliar damage leading to a drastic reduction in the productive life span of grapevines (Jogaiah, 2016). These significant factors severely impair vine growth and cause heavy foliar damage leading to a reduction in

berry quality and yield (Jogaiah, 2016; Serra et al., 2014; Shani and Ben-Gal, 2005; Stevens and Partington, 2013).

Salinity also affects fruitfulness and berry maturation in grapevines (Hawker and Walker, 1978; Walker et al., 2000). Maharashtra is the leading grape-producing state in India, followed by Karnataka and Tamil Nadu (Saxena et al., 2015; **Table 1.1**). However, the major problems in this region are soil and water salinity and drought. Salinity injury due to the salinity of soils and irrigated water is common in Maharashtra and northern Karnataka (Sharma and Singh, 2015; Shikhamany, 2001). Hence, the early death of grapevines due to prolonged salinity stress causes significant loss to the farmer. This further discourages the establishment of new vineyards limiting the total grape production and the export of the country.

Table 1.1 Percent share of grape production from various states in India (APEDA, 2015-2016)

Sr No.	State	Production (thousand tonnes)	Share (%)
1	Maharashtra	2,048.11	79.08
2	Karnataka	429.77	16.59
3	Tamil Nadu	34.09	1.32
4	Mizoram	22.55	0.87
5	Kerala	15.50	0.60
6	Andhra Pradesh	14.64	0.57
7	Telangana	13.54	0.52
8	Punjab	8.49	0.33
9	Madhya Pradesh	2.20	0.08
10	Nagaland	0.50	0.02

1.3 Soil salinity

1.3.1 Soil salinity across the globe

Globally, about 1128 million hectares of land is affected by salinity and sodicity stresses (**Figure 1.4**; Wicke et al., 2011). In India, the area under salt-affected soils is about 6.73 million-hectares (**Figure 1.5**) with the states of Gujarat (2.23 m ha), Uttar Pradesh (1.37 m ha), Maharashtra (0.61 m ha), West Bengal (0.44 m ha), Rajasthan (0.38 m ha), and Karnataka (1893 ha) together accounting for almost 75% of saline and sodic soils in the country (Sharma and Singh, 2015). Soil salinity results from various factors such as the weathering of parent rocks, presence of shallow water table, poor soil drainage, congestion of water, use of excess fertilizers, and use of saline groundwater for irrigation. The last two are the major anthropogenic contributors to soil salinity.

The introduction of Green revolution for increased crop yield has led to the overuse of fertilizers and agricultural chemicals in the Asian countries leading to the degradation of the environment and soil fertility (Mazoyer and Roudart, 2000). At the same time, irrigation with saline groundwater and waterlogging due to faulty irrigation practices have been increasing uncontrollably with the intention of increasing the area under agricultural crops. Unfortunately, a strong link has been observed between excess irrigation and soil salinization (Ghassemi et al., 1995). Leaching of soils by rains is the only natural process to reduce its salt content. Lack of or little precipitation makes soil-salinity more adverse and irreversible.

Therefore, increasing soil-salinity is a significant problem in arid regions with less precipitation like Pakistan (Evenson, 2000), Northwestern China (Wang et al., 2016), and many parts of India (Datta and De Jong, 2002; Lorenzen et al., 2012; Sharma and Singh, 2015; Tanji and Kielen, 2002). In Australia, the composition of groundwater itself is very similar to that of seawater, which has led to a tremendous increase in soil salinity (Nielsen et al., 2003; Rengasamy, 2006) (**Figure 1.4**). Salinization of soils is increasing rapidly worldwide (Shrivastava and Kumar, 2015) and has been estimated to reach 50% of the arable land by the year 2050 (Jamil et al., 2011).

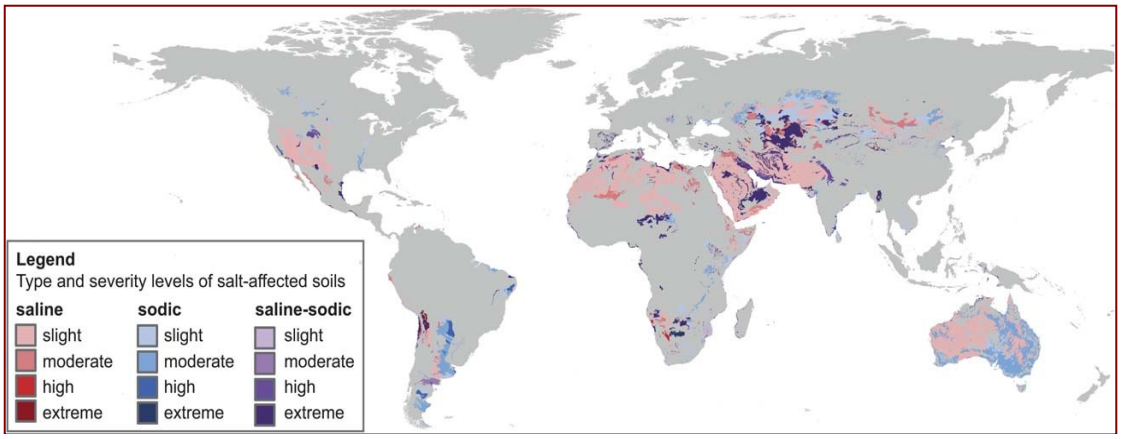


Figure 1.4 Global salt-affected soils, by type and severity (Adapted from Wicke et al., 2011)

1.3.2 Soil salinity and salt tolerance of crop plants

Rising soil salinity is a growing problem in irrigated agriculture systems in which accumulation of salts near the root zone severely impairs plant growth, limiting the productivity of crops with 20 to 50% yield loss. Soil salinity (EC_e) is measured in terms of electrical conductivity of a saturated soil paste extract taken from the root zone of a plant in units of deci Siemens per meter (dSm^{-1}). The soils with EC_e greater than 4 dSm^{-1} are considered unsuitable for crop cultivation (Shannon and Grieve, 1998). Majority of the crops are glycophytes in nature, and their salinity tolerance varies from species to species and variety to variety. There is an 8 to 10-fold range in salt tolerance of crops (Ayers, 1985).

The salt tolerance of a crop is assessed based on three main criteria – the ability to survive in saline soils, the crop yield on saline soils and the relative crop yield on saline soil as compared to that on non-saline soil under similar growing conditions. FAO has classified crops into four categories as sensitive, moderately sensitive, moderately tolerant and tolerant (**Figure 1.6**) based on their relative crop yield on a range of soils with varying salinity. According to this classification, grapevines fall into the moderately sensitive category with a yield potential of 75% in 4.1 dSm^{-1} of soil salinity or 2.7 dSm^{-1} of water salinity (**Table 1.2**). However, this cannot be generalized since different *Vitis* species have different salt stress tolerance.

Figure 1.5 Distribution of salt-affected soils in India (Adapted from Dagar, 2014)

Table 1.2 Crop tolerance and yield potential as influenced by irrigation water salinity (EC_w) or soil salinity (EC_e) (Adapted from Ayers, 1985)

Yield Potential	**100%**		**75%**		**50%**	
	EC_e	EC_w	EC_e	EC_w	EC_e	EC_w
Barley (*Hordeum vulgare*)	8.0	5.3	13.0	8.7	18.0	12.0
Date palm (*Phoenix dactylifera*)	4.0	2.7	11.0	7.3	18.0	12.0
Rice (paddy) (*Oryza sativa*)	3.0	2.0	5.1	3.4	7.2	4.8
Grape (*Vitis vinifera*)	1.5	1.0	4.1	2.7	6.7	4.5
Peach (*Prunus persica*)	1.7	1.1	2.9	1.9	4.1	2.7
Corn (maize) (*Zea mays*)	1.7	1.1	3.8	2.5	5.9	3.9

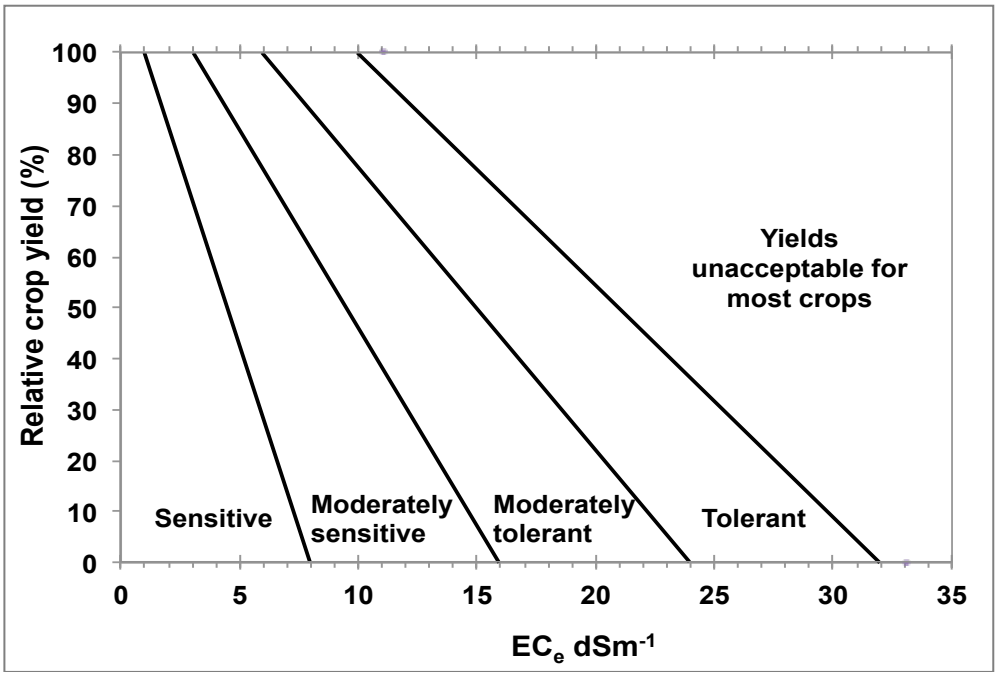


Figure 1.6 Classification of crop tolerance to salinity

(Adapted from Tanji and Kielen, 2002)

1.4 Salt stress responses in plants

Uptake of water by plant roots is primarily an osmotic process where water moves from high water potential to low water potential. This movement of water is hampered with an increase of salts in the soil, thus leading to osmotic stress on the plant, causing inhibition of cell expansion and shoot bud growth. When plants continue to grow under prolonged saline conditions, they start taking up more salts along with the water, which then accumulate in the leaves causing adverse effects of ionic toxicity like premature aging and chlorosis of leaves. Thus, plant's responses to salinity stress have been broadly characterized as initial osmotic phase and later ionic phase (Munns, 2002). These conditions cause various changes from physiological to biochemical to molecular alterations in the stressed plants.

1.4.1 Physiological traits affected by salt stress

Several physiological traits such as stomatal conductance, mesophyll conductance, water use efficiency (WUE), CO_2 assimilation, root water conductance, and xylem hydraulics have been extensively phenotyped in salinity stress studies (Negrão et al., 2017). Closure of stomata or decrease in stomatal conductance has been reported as an

essential response to drought or salinity stress in many plants (Hu et al., 2006; Huang et al., 2009; Jung et al., 2007; Romero-Aranda et al., 2001). The initial phase of osmotic stress signals plant to close the stomata to prevent loss of water due to transpiration. This, in turn, reduces the gaseous exchange of leaves, leading to increase in leaf temperature and decrease in CO_2 assimilation or photosynthesis (Delfine et al., 1998; Farquhar and Sharkey, 1982). Under such conditions of reduced photosynthesis and leaf growth, an increase in WUE is observed in salinity tolerant plants (Ahmed et al., 2013; Omamt et al., 2006).

Season-long WUE is the amount of dry biomass produced per unit season-long water use, and the instantaneous WUE is the ratio of CO_2 assimilation rate to transpiration rate (Martin and Thorstenson, 1988). The increased or stable WUE is considered as a critical trait that confers drought and salinity tolerance to plants (Davies et al., 2002; Karaba et al., 2007; Martin and Thorstenson, 1988). Reduction in hydraulic conductance of plant is another problem under extreme conditions, which is a result of uncontrolled transpiration that leads to formation of air bubbles in the xylem vessels, also known as xylem cavitation or xylem embolism (Brodersen et al., 2010; Rewald et al., 2011; Salleo et al., 2000). Studies in some plants have shown that the resistance to xylem cavitation can be associated with the strengthening of the xylem vessels by xylem lignification and thickening of xylem cell walls (Fernandez-Garcia et al., 2011; Neves et al., 2010; Stiller, 2009). The plants with salt stress thus have to maintain a proper balance between water transpiration and xylem conductance to avoid the adverse effects.

1.4.2 Oxidative stress and metabolic pathways affected by salt stress

Oxidative stress resulting due to the production of reactive oxygen species (ROS) is another common metabolic constraint on the plant during limiting CO_2 conditions (Hossain and Dietz, 2016). Plants produce several ROS-scavenging enzymes and secondary metabolites as a protective response against damaging levels of ROS in the cells. Enzymatic ROS-scavengers include superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, glutathione S-transferase, and peroxiredoxin (You and Chan, 2015). Non-enzymatic antioxidants like glutathione, ascorbic acid, carotenoids, tocopherols, and flavonoids are also crucial for ROS homeostasis in plants.

Similarly, metabolic or biochemical changes are also involved in adapting to stress conditions through oxidative stress tolerance, osmotic regulation and energy metabolism. For example, accumulation of osmolytes such as, proline, mannitol, sorbitol and glycine betain takes place in response to the osmotic stress, which may sustain throughout the salt stress. A multi-species study on correlation between physiological parameters to metabolite profiles has highlighted certain conserved patterns across different species and some species specific patterns (Gago et al., 2016). It is, therefore, necessary to understand the molecular responses that are involved in regulating these physiological processes.

1.4.3 Ionic stress mitigation by plants

Accumulation of metal ions in the cytoplasm of cells mostly in shoots and old leaves causes toxicity that interferes with essential cellular processes like photosynthesis, enzyme activity, and protein synthesis. The ionic stress tolerance depends on the plant's ability to prevent the ions from reaching the toxic levels in the cytoplasm. Under saline conditions, Na^+ competes with K^+ for binding sites on the plasma membrane, thus reducing K^+/Na^+ ratio (Yamada et al., 2011). Salt tolerant species usually maintain higher K^+ as compared to salt-sensitive species. Multiple mechanisms, including morphological and biochemical adaptations, are required for maintaining a low cytoplasmic Na^+/K^+ ratio. This is achieved by selective exclusion of ions from uptake into xylem or by sequestration of excess ions in the leaf vacuoles. However, both mechanisms can operate only up-to specific limits. Selective exclusion of metal ions from uptake into roots leads to increased accumulation of ions around the roots adding to the osmotic stress in the root zone, while accumulation in the leaves leads to their early senescence (Munns, 2002).

Salinity studies in the model plant Arabidopsis have revealed the mechanism of salt sensing in plants. The salt overly sensitive (SOS) pathway is triggered in response to ionic stress in the roots of salt-stressed plants, which is involved in Na^+ ion exclusion and ion homeostasis. SOS3 is a Calcineurin B-like protein (CBL4). Cytosolic perturbations of Ca^{2+} ion during salinity stress is sensed by CBLs through activation of calcium-dependent serine/threonine protein kinases (SOS2) which bind SOS3 and activate Na^+/H^+ antiporters (SOS1). Activation of plasma membrane-localized Na^+/H^+ antiporter effluxes the sodium ions from cytoplasm while the vacuolar antiporter

sequesters the ions into vacuoles (Halfter et al., 2000). Further, this protein kinase complex also downregulates AtHKT1, a low-affinity Na^+ transporter, which is involved in the transport of Na^+ ions in root cells. Additionally, SOS2 is found to interact with vacuolar Na^+/H^+ antiporter influencing the exchange of ions across the vacuolar membrane and hence sequestering it into the vacuole. All these physiological and metabolic changes in plants are tightly regulated by salinity induced stress hormones and other signaling molecules (Fujita et al., 2006; Julkowska and Testerink, 2015).

1.4.4 Salt stress-induced hormonal signaling pathways

Several physical and chemical changes resulting due to salinity stress, like change in hydraulic conductance, decrease in cell turgor, increased levels of ROS, decrease in cytoplasmic CO_2, etc., lead to the synthesis of stress hormone, abscisic acid (ABA), in the stressed cells (Urano et al., 2009). The stress-induced rise of ABA further activates expression of downstream genes involved in osmotic and redox homeostasis through transcription factors like ABA-responsive element-binding protein (AREB1) and ABA-insensitive 5 protein (ABI5) (Umezawa et al., 2013). Apart from the ABA-dependent signaling, several other pathways like phospholipid signaling and ABA independent transcription factors like ABRE-binding factors (ABFs) and dehydration-responsive element-binding protein 2 (DREB2) transcription factors are also activated due to osmotic stress (Munnik and Vermeer, 2010; Yoshida et al., 2014). Both ABA-dependent and -independent osmotic stress signaling pathways work together to induce the expression of early response transcriptional activators, which further activates the downstream effector genes leading to various physiological changes.

Other hormones such as cytokinins, auxins, and gibberellins (GA) also impart abiotic stress tolerance through modulation of growth (Colebrook et al., 2014; Zwack and Rashotte, 2015). Priming of plants with a small concentration of salicylic acid (SA) also helps in alleviating abiotic stress in many plants (Miura and Tada, 2014). Biotic stress hormones like jasmonic acid (JA) and ethylene participate in salinity stress tolerance signaling pathways through JAZ proteins (Kazan, 2015). Brassinosteroids (BR), strigolactones, and nitric oxide (NO) are also involved in the regulation of plant's response to abiotic stress (Gudesblat et al., 2012; Kohli et al., 2013). Simultaneous modulation of different hormonal levels thus suggests complex crosstalk between them.

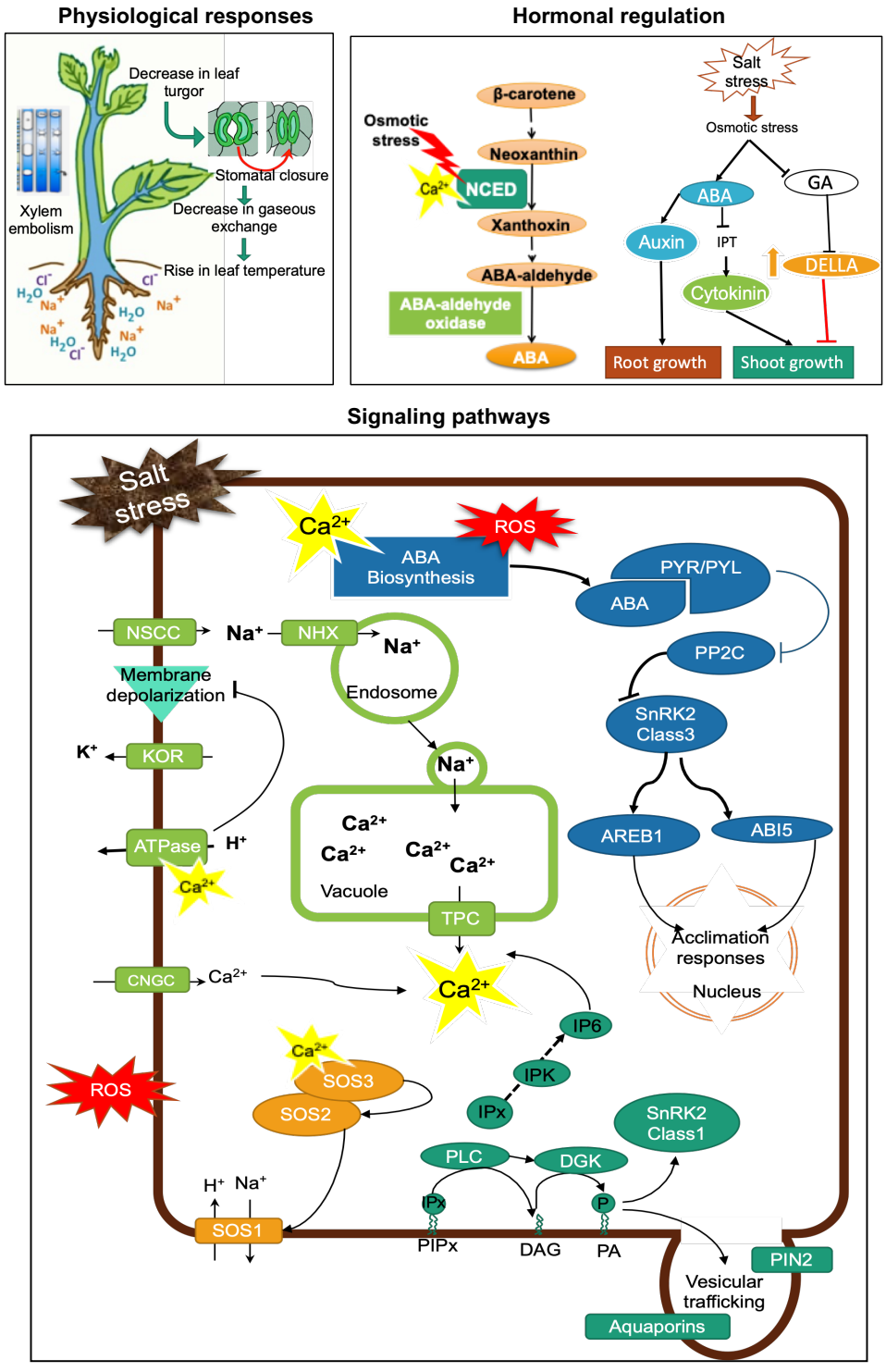


Plate 1: General salt-stress responses in plants

ABI5 – ABA insensitive, 5, AREB - ABA-responsive element-binding protein, CNGC - cyclic nucleotide-gated Ca^{2+} channel, DAG - diacylglycerol, DELLA - aspartate-glutamate-leucine-leucine-alanine or D-E-L-L-A motif containing protein, DGK - diacylglycerol kinases, IPK - Inositol phosphate kinase, IPT - Adenylate isopentenyltransferase, IPx - inositol x-phosphate, KOR - Potassium outward rectifying channel, NCED - 9-cis-

epoxycarotenoid dioxygenase, NHX - Na+:H+ antiporter, NSCC - non-selective cation channels, PA - phosphatidic acid, PIN2 - Auxin efflux carrier component 2, PIPx - phosphatidylinositol x-phosphate, PLC - Phospholipase C, PP2C - ABA-dependent protein phosphatase 2C, PYR/PYL - pyrabactin resistance (PYR) like (PYL) receptors, SnRK - plant-specific serine/threonine-protein kinase, SOS - Salt Overly Sensitive, SOS1 - sodium:proton exchanger 7 (NHX7), SOS2 - calcium-dependent serine/threonine protein kinases , SOS3 - Calcineurin B-like protein, TPC - Two-pore channels. Signaling pathway figure adapted from Julkowska and Testerink (2015).

The basic crosstalk between ABA and GA takes place through regulation of DELLA proteins. The increase in ABA levels downregulates GA and stabilizes DELLA proteins leading to suppression of growth-related gene expression in response to abiotic stress (Colebrook et al., 2014). These DELLA proteins also interact with auxin, ethylene, and JA signaling pathways. The complexity of the interaction network of phytohormone crosstalks thus suggests that not a single hormone, but a combination of phytohormone ratios in the cell regulates the gene expression in response to stress. Most of these salinity responsive genes and pathways are discovered in herbaceous or annual plants, such as Arabidopsis and rice. Grapevines being woody perennial plants, lack such transgenic studies. The investigation of salt stress responses in grapevines mostly includes physiological studies followed by some multi-omics studies.

1.5 Grapevines

1.5.1 Genetic diversity of grapevines

The genus *Vitis* contains more than 60 species (Emanuelli et al., 2013). These species are inter-fertile and have no genetic barriers within the genus, and therefore, they are also called as ecospecies. They are primarily distributed in the temperate zone of the northern hemisphere. A large number of grapevines are indigenous to North America and East Asia. *V. vinifera* is the most commonly cultivated species across the globe for both table and wine grape production. The genetic variation of the wild *V. vinifera* species has diminished due to loss of natural habitat. The only wild form *V. vinifera* L. ssp. *sylvestris* is naturally found in Europe. Several other wild *Vitis* species are only peripherally used for human consumption but are of great economic importance as a source for resistance breeding and as rootstocks for the highly susceptible *V. vinifera*. The classification of grapevines is presented in **Table 1.3**.

1.5.2 Grafting practices in viticulture

Since the beginnings of cultivation, desirable forms of the wild grapevine and spontaneous mutants within cultivated populations have been selected and preserved by vegetative propagation. Additional cultivars have been developed by both deliberate and spontaneous interspecific as well as intraspecific breeding (This et al., 2006). However, at the end of the 19th century, massive destruction of many European vineyards resulted in a significant reduction in genetic diversity of both, cultivated and

wild grapevines. It was due to the infestation of roots by the *Phylloxera* insect that had reached Europe from America. European vineyards were saved from extinction by the introduction of several native American, non-vinifera *Vitis* species, which were used as rootstocks for cultivating the commercial species (Olmo, 1971). Since then, the grafting of grapevines onto a suitable rootstock is commonly practiced in viticulture in order to resist root infestation by pathogens such as *Phylloxera* and nematodes or to improve the scion vigor as well as to overcome stress conditions such as soil salinity and drought stress (Granett et al., 1987; Walker et al., 2010, 2002, 2000). Vines are raised on their own roots in India due to the non-prevalence of *Phylloxera* or nematodes. However, in recent years, the 'Dogridge' rootstock is being employed to combat soil and water salinity problems (Shikhamany, 2001).

Table 1.3 Taxonomy of grapevine

Kingdom	**: Plantae**
Clade	: Angiosperms
Clade	: Eudicots
Clade	: Rosids
Order	: Vitales
Family	: Vitaceae
Subfamily	: Vitoideae
Genus	: *Vitis*
Species	: *vinifera*

1.6 Salt stress in grapevines

1.6.1 Physiological and biochemical response of grapevines to salt stress

The nature of salinity stress in field-grown grapevines is far more different than that in laboratory-grown Arabidopsis. Grapevines display two types of stomatal behaviours in response to water stress arising from either salt or drought stress conditions. Plants are

defined as isohydric if they can maintain constant midday leaf water potential (Ψ_l) regardless of soil water availability, or anisohydric if Ψ_l significantly declines with evaporative demand during the day. Based on stomatal kinetics under water stress conditions, isohydric cultivars prevent significant drops in Ψ_l by early stomatal closure whereas anisohydric cultivars maximize photosynthetic gain by keeping the stomata open despite significant decreases in Ψ_l (Tardieu and Simonneau, 1998). Some examples of isohydric grapevines include hybrids like *V. labruscana* (*Vitis labrusca* x *V. vinifera*), 110-Richter (*Vitis berlandieri* x *Vitis rupestris*) and *V. vinifera* cultivars like Grenache, Trincadeira Preta and Tempranillo; while anisohydric grapevines include the species *V. labrusca* and *Vitis californica* Benth., as well as many *V. vinifera* cultivars, including Chardonnay, Cabernet Franc, Cabernet Sauvignon, Syrah, Riesling, Carignan, Muscat, Thomson seedless and Touriga Nacional (Lovisolo et al., 2010). However, recent studies state that the classification of grapevine varieties into isohydric and anisohydric categories is still premature and maybe inappropriate (Santo et al., 2016). The stomatal opening in plants is largely determined by regulation of localized ABA levels in the leaf tissues leading to appropriate ABA signaling pathways. Therefore, the ABA-dependent stomatal regulation shows much variability in salinity stressed grapevines. Thus, it is suggested that the difference in xylem ABA concentrations among rootstocks is not only due to their ability to synthesize ABA but also due to the difference in water constraints sensed by the rootstock (Soar et al., 2004).

Additionally, the diversity in anatomy and architectures of root systems also contributes to the differences in salt tolerance within grapevine cultivars. Furthermore, the complexity of water uptake hydraulics becomes more complex in grafted grapevines, where the anatomy of the stem and root xylem tissues is governed by two different genotypes with contrasting isohydric and anisohydric behaviors. It is known that grafting induces extensive transcriptional modifications in the shoot apical meristem of the grape scion (Cookson et al., 2013). However, the influence of rootstock on the molecular mechanisms associated with the salt stress response in the grafted scion, such as regulation of stomatal behavior and ABA synthesis, are still poorly understood. It is, therefore, necessary to investigate in detail the synthesis and systemic distribution of ABA in regulating the stomatal behavior in grafted grapevines to establish a proper correlation between grape genotypes and their mechanisms of salt tolerance.

1.6.2 A systemic view of ABA synthesis in grapevines

Stomatal closure is one of the early responses to osmotic stress during salinity stress, which is signaled by the rise of ABA in guard cells. Earlier it was believed that osmotic stress is sensed by the roots resulting in localized ABA synthesis followed by its transport to shoots for signaling the closure of stomata and other adaptation processes (Wilkinson and Davies, 2002). However, later studies have shown that ABA transport from root to shoot is not necessary for informing the shoots about water stress. Instead, it is the hydraulic signals in the form of drop in vapor pressure deficit and cell turgor of leaf cells that signals the synthesis of ABA in the shoot (Christmann et al., 2007; McAdam and Brodribb, 2016). Drought-induced expression of a key enzyme of ABA biosynthesis pathway, 9-cis-epoxycarotenoid deoxygenase 3 (NCED3), is upregulated in the vascular tissues of both, root and shoot including the vascular parenchyma cells of leaves (Behnam et al., 2013; Endo et al., 2008; Rasheed et al., 2016). This confirms that the hydraulic stress-dependent induction of ABA biosynthesis in the leaves is the primary source of ABA in the shoots. However, root to shoot signaling of ABA is more complicated in grapevines. It is argued that root to shoot transport of ABA synthesized in the wild rootstocks is responsible for imparting water stress tolerance to the scion (Speirs et al., 2013).

On the contrary, other studies report the localized synthesis of ABA in leaves independent of long-distance signals coming from root or shoot apex (Christopher J Soar et al., 2004; Tombesi et al., 2015). Many such studies have been reviewed describing the localized ABA synthesis and its actions on corresponding organs (Lovisolo et al., 2016). Localized synthesis of ABA in the vascular tissues of shoot and root is, thus equally important in grapevines for local as well as long-distance signaling for systemic homeostasis and adaptation to prolonged salinity stress. Furthermore, the role of ABA also depends on its intracellular levels determined by the ABA transporter proteins.

Since ABA is synthesized in the vascular tissues, plants have developed different transporters for selective uptake and exclusion of ABA in different cells (**Figure 1.7**). Two transporter proteins ABCG25 and ABCG40 of ATP-binding cassette (ABC) family (Kuromori et al., 2011) and one AtNPF4.6 transporter of NRT1/PTR FAMILY (NPF) (Kanno et al., 2013) from Major Facilitator Superfamily

(MFS) (Reddy et al., 2012) have been identified and characterized as ABA transporters in plants. The transporter ABCG25 is an efflux protein expressed mainly in vascular tissues, while the ABACG40 and NPF4.6 are the importers on the plasma membrane of guard cells and vascular parenchyma cells, respectively (Boursiac et al., 2013). A recently identified ABA efflux transporter, AtDTX50 of DTX/Multidrug and Toxic Compound Extrusion (MATE) family, localized to guard cells, mesophyll cells and vascular tissues, is induced by exogenous application of ABA while its mutant has smaller stomatal opening and is highly sensitive to ABA-induced wilting (Zhang et al., 2014). This indicates that expression of ABA exporters is essential for the removal of excess ABA from the target cells. This is also applicable to salt or osmotic stress conditions where resumption of growth and stomatal opening requires removal of ABA from the cell cytoplasm. Overall, these studies suggest that it is the localized ABA levels synthesized in response to localized osmotic stress that triggers the molecular changes in woody plants like grapevines.

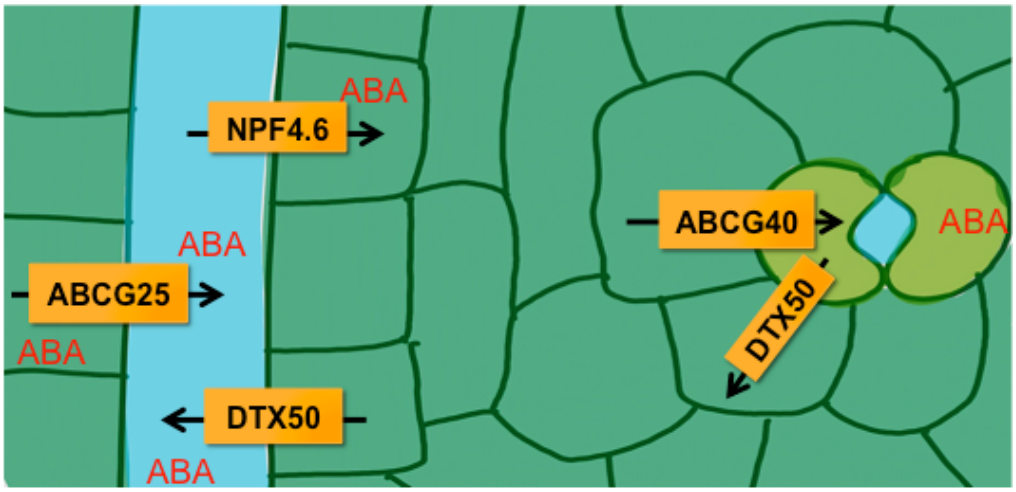


Figure 1.7 Schematic representation of ABA transporters in different plant cells

1.6.3 Salinity tolerance through grafting in grapevines

Majority of the commercially grown grapevines are grafted on the hybrid rootstock of American wild varieties that impart tolerance to biotic and abiotic stresses (Berdeja et al., 2015). The grafting of grapevines onto wild rootstocks was initially introduced for overcoming *Phylloxera* infestation of roots. Many of these wild grape rootstocks are known to possess drought- and salt-tolerant traits and are gaining popularity for cultivation under drought and salt stress conditions. Currently, most of the vineyards over the world are grafted on rootstocks, which are hybrids of three species: *V.*

berlandieri, V. riparia, and *V. rupestris* (Shikhamany, 2001). Some wild rootstocks also play a crucial role in salt tolerance by reducing the uptake of salts as well as the rate of salt accumulation in grapevines. A hybrid grapevine (*V. ruperstris* x *V. berlanderie*) rootstock, 110-Richter (110R), has been reported to be the best drought tolerant rootstock (Serra et al., 2014) (**Table 1.4**) and is also identified as the most potent salinity tolerant rootstock (Upadhyay et al., 2013). Though the vast differences in drought tolerance within grapevine species are due to anatomical and architectural differences of the root systems (Serra et al., 2014), the molecular mechanisms behind these differences are still unknown.

Table 1.4 Rootstock classification based on adaptation to drought

Proposed by Samson and Casteran, 1971, Fregoni, 1977 and Carbonneau, 1985 (Adapted from Serra et al., 2014)

Name	Crossing	Samson and Castéran	Fregoni	Carbonneau
110R	*V. rupestris* × *V. cinerea* var. helleri 'Resseguier#2'	Good	High resistance	High resistance
140Ru	*V. rupestris* × *V. cinerea* var. helleri 'Resseguier#2'	Average	High resistance	High resistance
44Đ53M	*V. riparia* × *V. cordifolia-V. rupestris*	Good	High resistance	High resistance
1103P	*V. rupestris* × *V. cinerea* var. helleri 'Resseguier#2'	Good	High resistance	Resistance
SO4	*V. riparia* × *V. cinerea* var. helleri 'Resseguier#2'	Weak	Weak resistance	Resistance
99R	*V. rupestris* × *V. cinerea* var. helleri 'Resseguier#2'	Average	Average resistance	Resistance
3309C	*V. riparia* × *V. rupestris*	Good	Weak resistance	Sensitive
420A MGt	*V. riparia* × *V. cinerea* var. helleri 'Resseguier#2'	Weak	Weak resistance	Sensitive
5BB	*V. riparia* × *V. cinerea* var. helleri 'Resseguier#2'	Bad	Weak resistance	Sensitive
41B MGt	*V. cinerea* var. helleri 'Resseguier#2' × *V. vinifera*	Average	High resistance	Sensitive
Rupestris du Lot	*V. rupestris*	Bad	Weak resistance	Sensitive
101-14 Mt	*V. riparia* × *V. rupestris*	Bad	Weak resistance	Very sensitive
Riparia Gloire de Montpellier	*V. riparia*	Bad	Weak resistance	Very sensitive
333EM	*V. cinerea var. helleri 'Resseguier#2'* × *V. vinifera*	Good	Mild resistance	Very sensitive

1.7 Salt stress response revealed through transcriptomic and proteomic studies

1.7.1 Transcriptomic and proteomic studies for gene expression analysis in plants

The recent developments in high throughput technologies like microarrays, RNA sequencing and high-pressure liquid chromatography coupled mass spectrometry have enabled scientists to look into the molecular dynamics of gene expression in the form of qualitative and quantitative measurements of RNA, proteins, and metabolites (Voelckel et al., 2017). This has been possible due to the sequencing of genomes followed by functional annotation of the predicted genes. These technologies have generated immense data on various aspects of plant biology like developmental biology, circadian rhythm, and plant-environment interactions (Urano et al., 2010). The latter includes diverse biotic and abiotic stress conditions. These data are interpreted primarily based on the functional classification of stress-responsive RNA transcripts or proteins using various annotation databases like Gene Ontology, KEGG pathways, The Munich Information Center for Protein Sequences (MIPPS) classification (Mewes et al., 2002), Protein families (Pfam), etc.

Changes in carbohydrate metabolism are significant in many salinity stress studies. The effect of salt stress on the expression of carbohydrate metabolism genes like enolase, fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, aconitate hydratase, etc. has been identified through several transcriptomic and proteomic studies of salinity stress in many plants, such as tomato, barley, wheat, *Arabidopsis* and grape (Brumós et al., 2009). Differential expression of hexokinases and phosphatases are also common during salt stress indicating an active exchange of high-energy phosphorylated sugars between energy assimilation and breakdown pathways like glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway, and photorespiration. The metabolome of salinity stressed Arabidopsis and Poplar leaves have shown a varied regulation in the metabolic flux of carbohydrates like glucose, fructose, maltose and starch (Janz et al., 2010; Kempa et al., 2008; Ottow et al., 2005).

1.7.2 The biological processes affected by salt stress as revealed through transcriptomic and proteomic studies

There have been a large number of omics studies on salinity stress in various plant species (**Table 1.5**). These studies have identified a large number of differentially regulated salinity stress-responsive genes from various metabolic pathways and molecular processes, such as carbon metabolism, flavonoid biosynthesis, serine metabolism, transcription, protein synthesis, ion transport, redox homeostasis, protein fate, DNA processing, etc. (**Table 1.6**). Most of these studies compare the molecular processes between salinity tolerant and salinity sensitive plant species; however, they lack the information about the corresponding physiological status of plant stress.

1.7.3 Transcriptomic and proteomic studies in grapevine

The availability of *V. vinifera* genome since 2007 has enabled several transcriptomic and proteomic investigations in grapevine (Cramer et al., 2013, 2006; Grimplet et al., 2009; Jaillon et al., 2007). There have been many transcriptomic studies on grapevines unraveling the genes involved in salinity stress response (Cramer et al., 2006; Daldoul et al., 2010; Das and Majumder, 2018; Upadhyay et al., 2018). A comparative study between water-deficit and equivalent salinity stress in cv. Cabernet Sauvignon revealed salinity specific modulation of genes from biological processes like transcription, protein synthesis, and protein fate in addition to water-deficit responsive genes involved in energy metabolism and nitrogen assimilation, particularly photosynthesis, gluconeogenesis, and photorespiration (Cramer et al., 2006). Another comparative study between salt-tolerant and sensitive grapevine cultivars Razegui and Syrah, respectively identified salt tolerance related genes like glutamate dehydrogenase, Dehydrin and pyrroline-5-carboxylate synthase using suppression subtractive hybridization (SSH) and microarray approach (Daldoul et al., 2010).

A recent study on salinity stressed grapevine cv. Thompson Seedless revealed that a large number of genes from metabolic pathways like carbohydrate metabolism, signal transduction, energy metabolism, amino acid metabolism, biosynthesis of secondary metabolites, and lipid metabolism are modulated in response to salt stress (Das and Majumder, 2018). On similar line, another study identified differential expression of many transcription factors from WRKY, EREB, MYB, NAC and bHLH families and genes from pathways like metabolic pathways, biosynthesis of secondary metabolites,

membrane transport development, etc. in salt-stressed Thompson seedless grafted on salinity tolerant 110R rootstock (Upadhyay et al., 2018). Apart from transcriptomic studies, a few proteomic studies have also been conducted to determine the salt stress responses in grapevines.

The proteomic makeup of tissue gives a more direct and closer glance of the molecular mechanisms underlying the physiological changes. The first proteomic study on the grapevine investigated the differences between *V. vinifera* cv. Chardonnay and cv. Cabernet Sauvignon and their responses to water deficit and salinity using Two-dimensional gel electrophoresis (2D-GE) based proteomics approach (Vincent et al., 2007). It revealed downregulation of proteins involved in photosynthesis, protein synthesis, and protein destination in grapevines subjected to water and salinity stress for 8 and 16 days. Another proteomic study on salt-tolerant Tunisian grapevine cultivar Razegui identified over-expression of a pathogenesis-related protein 10 in response to salt stress on the 15th day (Jellouli et al., 2008). However, these studies are restricted to a small number of proteins due to their limited separation on 2D-GE. Most of these omics studies are based on late time points and have missed the early response such as those observed in a water stress study (Cramer et al., 2013) wherein several metabolic pathways were observed to be altered in the proteome before inhibition of growth and photosynthesis.

Table 1.5 Omics studies on salinity stress response in different plants

Plant	Omics	Reference
Tomato	transcriptomics	(Sun et al., 2010)
Foxtail millet	transcriptomics	(Puranik et al., 2011)
Alfalfa	transcriptomics	(Postnikova et al., 2013)
Sunflower	transcriptomics	(Fernandez et al., 2008)
Barley	proteomics	(Fatehi et al., 2012)
Poplar	transcriptomics, metabolomics	(Janz et al., 2010)
Wheat	proteomics	(Peng et al., 2009)

Plant	Omics	Reference
Soybean	proteomics	(Aghaei et al., 2009)
Rice	proteomics	(Yan et al., 2005)
Grape	transcriptomics	(Corso et al., 2015)
Grape	transcriptomics, metabolomics	(Cramer et al., 2007)
Barley	transcriptomics	(Hill et al., 2016)
Grape	transcriptomics	(Dal Santo et al., 2016)
Arabidopsis	transcriptomics	(Chan et al., 2011)
Rice	transcriptomics, metabolomics	(Maruyama et al., 2014)
Grape	transcriptomics	(Daldoul et al., 2010)
Rice	transcriptomics	(Mizuno et al., 2010)
Rice	proteomics	(Shu et al., 2010)
Grape	transcriptomics	(Henderson et al., 2014)
Poplar	transcriptomics	(Royer et al., 2016)
Grape	transcriptomics, metabolomics	(Savoi et al., 2016)
Poplar	transcriptomics	(Beritognolo et al., 2011)
Cabbage	proteomics	(FERNANDEZ-GARCIA et al., 2011)
Arabidopsis	proteomics	(Pang et al., 2010)

1.7.4 Salt tolerance related *Vitis* genes imparting stress tolerance in other plants

The outcomes from transcriptomics and gene expression studies have been validated through the development of transgenics in *Arabidopsis* and tobacco (*Nicotiana tabacum*) using specific genes (**Table 1.7**). Most of these genes include transcription factors, along with some enzymes and signaling proteins. The Arabidopsis plants overexpressing *VvbHLH1* transcription factor imparted salt tolerance through

upregulation of flavonoid and ABA synthesis genes (F. Wang et al., 2016) while overexpression of mitochondrial aldehyde dehydrogenase (*VvALDH2B8*) resulted into decrease in ROS generated during salt stress (Xu et al., 2013). Both the transgenic plants displayed increased root growth under salt stress conditions of 200mM and 250mM NaCl, respectively.

Similarly, transgenic Arabidopsis overexpressing a calcium-dependent protein kinase, *VaCPK21*, from *Vitis amurensis* displayed enhanced salt stress tolerance through upregulation of several stress-related genes, namely *AtCOR15, AtCOR47, AtCAT1, AtCSD1, AtNHX1, AtKIN1, AtRD26*, and *AtRD29B* under salt stress condition (Dubrovina et al., 2015). The *VaCPK29* overexpression displayed salt stress tolerance in *V. amurensis* callus cell culture but not in transgenic Arabidopsis (Dubrovina et al., 2016). Another transgenic Arabidopsis overexpressing *VlWRKY3*, a transcription factor from *V. labruscanana*, displayed improved salt tolerance during germination, seedling and mature plant stages when treated with 200 mM NaCl for 7 days (d) (Guo et al., 2018).

The overexpression of any stress-related gene could impart stress tolerance, however; lack of positive control in these studies makes it difficult to comment on the superiority of these *Vitis* genes over their orthologs in original genomes of transgenic plants. It is, therefore, necessary to compare the stress tolerance imparted by the overexpression of a foreign gene with that imparted by the overexpression of a self-ortholog in the transgenic plant. Furthermore, the salt tolerance phenotype of a gene cannot be confirmed until field experiments on plant yield are conducted.

Table 1.6 Differentially expressed genes in salinity stress studies identified using proteomics (P) and transcriptomics (T) techniques

Gene	Expression regulation	Plant	Organ	Technique	Reference
Carbohydrate metabolism					
Glyceraldehyde 3 phosphate dehydrogenase	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Aconitate hydratase	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Cytosolic malate dehydrogenase	up	Grape	Leaf	T	(Cramer et al., 2007)
Phosphoenol pyruvate carboxylase	up	Grape	Leaf	T	(Cramer et al., 2007)
Ribulose-5-phosphate-3-epimerase	up	Grape	Leaf	T	(Cramer et al., 2007)
Sedoheptulose-1,7-bisphosphatase	up	Grape	Leaf	T	(Cramer et al., 2007)
Photosynthesis					
Rubisco large subunit	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Chlorophyll a-b binding protein 2	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
RuBisCo activase	up	Grape	Leaf	T	(Cramer et al., 2007)
Photosystem II CP43 protein	up	Grape	Leaf	T	(Cramer et al., 2007)
Redox homeostasis					
Peroxidase	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Alkyl hydroperoxide reductase	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Atypical CYS His-rich thioredoxin 5	up	Arabidopsis	Leaf	T	(Chan et al., 2011)

Gene	Expression regulation	Plant	Organ	Technique	Reference
Superoxide dismutase	up	Arabidopsis	Leaf	T	(Chan et al., 2011)
Copper/zinc superoxide dismutase	up	Arabidopsis	Leaf	T	(Chan et al., 2011)
Transcription and translation					
50S Ribosomal protein L21	down	Arabidopsis	Leaf	P	(Chan et al. 2011)
Eukaryotic initiation factor 4A	down	Arabidopsis	Leaf	P	(Kempa et al., 2008)
DEAD-box ATP-dependent RNA helicase 56	down	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Ribosomal biogenesis	up	Arabidopsis	Root	T	(Geng et al., 2013)
rRNA processing	up	Arabidopsis	Root	T	(Geng et al., 2013)
Cell cytoskeleton					
Fibrillin-like protein	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Actin-binding, cofilin/tropomyosin type domain-containing protein	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Defense					
Class II Heat-shock protein	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
DNA-damage- repair/ toleration protein-like	up	Arabidopsis	Leaf	P	(Kempa et al., 2008)
Xylem lignification					
Casparian strip formation	up	Arabidopsis	Root	T	(Geng et al., 2013)
Suberin biosynthesis	up	Arabidopsis	Root	T	(Geng et al., 2013)
Laccase activity	up	Arabidopsis	Root	T	(Geng et al., 2013)

Table 1.7 *Vitis* genes imparting salt and osmotic stress tolerance in transgenic plants

	Species	Gene	Name	Transgenic plant	Phenotype	Reference
1	*V. vinifera*	*VvSDIR1*	Salt and drought-induced RING finger 1	*N. tabacum*	Salt and osmotic stress tolerance	(Tak and Mhatre, 2012)
2	*V. vinifera*	*VvALDH2B8*	Aldehyde dehydrogenase	*A. thaliana*	Salt tolerance	(Xu et al., 2013)
3	*V. vinifera*	*VvRD22*	Dehydration responsive	*N. tabacum*	Salt tolerance	(JARDAK JAMOUSSI et al., 2014)
4	*V. vinifera cv.* Muscat Hamburg	*VvICE1*	bHLH transcription factor	*A. thaliana*	Salt and drought tolerance	(Li et al., 2014)
5	*V. vinifera*	*VvMBF1*	Multiprotein bridging factor 1	*A. thaliana*	Drought tolerance	(Yan et al., 2014)
6	*V. labruscanana*	*VlAP17*	Group C aspartic protease	*A. thaliana*	Osmotic stress tolerance	(Guo et al., 2014)
7	*V. amurensis*	*VaCPK21*	Calcium dependent protein kinase	*A. thaliana*	Salt tolerance	(Dubrovina et al., 2015)
8	*V. quinquangularis*	*VqSTS21*	Stilbene synthase	*A. thaliana*	Resistance to osmotoc stress	(Huang et al., 2016)
9	*V. labruscana*	*VlbZIP36*	bZIP transcription factor	*A. thaliana*	Drought tolerance	(Tu et al., 2016a)
10	*V. quinquangularis*	*VqbZIP39*	bZIP transcription factor	*A. thaliana*	Salt and drought tolerance	(Tu et al., 2016b)
11	*V. vinifera*	*VvbHLH1*	bHLH transcription factor	*A. thaliana*	Salt and drought tolerance	(F. Wang et al., 2016)

	Species	Gene	Name	Transgenic plant	Phenotype	Reference
12	*V. amurensis*	*VaCPK29*	Calcium-dependent protein kinase	*A. thaliana*	Osmotic stresses and heat tolerance	(Dubrovina et al., 2016)
13	*V. vinifera*	*VvXDH*	Xanthine dehydrogenase	*A. thaliana*	Salt tolerance	(You et al., 2017)
14	*V. vinifera* cv. Razegui	*Vv-α-gal/SIP*	Alkaline alpha-galactosidase	*N. tabacum*	Salt and dessication tolerance	(Daldoul et al., 2017)
15	*V. labruscanana*	*VlWRKY3*	WRKY transcription factor	*A. thaliana*	Salt and drought tolerance	(Guo et al., 2018)
16	*V. pseudoreticulata*	*VpSBP16*	SBP-Box Gene	*A. thaliana*	Salt and drought tolerance	(Hou et al., 2018)
17	*V. vinifera*	*VvWRKY2*	WRKY transcription factor	*N. tabacum*	Salt and osmotic stress tolerance	(Mzid et al., 2018)
18	*V. vinifera*	*VvGSTF13*	Glutathione S-transferase	*A. thaliana*	Salt, drought, and methyl viologen stress	(Xu et al., 2018)
19	*V. labruscana*	*VlWRKY48*	WRKY transcription factor	*A. thaliana*	Drought tolerance	(Zhao et al., 2017)
20	*V. vinifera*	*VvABF2*	bZIP transcription factor	*A. thaliana*	Osmotic stress tolerance	(Liu et al., 2019)

1.8 Genesis of the thesis

Thompson Seedless is a popularly grown cultivar of *V. vinifera* for the production of table grapes and resins. Grapevines are perennial woody vines with a lifespan of 10 to 15 years. Their productivity largely depends on cultivation practices such as canopy management, pruning schedules, cluster thinning, application of plant growth regulators, and soil fertility. Above all, the climate and environmental stresses such as pathogen attack and drought and salt stress conditions severely decrease their yield. Rising soil salinity is a severe problem in irrigated agriculture systems wherein; accumulation of salts near the root zone severely hampers plant growth leading to a decrease in the productive lifespan of grapevine causing heavy yield loss. The own-rooted Thompson Seedless grapevines are highly susceptible to salt stress.

Salinity stress causes early aging and sometimes shows the burning of leaves under extreme conditions in grapevines. Whereas, grafting of Thompson Seedless onto the rootstock of hybrid wild cultivars, such as Dogridge and 110-Richter protects the vines from adverse effects of water and salt stress conditions and increases their lifespan and yield. Thus, the root system of grapevines is said to play a significant role in imparting tolerance to water and salt stress conditions. However, the differential response of the same scion, but having different root stocks, to salt stress have not been studied yet. Therefore in the present study, we investigated the differences in response to salinity between own-rooted Thompson Seedless (TSOR), and 110R grafted Thompson Seedless (TS110R) grapevines; wherein, TS110R is relatively salt tolerant. The grapevines were subjected to salt stress by treating them with a 150 mM NaCl solution.

These classical gene responses may or may not co-occur inside a cell. The chronology of these processes varies. For instance, the salinity induced-decrease in plant growth rate is not a single step process but a highly resolved four-phase process. Plant's first encounter with saline condition results into initial decline in growth rate (stop phase) leading to a quiescent phase of slow growth followed by a growth recovery phase and finally reaching a homeostasis phase with stable growth rate but less than the original growth rate (Geng et al., 2013; Skirycz et al., 2011, 2010). The temporal dynamics of stress-induced genes in *Arabidopsis* roots have also revealed that ABA

related gene expression increases during the quiescent phase and decreases during the recovery phase (Geng et al., 2013). This study indicated that the salt stress response varies with the duration of stress and displays temporal differences at the molecular level. Therefore, a temporally resolved salt stress study was undertaken to cover early stress response at 6 hours (h), mid-phase (24 h and 48 h) and late stage of stress on 7 days (d) of stress in the present Ph.D. thesis work.

The studies on salinity stress in plants have identified a significant role of ion transporters for tolerating the stress. The ion transporters are well known to regulate the osmotic stress-induced uptake of Na^+ and Cl^- ions in the stressed plants. At the same time, some sugar and nitrate transporters have also been reported to be involved in salinity tolerance. Most of these sugar and nitrate transporters are major facilitator family proteins; however, very little information is available on their diversity and regulation in response to salt stress. In the present work, we have tried to look into these details by undertaking identification and annotation study of major facilitator superfamily proteins in grapevines in reference to Arabidopsis and by studying their expression profiles in response to salt stress.

The present thesis has been organized in the following manner

Chapter 1: Introduction and review of literature

Chapter 2: Comparative proteomics unravels the differences in salt stress response of own-rooted and 110R grafted Thompson Seedless grapevines

Chapter 3: Transcriptomic regulation of salt stress response in 110R grafted Thompson Seedless grapevines

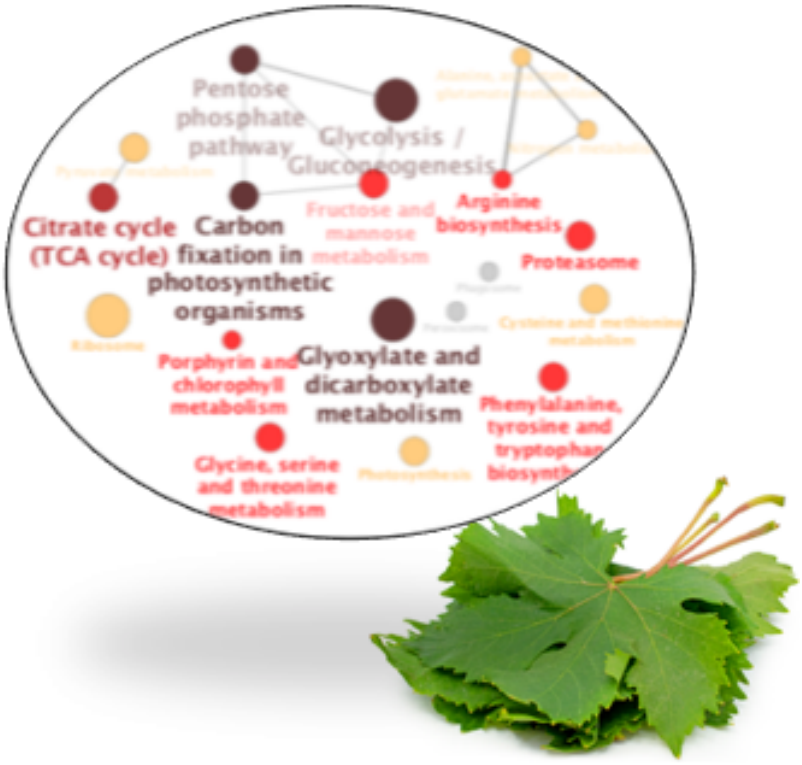
Chapter 4: Global study of MFS superfamily transporters in Arabidopsis and grapes reveals their functional diversity in response to salt stress

Summary and future directions

Bibliography

Chapter 2

Comparative proteomics unravels the differences in salt stress response of own rooted and 110R grafted Thompson Seedless grapevines



The contents of this chapter have been published as a research article--

Patil, Sucheta, et al. "Comparative Proteomics Unravels the Differences in Salt Stress Response of Own Rooted and 110R Grafted Thompson Seedless Grapevines." Journal of Proteome Research (2019). DOI: 10.1021/acs.jproteome.9b00420

Chapter 2 Comparative Proteomics Unravels the Differences in Salt Stress Response of Own Rooted and 110R Grafted Thompson Seedless Grapevines

2.1 Introduction

Soil salinity is one of the major environmental factors limiting the productivity of crops, with 20 to 50% yield loss. Soil salinization can be caused by several factors, such as high evaporation of soil water, insufficient rains, use of poor-quality water for irrigation as well as indiscriminate use of fertilizers. A strong link has been established between excess irrigation and soil salinization (Ghassemi et al., 1995). Additionally, the lack of leaching of salts from the soil either due to poor drainage, or little or no precipitation, makes soil-salinity more adverse and irreversible. This is a significant problem in arid regions, such as Pakistan (Evenson, 2000), Northwestern China (Wang et al. 2016) and Northwestern parts of India (Datta and De Jong, 2002; Lorenzen et al., 2012; Sharma and Singh, 2015; Tanji and Kielen, 2002). Australia is also highly affected by salinity, mainly due to the high level of groundwater-table, leading to salinization of surface soils (Nielsen et al., 2003; Rengasamy, 2006). Salinization of soils is increasing rapidly worldwide (Shrivastava and Kumar, 2015) and has been estimated to reach staggering 50% of the arable land by the year 2050 (Jamil et al., 2011). Developing salt tolerant crops is thus essential for practicing sustainable agriculture under saline conditions. Several physiological and molecular studies of salinity stress in plants have revealed various mechanisms of salt stress tolerance, which include accumulation of compatible osmolytes to maintain turgor, maintenance of ionic homeostasis, increased water use efficiency, enhanced photosynthesis and alleviation of oxidative stress through antioxidant metabolism (Munns, 2002). These physiological and metabolic changes are a result of complex regulation of several enzymes, signaling pathways and structural molecules.

In order to unravel simultaneous variations in different metabolic pathways, transcriptomic and proteomic investigations are necessary to perform. Such efforts have become possible due to the availability of *V. vinifera* genome since 2007 (Jaillon et al., 2007). Two-dimensional gel electrophoresis (2D-GE) based proteomic approach was used to investigate the differences between *V. vinifera* cv. Chardonnay and cv. Cabernet Sauvignon in response to 8 and 16 days of water deficit and salinity stress (Vincent et

al. 2007). Another proteomic study was performed on salt-tolerant Tunisian grapevine cv. Razegui, which depicted over-expression of a pathogenesis-related protein 10 upon salt stress for 15 d (Jellouli et al., 2008). A study on early and late stages of the water-stressed grapevines showed enormous changes in several metabolic pathways even before inhibition of growth and photosynthesis (Cramer et al., 2013). However, these studies were mostly focused on the late stage of response. Moreover, 2D-GE approach lacks the depth of the comprehensive approach of using LC-MS/MS.

Rootstocks play a crucial role in salt tolerance by reducing the rate of salt accumulation in grapevines. The grafting of grapevines onto a suitable rootstock is commonly practiced in viticulture in order to overcome stress conditions such as soil salinity and drought stress (Walker et al., 2010, 2002, 2000). However, the influence of rootstock on the molecular mechanisms associated with the salt stress response in scion is still unknown. Recently, transcriptomic studies have shown that grafting can determine stock-specific transcript concentration changes in the grapevine scion and cause upregulation of stress-related genes at graft interface in hetero-grafts (Cookson et al., 2014, 2013). In the present study, we compared the proteomic changes in Thompson Seedless leaves of own-rooted (TSOR; salinity-sensitive) and 110-Richter (110R; salinity-tolerant) grafted (TS110R) grapevines. The protein expression patterns were profiled across three time-points, including the early and late stages of salt stress in both the vines using label-free shotgun proteomics approach. The differentially expressed proteins identified in the present study revealed distinct regulation of salt stress responses in the two grapevines.

2.2 Materials and methods

2.2.1 Plant material

Sixteen months old Thompson Seedless grapevines on its own roots (TSOR) and the same-aged vines of Thompson Seedless grafted on 110R rootstock (TS110R) were used for the study (**Figure 2.1A,B**). These plants were raised in 14” x 14” polybags filled with a potting mixture consisting of black cotton soil, farm yard manure (FYM), and sand in 1:1:1 proportion (v/v) and maintained in shade net under ambient conditions of temperature, moisture, and photoperiod. The ambient temperature ranged between 22 - 37°C, relative humidity ranged between 32.5% - 79.2% and average photoperiod was

11 h. During the growth phase, plants were irrigated with frequent and light irrigation of water to maintain the soil at field capacity. The potted vines were kept in healthy growth conditions by irrigating them with half-strength Hoagland's nutrient solution and by intermittent foliar application of 2 g/l of NPK fertilizer 19:19:19. These experiments were carried out at the National Research Centre for Grapes (NRCG), Pune, Maharashtra, India.

Figure 2.1 Experimental plant material

A – Sixteen-month-old TSOR grapevines. B – Sixteen-month-old TS110R grapevines. C – Young leaf samples collected for analysis.

2.2.2 Salinity stress experiment

The vines were subjected to salt stress by irrigating them with 150 mM NaCl solution (EC_w = 12.0 dSm^{-1}), and the control vines were irrigated with potable water (EC_w < 0.7 dSm^{-1}). The average temperatures during the experiment ranged between 24 and 37 °C, while the average relative humidity was between 32.5 and 79.2 %. The average day length was 11 h. The young leaf samples (**Figure 2.1C**) were collected from nine control and nine treated vines each at 6 h, 24 h, 48 h, 7 d and 15 d of salt treatment and were snap-frozen in liquid nitrogen and stored at -80 °C. The samples were collected between 10:00 and 10:30 am except for 6 h samples, which were collected at 4:00 pm. The leaves collected from a single vine were considered as one biological replicate.

2.2.3 Measurement of morphological and physiological parameters

The plant growth and the leaf gas exchange were measured at 3-day intervals on nine vines each of control and treated plants. Morphological parameters such as plant height, shoot length, leaf length, leaf width, and internode distance were measured using a geometrical scale. Gas exchange parameters *viz*. net assimilation rates (AN), stomatal conductance (gs), transpiration rate (E) were recorded on fully expanded fourth or fifth leaf on 0, 4^{th}, 7^{th}, 9^{th} , 13^{th}, 16^{th}, 19^{th}, and 23^{rd} days of treatment using Infra-Red Gas Analyser (IRGA, GFS 3000, Heinz Walz GmbH, Effeltrich, Germany) system in the morning hours (9-11 am). Therefore, the tissue sampling coincided with physiological measurements only on the 7th day of experiment. Instantaneous water use efficiency ($WUE_{instantaneous}$) was calculated as the ratio of CO_2 assimilation rate (A) and transpiration rate (E) and intrinsic WUE ($WUE_{intrinsic}$) as ratio AN/gs.

2.2.4 Estimation of Na^+ and Cl^- content

All the leaves on the shoot, except the young leaves that were used for RNA and protein extraction and the fourth or fifth leaf for other biochemical analysis, were collected for nutrient analysis at 6 h, 24 h, 48 h, 7 d, 15 d and 30 d after salt treatment. The leaves were detached from petioles, washed with distilled water and oven-dried at 70 °C. The leaves from three vines were pooled and taken as one replicate, and nutrient analysis was performed in triplicate. Leaf samples were ground in Cyclotec sample mill (Foss Tecator, Hillerod, Denmark) followed by digestion in block digester with H_2SO_4:H_2O_2 mixture. Sodium was estimated using Analyst 100 Atomic Absorption Spectrophotometer (Perkin Elmer, Waltham, MA, USA) in emission mode, while

Chloride was estimated using flow injection system (San++ Automated Wet Chemistry Analyzer, Skalar Analytical B.V., Breda, The Netherlands).

2.2.5 Protein extraction and sample preparation for untargeted proteomics

Protein was extracted from 100 mg of finely crushed leaf samples using a modified TCA – acetone phenol extraction protocol (Wang et al., 2008). In brief, the phenolics were removed by precipitation of proteins in 10% TCA/acetone (w/v) followed by washing of the pellet with 80% methanol in water (v/v). The pellet was resuspended in 2 ml of extraction buffer containing 0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl, pH 7.5 and 50 mM EDTA. The beta-mercaptoethanol to a final concentration of 2% (v/v) was freshly added in every solution at each step to maintain the reduced conditions. Samples were vortexed and incubated with constant shaking for 10 min on ice. The proteins from the cell lysate were extracted into the phenol phase with the addition of an equal volume of Tris-buffered phenol (pH = 8.0). The proteins were precipitated out from phenol phase by carefully transferring it to a tube containing four volumes of 0.1 M ammonium acetate in ice-cold 80% methanol. The samples were incubated overnight at – 20 °C. The precipitated protein pellets were washed with ice-cold 80% methanol in water (v/v) and 80% acetone in water (v/v), thrice each. The protein pellets were air-dried and stored at -80 °C until further use. Protein samples were solubilized in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest (Waters, Milford, MA, USA) and quantified using Bradford protein assay. Protein samples were diluted to 1 µg/µl protein concentration of which 100 µl was used for in-solution trypsin digestion. The dissolved proteins were reduced and alkylated using DTT and iodoacetamide respectively, followed by overnight tryptic hydrolysis at 37°C using sequencing grade Trypsin-Gold (Promega, Madison, WI, USA). The tryptic peptide samples were cleaned using ZipTip (Merck-Millipore, Burlington, MA, USA) before injecting them into the LC-MS^E workflow.

2.2.6 Untargeted shotgun proteomics on Synapt HDMS G1

Digested protein samples were analyzed using a NanoAcquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a SYNAPT-G1 high definition mass spectrometer (Waters, Milford, MA, USA). The binary solvent system used in the UPLC system comprised of 99.9% water and 0.1% formic acid

(mobile phase A) and 99.9% acetonitrile with 0.1% formic acid (mobile phase B). The trypsin digested protein samples were spiked with 100 fmol of yeast enolase (P00924) digest and were initially pre-concentrated and desalted online at a flow rate of 5 µl/min using a Symmetry C18 trapping column (internal diameter 180 µm, length 20 mm) (Waters, Milford, MA, USA) with 0.1% B mobile phase. After trapping, the samples were resolved for 120 minutes on a C18 reverse-phase column (BEH-C18 1.7 µm particle size, internal diameter of 75 µm and length of 250 mm) (Waters, Milford, MA, USA) at a flow rate of 0.25 µl/min with a gradient of solvent B from 3% to 85% in solvent A (**Table 2.1**).

Table 2.1 Water-acetonitrile gradient profile of 120 minutes

Time (min)	Flow rate (µl/min)	Water %	Acetonitrile %
0	0.25	97	3
5	0.25	90	10
95	0.25	65	35
99	0.25	60	40
102	0.25	15	85
108	0.25	15	85
110	0.25	97	3
120	0.25	97	3

The peptides were eluted into the NanoLockSpray ion source and were acquired in a positive V-mode at 9000 full width half maximum (FWHM) resolution with a scan time 0.75 s over 50-2000 m/z mass range with a constant low energy of 4V for MS mode and a step from 15 to 40 V of collision energy during high energy MS^E mode scans. The lock mass correction and mass spectrometer calibration were done every 30s with

the MS/MS spectra of Glufibrinopeptide B (m/z 785.8426) that was supplied to the ionization source through an Auxiliary inlet.

2.2.7 Identification and quantitation of peptide mass spectra using Progenesis QI for proteomics

The raw files were imported into the Progenesis QI for proteomics software (v. 3.0.6; Nonlinear Dynamics, Durham, NC, USA). The runs were aligned with each other, and automatic peak picking was performed for the detection of peptide ions. The MS^E fragmentation data processing was performed with ion matching parameters wherein, the precursor, and product ion tolerance was set to automatic, minimum number of fragment ion matches per peptide 3, minimum number of fragment ion matches per protein 5 and minimum peptide matches per protein was 1. The number of missed cleavage sites was set to 1, along with carbamidomethylated Cys (C) residues as fixed and Met (M) oxidation as variable modifications. The false-positive rate was 5%. The *Vitis vinifera* protein database (ftp://ftp.ensemblgenomes.org/pub/plants/release-35/fasta/vitis_vinifera/pep/) was used for identification of proteins that were further quantitated based on the unique peptides using Absolute Quantitation Hi-N method.

2.2.8 Statistical and Bioinformatic analyses

The differential expression analysis was performed for quantifiable proteins identified with unique peptides using unpaired ANOVA implemented in the Progenesis QI for proteomics software (v. 3.0.6; Nonlinear Dynamics, Durham, NC, USA) by comparing the protein abundance values in control and treated samples for every time point of salt stress experiment. The proteins with p-value ≤ 0.05, q-value ≤ 0.05, and fold change ≥ 1.5 were considered as significantly differentially expressed proteins (DEPs). The expression pattern of DEPs across all stages of salt stress in the two vines was evaluated by clustering in ClustVis (https://biit.cs.ut.ee/clustvis) using correlation distance and Ward linkage. The Gene Ontologies (GO) of all the proteins in the *V. vinifera* proteome database were identified using Blast2GO v. 3.1.3 (Conesa et al., 2005). The resulting annotation file was used as a reference for GO enrichment analysis in BiNGO v. 3.0.3 (Maere et al., 2005). The GO terms were selected for classification of proteins only if there were more than five proteins in the test set and less than three thousand proteins in the reference set. KEGG pathway enrichment was performed using ClueGO v. 2.1.7 (Bindea et al., 2009) plugin in Cytoscape v. 3.1.1 (Shannon, 2003) by implementing

Right-sided hypergeometric statistical test and Bonferroni step down correction method.

2.2.9 Quantitative reverse-transcriptase PCR analysis

The RNA was extracted from the leaves of three biological replicates of each time-point using Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MI, USA). Two µg of RNA was used for first-strand cDNA synthesis using High Capacity cDNA reverse transcription kit (Applied Biosystem, Carlsbad, CA, USA). cDNA sample was diluted 20 fold with RNase free water, and 2 µl of diluted cDNA was used as the template for each reaction. Top five up and down-regulated proteins were selected for expression analysis and the primers were designed for their amplification using Primer3web v.4.0.0 (https://primer3plus.com/) (**Supplementary Table 1**). The amplification of genes was quantified using 7900HT Fast Real-Time PCR System (Applied Biosystems, California, USA) with initial denaturation at 95 °C for 10 min and subsequent 40 thermal cycles of 95 °C for 3 s and 60 °C for 30 s followed by a dissociation curve analysis of amplicons. Three biological replicates were used for each sample, and each reaction was performed in duplicate. Relative quantification was performed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Elongation factor 1-α (EF1α) was used as a reference gene for normalization and compared with corresponding control samples. The statistical significance was calculated using student's t-test.

2.3 Results

2.3.1 Growth retardation and physiological changes in salt-stressed vines

Salt stress affected both TSOR and TS110R vines causing a significant decrease in growth and water conductance. The details about the measurement of growth-related and gas exchange parameters are described in publication of this work (Upadhyay et al., 2018). The difference in the salt tolerance of TSOR and TS110R vines was evident from the decrease in plant height and physiological changes of salt-stressed and control vines (**Figure 2.2A**, **Figure 2.3**). The shoot growth of TS110R was unaffected until 10 d of stress while the shoot of TSOR was aborted within 4 d, indicating the ability of TS110R vines to tolerate the stress for a relatively long period. A consistent rise of sodium and chloride ions into the shoots was observed in TSOR; while TS110R

suppressed it till 15 d, suggesting exclusion of ions by the rootstock which could be the primary mechanism of salt tolerance in them (**Figure 2.2B**).

The CO_2 assimilation rate of TSOR increased in response to salt stress up to 10 d with a simultaneous rise in transpiration rate and water conductance. Whereas, in TS110R the transpiration rate and water conductance initially dropped till 4 d of stress and were stabilized to normal by 7 d with a simultaneous rise in CO_2 assimilation rate leading to increased water use efficiency (WUE).

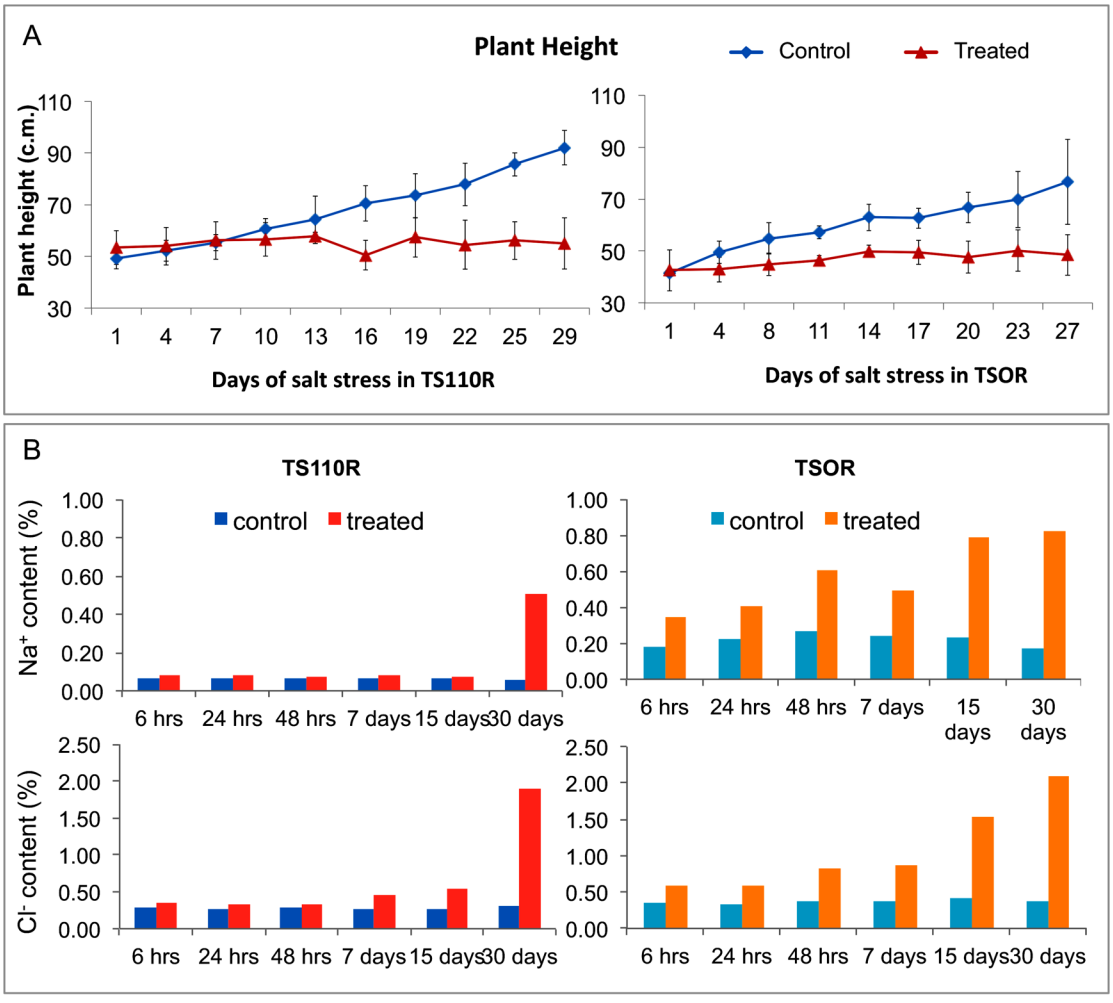


Figure 2.2 Effect of salt stress on TS110R and TSOR grapevines

A – Effect of salt treatment on plant height. B – The sodium and chloride content of grapevine leaves at different time-points of salinity experiment

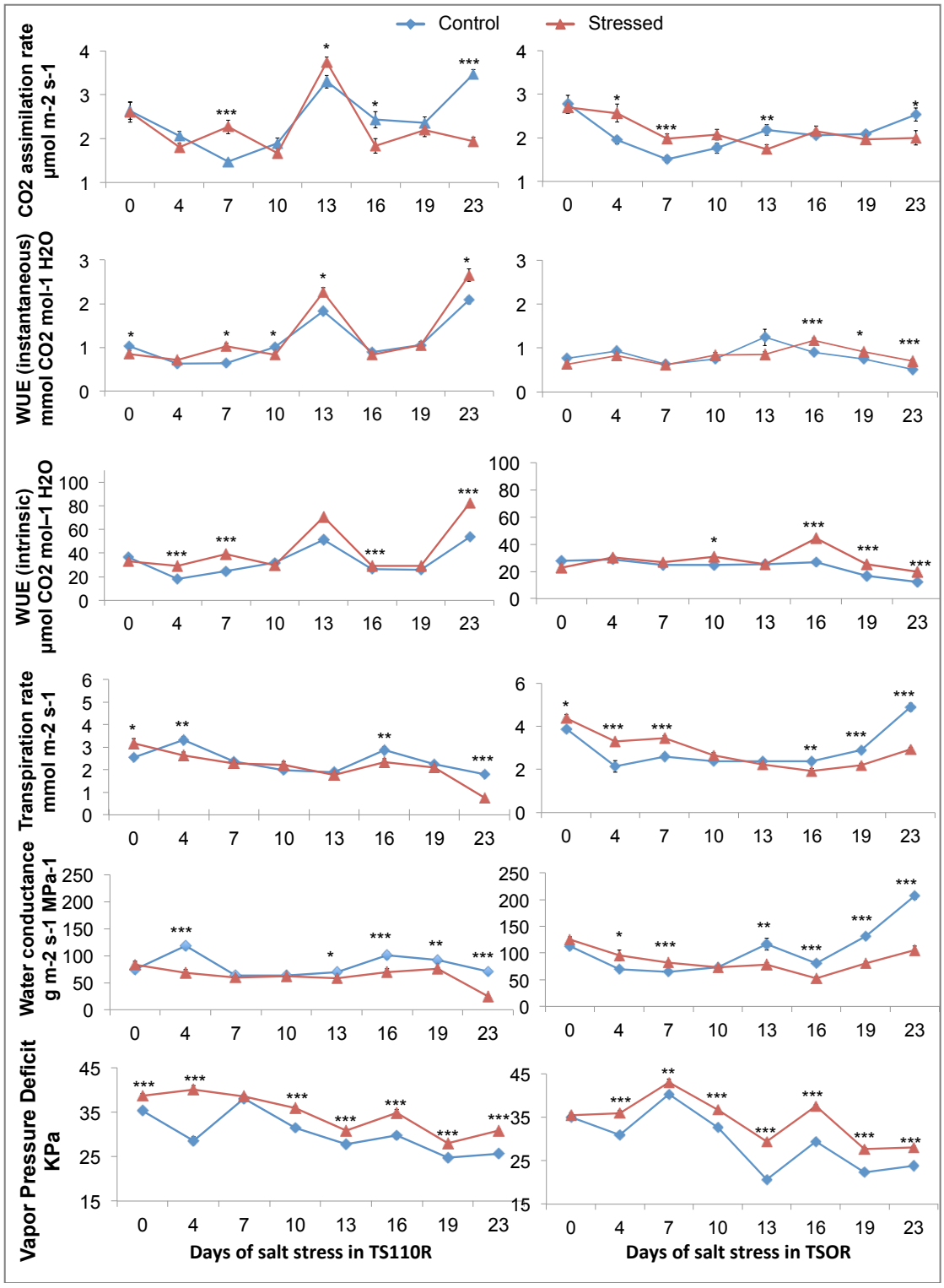


Figure 2.3 Physiological observations of control and salt-stressed grapevines

The overall WUE of TS110R was higher than that of TSOR vines. In total, exclusion of ions from shoot and rise in WUE contributed to salinity tolerance in TS110R with unhampered shoot elongation for a longer period; while TSOR lacked the ability to

exclude Na^+ and Cl^- ions and responded to the salt-stress by increasing CO_2 assimilation rate along with transpiration rate.

2.3.2 Proteome identification and its functional classification

The salt-stressed potted grapevines showed a decrease in growth rate with complete burning of shoot tip by 15th day of the stress. The effect of salt stress at early (6 h), mid (48 h) and late (7 d) stages was investigated in the newly emerged young leaves of control and stressed grapevines using untargeted shotgun proteomics approach. The extraction of proteins using modified TCA-acetone protocol resulted in an average yield of 12.5 mg/g and 7.6 mg/g of tissue from the leaves of 110R grafted and own-rooted Thompson Seedless grapevines, respectively. The protein samples were digested using trypsin, and their mass spectra were acquired using LC-MS^E approach. All 108 raw mass spectral files generated from three technical replicates of each biological replicate for six samples of TSOR and six of TS110R were aligned together using Progenesis QI for proteomics (v. 3.0.6; Nonlinear Dynamics, Durham, NC, USA). A total of 2793 protein groups were identified from the analysis (**Supplementary Table 2**) of which, 513 were identified with unique peptides and were used for the statistical analysis.

The qualitative analysis was conducted using representative protein from each protein group. The Gene Ontology (GO) based classification of proteins into different Biological Processes (BP), Cellular Components (CC) and Molecular Functions (MF) were conducted using BiNGO. The total identified proteins represented several subcellular components with the highest number from nucleus followed by cytosol, plastid, plasma membrane, ribosome, mitochondria, thylakoid, vacuole, cell wall, Golgi apparatus, endoplasmic reticulum, cytoskeleton, nucleoplasm, nucleolus, peroxisome and nuclear envelope (**Figure 2.4A**). The enrichment analysis of total quantifiable proteins revealed a significant representation of proteins from cytosol followed by plastid, ribosome, mitochondrion, and thylakoid (**Figure 2.4B**). These proteins carried out different molecular functions and were involved in 23 biological processes of which catabolic process, cellular amino acid derivative metabolic process, translation, carbohydrate metabolic process, generation of precursor metabolites and energy, cellular homeostasis and photosynthesis were significantly over-represented terms.

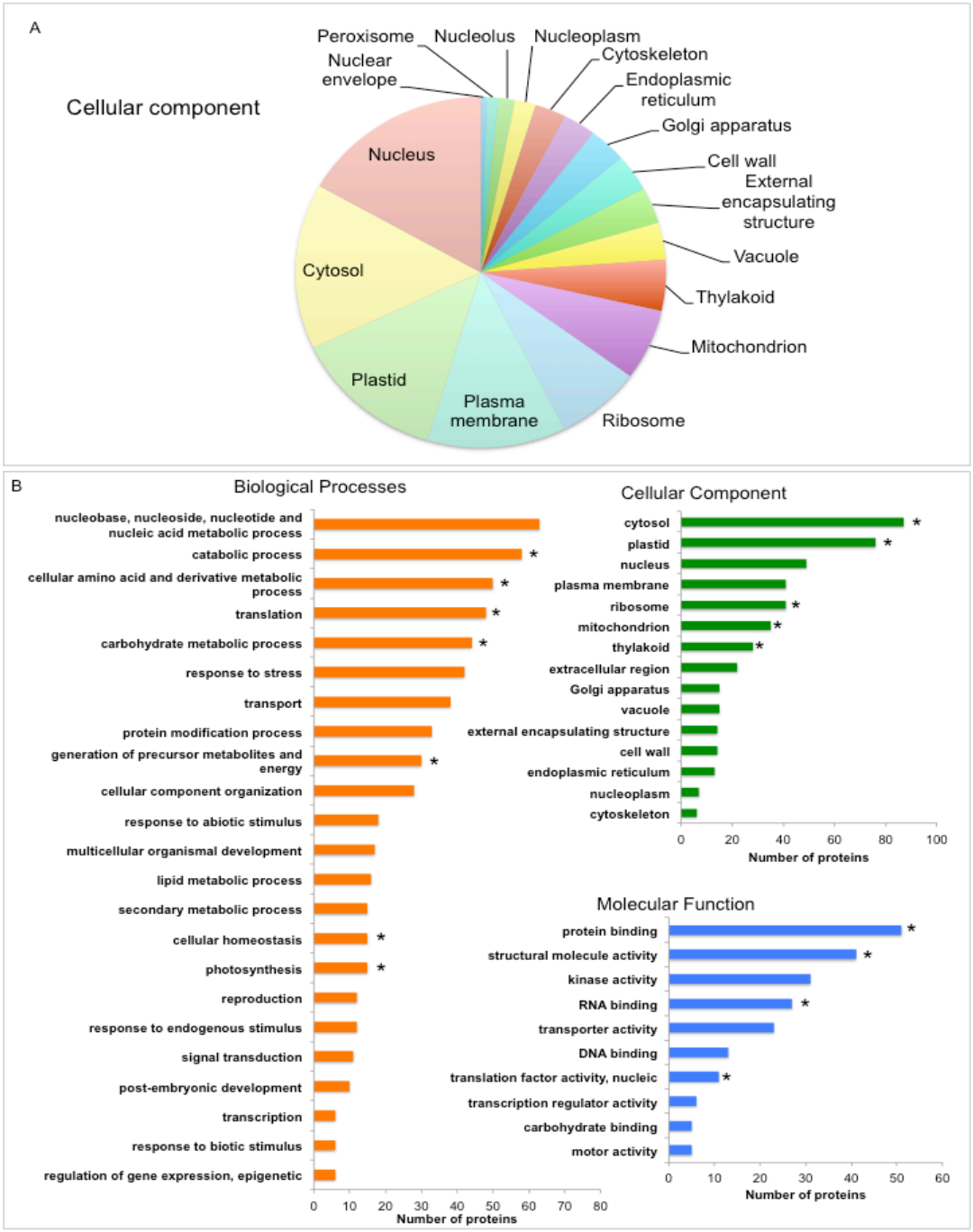


Figure 2.4 GO term enrichment of identified and quantifiable proteins

A – Cellular component classification of all the proteins identified in the proteome of grapevine leaves based on GOSlim_Plant annotation. B – Classification of quantifiable proteins into biological processes, cellular components, and molecular functions. Significantly enriched (p-value < 0.05) GO terms are marked with an asterisk

2.3.3 Differences between TSOR and TS110R

The own-rooted TSOR vines had a slower growth rate compared to that of grafted TS110R vines. The percent increase in plant height of TS110R vines was 88% in 29 days, and that of TSOR vines was 80% in 27 days (**Figure 2.2**). The comparison between proteomes of control vines, TS110R, and TSOR displayed a greater number of differentially abundant proteins in TS110R than in TSOR (**Figure 2.5A, B**). The TSOR specific proteins had significant enrichment of primary metabolic processes, while TS110R had oxidation-reduction and biosynthesis of cellular nitrogen compounds (**Figure 2.5C**). This indicated that TS110R vines were able to support faster growth through the efficient synthesis of nitrogenous compounds despite the grafting induced oxidative stress.

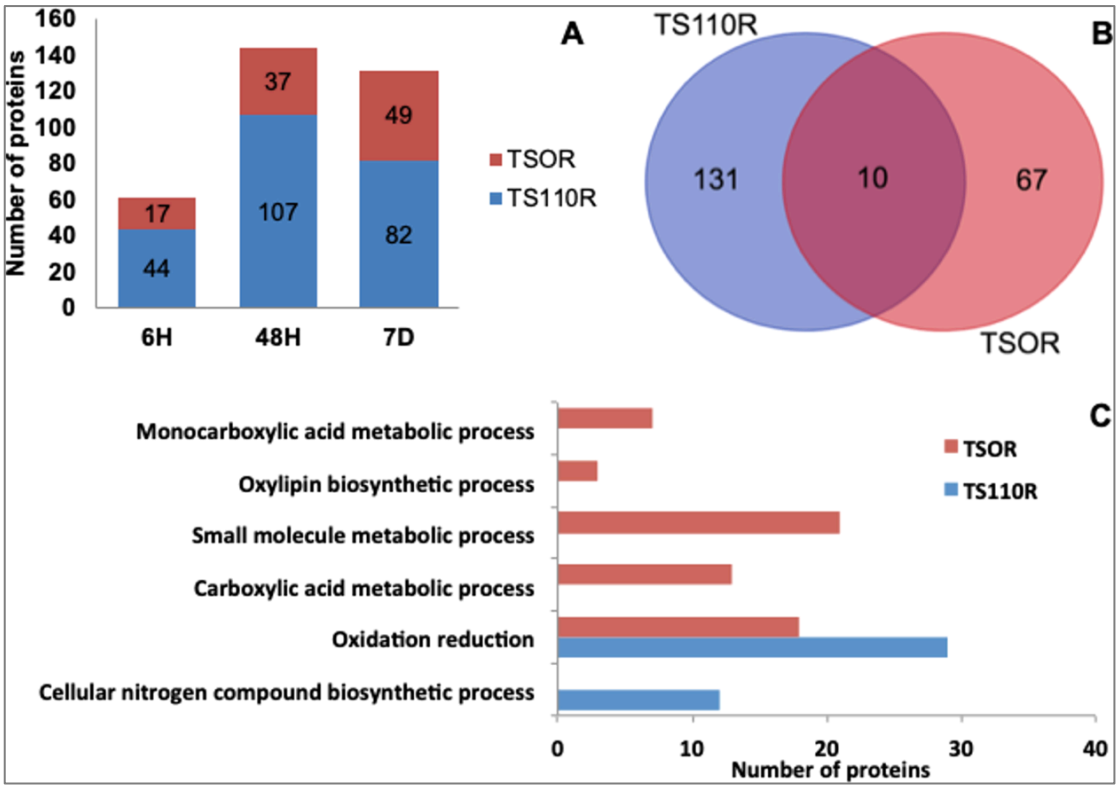


Figure 2.5 Differences between proteomes of control vines – TSOR and TS110R

A – Number of differentially abundant proteins at three stages of salt stress in the two vines. B – Number of proteins uniquely abundant in the two vines. C – Significant biological processes represented in the unique sets of proteins of TSOR and TS110R vines

2.3.4 Differentially altered protein expression in salt-stressed own-rooted and 110R grafted grapevines

Statistical analysis of protein expression changes between control and stressed grapevines identified the proteins significantly affected by salt stress in TSOR and TS110R. A total of 246 proteins were significantly differentially regulated (**Supplementary Table 3**), 214 in TSOR, and 151 in TS110R. The number of differentially expressed proteins (DEPs) decreased from 80% at an early stage to 27% at a late stage in TSOR vines, suggesting a constant drop in molecular response with increasing salt stress (**Figure 2.6A**). The TS110R vines displayed huge response to early stress with upregulation of nearly 50% DEPs followed by a drop in the stress response to 13% DEPs at mid-stage and suppression at a late stage with downregulation of about 60% DEPs. Twenty-four proteins were differentially regulated throughout the stress duration in TSOR; while in TS110R, 42 DEPs were common to the early and late stages of stress and none were common between early and mid-stage (**Figure 2.6B**).

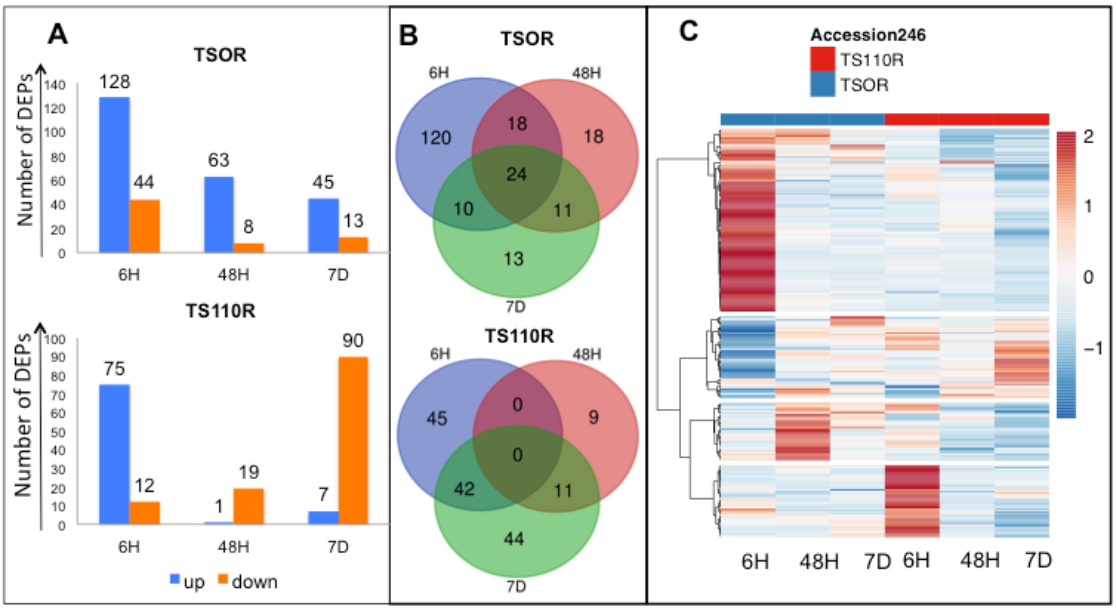


Figure 2.6 Differentially expressed proteins

A – Number of significantly differentially expressed proteins (DEPs) with p-value ≤ 0.05, q-value ≤ 0.05 and fold change ≥ 1.5 at 6 h (6H), 48 h (48H) and 7 d (7D) in TSOR and TS110R vines. B – Venn of DEPs at different stages of stress in TSOR and TS110R. C - Major protein expression patterns associated with the DEPs - Heat map of log-transformed fold change values of all 246 DEPs after unit variance scaling of rows

The clustering of expression patterns of 246 DEPs revealed distinct regulation of DEPs in the two grapevines (**Figure 2.6C**) highlighting the difference in their response to salt stress. The cluster of upregulated proteins at an early stage in TSOR was distinct and larger than that in TS110R. Similarly, distinct clusters of DEPs were identified for other time-points as well. The second cluster showed that the proteins, which were downregulated at an early stage in TSOR, were mostly upregulated at a late stage in TSOR and TS110R. However, the expression was more predominant in TS110R. The proteins upregulated only in TS110R on the 7th day of stress possible for salt tolerance in TS110R were bHLH47 (VIT_17s0000g06000.t01), protochlorophyllide reductase (VIT_12s0059g00270.t01), RAS-related protein RABC1 (VIT_18s0001g15090.t01), polycomb group protein (VIT_19s0014g05210.t01) and linoleate 13s-lipoxygenase-2 chloroplastic (VIT_13s0064g01490.t01).

2.3.5 Biological processes affected by salt stress

The biological processes affected by differential expression of proteins at different stages of salt stress were identified through GO enrichment of up-regulated and down-regulated DEPs at individual time-points (**Figure 2.7**). The two grapevines responded differently to the salt stress. Processes like photosynthesis and generation of precursor metabolites and energy were significantly upregulated at an early stage in TSOR but were unaffected in TS110R. These processes were suppressed or downregulated at a late stage in both the vines. The photosynthesis-related proteins like photosystem II (PSII) protein D1, oxygen-evolving enhancer protein, Photosystem I (PSI) reaction center subunit III, plastocyanin, chlorophyll a-b binding protein, ribulose bisphosphate carboxylase (RuBisCO), RuBisCO activase were upregulated in TSOR indicating increased photosynthesis which correlated with the increase in CO_2 assimilation rate of TSOR vines during early phase of salt stress. Alternatively, early stress in TS110R resulted in upregulation of translation and cellular amino acid and derivative metabolic processes, while TSOR displayed delayed upregulation of translation-related proteins at mid-stage. Proteins like cysteine tRNA ligase, cytoplasmic and mitochondrial glycine tRNA ligase were upregulated at an early stage in TS110R and were highly upregulated at mid-stage in TSOR. Additionally, proteins involved in the regulation of gene expression, epigenetic process were significantly upregulated at mid-stage in TSOR. The stress-related functions of proteins from these biological processes are discussed later.

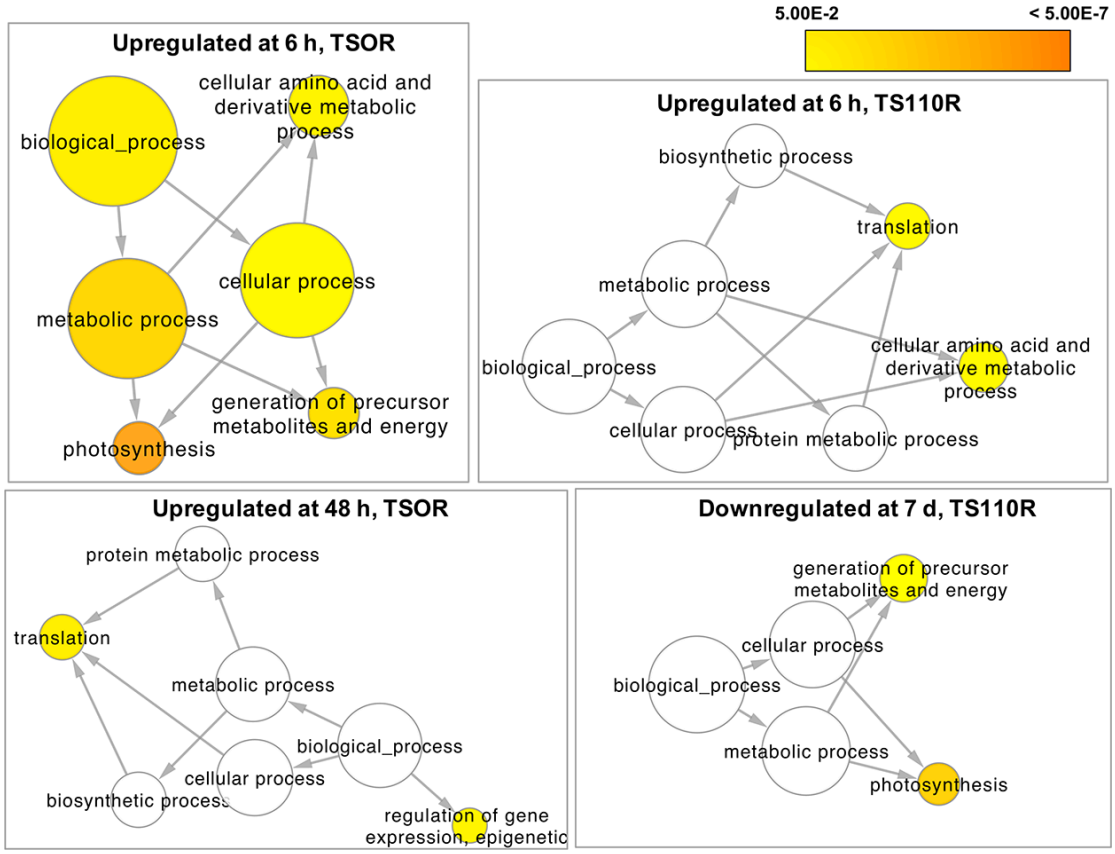


Figure 2.7 Gene ontology enrichment of DEPs at different stages of stress

The color gradient indicates the p-value of statistical enrichment while the size of the circle indicates the number of enriched proteins in each process

2.3.6 Metabolic pathways affected by salt stress

The metabolic pathways affected during salinity stress were identified from the representative enzymatic proteins within DEPs. The KEGG pathway enrichment analysis of all 246 DEPs resulted in 19 clusters, 11 of which were significantly enriched with p-value < 0.05 (**Figure 2.8**). These included ten primary metabolic pathways and a proteasomal pathway. The primary metabolic pathways were from carbon metabolism, amino acid metabolism, and chlorophyll biosynthesis. The carbon metabolism pathways included Carbon fixation in photosynthetic organisms, Glycolysis/ Gluconeogenesis, Fructose, and Mannose metabolism, Glyoxylate and Dicarboxylate metabolism, Citrate (TCA) cycle, and Pentose phosphate pathway (PPP). The amino-acid metabolism pathways included glycine, serine, and threonine metabolism (QST metabolism), Phenylalanine, tyrosine and tryptophan biosynthesis, and Arginine biosynthesis. Metabolic pathways are interconnected through several

common metabolites, and hence, most of the enzymes in Carbon metabolism were shared with multiple pathways (**Figure 2.9**).

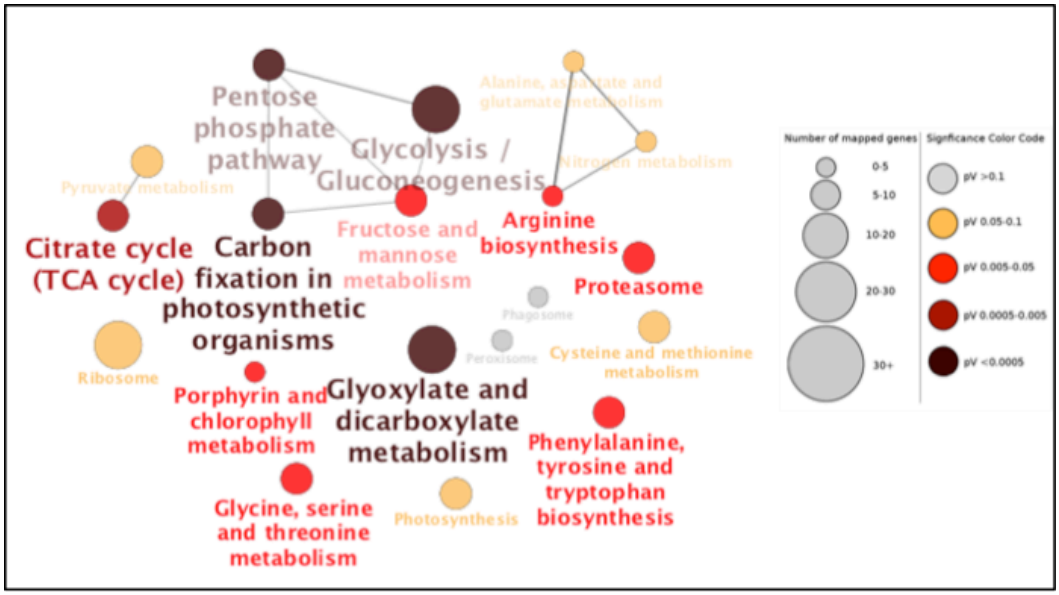


Figure 2.8 KEGG pathway enrichment from 246 DEPs

The color gradient indicates the p-value of statistical enrichment, while the size of the circle indicates the number of enriched proteins in each process

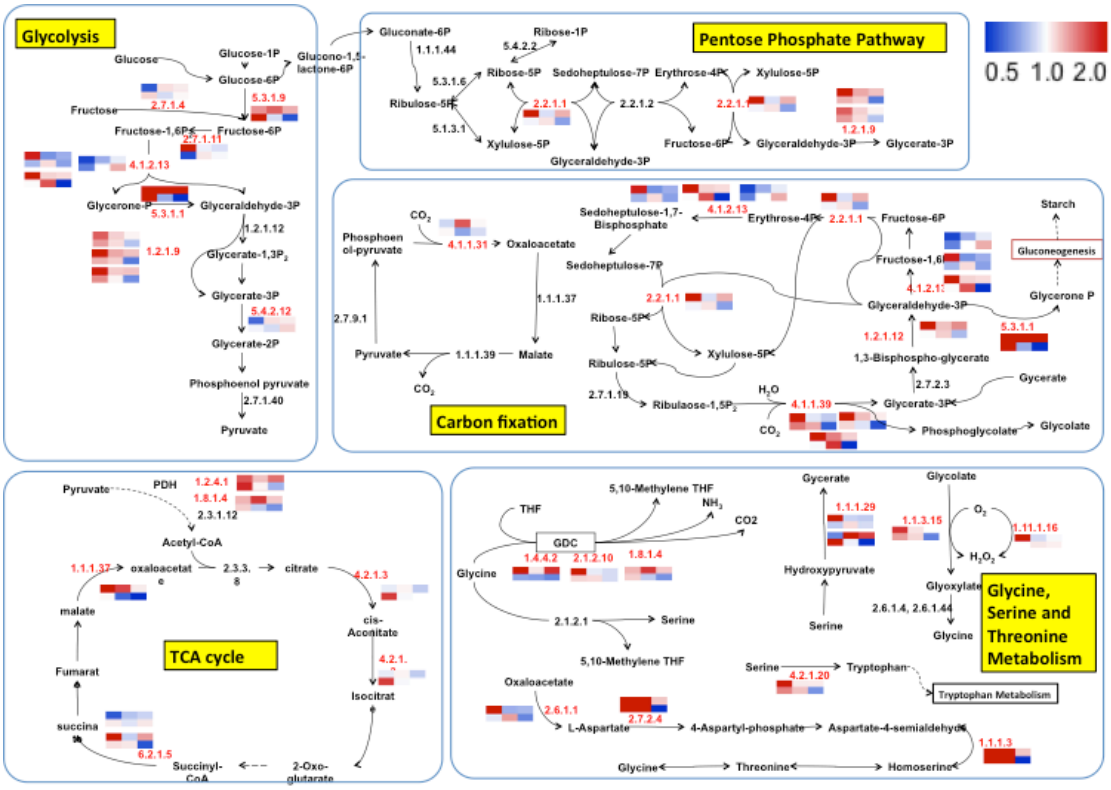


Figure 2.9 Metabolic pathways affected by salt stress

The heat maps denote the fold change values for TSOR on the upper row and for TS110R on the lower row at three time-points – 6 h, 48 h and 7 d of salt stress. Enzymes denoted by the EC numbers: 1.1.1.29 – glycerate dehydrogenase/ hydroxyphenylpyruvate reductase, 1.1.1.3 – homoserine dehydrogenase, 1.1.1.37 – malate dehydrogenase chloroplastic, 1.1.3.15 – (S)-2-hydroxy-acid oxidase GLO1 peroxisomal, 1.2.1.12 - glyceraldehyde-3-P-dehydrogenase chloroplastic, 1.2.1.9 – glyceraldehyde-3-phosphate dehydrogenase (NADP+), 1.2.4.1 – pyruvate dehydrogenase, 1.4.4.2 – glycine dehydrogenase mitochondrial, 1.8.1.4 – dihydrolipoyl dehydrogenase mitochondrial, 2.1.2.10 – aminomethyltransferase, mitochondrial, 2.2.1.1 – transketolase chloroplastic, 2.6.1.1 – aspartate transaminase chloroplastic, 2.7.1.4 – fructokinase, 2.7.2.4 – aspartate kinase, 4.1.1.31 – phosphoenolpyruvate carboxylase, 4.1.1.39 – ribulose-bisphosphate carboxylase, 4.1.2.13 – fructose-bisphosphate aldolase chloroplastic, 4.2.1.20 – tryptophan synthase, 4.2.1.3 – aconitate hydratase, 5.3.1.1 – triose-phosphate isomerase cytosolic, 5.3.1.9 – glucose-6-phosphate isomerase chloroplastic, 5.4.2.12 – phosphoglycerate mutase, 6.2.1.5 – succinyl-ligase, 6.4.1.2 – acetyl-CoA carboxylase

Glycolysis shared enzymes with PPP and QST metabolism. PPP pathway enzymes were also a part of Fructose and mannose metabolism. Glyoxylate metabolism pathway proteins were shared with QST metabolism and TCA cycle. Apart from KEGG pathways, the modulation of photorespiratory pathway was evident from the differential expression of proteins such as mitochondrial dihydrolipoyl dehydrogenase, glyoxylate/ hydroxyphenylpyruvate reductase-like, catalase isozyme 1, mitochondrial Aminomethyltransferase, RuBisCO small subunit, peroxisomal (S)-2-hydroxy-acid oxidase GLO1, glutamine synthetase and partial RuBisCO large subunit (**Figure 2.10, Figure 2.11**), which are also involved in glyoxylate metabolism and QST metabolism. Most of these enzymes were upregulated at an early stage in both the vines **(Figure 2.11**). Among all these enzymes, glutamine synthase was upregulated with a very high fold change in both the vines.

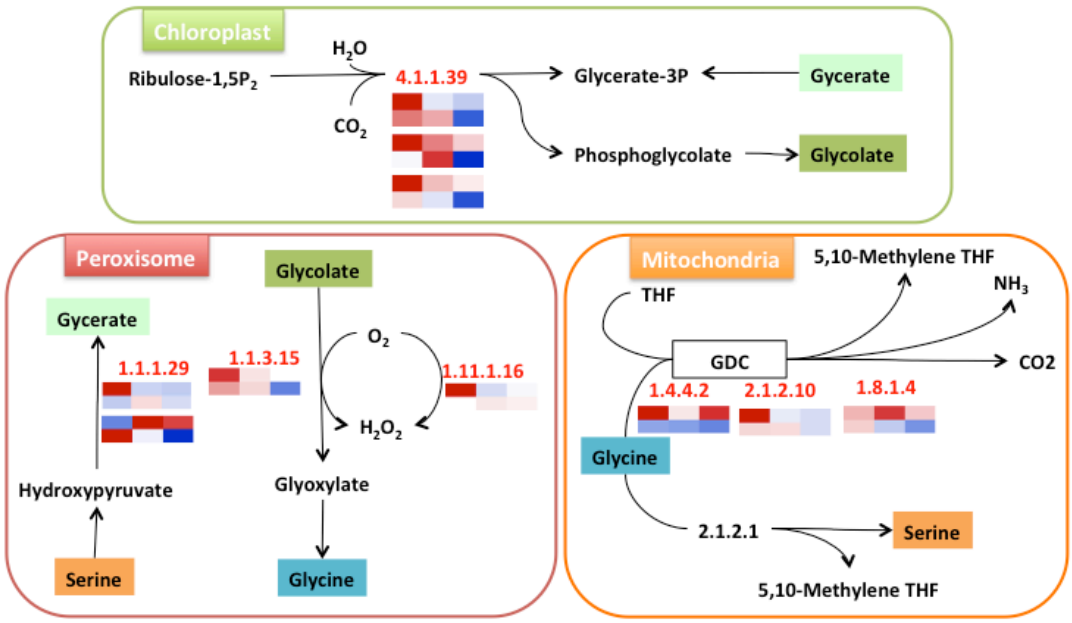


Figure 2.10 Photorespiration related DEPs

The heat maps denote the fold change values for TSOR on the upper row and TS110R on the lower row at three time- points – 6 h, 48 h, and 7 d of salt stress. GDC – Glycine Dehydrogenase Complex. Enzymes denoted by the EC numbers: 4.1.1.39 – ribulose-bisphosphate carboxylase, 1.1.1.29 – glycerate dehydrogenase/ hydroxyphenylpyruvate reductase, 1.1.3.15 – (S)-2-hydroxy-acid oxidase GLO1, peroxisomal, 1.11.1.16 – versatile peroxidase, 1.4.4.2 – glycine dehydrogenase mitochondrial, 2.1.2.10 – aminomethyltransferase, mitochondrial, 1.8.1.4 – dihydrolipoyl dehydrogenase mitochondrial

2.3.7 Validation of stress-induced gene expression changes using qRT-PCR

The transcript expression levels determine the variations in protein expression patterns. Hence, the expression patterns of 24 DEPs were compared with their transcript expression patterns using cDNA samples and qRT-PCR technique (**Figure 2.12**). In TS110R, positive correlation was observed between the transcript and protein expression patterns for eight genes - POR2, GDH, GRS, RH37L, GS3, SREM, BADH, and P5L. Similarly, in TSOR, positive correlation was observed for seven genes – COP1, FBA, GS1, GS3, POR2, RCAA1, and TPI. The results thus identified the genes having similar and dissimilar expression patterns in transcriptome and proteome of the plant tissue during the stress conditions.

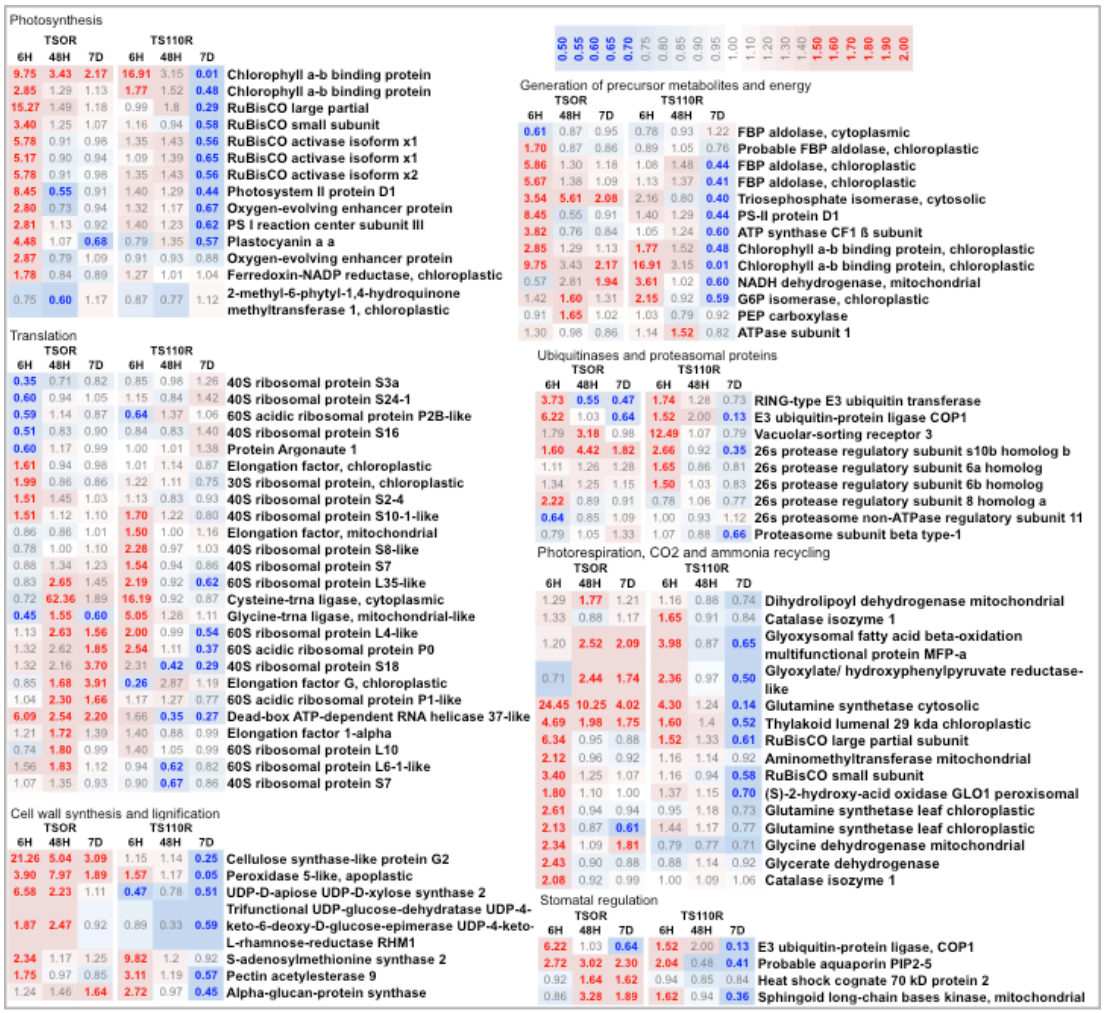


Figure 2.11 Expression patterns of DEPs from various biological processes affected by salt stress in TSOR and TS110R grapevines

The values in bold red and bold blue are the significantly (p-value ≤ 0.05, q-value ≤ 0.05) up and down-regulated fold change values (fold change ≥ 1.5 or ≤ 0.7), respectively

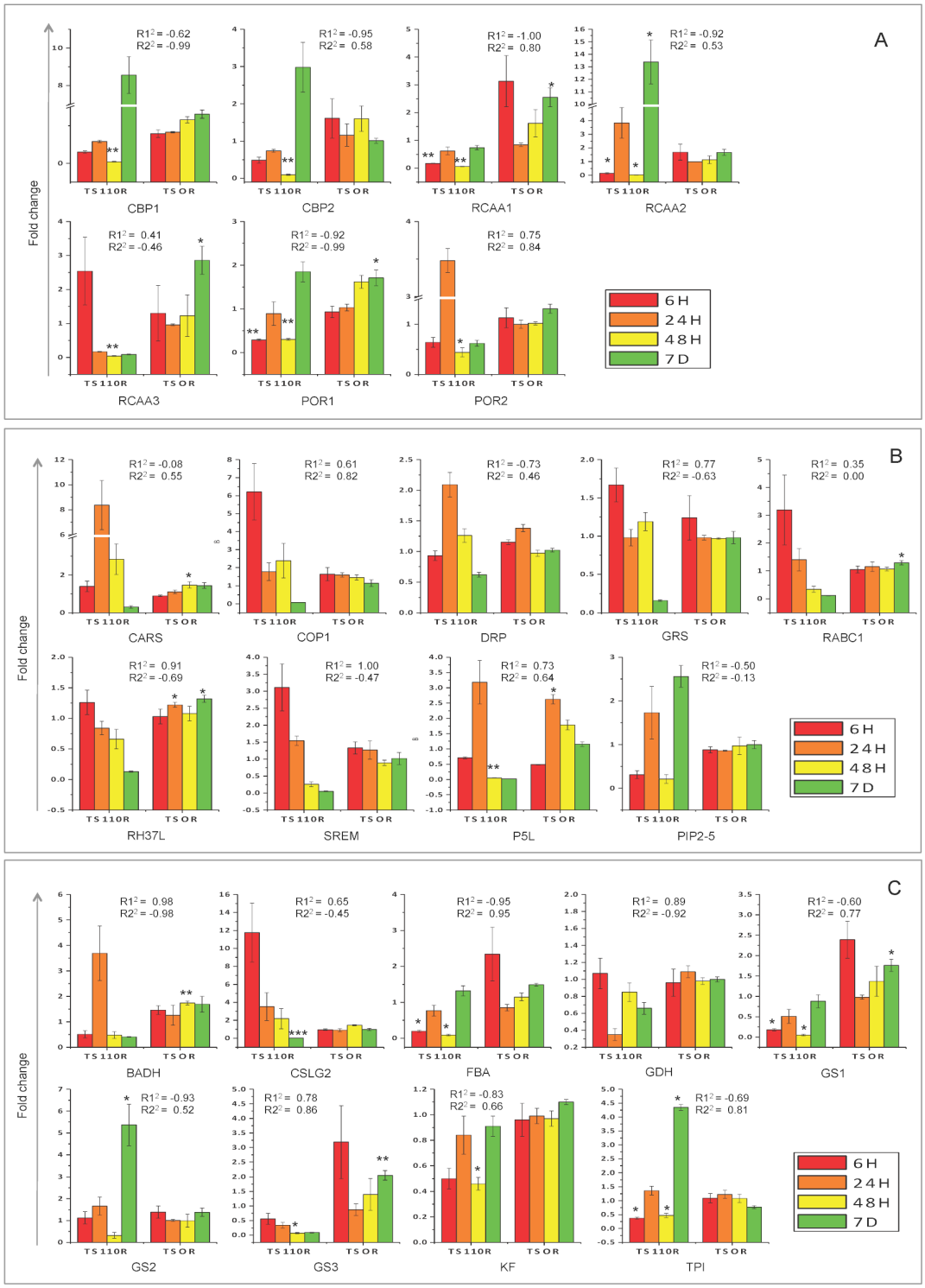


Figure 2.12 Fold change of transcripts obtained from qRT-PCR data at 6 h, 24 h, 48 h and 7 d of salt stress

A – Photosynthesis and carbon assimilation related genes. B – Stress-related genes. C – Amino-acid metabolism-related genes. $R1^2$ – Correlation between protein and transcript expression patterns determined through proteomic and qRT-PCR techniques in TS110R samples. $R2^2$ –

Correlation between protein and transcript expression patterns determined through proteomic and qRT-PCR techniques in TSOR samples. Gene names: BAHD – bifunctional aspartokinase homoserine dehydrogenase chloroplastic-like, CARS – cysteine-tRNA ligase cytoplasmic, CBP1 – chlorophyll a-b binding protein chloroplastic, CBP2 – chlorophyll a-b binding protein chloroplastic, COP1 – Constitutive photomorphogenic 1/ E3 ubiquitin-protein ligase, CSLG2 – cellulose synthase-like protein g2, DRP – desiccation-related protein at2g46140, FBA – fructose-bisphosphate aldolase chloroplastic, GDH – glutamate dehydrogenase 1, GRS – glycine-tRNA ligase mitochondrial-like, GS1 – glutamine synthetase cytosolic, GS2 – glutamine synthetase leaf chloroplastic, GS3 – glutamine synthetase leaf chloroplastic, KF – kynurenine formamidase, P5L – peroxidase 5-like, apoplastic, PIP2-5 – probable aquaporin pip2-5, POR1 – protochlorophyllide oxidoreductase chloroplastic, POR2 – protochlorophyllide oxidoreductase-like, RABC1 – RAS-related protein RABC1, RCAA1 – RuBisCO activase isoform x1, RCAA2 – RuBisCO activase isoform x1, RCAA3 – RuBisCO activase isoform x2, RH37L – dead-box ATP-dependent RNA helicase 37-like, SREM – serrate RNA effector molecule-like, TPI – triosephosphate isomerase cytosolic

2.4 Discussion

Shotgun proteomics is a well-established high-throughput technology that enables identification of thousands of proteins for the elucidation of qualitative and quantitative changes in the proteome. Large-scale proteomic approaches have been used in grapevines to study various conditions like drought stress (Cramer et al., 2013; Grimplet et al., 2009), apoplastic proteome (Delaunois et al., 2013), heat stress (Liu et al., 2014), downy mildew infection (Palmieri et al., 2012), ABA treatment (Rattanakan et al., 2016), cold stress (Deng et al., 2017), etc. Similarly, there have been several shotgun proteomic studies on salt stress in various plants (Vincent et al. 2007; Jiang et al. 2007; Cui et al. 2015; Vítámvás et al. 2015; Fercha et al. 2016). The present study provides comparative insight into the proteomic responses to salt stress in own-rooted and 110R-grafted grapevines.

In salt-stressed grafted grapevines, a complex molecular response is triggered through communication between the rootstock and the scion. The grapevine species such as, *V. champini, V. rupestris* and hybrids of *V. rupestris, V. riparia, V. berlandieri and V. longii,* are salinity tolerant due to the ability of their roots to prevent uptake of salts or root to shoot transport of salts (Henderson et al., 2014; Sauer, 1968). In our

study, TS110R also displayed salt exclusion from the shoot; however, preventing or reducing the uptake of salts did not alleviate the stress response in leaves. Instead, changes in the leaf proteome were observed despite the exclusion of salts from TS110R shoots, suggesting the presence of root to shoot signaling. While in TSOR vines, the immediate rise in transpiration rate along with uptake of salts into the shoot indicated that the proteomic changes were triggered due to salts stress in the shoot. Thus, the differences in protein expression patterns observed in TSOR and TS110R vines were due to the difference in the salt accumulation of the shoots and the difference in signaling from the roots of the two plants.

2.4.1 Protection of photosystem is more crucial than upregulation of photosynthesis

Photosynthesis and cell growth are the primary processes affected by salt stress, as reported in several plant systems (Chaves et al., 2009). However, reports on the immediate effects of salt stress on photosynthesis and its relation to salinity tolerance are scarce (Daldoul et al., 2010). In our study, the early phase of salt stress resulted in significant upregulation of photosynthesis-related proteins in salinity sensitive TSOR vines (**Figure 2.11**), which correlated well with the rise in their CO_2 assimilation rate. The early upregulation of three RuBisCO activase isozymes (VIT_06s0004g05180.t01, VIT_08s0007g00840.t01, VIT_13s0019g02050.t01) in TSOR may be responsible for the increased CO_2 assimilation rate. The main role of this activase is to maintain the catalytic activity of RuBisCO by removal of inhibitory sugars from the active site of uncarbamylated and carbamylated RuBisCO (Portis, 2003). Several, proteomic studies on salt stress in plants have shown upregulation of RuBisCO activase in many *Triticum* and *Brassica juncea* studies (Caruso et al., 2008; Yousuf et al., 2016).

The increase in photosynthetic rate is sometimes associated with the salinity tolerance of plants for providing the energy necessary for tolerating the stress (Kawasaki et al., 2001; Ma et al., 2012). However, the instant decrease in shoot growth observed in TSOR vines indicated that the increase in photosynthesis alone does not assert salinity tolerance in perennial plants. The prolonged salt stress led to the closure of stomata and results in decreased photosynthesis and increased photorespiration in grapevines (Downton 1977; Vincent et al. 2007). The impaired repair and replenishment of damaged PSII under salt stress is responsible for the decline in

photosynthesis (Takahashi and Murata, 2008). The upregulation of PSII in TSOR indicated the preparation for the replenishment of the damaged protein. Whereas, TS110R vines did not alter the PSII expression levels; instead, they displayed upregulation of chlorophyll a-b binding proteins with a relatively greater fold change compared to the TSOR vines. These proteins are associated with chlorophyll and xanthophyll in the light-harvesting complex of PSII and serve as antenna proteins to trap and transfer the light energy to PSII core complexes for initiating the photosynthetic electron transport (Jansson, 1999, 1994). This clearly indicated that in TS110R, the PSII was protected from oxidative damage due to increased abundance of chlorophyll a-b binding proteins.

Furthermore, the early upregulation of chlorophyll biosynthesis proteins like protochlorophyllide reductase (**Figure 2.13**) provided additional protection from oxidative stress in chloroplasts. The increased levels of the long-wavelength form of protochlorophyllide contribute to protective mechanisms against salt stress (Abdelkader et al., 2010). Additionally, certain enzymes that were upregulated at late stages of stress in TSOR, such as NADH dehydrogenase and chloroplastic glucose-6-phosphate isomerase (G6P isomerase), were upregulated at an early stage in TS110R. The chloroplastic G6P isomerase is required for the synthesis of starch in leaves (Geigenberger, 2011; Yu et al., 2000) and its upregulation in response to salt stress in *Dunaliella salina* (Cui et al., 2009) signifies its role in salt stress tolerance. The decrease in shoot growth of TSOR vines despite increased CO_2 assimilation rate might be due to the partitioning of photosynthates towards stress tolerance; whereas TS110R vines maintained normal CO_2 assimilation rate similar to that of control vines, while preventing the oxidative damage to photosystem. Hence, the prevention of photo-oxidation of PSII along with efficient and earlier upregulation of starch synthesis might be responsible for salt tolerance in TS110R vines.

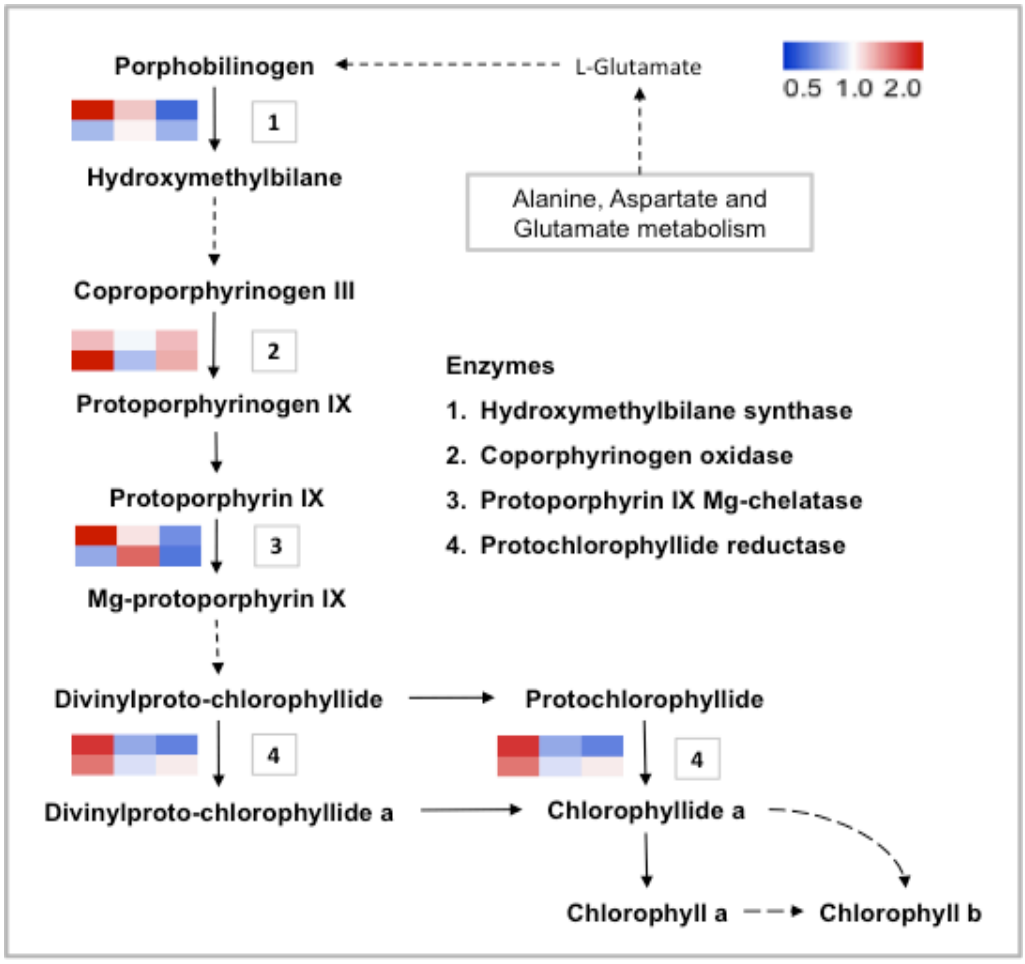


Figure 2.13 DEPs from chlorophyll biosynthesis pathway

The heat maps denote the fold change values for TSOR on the upper row and TS110R on the lower row at three time points – 6 h, 48 h and 7 d of salt stress

2.4.2 Oxidative stress signaling in TSOR vines

In the present study, the proteins involved in the generation of precursor metabolites and energy were significantly upregulated in both the grapevines (**Figure 2.11**). However, their expression patterns were not the same. These proteins were from processes like glycolysis, TCA cycle, photosynthesis, and ATP generation. A glycolysis pathway enzyme - triosephosphate isomerase (TPI) was upregulated through all the three stages of salt stress in TSOR vines (**Figure 2.9**). It catalyzes reversible isomerization of triose phosphates, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate, and is essential for efficient energy production. The concentration of methylglyoxal, a transition-state intermediate of the two triose-phosphates, increases under salinity stress conditions (Yadav et al., 2005). A study on the external application

of methylglyoxal shows upregulation of TPI with increasing concentration of methylglyoxal (Sharma et al., 2012).

Additionally, several proteomic studies have shown upregulation of TPI in response to salt stress conditions (Caruso et al., 2008; Gao et al., 2011). This suggests that positive regulation of TPI in our experiments could be due to increased methylglyoxal levels under salt stress conditions. Furthermore, four fructose-bisphosphate aldolase (FBP aldolase) isozymes were affected by salt stress in TSOR wherein the cytoplasmic isozyme was downregulated while three plastidial isozymes were upregulated. The cytoplasmic FBP aldolases are involved in glycolysis and gluconeogenesis, while the plastidial isozymes are involved in the TCA cycle (Lu et al., 2012). These two enzymes, TPI and plastidial FBP aldolase, have been identified as the major targets of S-glutathionylation in *Arabidopsis* (Ito et al., 2003).

Protein glutathionylation protects proteins from irreversible oxidation and acts as a redox signaling mechanism in plants (Dalle-Donne et al., 2009; Ito et al., 2003). The exogenous application of glutathione decreases the methylglyoxal levels in the plant (Yadav et al., 2005), wherein glyoxylase I plays an important role in detoxification of methylglyoxal with the help of reduced glutathione (Ghosh et al., 2016). However, in the present study, the glyoxalase I family protein lactoylglutathione lyase was downregulated in TSOR, which indicated downregulation of methylglyoxal detoxification to maintain free glutathione levels for glutathionylation of proteins. The upregulation of glutathione synthesis is known to be necessary for protecting plants under oxidative stress (Cheng et al., 2015). Hence, these energy-generating proteins TPI, plastidial FBP aldolase, and lactoylglutathione lyase appear to be involved in signaling of redox stress through methylglyoxal and glutathione levels in TSOR vines. Whereas, in TS110R vines, these enzymes were unaffected and downregulated at a late stage, suggesting the absence of redox stress.

Furthermore, among all the enzymes from amino acid biosynthetic pathways, kynurenine formamidase was upregulated throughout the salt stress in TSOR vines only (**Supplementary Table 3**). It synthesizes L-Kynurenine through tryptophan metabolic pathway. The synthesis of L-Kynurenine and early upregulation of tryptophan synthase suggested an essential role of L-Kynurenine in salt stress. The kynurenine pathway is necessary for the synthesis of NAD, which is present in monocots but absent in dicots

(Katoh, 2006). However, the gene kynurenine formamidase is present in many dicots (Katoh, 2006), indicating its alternate role in them. A chemical screening study has identified kynurenine as an inhibitor of ethylene responses in *Arabidopsis* (He et al., 2011). While another proteomic study showed drought-induced upregulation of this protein in grapevine leaves (Król and Weidner, 2017). Hence, the upregulation of kynurenine synthesis in salt-stressed TSOR vines could be associated with suppression of ethylene-induced leaf senescence and abscission. However, the expression of this protein remained unaltered throughout the stress in TS110R vines suggesting the absence of stress-induced ethylene signaling.

2.4.3 Differences in the regulation of translation in TSOR and TS110R vines

Translation of mRNAs into polypeptides is an energy-dependent biosynthetic process, which is severely affected by salt stress. The abundance of several ribosomal proteins was affected due to salinity stress (**Figure 2.11**). The upregulation of aminoacyl tRNA ligases along with enzymes associated with amino acid biosynthesis indicated the upregulation of translation process in both the vines. Several individual ribosomal proteins are associated with abiotic stress tolerance such as salt tolerance in yeast and plants by RPS3aE (Liang et al., 2015) and water use efficiency and drought tolerance by RPL23A (Moin et al., 2017). Hence, differential regulation of individual ribosomal subunit proteins may be involved in salt tolerance through unknown mechanisms. Other translation-related upregulated proteins included elongation factors, tRNA ligases, and DEAD-box helicase. DEAD-box helicases catalyze the ATP-dependent unwinding of RNA duplexes (Andreou and Klostermeier, 2013). Therefore, the constitutive upregulation of a DEAD-box helicase 37 like protein in TSOR may be responsible for the initiation of translation. However, significant upregulation of translation was delayed in TSOR (**Figure 2.13**); whereas, significant early upregulation of translation in TS110R followed by downregulation at later stages might have resulted in timely and increased expression of the salt tolerance genes in TS110R, which could have resulted in adaptation to stress at the early phase.

2.4.4 Proteolytic and non-proteolytic role of ubiquitination and proteasomal subunits

Ubiquitination plays a critical role in regulating plant responses to abiotic stresses. Plant growth and development is primarily influenced by ubiquitin-mediated regulation of protein stability. E.g., degradation of the microtubule-stabilizing protein SPIRAL1 by the 26S proteasome is vital for salt stress tolerance (Wang et al., 2011). Most stress-related E3 ubiquitin ligases identified to date facilitate responses to environmental stimuli by modulating the abundance of crucial downstream stress-responsive transcription factors (Lyzenga and Stone, 2011). Upregulation of ubiquitin transferase and ubiquitin ligase at 6 h indicated the onset of proteasomal degradation of specific proteins in the salt-stressed vines (**Figure 2.11**). However, the proteolytic subunit of the proteasome was not upregulated. Instead, the 19S regulatory subunit proteins were upregulated (**Figure 2.11**). In eukaryotes, 26S proteasomal complexes contain a 20S proteolytic core covered by two 19S regulatory complexes (Voges et al., 1999).

Apart from proteasomal regulation, the 19S regulatory subunits play a crucial role in dissociating stalled RNA polymerase II (RNAPII) from the DNA strands to release the DNA for binding of new RNAPII proteins (Ferdous et al., 2002; Gillette et al., 2004; Somesh et al., 2005). Some of these 19S regulatory subunits like S10b and S6 play a regulatory role in the transcription of mammalian genes (Truax et al., 2010). Thus, in the present study, the upregulation of 19S subunits -S10b, S6a, S6b, and S8 followed by downregulation of a proteolytic subunit indicated the possibility of the non-proteolytic role of these proteins. The upregulation of regulatory subunit proteins in TS110R was observed only at an early phase. Ubiquitination is also required for subcellular transport of proteins. For instance, vacuolar sorting receptors (VSRs) are a family of proteins involved in ubiquitination mediated sorting and packaging of vacuolar proteins into transport vesicles to vacuoles (Lee et al., 2013). For instance, VSR1, VSR3, and VSR4 are involved explicitly in the sorting of lytic proteins and vacuolar storage proteins to vacuoles in vegetative cells (Lee et al., 2013). Therefore, the parallel upregulation of VSR3 and ubiquitinating enzymes may be carrying out trafficking of proteins to vacuoles in stressed vines.

2.4.5 Photorespiration and recycling of ammonia

The CO_2 limiting conditions created due to the closure of stomata during osmotic stress enhances the oxygenase activity of RuBisCO, leading to increased photorespiration in stressed leaves. Expression of several enzymes from this pathway is upregulated by salt stress (Abogadallah, 2011; Hoshida et al., 2000; Srivastava et al., 2008). Photorespiration is an energy-consuming protective process for replenishing of carbon lost during the oxygenase activity of RuBisCO. It includes several metabolic intermediates from glycolate to glycerate-3-phosphate passing through various conversions through chloroplast, mitochondria, and peroxisome (**Figure 2.10**) (Noctor et al., 2002; Peterhansel et al., 2010). The upregulation of photorespiration related proteins, specifically in TSOR vines, indicated the occurrence of severe oxidative stress in them. During photorespiration, a large amount of ammonia generated by oxidative stress is recycled into amino acids such as, serine and glycine. Serine and glycine produced during photorespiration play an important role in protecting photosynthesis from photoinhibition. Whereas, in TS110R, these proteins went on decreasing with increasing stress and were mostly downregulated at late stage of stress implying probable amelioration of oxidative stress-induced during early stages of salinity treatment.

The glutamate dehydrogenase (GDH) expression is associated with salt stress tolerance through the production of glutamate and proline from the high concentrations of intracellular ammonia generated by the oxidative stress (Skopelitis et al., 2006). Arginine is another critical amino acid involved in nitrogen assimilation; it has the highest nitrogen to carbon ratio. Photosynthetic organisms store nitrogen as arginine by controlling the feedback inhibition of N-acetyl glutamate kinase (NAGK) (Llácer et al., 2008). Interestingly, the NAGK and GDH were upregulated only in TS110R vines (**Supplementary Table 3**) suggesting their role in efficient nitrogen assimilation. Salinity induced upregulation of GDH is also reported in salt-tolerant grapevine cultivar, Razegui (Daldoul et al., 2010). Glutamine synthetase (GS) is another enzyme crucial for the remobilization of protein derived nitrogen (Bernard and Habash, 2009).

Glutamine is the primary amino acid in the xylem sap, which decreases under salt stress conditions in *Brassica oleracea* (Fernandez-Garcia et al., 2008). Several studies have reported tissue-specific altered activity or expression of GS in response to

salinity stress (Rana et al., 2008; Silveira et al., 2003). Transgenic rice plants overexpressing glutamine synthetase had increased photorespiratory activity leading to protection of photosystem and thus imparting tolerance to salt stress (Hoshida et al., 2000). In our study, three isozymes of GS were upregulated in TSOR vines (**Figure 2.11**). The cytosolic GS1 was highly upregulated in TSOR throughout the stress while in TS110R; it was increased only at early stage. This indicated higher degree of assimilation of cytosolic nitrogen into glutamine in TSOR vines. The upregulation of two chloroplastic GS at early stage suggested the presence of severe photooxidative stress in TSOR, which was not seen in TS110R.

2.4.6 Anisohydric behavior of TSOR vines not seen in TS110R vines

Photosynthesis and transpiration are regulated by the stomatal opening in plants, which affects the plant growth under salt stress conditions. Stomatal closure in response to osmotic stress leads to a decrease in transpiration rate and photosynthesis. The rise in transpiration rate of salt-stressed TSOR vines correlates well with its anisohydric phenotype (Lovisolo et al., 2010). However, salinity induced decrease in transpiration rate and water conductance in TS110R vines, which is a characteristic of isohydric behavior, suggested that grafting on 110R rootstock had transformed the stomatal behavior of Thompson Seedless vines. The drop in transpiration rate in salt-stressed TS110R vines indicated a complex regulation by several proteins such as, Constitutive photomorphogenic 1 (COP1), PIP2 aquaporin, Heat shock cognate 70 kD protein (HSP70) and Sphingoid long-chain base kinase (LCBK) (**Figure 2.11**), which are reported to regulate stomatal opening in plants (Chaumont and Tyerman, 2014; Doubnerová and Ryslava, 2013; Mao et al., 2005; Ng et al., 2001).

The LCBKs are involved in the production of bioactive metabolites like sphingosine 1-phosphate (S1P) and phyto-S1P (Guo and Wang, 2012; Imai and Nishiura, 2005), which are involved in various signaling pathways in plants (Pata et al., 2010) such as, drought-induced rise in S1P for regulation of stomatal opening by modulating guard cell turgor (Ng et al., 2001). The COP1 is a E3-ubiquitin-protein ligase that suppresses photomorphogenesis and stomatal opening (Jang, 2005; Mao et al., 2005). The upregulation of COP1 with a higher fold change in TSOR than that in TS110R may be responsible for the early inhibition of shoot elongation in TSOR through downstream suppression of photomorphogenesis related transcription factors;

however, its upregulation did not suppress transpiration rate. Aquaporins modify the water conductance of the whole plant through key "gatekeeper" cell layers in different organs, which, together with stomatal regulation determines the degree of isohydry/anisohydry (Chaumont and Tyerman, 2014). Therefore, the increased abundance of PIP2 aquaporin throughout the seven days of stress in TSOR could be responsible for its increased transpiration rate. Whereas, its decreasing expression with increasing stress in TS110R may be causing the decrease in transpiration rate leading to less uptake of saline water.

2.4.7 Cell wall synthesis and lignification

Lignification is a part of the normal cell differentiation process, but it can also be triggered by several biotic and abiotic stresses (Barros et al., 2015). Increased activity of extracellular peroxidases in response to salt stress is reported in many plants (Choi and Hwang, 2011; Meloni et al., 2003; Mutlu et al., 2009). The mechanism of salt tolerance imparted by peroxidases involves lignification of xylem vessels (Fernandez-Garcia et al., 2008). Several studies have shown that cell wall peroxidases are involved in lignin biosynthesis (Blee et al., 2003; Fagerstedt et al., 2010; Fernández-Pérez et al., 2015; Herrero et al., 2013). In grapevines, extracellular peroxidase activity is also associated with cell wall lignification (Barceló et al., 2003). Xylem lignification has been observed in several species under salt stress wherein the phi cell thickening controls the movement of ions from cortex to xylem and lignification of xylem vessels increases their capillarity in roots (Cachorro et al. 1993; Jbir et al. 2001; Sánchez-Aguayo et al. 2004; Fernandez-Garcia et al. 2008; Neves et al. 2010).

Apart from lignification, salt stress also affects synthesis of cell wall. Several cell wall synthesis related proteins were affected by salt stress in both the vines (**Figure 2.11**). According to a study, a mutation in cellulose synthase-like D5 (CSLD5) made plants hypersensitive to salt stress and osmotic stress (Zhu et al., 2010). Thus, increased abundance of cellulose synthase-like protein G2 (CSLG2) throughout the stress in TSOR vines implies its role in tolerating the stress in these vines. The increased abundance of extracellular peroxidases along with cell wall synthesis proteins indicated the upregulation of cell wall synthesis along with cell wall lignification process.

2.5 Conclusions

The effect of salt stress on grapevines was studied across three phases of stress: early, mid, and late. The early phase of salt stress displayed more differential protein expression in both the grapevines, wherein photosynthesis was highly upregulated along with photorespiration in TSOR vines; while translation and amino acid metabolism were upregulated in TS110R vines. The upregulation of chlorophyll biosynthesis along with the higher expression of chlorophyll a-b binding proteins in TS110R suggested the protection of PSII from photo-oxidative damage with more efficient trapping of light energy under stress conditions. This has been supported by the suppression of ROS species in TS110R in the present study.

The rise in methylglyoxal and glutathione levels predicted from upregulation of TPI and downregulation of lactoylglutathione lyase respectively, revealed their role in the signaling of redox stress in TSOR while their normal expression in TS110R suggested the absence of redox stress. Furthermore, the increased abundance of oxidation-reduction proteins observed in unstressed TS110R vines indicated their preconditioning to grafting induced redox stress, which must have helped the salt-stressed vines in tolerating the stress. Overall, the decrease in shoot growth of TSOR vines despite increased CO_2 assimilation rate and photosynthesis was possibly due to the partitioning of generated energy towards redox stress tolerance. Whereas, the TS110R grapevines displayed early adaptation to oxidative stress with efficient utilization of energy and hence prevented the immediate inhibition of shoot growth under salt stress conditions.

Chapter 3

Transcriptomic regulation of salt stress response in 110R grafted Thompson Seedless grapevines

Chapter 3 Transcriptomic regulation of salt stress response in 110R grafted Thompson Seedless grapevines

3.1 Introduction

The grapevine cultivation is severely hampered by environmental stresses among which drought and salinity are the major problems in arid and semi-arid regions with scanty or no rainfall (Stevens and Walker, 2002). In India, a majority of the grape production comes from tropical regions, around 80% of which comes from Maharashtra (Shikhamany, 2001). However, these states are showing a rise in soil salinity due to irrigation with salty groundwater (Shikhamany, 2001). The increase in soil salinity has an adverse effect on grape cultivation (Keller, 2010). The salt-stressed plants display changes in stomatal conductance, photosynthesis, leaf turgor, chlorophyll content, and leaf ion content. Apart from these physiological problems, salt stress also causes several molecular changes such as the formation of reactive oxygen species (ROS), disorganization of cellular membranes, increase in ionic toxicity and induction of stress-specific plant hormones, particularly ABA (Cramer et al., 2006). These physiological and molecular changes together affect the regulation of gene expression patterns in stressed plants. Therefore, understanding of these gene expression changes in the transcriptome will enable the recognition of biological processes associated with the stress response.

Transcriptomics is a technique used for the understanding of plant molecular responses under different developmental and stress conditions. The transcriptome of a cell is defined as the total pool of transcribed genes present in a cell at a given time-point and a particular condition. The same applies to the transcriptome of a tissue. The main objective of transcriptomics is to generate qualitative and quantitative information about the entire pool of messenger RNAs (mRNAs) present in the tissue. Transcriptome consists of different RNA species like mRNAs, ribosomal RNAs, microRNAs, and transfer RNAs. The mRNAs are the direct intermediates of protein-coding genes, and hence transcriptomic studies focus mostly on the dynamics of mRNAs. Earlier, the techniques like northern blotting were used for RNA quantification, but they were highly prone to RNA degradation and were not suitable for high-throughput analysis of a pool of RNA molecules. Therefore, the synthesis of complementary DNA strands

(cDNAs) of mRNAs was introduced to increase the stability of samples for quantitative studies. Diverse techniques were developed based on the analysis of these cDNA libraries of which expressed sequence tag (EST) analysis, serial analysis of gene expression (SAGE), RNA microarrays and RNA sequencing were the most popular ones (Lowe et al., 2017). The EST sequencing approach uses fragments of mRNA sequences derived through single sequencing reactions performed on randomly selected clones from cDNA libraries (Parkinson and Blaxter, 2009).

Suppression subtractive hybridization (SSH) is another approach that allows the PCR-based amplification of only those cDNA fragments that differ between control and experimental transcriptomes. Whereas, the microarray approach is based on the hybridization of reverse-transcribed mRNAs (cDNAs) to gene-specific DNA probes that are impregnated on a solid support. Until 2012, the microarray technique was highly popular, owing to its high throughput capacity at a cheaper cost. However, it had limitations such as cross-hybridization of related sequences, signal saturation, and insensitivity because of high background. Moreover, it was applicable only for organisms with sequenced genomes (Okoniewski and Miller, 2006; Royce et al., 2007). Later, the advancement in sequencing technologies tremendously lowered the sequencing costs, making RNA sequencing the cheapest and widely used approach (Lowe et al., 2017). Additionally, its high sensitivity allows the detection of single nucleotide changes even in high-throughput analyses (Wang et al., 2009).

The previous transcriptome studies on salinity stress in grapevines have identified several genes involved in salt stress response in different cultivars of grapevines (Cramer et al., 2006; Daldoul et al., 2010). A microarray-based transcriptomic study of cv. Cabernet Sauvignon identified the genes and the biological processes that were specifically altered by salt stress when compared with similar water stress condition for 16 d (Cramer et al., 2006). Another comparative study based on SSH and microarray approach identified the genes responsible for salt tolerance in Razegui grapevines when compared with a salt-sensitive variety, Syrah (Daldoul et al., 2010). Similarly, large-scale expression analyses of drought (Savoi et al., 2016), heat (Rienth et al., 2016, 2014) and UV radiation (Xi et al., 2014) stress using microarray and RNA sequence in wine grapes have provided a better understanding of the stress response in grape. The high throughput nature of these studies identifies a large number

of differentially expressed genes and hence recognizes the biological processes in which they are involved. A recent study on cv. Thompson Seedless reveals a large number of metabolic pathways affected by salt stress (Das and Majumder, 2018). The present study provides further insights into the salt stress response of Thomson Seedless grapevines when grafted onto a salinity tolerant 110R rootstock as the proteomics analysis of 110R grafted, and own-rooted Thompson Seedless grapevine, detailed in the previous chapter, supported better salt tolerance in 110R grafted Thompson Seedless vines. The next generation sequencing (NGS) approach was used for studying the transcriptomes of salt-stressed and control grapevines at early (6 h), mid (24 h) and late (7 d) stages of stress.

3.2 Materials and methods

3.2.1 Plant material and salinity stress experiment

The growing and collection of tissue of Thompson Seedless grafted on 110R roots were performed as detailed in Sections 2.2.1 and 2.2.2. The young leaf tissue samples were collected from nine control and nine treated vines each at 6 h, 24 h, 48 h, 7 d, and 15 d of salt treatment. They were snap-frozen in liquid nitrogen and stored at -80°C until further use. The leaves collected from a single vine were considered as one biological replicate.

3.2.2 RNA isolation and sequencing

The young leaf samples of TS110R grapevines from salinity stress experiment collected at 6 h, 24 h, and 7 d were used for the transcriptome analysis. Frozen leaves were ground in liquid nitrogen, and an equal quantity of finely crushed tissue from three biological replicates was pooled for each sample of control and treated vines. Thus, a total of six samples were processed for RNA sequencing on Illumina HiSeq 2000 sequencer (Illumina, USA). The Spectrum™ Plant Total RNA kit (Sigma-Aldrich, MO) was used for the extraction of total RNA following the instructions given in the manufacturer's protocol. The RNA was subjected to on-column DNase treatment before elution from the column. The quality of RNA samples was assessed using an RNA 6000 Nano kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The high-quality RNA samples having RIN value > 8.0 were used for RNA sequencing. About 2.5 µg of high-quality total RNA from each sample was used for the isolation of

poly(A) mRNA. The non-directional Illumina RNA-seq library was prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA). The quality of the library was checked with a High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) and was sequenced using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). The paired-end sequences of length 101 base pair were generated. The isolation of RNA and sequencing were performed in collaboration with the National Research Centre for Grapes (NRCG), Pune, Maharashtra, India.

3.2.3 Trimming and mapping of reads

The quality of fastq files containing raw Illumina reads was assessed using FastQC (v. 0.11.3) computational tool. These reads were trimmed to remove poor-quality bases generated by Illumina sequencing. The low-quality bases with Phred score < 30 were trimmed from the ends of Illumina reads using a Perl script to obtain good quality sequences of an average length of 87 bases. The trimmed sequences were used for genome-guided assembly, wherein the reads were first mapped onto the *Vitis vinifera* 12X genome (ftp://ftp.ensemblgenomes.org/pub/plants/release-19/fasta/vitis_vinifera/) using TopHat2 (v. 2.1.1; Kim et al., 2013) followed by the genome-guided assembly on Trinity (v. 2.8.5; Grabherr et al., 2011). The gene annotation files were downloaded from http://plants.ensembl.org/Vitis_vinifera/Info/Index. Quality of assembled Trinity transcripts was evaluated using a Perl script in Trinity tools.

3.2.4 Quantification of transcripts

The expression of transcripts was estimated using two different programs. Firstly, the transcripts were quantified as fragments per kilobase per million mapped reads (FPKM) using Cufflinks (v. 2.2.1; http://cole-trapnell-lab.github.io/cufflinks/) by mapping the reads to the gene models provided by the genome annotation. The differential expression analysis was performed using the Cuffdiff program (v. 2.2.1; http://cole-trapnell-lab.github.io/cufflinks/) with blind dispersion method, which considers all samples as replicates of a single global condition. Since the RNA from three biological replicates was pooled for sequencing, the statistical analysis was not possible. Therefore, to increase the confidence of differential expression analysis, secondary quantification of transcripts was performed using Genome Guided Trinity (GTT) method wherein the transcripts were assembled into Trinity transcripts and then quantified by mapping RNAseq reads onto them to get their FPKM values. A

normalized matrix of FPKM values of assembled transcripts of all six samples was generated by RSEM method (RNA-Seq by Expectation-Maximization) (Li and Dewey, 2011), and the differential expression analysis was performed using the edgeR package from Bioconductor (Robinson et al., 2010). The genes having log2 fold change ≥ 1.3 or ≤ -1.3 in both Cuffdiff and GTT analyses were considered as differentially expressed genes.

3.2.5 Bioinformatics analysis

The expression pattern of differentially expressed genes (DEGs) across three stages of salt stress was evaluated by clustering in ClustVis (https://biit.cs.ut.ee/clustvis) using correlation distance and Ward linkage. The Blast2GO (v. 3.1.3) (Conesa et al., 2005) was used for functional annotation of the expressed transcripts. The resulting annotation file was used as a reference for Gene Ontology (GO) enrichment analysis using BinGO (v. 3.0.3) (Maere et al., 2005). The KEGG pathway enrichment was performed using ClueGO v. 2.1.7 (Bindea et al., 2009) plugin in Cytoscape v. 3.1.1 (Shannon, 2003), by implementing Right-sided hypergeometric statistical test and Bonferroni step down correction method.

3.2.6 Quantitative reverse-transcriptase PCR analysis

These experiments were performed as detailed in Section 2.2.9.

3.3 Results

3.3.1 Transcriptome analysis

The RNA samples with RIN value ≥ 8 were sequenced for transcriptome analysis using Illumina HiSeq 2000 at SciGenome, Kerala, India. A total of six sequencing libraries were prepared from three control samples (Control 6 h, Control 24 h, and Control 7 d) and three NaCl-treated samples (Salt-stressed 6 h, Salt-stressed 24 h and Salt-stressed 7 d) to study the response of grape variety Thompson Seedless grafted on 110R roots to salt-stress at transcriptome level. The number of raw reads per sample ranged from 74.81 to 82.97 million. The average length of these raw reads was 101 bp. Among all the raw reads, more than 91% had a Phred score of >=30. The reads passing the quality filtering were used for reference-based alignment using TopHat2 (v. 2.1.1; Kim et al., 2013). More than 89% (**Table 3.1**) of the reads aligned to the reference genome.

Table 3.1 Read mapping rate

	Read mapping rate			Concordant pair alignment rate
	Left reads	Right reads	Overall	
Control 6 h	91.0%	88.5%	89.7%	84.1%
Control 24 h	90.8%	88.5%	89.7%	84.3%
Control 7 d	90.9%	88.4%	89.6%	84.1%
Salt-stressed 6 h	90.6%	88.3%	89.4%	83.9%
Salt-stressed 24 h	90.3%	88%	89.1%	83.6%
Salt-stressed 7 d	91.1%	88.5%	89.8%	84.2%

3.3.2 Transcript quantification

The gene expression analysis was performed using Cufflinks and genome guided Trinity tools. In the cufflinks pipeline, transcripts were quantified by mapping the reads to the genes, while the GTT pipeline first assembled the reads into Trinity transcripts followed by merging and annotation into genes. The cufflinks pipeline identified 29,288 gene transcripts, while GTT identified 16,400 genes with 16,160 genes common in both the approaches (**Figure 3.1**). On average, about 18,491 and 16,388 transcripts from cufflinks and GTT, respectively had FPKM value ≥ 1 (**Table 3.2**). The use of more stringent parameters for trimming of transcripts resulted in 2% lesser transcripts being identified than those identified in our previous publication (Upadhyay et al., 2018).

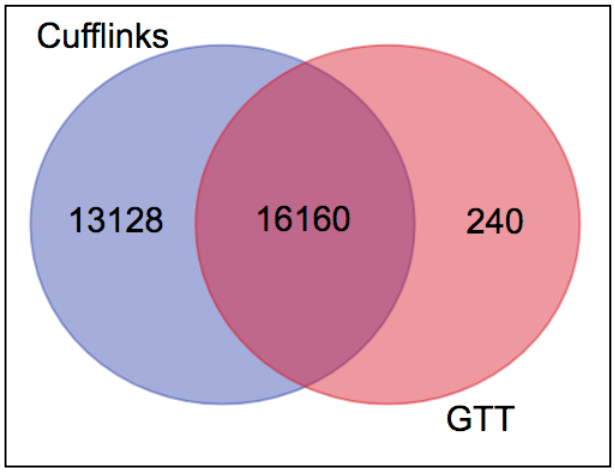


Figure 3.1 Total genes identified by Cufflinks and GTT pipelines

Table 3.2 Number of transcripts identified by Cufflinks and GTT pipelines

	Total transcripts	Transcripts with FPKM ≥ 1					
		Control 6H	Control 24H	Control 7D	Stressed 6H	Stressed 24H	Stressed 7D
Cufflinks	29288	18472	18398	18388	18696	18432	18566
GTT	16400	16385	16392	16377	16393	16389	16394

3.3.3 Differential expression of genes in response to salt stress

The gene expression profiles of the control and salt-stressed samples were compared and analyzed to identify the stress-responsive genes. Stringent values of log2 fold change ≥ 1.3 or ≤ -1.3 and FPKM value ≥ 2.5 in at least one of the samples were used as thresholds to enlist the differentially expressed genes at each time point. The differential expression of genes was studied using two tools – Cufflinks and GTT, which use different algorithms for quantification of genes. Therefore, only the genes with the same expression patterns from both the tools were considered as significantly differentially expressed genes (DEGs). These genes displayed positive correlations of ≥ 96% between the fold change values derived from the two protocols (**Figure 3.2A, B, C**).

At the early stage of 6 h of salt stress, 196 DEGs were upregulated, and 55 were downregulated. A total of 509 genes were significantly altered in response to salt stress

(**Supplementary Table 4**). The number of DEGs dropped down to 34 upregulated and 55 downregulated genes at 24 h of stress. The late stage of stress displayed abundant upregulation of 262 DEGs and downregulation of 20 DEGs after 7 days (7 d) of salt stress (**Figure 3.3A**). The Venn diagram (**Figure 3.3B**) indicated that only 11 genes were shared between 6 h, 24 h, and 7 d. The number of common DEGs was more between early and late stress stages than those with mid-stage. The heat map showed that among the 11 common DEGs, four were upregulated throughout the stress while five were downregulated only at 24 h (**Figure 3.3C**). The class IV chitinase was initially downregulated at 6 h and subsequently upregulated at 24 h and 7 d. An ethylene-responsive transcription factor and a probable WRKY 40 were upregulated throughout the stress, while NAC transcription factor 29 and Dehydrin 1 were upregulated at early and late stages with intermediate downregulation at 24 h.

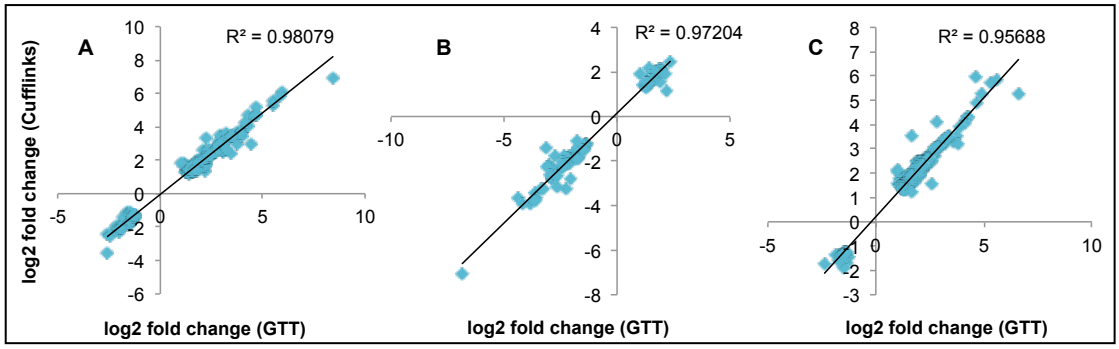


Figure 3.2 Correlation of gene expression patterns obtained from Cufflinks and Genome Guided Trinity (GTT) pipelines

Correlation between log2 fold change values of common DEGs generated by Cufflinks and GTT at three time-points 6 h (A), 24 h (B) and 7 d (C).

3.3.4 Functional classification of DEGs

The Gene Ontology (GO) based enrichment of 509 DEGs was performed using BiNGO against the background of whole transcriptome annotation containing 22,316 genes. The clustering of GO terms was based on GOSlim_plants reference ontology. The Hypergeometric statistical test was implemented along with Benjamini & Hochberg False Discovery Rate (FDR) correction for determining the significance of GO enrichment. Out of 509 DEGs, 420 were classified into different biological processes (BP), molecular functions (MF), and cellular components (CC) (**Figure 3.4**).

Transcription was the most significantly enriched BP, and transcription factor activity was the most significant MF. The cellular components included the nucleus, extracellular region, and thylakoid. Similarly, the KEGG pathway enrichment of individual sets of up and down-regulated DEGs revealed significant upregulation of plant hormone signal transduction and diterpenoid biosynthesis at the early stage of stress while photosynthesis was significantly upregulated at the late stage of stress (**Figure 3.5**).

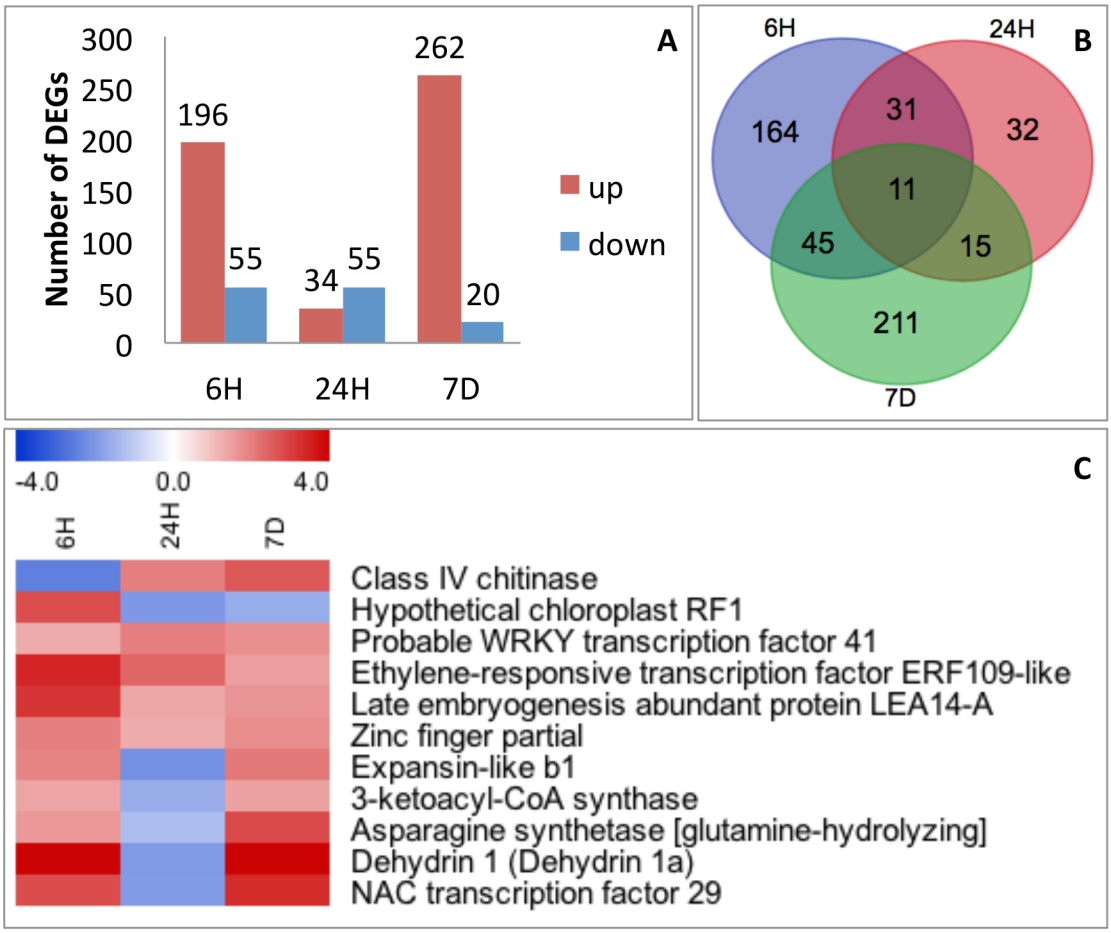


Figure 3.3 Overview of differentially expressed genes across three time-points of salt stress

A – The number of up and down-regulated significantly differentially expressed genes (DEGs) at 6 h (6H), 24 h (24H) and 7 d (7D) of salt stress. B – Venn diagram of total DEGs at three stages of stress. C – The heat map of log_2 fold change values of 11 common DEGs.

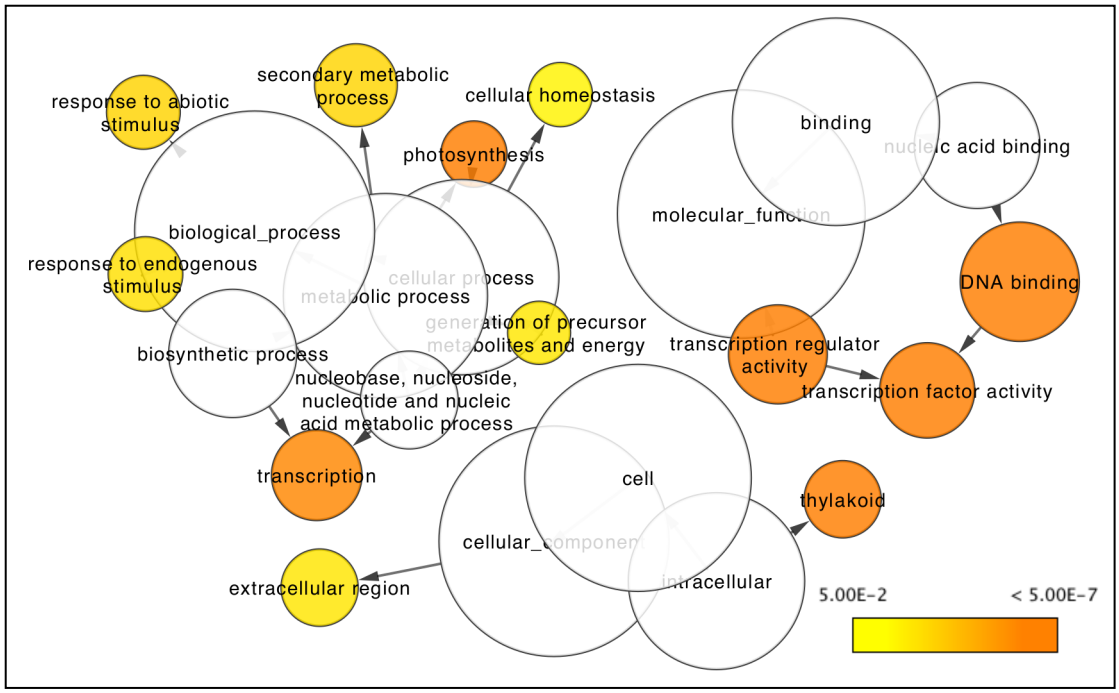


Figure 3.4 Gene ontology enrichment of 509 DEGs

The size and color of nodes in the network plot correspond to the number of enriched genes and the p-value of GO terms, respectively

3.3.5 Stage-specific salt stress response

The statistical analysis of each set of DEGs from **Figure 3.3A** resulted in the enrichment of different GO terms at different stages of stress (**Figure 3.6**). The response to the abiotic stimulus was upregulated at both 6 h and 7 d of stress, while the response to the biotic stimulus was upregulated only at 7 d of stress. A significant upregulation of carbohydrate metabolic process was seen at 24 h of stress with a simultaneous significant downregulation of extracellular region, cell wall, and external encapsulating process, which mainly constituted of peroxidases and xyloglucan endotransglucosylase/hydrolase (**Figure 3.7**). The ripening term was significantly downregulated at 24 h of stress, which constituted of a single NAC transcription factor 29. Photosynthesis was significantly downregulated at an early stage while it was significantly upregulated at the late stage of stress. Interestingly, the transcription factor activity was highly upregulated at all three stages of stress (**Figure 3.6**).

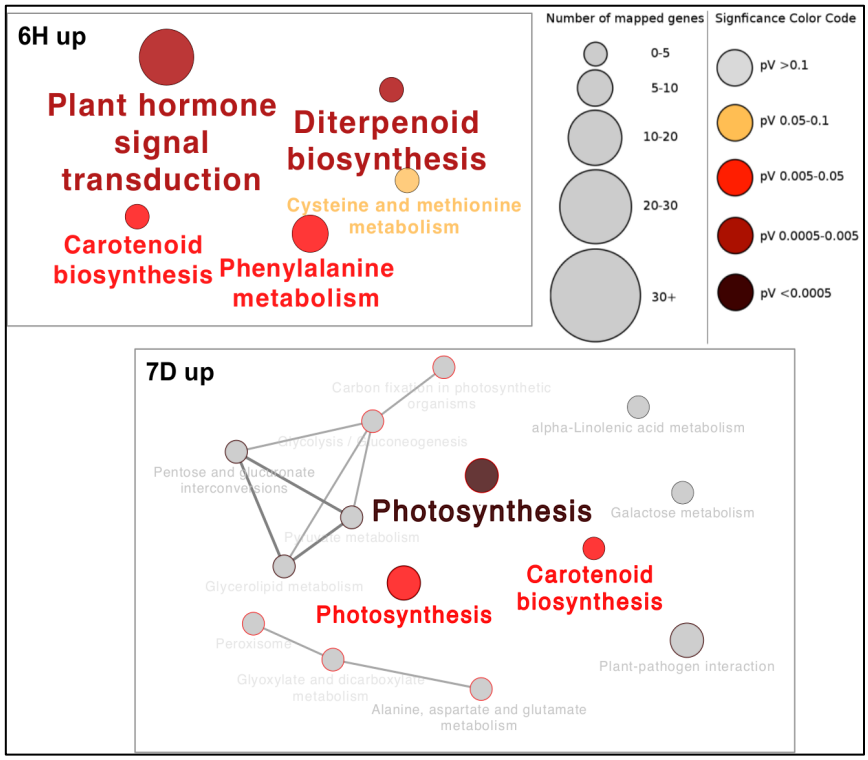


Figure 3.5 KEGG pathway enrichment analysis

The size and color of the enriched nodes correspond to the number of mapped genes and the significance of enrichment, respectively

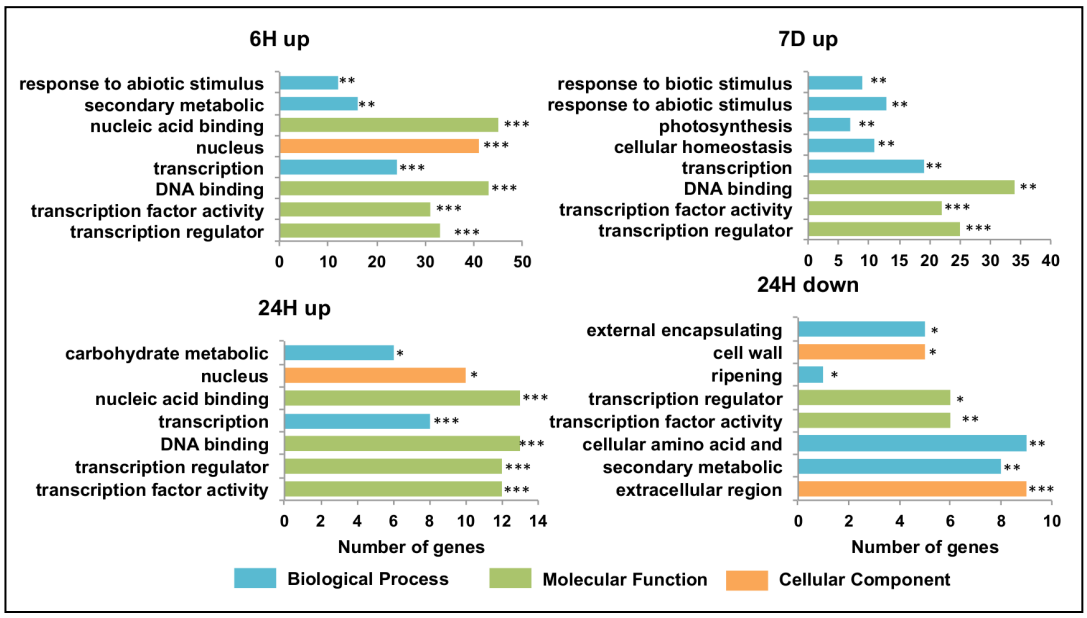
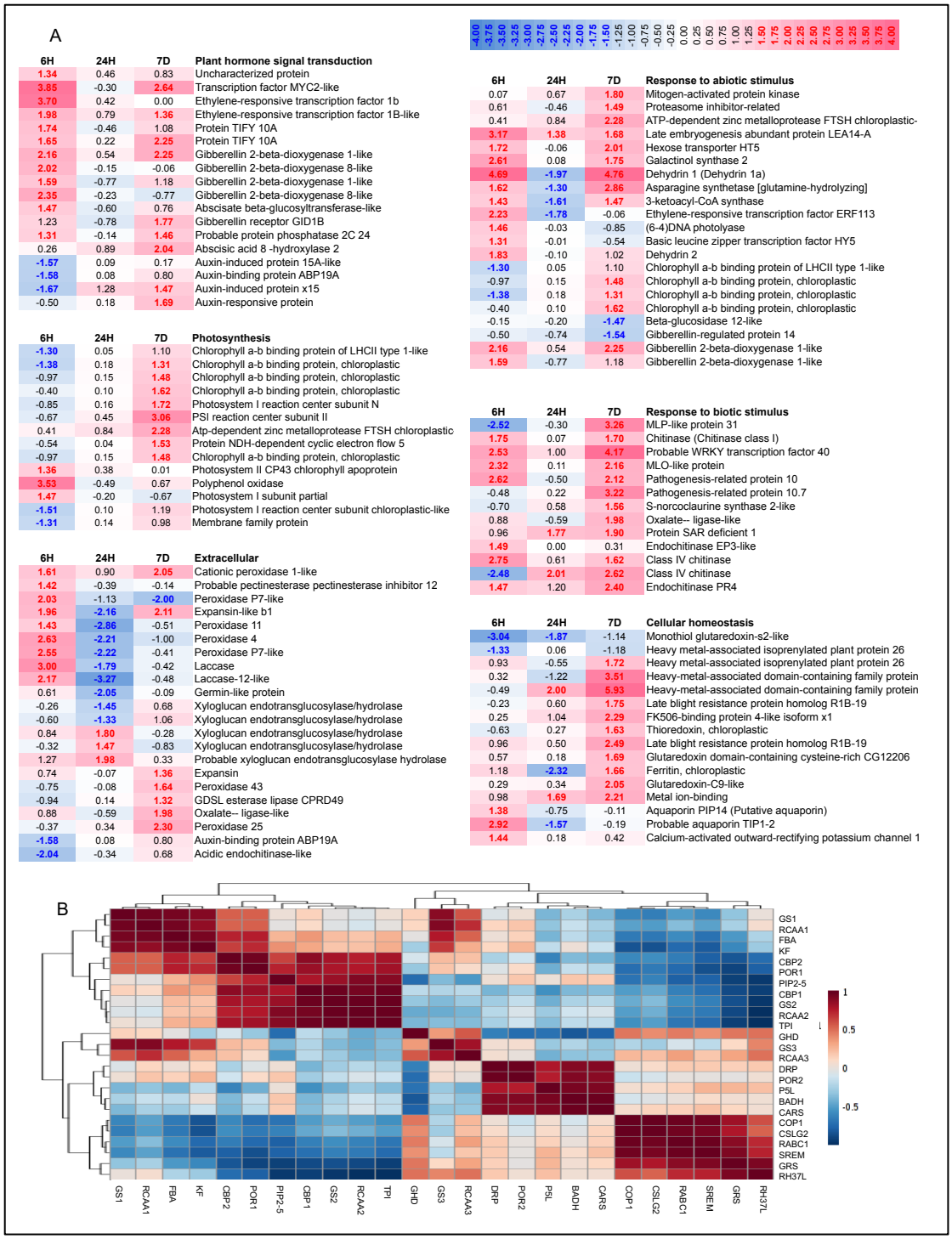


Figure 3.6 Temporal differences in biological processes, molecular functions and cellular components affected by salt stress

The bar plots display the number of genes significantly enriched in each GO category.



6H	24H	7D	Plant hormone signal transduction
1.34	0.46	0.83	Uncharacterized protein
3.85	-0.30	2.64	Transcription factor MYC2-like
3.70	0.42	0.00	Ethylene-responsive transcription factor 1b
1.98	0.79	1.36	Ethylene-responsive transcription factor 1B-like
1.74	-0.46	1.08	Protein TIFY 10A
1.65	0.22	2.25	Protein TIFY 10A
2.16	0.54	2.25	Gibberellin 2-beta-dioxygenase 1-like
2.02	-0.15	-0.06	Gibberellin 2-beta-dioxygenase 8-like
1.59	-0.77	1.18	Gibberellin 2-beta-dioxygenase 1-like
2.35	-0.23	-0.77	Gibberellin 2-beta-dioxygenase 8-like
1.47	-0.60	0.76	Abscisate beta-glucosyltransferase-like
1.23	-0.78	1.77	Gibberellin receptor GID1B
1.31	-0.14	1.46	Probable protein phosphatase 2C 24
0.26	0.89	2.04	Abscisic acid 8 -hydroxylase 2
-1.57	0.09	0.17	Auxin-induced protein 15A-like
-1.58	0.08	0.80	Auxin-binding protein ABP19A
-1.67	1.28	1.47	Auxin-induced protein x15
-0.50	0.18	1.69	Auxin-responsive protein

6H	24H	7D	Photosynthesis
-1.30	0.05	1.10	Chlorophyll a-b binding protein of LHCII type 1-like
-1.38	0.18	1.31	Chlorophyll a-b binding protein, chloroplastic
-0.97	0.15	1.48	Chlorophyll a-b binding protein, chloroplastic
-0.40	0.10	1.62	Chlorophyll a-b binding protein, chloroplastic
-0.85	0.16	1.72	Photosystem I reaction center subunit N
-0.67	0.45	3.06	PSI reaction center subunit II
0.41	0.84	2.28	Atp-dependent zinc metalloprotease FTSH chloroplastic
-0.54	0.04	1.53	Protein NDH-dependent cyclic electron flow 5
-0.97	0.15	1.48	Chlorophyll a-b binding protein, chloroplastic
1.36	0.38	0.01	Photosystem II CP43 chlorophyll apoprotein
3.53	-0.49	0.67	Polyphenol oxidase
1.47	-0.20	-0.67	Photosystem I subunit partial
-1.51	0.10	1.19	Photosystem I reaction center subunit chloroplastic-like
-1.31	0.14	0.98	Membrane family protein

6H	24H	7D	Extracellular
1.61	0.90	2.05	Cationic peroxidase 1-like
1.42	-0.39	-0.14	Probable pectinesterase pectinesterase inhibitor 12
2.03	-1.13	-2.00	Peroxidase P7-like
1.96	-2.16	2.11	Expansin-like b1
1.43	-2.86	-0.51	Peroxidase 11
2.63	-2.21	-1.00	Peroxidase 4
2.55	-2.22	-0.41	Peroxidase P7-like
3.00	-1.79	-0.42	Laccase
2.17	-3.27	-0.48	Laccase-12-like
0.61	-2.05	-0.09	Germin-like protein
-0.26	-1.45	0.68	Xyloglucan endotransglucosylase/hydrolase
-0.60	-1.33	1.06	Xyloglucan endotransglucosylase/hydrolase
0.84	1.80	-0.28	Xyloglucan endotransglucosylase/hydrolase
-0.32	1.47	-0.83	Xyloglucan endotransglucosylase/hydrolase
1.27	1.98	0.33	Probable xyloglucan endotransglucosylase hydrolase
0.74	-0.07	1.36	Expansin
-0.75	-0.08	1.64	Peroxidase 43
-0.94	0.14	1.32	GDSL esterase lipase CPRD49
0.88	-0.59	1.98	Oxalate-- ligase-like
-0.37	0.34	2.30	Peroxidase 25
-1.58	0.08	0.80	Auxin-binding protein ABP19A
-2.04	-0.34	0.68	Acidic endochitinase-like

6H	24H	7D	Response to abiotic stimulus
0.07	0.67	1.80	Mitogen-activated protein kinase
0.61	-0.46	1.49	Proteasome inhibitor-related
0.41	0.84	2.28	ATP-dependent zinc metalloprotease FTSH chloroplastic-
3.17	1.38	1.68	Late embryogenesis abundant protein LEA14-A
1.72	-0.06	2.01	Hexose transporter HT5
2.61	0.08	1.75	Galactinol synthase 2
4.69	-1.97	4.76	Dehydrin 1 (Dehydrin 1a)
1.62	-1.30	2.86	Asparagine synthetase [glutamine-hydrolyzing]
1.43	-1.61	1.47	3-ketoacyl-CoA synthase
2.23	-1.78	-0.06	Ethylene-responsive transcription factor ERF113
1.46	-0.03	-0.85	(6-4)DNA photolyase
1.31	-0.01	-0.54	Basic leucine zipper transcription factor HY5
1.83	-0.10	1.02	Dehydrin 2
-1.30	0.05	1.10	Chlorophyll a-b binding protein of LHCII type 1-like
-0.97	0.15	1.48	Chlorophyll a-b binding protein, chloroplastic
-1.38	0.18	1.31	Chlorophyll a-b binding protein, chloroplastic
-0.40	0.10	1.62	Chlorophyll a-b binding protein, chloroplastic
-0.15	-0.20	-1.47	Beta-glucosidase 12-like
-0.50	-0.74	-1.54	Gibberellin-regulated protein 14
2.16	0.54	2.25	Gibberellin 2-beta-dioxygenase 1-like
1.59	-0.77	1.18	Gibberellin 2-beta-dioxygenase 1-like

6H	24H	7D	Response to biotic stimulus
-2.52	-0.30	3.26	MLP-like protein 31
1.75	0.07	1.70	Chitinase (Chitinase class I)
2.53	1.00	4.17	Probable WRKY transcription factor 40
2.32	0.11	2.16	MLO-like protein
2.62	-0.50	2.12	Pathogenesis-related protein 10
-0.48	0.22	3.22	Pathogenesis-related protein 10.7
-0.70	0.58	1.56	S-norcoclaurine synthase 2-like
0.88	-0.59	1.98	Oxalate-- ligase-like
0.96	1.77	1.90	Protein SAR deficient 1
1.49	0.00	0.31	Endochitinase EP3-like
2.75	0.61	1.62	Class IV chitinase
-2.48	2.01	2.62	Class IV chitinase
1.47	1.20	2.40	Endochitinase PR4

6H	24H	7D	Cellular homeostasis
-3.04	-1.87	-1.14	Monothiol glutaredoxin-s2-like
-1.33	0.06	-1.18	Heavy metal-associated isoprenylated plant protein 26
0.93	-0.55	1.72	Heavy metal-associated isoprenylated plant protein 26
0.32	-1.22	3.51	Heavy-metal-associated domain-containing family protein
-0.49	2.00	5.93	Heavy-metal-associated domain-containing family protein
-0.23	0.60	1.75	Late blight resistance protein homolog R1B-19
0.25	1.04	2.29	FK506-binding protein 4-like isoform x1
-0.63	0.27	1.63	Thioredoxin, chloroplastic
0.96	0.50	2.49	Late blight resistance protein homolog R1B-19
0.57	0.18	1.69	Glutaredoxin domain-containing cysteine-rich CG12206
1.18	-2.32	1.66	Ferritin, chloroplastic
0.29	0.34	2.05	Glutaredoxin-C9-like
0.98	1.69	2.21	Metal ion-binding
1.38	-0.75	-0.11	Aquaporin PIP14 (Putative aquaporin)
2.92	-1.57	-0.19	Probable aquaporin TIP1-2
1.44	0.18	0.42	Calcium-activated outward-rectifying potassium channel 1

Figure 3.7 Expression patterns of DEGs

A - The heat maps of different GO terms display the log2 fold change values of DEGs at all three time-points of stress. The values in bold red and bold blue are the log2 fold change values for significantly up and down regulated DEGs, respectively. B – Correlation of gene expression patterns obtained from RNASeq data and qRT-PCR data.

3.3.6 Validation of DEGs

The fold change patterns of differentially expressed genes obtained from NGS data analyses were compared with those obtained from the qRT-PCR analysis. The expression pattern of 15 out of 25 genes across three time-points of stress displayed a positive correlation of ≥ 70% (**Table 3.3**).

Table 3.3 Correlation of gene expression patterns of 25 DEGs obtained from RNA Seq data and qRT-PCR

Gene	RNAseq fold changes			qPCR fold changes			Correlation
	6H	24H	7D	6H	24H	7D	
BAHD	0.97	1.01	0.87	0.51	3.69	0.41	0.75
CARS	0.88	0.94	0.90	1.40	8.37	0.31	0.92
CBP1	0.37	1.28	2.58	0.30	0.58	8.56	0.93
CBP2	0.59	1.17	2.46	0.49	0.74	2.98	0.98
COP1	1.15	0.91	0.89	6.21	1.77	0.07	0.98
CSLG2	1.07	0.88	1.04	11.7	3.53	0.03	0.39
DRP	0.69	0.94	1.30	0.93	2.09	0.62	-0.29
FBA	0.82	1.26	2.44	0.20	0.77	1.32	0.97
GDH	0.79	0.88	0.87	1.07	0.35	0.66	-0.96
GRS	1.14	0.92	0.91	1.67	0.98	0.16	0.86
GS1	1.05	1.27	2.29	0.18	0.51	0.88	0.95
GS2	0.81	1.18	1.77	1.12	1.66	5.37	0.96
GS3	1.10	1.24	2.96	0.56	0.34	0.09	-0.91
KF	0.86	0.90	1.18	0.50	0.84	0.91	0.71
P5L	1.43	0.74	0.38	0.71	3.18	0.02	0.04
PIP2-5	1.12	0.89	1.12	0.31	1.73	2.56	-0.17
POR1	0.50	0.95	1.55	0.29	0.89	1.85	1.00
POR2	0.54	1.08	1.88	0.64	3.48	0.62	-0.12
RABC1	0.91	0.99	1.06	3.19	1.40	0.12	-1.00
RCAA1	0.69	1.26	2.80	0.17	0.62	0.74	0.83
RCAA2	0.73	1.14	2.28	0.15	3.83	13.39	1.00
RCAA3	0.40	1.11	4.72	2.54	0.17	0.09	-0.65
RH37L	1.07	0.94	1.04	1.26	0.84	0.13	0.07
SREM	1.22	1.05	0.78	3.11	1.54	0.05	0.99
TPI	0.69	1.08	1.28	0.37	1.36	4.35	0.89

BAHD - bifunctional aspartokinase homoserine dehydrogenase chloroplastic-like, CARS - cysteine--tRNA ligase cytoplasmic, CBP1 - chlorophyll a-b binding protein chloroplastic, CBP2 - chlorophyll a-b binding protein chloroplastic, COP1 - e3 ubiquitin-protein ligase cop1, CSLG2 - cellulose synthase-like protein g2, DRP - desiccation-related protein at2g46140, FBA - fructose-bisphosphate aldolase chloroplastic, GDH - glutamate dehydrogenase 1, GRS -

glycine--tRNA ligase mitochondrial-like, GS1 - glutamine synthetase, GS2 - glutamine synthetase leaf chloroplastic, GS3 - glutamine synthetase leaf chloroplastic, KF - kynurenine formamidase, P5L - peroxidase 5-like, apoplastic, PIP2-5 - probable aquaporin pip2-5, POR1 - protochlorophyllide oxidoreductase chloroplastic, POR2 - protochlorophyllide oxidoreductase-like, RABC1 - ras-related protein rabc1, RCAA1 - ribulose bisphosphate carboxylase oxygenase activase chloroplastic isoform x1, RCAA2 - ribulose bisphosphate carboxylase oxygenase activase chloroplastic isoform x1, RCAA3 - ribulose bisphosphate carboxylase oxygenase activase chloroplastic isoform x2, RH37L - dead-box ATP-dependent RNA helicase 37-like, SREM - serrate RNA effector molecule-like, TPI - triosephosphate isomerase cytosolic

3.4 Discussion

3.4.1 Response to abiotic stimulus

The response to abiotic stimulus included upregulation of several osmotic and drought stress tolerance related genes (**Figure 3.7**). The synthesis of osmoprotectants was evident from the upregulation of galactinol synthase at 6 h of stress. Galactinol synthase catalyzes the first step in the synthesis of raffinose family oligosaccharides (Taji et al., 2002). Both raffinose and galactinol act as osmoprotectants in cold, drought, and salt-stressed plants and protect them from oxidative damage (Nishizawa et al., 2008). The salt stress-induced enhanced accumulation of reactive oxygen species can cause DNA damage; hence, the early upregulation of DNA photolyase, a DNA repair enzyme (Schleicher et al., 2007), suggested the early onset of DNA repair mechanism in stressed grapevine plants. The upregulation of dehydrins (DHNs) and late embryogenesis abundant (LEA) proteins also implied their protective role in response to cellular dehydration, but their precise mechanism is still not known (Hanin et al., 2011; Singh et al., 2005).

Among transporters, a hexose transporter, HT5, from the major facilitator superfamily (MFS) was upregulated in our study, which correlated well with the previous report on water stress in grapevines (Medici et al., 2014). Hexose transporters from MFS are mostly involved in regulating the transport of sugars from source to sink tissues (Roitsch, 1999). Therefore, the stress-specific upregulation of hexose

transporter may be involved in redirecting the sugars to stressed cells. Interestingly, most of these abiotic stress-related genes were downregulated at 24 h and again upregulated at the late stage of stress. The genes that were initially downregulated but gradually upregulated at late stage also included the chloroplastic protein-coding genes. The chlorophyll a-b binding proteins are associated with chlorophyll and xanthophyll in the light-harvesting complex of PSI and PSII and serve as antenna proteins to trap and transfer the light energy for initiating the photosynthetic electron transport (Jansson, 1999, 1994). Therefore, the delayed upregulation of three chlorophyll a-b binding protein-coding genes indicated the onset of photosystem protection from oxidative damage and degradation due to increased stress.

3.4.2 Photosynthesis

Salt stress primarily affects the photosynthetic efficiency of plants leading to decreased growth rate and productivity. This mainly occurs through the photoinhibition of the photosystem, which is enhanced during environmental stress conditions due to ROS mediated impaired replenishment of damaged proteins (Murata et al., 2007). Prolonged salinity stress induces chlorophyll degradation, destruction of chloroplast structure and destabilization of pigment complexes in salinity sensitive plants (Abdelkader et al., 2010; Santos, 2004; Zaidi et al., 2014). These damaged proteins are degraded by FTSH metalloprotease in Arabidopsis and replaced by a new copy (Lindahl et al., 2000), while the chlorophyll a-b binding proteins prevent the photodamage of PSII. Hence, the upregulation of FTSH metalloprotease along with increased expressions of PSI subunits and chlorophyll a-b binding proteins in our study indicated the recycling of damaged photosystem subunit proteins. The early downregulation, followed by gradual upregulation of these genes, suggested the beginning of new protein synthesis to replenish the damaged photosystems.

The thylakoid polyphenol oxidase (PPO) was also upregulated at the early stage of stress. However, it is not associated with an increase in photosynthetic ability; but may be involved in regulating the local levels of ROS (Boeckx et al., 2015). Its increased activity in intact cells was associated with increased vigor in red clover plants (Boeckx et al., 2017). PPOs are typically associated with the browning of fruits due to the production of highly reactive o-quinones in damaged cells or tissues. Therefore, its

gradual suppression at the later stages of stress indicated the recovery of the grapevines from oxidative damage.

Additionally, the transfer of electrons from NADH to ubiquinone during the electron transport chain by NAD(P)H dehydrogenase (NDH) complex might have reduced plastoquinones in thylakoid membranes. It is the primary source of ROS in cells. The NDH-dependent cyclic electron flow-5 (NDF5) is required for maintaining the stability and activity of NDH complex in *Arabidopsis* (Ishida et al., 2009). Therefore, the late upregulation of NDF5 might be protecting the electron transport chain under stress conditions in our study.

3.4.3 Response to biotic stimulus

The pathogenesis-related (PR) proteins like chitinases and glucanases were upregulated at different stages of stress in our study. Plant chitinases are commonly known as defense proteins produced by plants upon pathogen attack. Their overexpression imparts higher resistance to pathogens in transgenic plants (Brogue et al., 1991; Schlumbaum et al., 1986). However, the role of chitinases has now been expanded to many other physiological processes, such as development (Samac and Shah, 1991), cell wall regeneration (Kwon et al., 2005) and tolerance to environmental stresses like osmotic stress, drought stress and salt stress (Pinheiro et al., 2001; Takenaka et al., 2009; Tateishi et al., 2001; Yun et al., 1996). Therefore, upregulated chitinases in our study could be the potential mediators of salinity tolerance in grapevines. The transcription regulator SAR deficient 1 (SARD1) is involved in regulating salicylic acid biosynthesis in response to biotic and abiotic challenges (Truman and Glazebrook, 2012). Similarly, several other PR proteins have been associated with abiotic stress tolerance in plants (Liu and Ekramoddoullah, 2006; Rodrigo et al., 1991). Transcriptomics studies have also revealed modulation of PR proteins in response to abiotic stress in other plants (Ergen et al., 2009; Sahi et al., 2006). However, their primary mechanisms of stress tolerance are still elusive.

3.4.4 Transcription factors: Key regulators of gene expression

Transcription factors (TFs) play vital roles in plant development and stress response by temporal and spatial regulation of the transcription of their target genes. Grapevine genome encodes for a large number of transcription factors (TF), around 1276 TFs,

which are classified into 58 families (Jin et al., 2013) (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Vvi) (**Supplementary Table 5**). The modulation of several transcription factors under the biological process of transcription could be the key regulators of stress-responsive transcript expression in the plant. There are 74 NAC genes in *V. vinifera* genome (Wang et al., 2013), which are involved in various developmental processes. The upregulation of seven NAC domain-containing proteins at different stages of stress in our study suggested their possible role in the transcription of stress-related genes. Abiotic stress-responsive NAC TFs have been reported in many plants (Nakashima et al., 2012). For instance, NAC29, a transcription activator enhances the transcription of abscisic aldehyde oxidase3 (AAO3), which induces the biosynthesis of hormone abscisic acid (ABA) that further induces the degradation of chlorophyll in Arabidopsis leaves (Yang et al., 2014). Hence, the strong upregulation of *NAC29* homolog in grapevine at early and late stages of salt stress indicated the onset of ABA synthesis and chlorophyll degradation in stressed leaves.

Ethylene is a crucial mediator of biotic and abiotic stress responses in plants, and its signaling involves several Ethylene Response Factors (ERFs), which belong to the transcription factor family APETALA2/ERF (Müller and Munné-Bosch, 2015). *Vitis* has 149 AP2/ERF family transcription factors (Licausi et al., 2010), of which 13 were upregulated in the salt-stressed grapevines - seven at the early stage and seven at the late stage of stress. ERF190-like was upregulated throughout the stress. Though ERFs are mostly known to regulate the defense against pathogen attack, they are also identified as key regulatory hub for integrating ethylene, abscisic acid, jasmonate, and redox signaling in the plant response to several abiotic stresses (Müller and Munné-Bosch, 2015). Therefore, modulation of a large number of ERFs supports the probable crosstalk between response to biotic and abiotic stimulus observed in our study.

A bZIP transcription factor HY5 is involved in photomorphogenic development in *Arabidopsis* by promoting the expression of negative regulators of auxin signaling (Cluis et al., 2004). Therefore, early upregulation of HY5 might have resulted in decreased expression of auxin-responsive proteins in the present study. A previous study has shown that upregulation of C-repeat binding protein-4 (VvCBP-4) in response to freezing stress in *Vitis vinifera* imparts cold tolerance through regulation of genes involved in cell wall structure formation, lipid metabolism, epicuticular wax

formation and stress-responses (Tillett et al., 2012). In our study, CBP-4/DREBA1 was upregulated at 24 h and 7 d of stress while cell wall modification-related genes were mostly affected at 24 h of stress thus, supporting a probable regulatory function of this transcription factor.

The transcription factor ZAT10 has been associated with salt tolerance in several studies (Nguyen et al., 2016; Xie et al., 2012). It was found to enhance the tolerance of *Arabidopsis* to salinity, heat, and osmotic stress by increasing the expression of reactive oxygen-defense transcripts (Mittler et al., 2006). Thus, early upregulation of this transcription factor in our study might be associated with salt tolerance of TS110R grapevines. Homeobox leucine zipper protein, ATHB12, is an ABA inducible transcription factor that is highly upregulated in Arabidopsis under water stress conditions and its overexpression makes the plant hypersensitive to ABA (Olsson et al., 2004). Hence, the increased expression of this transcription factor at the late stage of salt stress might be associated with dehydration responses in our study.

3.4.5 Plant hormone signal transduction

Several plant hormones, such as ethylene, abscisic acid, jasmonates, salicylic acid, gibberellins, cytokinins, auxin, and brassinosteroids, have been reported to be involved in stress signaling (Müller and Munné-Bosch, 2015). The early stress response in our study displayed a significant hormonal modulation through the upregulation of genes involved in plant hormone regulation. Abscisic acid 8'-hydrolase 2 is involved in the oxidative degradation of abscisic acid (Kushiro et al., 2004). Its early downregulation followed by upregulation at the late phase in our study indicated the temporal regulation of ABA levels in response to stress allowing increased levels of ABA at the early stage with a subsequent decrease at a late stage. The levels of Gibberrelin (GA) are downregulated through the catabolic activity of Gibberellin 2-beta-dioxygenase 1 (*G2OX1*), which deactivates various GAs by catalyzing the 2-beta-hydroxylation of biologically active GAs (Thomas et al., 1999). The early upregulation of four *G2OX* genes thus indicated the suppression of GA signaling pathways in salt-stressed grapevines.

Since gibberellin regulates major aspects of plant growth and development; suppression of GA signaling signified the stalling of growth at the early stage of stress. Whereas, the genes encoding GA receptors like Gibberellin receptor GID1B and

protein phosphatase 2C were upregulated at a late stage, suggesting plant growth recovery. The TIFY 10a is a JAZ family protein that acts as a repressor of Jasmonate signaling in Arabidopsis (Chini et al., 2007; Chung and Howe, 2009) and Jasmonate is induced in response to the biotic stimulus. The upregulation of two TIFY10 genes at an early stage of stress in the present study suggested the suppression of jasmonate signaling.

3.4.6 Role of genes encoding extracellular proteins

The genes encoding extracellular proteins such as expansin, laccase, peroxidase, and xyloglucan endotransglucosylase/ hydrolase are associated with the cell wall and lignin synthesis. The modulation of several laccases and Dirigent protein-coding genes could be involved in lignin biosynthesis. Some *Arabidopsis* laccases have been identified to be involved in stem lignification by catalyzing lignin polymerization through oxidization of monolignols leading to lignification of cell walls (Berthet et al., 2012; Liu et al., 2017). The dirigent (DIR) proteins determine the stereochemistry of the compounds synthesized by other enzymes. A bioinformatic study has identified several dirigent proteins modulating the lignin and lignin biosynthesis in response to biotic and abiotic stresses (Paniagua et al., 2017). Though DIRs were downregulated throughout the stress, two laccases were upregulated at an early stage in stressed grapevines in our study suggesting induction of lignin polymerization.

The synthesis of suberin aromatics could also be upregulated due to increased expression of Omega-hydroxypalmitate O-feruloyl transferase (Molina et al., 2009) at an early stage. Hence, the early synthesis of lignin and suberin could be predicted to be involved in the protective mechanism to decrease the evaporation of water. Expansins are the non-enzymatic proteins present in plant cell walls that are involved in cell elongation through the loosening of cell walls (Cosgrove, 2000).

Similarly, xyloglucan endotransglucosylases are involved in cutting and rejoining of xyloglucan, an essential polymer that holds together cellulose microfibrils (Cosgrove, 2005). Hence, the upregulation of these two genes indicated the possibility of the increased flexibility of cell walls for continuing the plant growth under salt stress conditions in the present study. In a previous study, the constitutive overexpression of a pepper xyloglucan endotransglucosylase/hydrolase has displayed enhanced tolerance to salt stress in transgenic Arabidopsis and tomato plants (Cho et al., 2006; Choi et al.,

2011). Stress-induced synthesis of lignin indicates the onset of thickening of cell walls in plants (Berthet et al., 2012). The mechanism of salt tolerance though cell wall lignification, is associated with the modification of vascular tissues to enhance their capillarity (Fernandez-Garcia et al., 2008). Some plant laccases are involved in the production of higher-order lignin through oxidization of monolignols leading to lignification of cell walls (Liu et al., 2017).

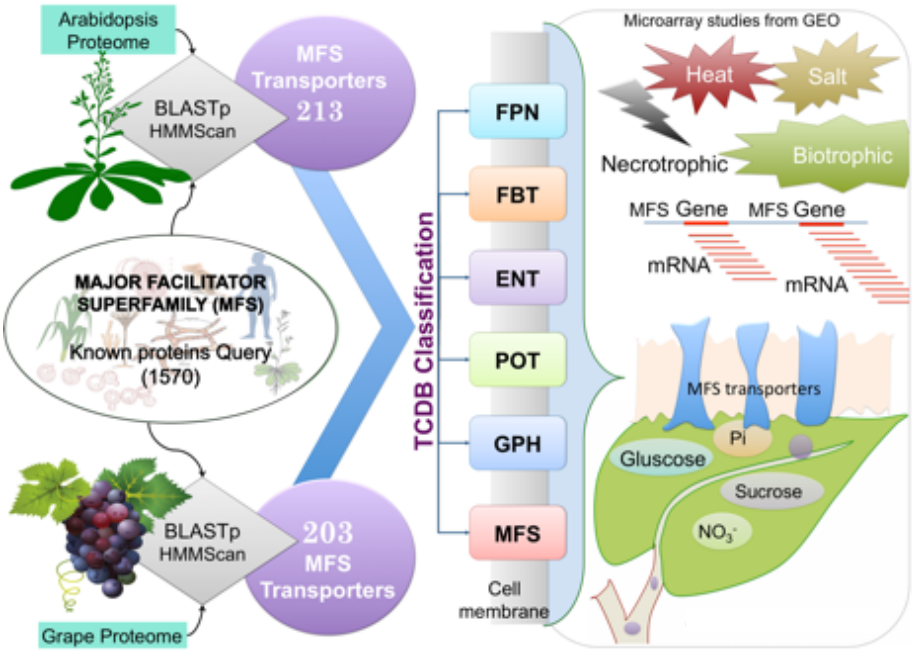
Furthermore, overexpression of extracellular peroxidases is also associated with salt tolerance by participating in lignin biosynthesis and cell wall synthesis in several plants (Blee et al., 2003; Choi and Hwang, 2011; Fagerstedt et al., 2010). Therefore, the upregulation four peroxidases at an early stage along with two laccases and one expansin might have supported the induction of cell wall elongation and lignification process in response to salt stress in grapevines; while their downregulation at 24 h, but upregulation of three xyloglucan endoglucosylases indicated the increase in flexibility of cell walls at mid stage of stress.

3.5 Conclusions

The 110R grafted Thomson seedless vines responded to salt stress through expression modulation of a large number of genes. A total of 509 genes were significantly differentially expressed. A colossal response of transcription factor modulation indicated the onset of change in normal gene expression patterns to adapt to the stress condition. Several abiotic stress-related genes such as Dehydrins and LEAs were upregulated by the stress. Hormonal regulation also played a significant role in response to salt stress as evident from the change in expression of ABA, GA, and Auxin signaling related genes. Temporal differences were observed in cell wall lignification and expansion related genes. The steady increase in photosynthetic machinery related genes was observed as an adaptation of vines to increase their photosynthetic efficiency with increasing duration of stress. Overall, the enhanced ABA related signaling and suppressed GA signaling in response to early stress resulted into growth retardation phase; while, the upregulation of photosynthesis at the late stage of stress indicated the growth recovery phase.

Chapter 4

Global study of MFS superfamily transporters in Arabidopsis and grapes reveals their functional diversity in response to salt stress



Contents of this chapter have been published as a research article …

Patil Sucheta et al., "Global study of MFS superfamily transporters in arabidopsis and grapes reveals their functional diversity in plants." Plant Gene (2019): 100179. https://doi.org/10.1016/j.plgene.2019.100179

Chapter 4 Global study of MFS superfamily transporters in Arabidopsis and grapes reveals their functional diversity in response to salt stress

4.1 Introduction

Plants display various molecular and biochemical mechanisms to tolerate salt stress. Salinity stress in plants consists of initial osmotic stress followed by ionic toxicity arising from the accumulation of ions in order to overcome the osmotic stress. The rise in soil salinity decreases the osmotic potential of water and reduces its availability to plant roots and hence, plants tend to uptake the abundantly available Na^+ and Cl^- ions to lower the tissue-osmotic potential to overcome the osmotic stress. Therefore, the studies on transporters in salt stress mainly focus on the uptake, extrusion, long-distance transport, and compartmentalization of Na^+, K^+ and Cl^- ions in the cells and the tissues of stressed plant (Brini and Masmoudi, 2012). These ion transporters also act during transport through vascular tissues in stem and leaves wherein they sequester the ions in the vacuoles of cells for excluding them from the main circulating fluid. At the same time, the production of osmolytes such as mannitol, glycine betaine, sorbitol, and proline also act as antioxidants and protect from osmotic stress (Brini and Masmoudi, 2012).

The expression of specific sugar and amino acids transporters induced in response to salinity stress are reported to be involved in regulating the transport of sugar alcohols and nitrates in a stressed plant. A gene profiling study on sugar transporters in grapevines has shown upregulation of transporters like *VvHT5*, a hexose transporter, and *VvSUC11*, a sucrose transporter and strong downregulation of *VvHT1*, a hexose transporter under water deficit condition (Medici et al., 2014). A knockout mutation of *Arabidopsis* root stele transporter *AtNPF2.3* reduces the shoot biomass under salt stress conditions by reducing the transport of nitrates to shoot (Taochy et al., 2015).

Similarly, the accumulation of Cl^- into *Arabidopsis* shoots during salt stress is also regulated by root transporters: NPF2.4 loads Cl^- into the root xylem, while NPF2.5 is involved in chloride exclusion by modulating the efflux of Cl^- ions from the roots (Li et al., 2017). These transporters belong to a huge family of diverse transporters called Major Facilitator Superfamily (MFS) (Nour-Eldin et al., 2012; Reddy et al., 2012).

Identification of gene families and studying their expression across different developmental and stress conditions has enabled identification of several new genes and their functions in different plants (Martin et al., 2010; Moin et al., 2017; Orsel et al., 2002). Many transporters from high-affinity potassium transporter (HKT) gene family are involved in the exclusion of Na^+ ions from the shoot (Munns and Tester, 2008). A phylogenetic and structural analysis of HKT gene family identifies even more salinity responsive HKT transporters, which can become potential leads for developing salt-tolerant transgenic plants (Waters et al., 2013). Similarly, a detailed investigation of MFS transporters in grapevines will reveal the number of transporters and their role in salinity stress tolerance.

Transporter proteins have a significant role during salinity stress in plants as they are involved in the uptake of nutrients, maintenance of membrane potential, extrusion of toxic compounds, etc. The studies on microbial transporter proteins have shown that about one-third of the proteins of a cell are embedded in biological membranes, and one-third of them function to catalyze the transport of molecules across the membranes (Paulsen et al., 1998). Similarly, in *Arabidopsis thaliana,* 2.5% of the proteome consists of transporter proteins (Arabidopsis Genome Initiative, 2000; Blattner et al., 1997), while in *Vitis vinifera,* 7% of the predicted genes are identified as transporter proteins (Grimplet et al., 2012). Thus, a small percentage of every genome encodes a repertoire of diverse transporter proteins that catalyze the transport of various substrates based on extra- and intracellular cues.

Transporter proteins are of two types, channel proteins, and carrier proteins. Channel proteins transport a solute down its electrochemical gradient, while the carrier proteins actively translocate substrates against the concentration gradient (Busch and Saier, 2002; Saier, 1999). Based on the source of energy utilized by the carrier proteins, they are classified as primary active transporters and secondary active transporters. The primary active transporters utilize the energy of ATP hydrolysis, photon absorption, substrate decarboxylation, or methyl transfer to transport solutes. Secondary active transporters use the energy stored in the concentration gradient of one solute to translocate another solute against its concentration gradient by symport or antiport mechanisms. The MFS Superfamily is the largest group of secondary active transporters, which transport a broad range of structurally diverse molecules like sugars,

amino acids, oligopeptides, nucleosides, drugs, etc. (Law et al., 2008). However, it also includes facilitators, and hence, it is defined as a family of secondary transporters that includes three distinct kinetic mechanisms – uniport, antiport and symport (Law et al., 2008; Paulsen et al., 1996). The MFS transporters are defined by the presence of a structural fold that acts as a scaffold for all MFS proteins, irrespective of their transport mechanism (Law et al., 2008).

The substrate specificity and structure of a transporter protein are determined by its aminoacyl sequence. Hence, the sequence homology between transporter proteins is used to classify them into families and superfamilies. The Transporter Classification Database (TCDB) was established for the classification of transporters from bacterial, archaeal, and eukaryotic genomes (Saier, 1999; Saier et al., 2013). The MFS Superfamily is present ubiquitously in all the classes of living organisms. The name 'major facilitator superfamily' was initially given to a group of structurally related five families that comprised the proteins from bacteria, lower eukaryotes, plants, and animals, *viz*. drug-resistance proteins, sugar facilitators, facilitators for Krebs cycle intermediates, phosphate-ester-phosphate antiporters and a distinct group of oligosaccharide-H+ symporters (Marger and Saier, 1993).

Based on the phylogenetic studies, this superfamily was further expanded to 74 families (Reddy et al., 2012). Currently, the MFS Superfamily (MFSS) in TCDB (http://www.tcdb.org/superfamily.php) has ten different families. However, the list of families is frequently updated to include the newly identified MFSS transporters from various organisms and thus, the number of MFSS subfamilies is expected to increase with functional characterization and annotation of genes from newly sequenced organisms. The previous literature states all the transporters of MFSS families as MFS transporters (Newstead, 2015; Quistgaard et al., 2016). However, to avoid the confusion due to similar naming of the families, we refer to the transporters of MFS (2.A.1) family as MFS transporters and those from the parent superfamily (MFS Superfamily), which also includes the MFS along with nine other families, as MFSS transporters.

The physiological significance of several MFSS transporters has been explored in various organisms. Prokaryotic genomes have more than 70 MFSS transporters representing nearly 25% of their transporter proteins (Law et al., 2008; Madej et al., 2013; Quistgaard et al., 2016; Saier, 1999), most of which are involved in multidrug

resistance (Prasad and Rawal, 2014). Likewise, in humans, about 100 MFSS transporters have been reported whose functions range from simple intestinal nutrient uptake to the regulation of drug pharmacokinetics (Quistgaard et al., 2016). Other examples include transport of glucose or fructose in various tissues and cell types by GLUTs, glucose transporters (Mueckler and Thorens, 2013) and uptake of docosahexaenoic acid across the blood-brain barrier by MFSD2a, major facilitator superfamily domain-containing 2a (Nguyen et al., 2014). In plants, stomatal movement and the salinity tolerance like phenotypes are regulated by the Zinc-Induced Facilitator-Like 1 (ZIFL1) protein (Remy et al., 2013) and a Phosphate Transporter (PHT4;6) (Cubero et al., 2009), respectively. However, despite the progress in genome sequencing and annotation in various organisms, there has been only one study on the MFS repertoire so far - in the eukaryotic model organism yeast (Gaur et al., 2008). Thus, considering the diverse and vital roles of these proteins, it is essential to understand their structural and functional diversity in higher plants.

Arabidopsis thaliana is a model plant for understanding gene functions in dicotyledonous plants. The sequencing of the *A. thaliana* genome (Arabidopsis Genome Initiative, 2000) revolutionized the functional studies in plants. More than a decade of these functional studies have generated a large amount of information that has enabled the homology-based exploration of gene functions in higher plants. Likewise, grape (*Vitis vinifera* L.) is a perennial woody vine cultivated across the world mainly for wine production and consumption of fresh grapes or processed raisins. However, viticulture has been facing various abiotic and biotic threats like climate change, salinity stress, drought stress, and pathogen attack. To address these issues, the research on grapevines has been focused on the development of new rootstocks and varieties with improved canopy structure (This et al., 1997), improved fungicides and agro-economic characters such as flavor and aesthetics. The sequencing of *V. vinifera* genome in 2007 has given a boost to a multitude of genomic studies, which has led to the correlation of these phenotypes to genes and gene expression patterns (Grimplet et al., 2012). Grape berries are the primary sink tissues of grapevines; hence, a detailed transportome analysis would enable understanding the nutrient distribution and metabolic flux in grapevines that can be implemented for improving the quality and yield of grapes.

In the present study, we identified 213 and 203 MFS Superfamily (MFSS) transporters in *Arabidopsis thaliana* and *Vitis vinifera*, respectively and classified them into six MFSS families and twenty subfamilies based on their transporter classification identifiers (TCIDs). Phylogenetic and gene duplication studies revealed the expansion of sugar porter and proton dependent oligopeptide transporter families in *Arabidopsis* and grape. The subcellular localization of the majority of the transporters was predicted to be in the plasma membrane. Furthermore, the microarray expression analysis of MFSS transporters from *Arabidopsis* and grapes revealed their multi-tissue-specificity and differential regulation under biotic and abiotic stress conditions. Studies of the transmembrane topology highlighted the presence of central cytoplasmic loop along with family-specific topological variations that were evident from phylogenetic analysis. Overall, this study adds to the knowledge of functional and structural diversity and evolution of MFSS transporters in the two plants and opens the scope for detailed physiological and functional studies on these proteins.

4.2 Materials and methods

4.2.1 Identification and annotation of putative MFSS proteins

We prepared a set of known MFSS proteins by combining 1055 MFS Superfamily proteins of TCDB and 1877 "MFS transporter superfamily" proteins from Swiss-Prot (http://www.uniprot.org/uniprot/?query=*&fil=reviewed%3Ayes, November 2017). For identification of MFS like proteins, these sequences were clustered into 1570 sequences using CD-HIT (http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit) at 90% sequence identity cut-off and were queried against the proteomes of *A. thaliana*, *V. vinifera*, *O. sativa* (var. Japonica), *S. moellendorffii*, *P. patens* and *C. reinhardtii* (http://plants.ensembl.org/index.html, October 2018) with an e-value threshold of $1e^{-5}$ for BLASTp search. The hits obtained were further screened for the presence of Hidden Markov Models (HMMs) for MFS domains using hmmscan of HmmerWeb ver. 2.19.0 (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) supported with reference HMMs from SUPERFAMILY protein domain databases. The sequences having the Major Facilitator Superfamily domain, SSF103473, were further shortlisted as probable MFSS proteins and were finally confirmed to be MFSS transporter proteins only if they had at least two transmembrane helices in them. The number of transmembrane helices was assessed using Phobius

(http://phobius.binf.ku.dk/). The proteins were annotated based on their top PSI-BLAST hits from the entire TCDB database (Novembr, 2017). The genomic locations of all the predicted MFSS genes were retrieved from the ensemble plant BioMart server (http://plants.ensembl.org/biomart/martview/) and were displayed on the chromosomes using MapInspect (http://mapinspect.software.informer.com/).

4.2.2 Phylogenetic analysis and gene duplication study

The predicted MFSS proteins were subjected to phylogenetic analysis using PhyML-SMS ver. 3.0, a maximum likelihood tree approach (Guindon et al., 2010). The sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and then submitted to the online PhyML-SMS tool (http://www.atgc-montpellier.fr/phyml/) having an option for automatic selection of amino-acid substitution model by SMS (Smart Model Selection). The SMS algorithm chose 'JTT model' as the best model for evolutionary analysis for the given set of sequences. SH-like approximate likelihood-ratio (SH-like aLRT) test was used to estimate the branch support values of the phylogenetic tree (Anisimova and Gascuel, 2006). The tree was visualized using the Interactive Tree of Life (iTOL) (https://itol.embl.de/), and the protein topology was displayed in the form of the domain architecture of TMH, cytoplasmic and non-cytoplasmic domains at the tip of every leaf. To identify the origins of duplicate genes, self-proteome BLASTp was performed for both *Arabidopsis* and grapes and the output was analyzed using the duplicate gene classifier provided in MCScanX software (http://chibba.pgml.uga.edu/mcscan2/).

4.2.3 Prediction of subcellular localization

Subcellular localizations of all the identified MFSS transporters were predicted using ProtComp ver. 9.0 (http://www.softberry.com). These subcellular locations were grouped family-wise to understand their representation across different families of MFS Superfamily.

4.2.4 Microarray studies

Previously published microarray datasets, available in Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/), were selected for analyzing the expression patterns of MFSS transporters in various plant tissues. Five *Arabidopsis* microarray studies (GSE5630, GSE5631, GSE5632, GSE5633, and GSE5634) and one

grape microarray study (GSE36128) covering various tissues at different developmental stages were selected. Similarly, the differential regulation of MFSS genes in response to abiotic and biotic stresses was studied using the microarray datasets GSE31677, GSE39956, GSE53409, GSE31594, GSE3220, GSE53824, GSE5684, and GSE52586. Statistical analysis of expression of the MFSS genes was performed on the pre-analyzed gene expression values provided in the series-matrix files of the respective datasets using Multiple Experiment Viewer (MeV v. 4.8; http://mev.tm4.org/). For tissue-specific expression analysis, the genes were considered as expressed if their expression values were higher than the threshold value. The microarray platforms were different for the selected datasets; the threshold value for *A. thaliana* Affymetrix array was five log2 expression units, and for *V. vinifera* NimbleGen array was 100 expression units. The stress-responsive genes were identified using the Student's t-test, and the expression patterns of significantly differentially expressed genes were visualized using heat maps.

4.3 Results

4.3.1 Identification and annotation of MFSS transporters

Homology search against the proteomes of *A. thaliana* and *V. vinifera* yielded 515 and 269 BLASTp hits, respectively. However, of the total of 784 hits, only 626 had the MFS domain (SSF103473) and a minimum of two transmembrane helices (TMH). After eliminating the protein isoforms of *A. thaliana*, we identified 213 MFSS genes in *A. thaliana* and 203 MFSS genes in *V. vinifera*. The Transporter Classification numbers (TCIDs) were annotated to these predicted MFSS transporters, which classified them into 20 categories based on their four-component-TCIDs (MFSS subfamilies) (**Figure 4.1**). A TCID is typically a number with five components: V.W.X.Y.Z. V, a number, corresponds to a transporter class like, channel, carrier, primary active transporter or group translocator; W, a letter, corresponds to transporter subclass; X, a number, corresponds to transporter family or superfamily; Y, a number, corresponds to family or subfamily, and Z corresponds to substrates transported.

For the convenience of discussion, the predicted MFSS proteins were renamed such that the first two letters depict the species followed by two to three letters of MFSS subfamily name and then a number corresponding to their ascending order of genomic

loci (**Supplementary Table 6**). For example, the six ACS family genes from *Arabidopsis*, AT2G29650, AT2G38060, AT3G46980, AT4G00370, AT5G20380 and AT5G44370 were renamed as AtACS1, AtACS2, AtACS3, AtACS4, AtACS5 and AtACS6 respectively; while the six ACS family genes from grape, VIT_03s0038g03910, VIT_06s0004g06040, VIT_06s0004g06220, VIT_08s0007g01810, VIT_11s0118g00410 and VIT_15s0046g02390 were renamed as VvACS1, VvACS2, VvACS3, VvACS4, VvACS5 and VvACS6, respectively. Hence, identical name of proteins from two plants does not refer to their orthology. A similar nomenclature system was also adopted in previous protein family studies (Léran et al., 2014; Verrier et al., 2008).

4.3.2 Families identified under MFS superfamily

A family of proteins is formed by a group of proteins from the same or different organisms having a common structure and function. Six of the eight MFSS families were present in both, *A. thaliana* and *V. vinifera*. The Major Facilitator Superfamily (MFS/ 2.A.1) was the largest of the six MFSS families (**Figure 4.1**) with 130 transporters from *A. thaliana* and 128 from *V. vinifera*. This 2.A.1 family contains 84 MFSS subfamilies in TCDB. However, only 13 of them (MDP, ACS, DHA1, Spinster, NAG-T, NNP, OCT, OPA, PHS, PCUP, SP, UMF15 and UMF13) were identified in *A. thaliana* and *V. vinifera* (**Figure 4.1**). The Proton Dependent Oligo-Peptide Transporter (POT) family (2.A.17) was the second-largest MFSS family with 53 and 57 transporters from *Arabidopsis* and grape, respectively followed by the Glycoside-Pentoside-Hexuronide (GPH): Cation Symporter family (2.A.2) with 11 transporters from *Arabidopsis* and six from the grape. Based on TCID annotation, the GPH family showed two subfamilies: Sucrose: H+ symporter (2.A.2.4) and pigment transporter in insects (2.A.2.7) and hence, they were renamed as GPH1 and GPH2, respectively. Similarly, the Folate Biopterin Transporter (FBT) family (2.A.71) had transporters with two different TCIDs - 2.A.71.1 and 2.A.71.3, and therefore, they were renamed as FBT1 and FBT2, respectively. All the MFSS subfamilies were considered individually for further analyses.

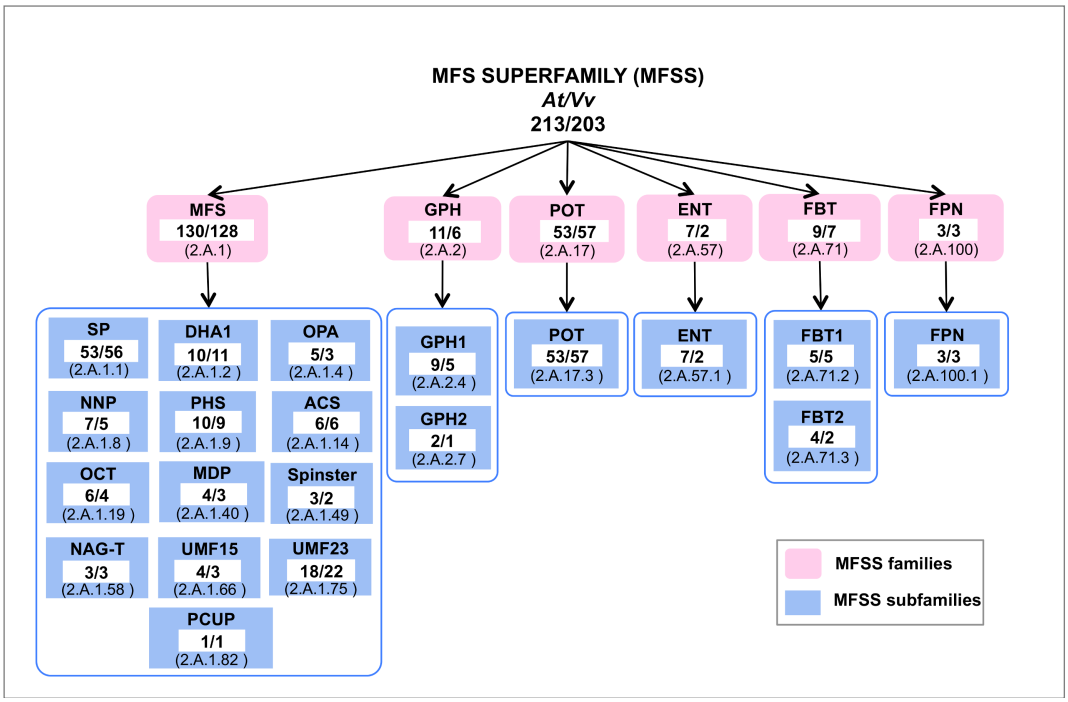


Figure 4.1 Classification of predicted MFSS proteins based on TCID annotation

ACS - Anion:Cation Symporter Family, DHA1 - Drug:H+ Antiporter-1 Family, ENT - Equilibrative Nucleoside Transporter Family, FBT1 - Folate-Biopterin Transporter Family 1, FBT2 - Folate-Biopterin Transporter Family 2, FPN - Ferroportin Family, GPH1 – Sucrose:H+ symporter, GPH2 - Pigment transport in insects, MDP - Major Facilitator Superfamily Domain-containing Protein Family, MFS – Major Facilitator Superfamily, NAG-T - N- Acetylglucosamine Transporter Family, NNP - Nitrate/Nitrite Porter Family, OCT - Organic Cation Transporter Family, OPA - Organophosphate:Pi Antiporter Family, PCUP - Plant Copper Uptake Porter, PHS - Phosphate:H+ Symporter Family, POT - Proton-dependent Oligopeptide Transporter Family, SP - Sugar Porter Family, Spinster - Endosomal Spinster Family, UMF15 - Unidentified Major Facilitator-15 Family, UMF23 - Unidentified Major Facilitator-23 Family.

4.3.3 Phylogenetic clustering and evolutionary origin

Phylogenetic analysis of the MFSS proteins from *Arabidopsis* and grapes (**Figure 4.2**) resulted in 20 distinct clusters of branch support values greater than 0.9. All families formed separate clusters except the DHA1, FPN and NAG-T families. The DHA1 family formed three separate clusters C1, C2, and C3; The FPN family was also split into two clusters C8 and C13, while the NAG-T family proteins remained at the root of the tree without forming any cluster. FBT1 and FBT2 families formed a single cluster, C14, while PCUP proteins and OCT family formed a cluster, C7. The SP and POT

families formed two large clusters in the tree indicating the possibility of expansion of these families during evolution. According to the gene duplication types assigned by MCScanX (Wang et al., 2012), about 40% of the MFSS genes originated through dispersed gene duplications; while more than 50% of the genes originated from tandem and whole genome duplications (WGD)/ segmental duplications and up to 7% genes originated from proximal duplications (**Figure 4.3A**). Tandem duplications were relatively more frequent in grape. The SP and POT family showed almost double the tandem duplication events in grape in comparison to *Arabidopsis* (**Figure 4.3B, C, Figure 4.4**). The MFSS genes were randomly distributed across all the chromosomes in both the plant genomes (**Figure 4.3B, C**). However, a dense distribution was observed in *Arabidopsis* than in the grape genome.

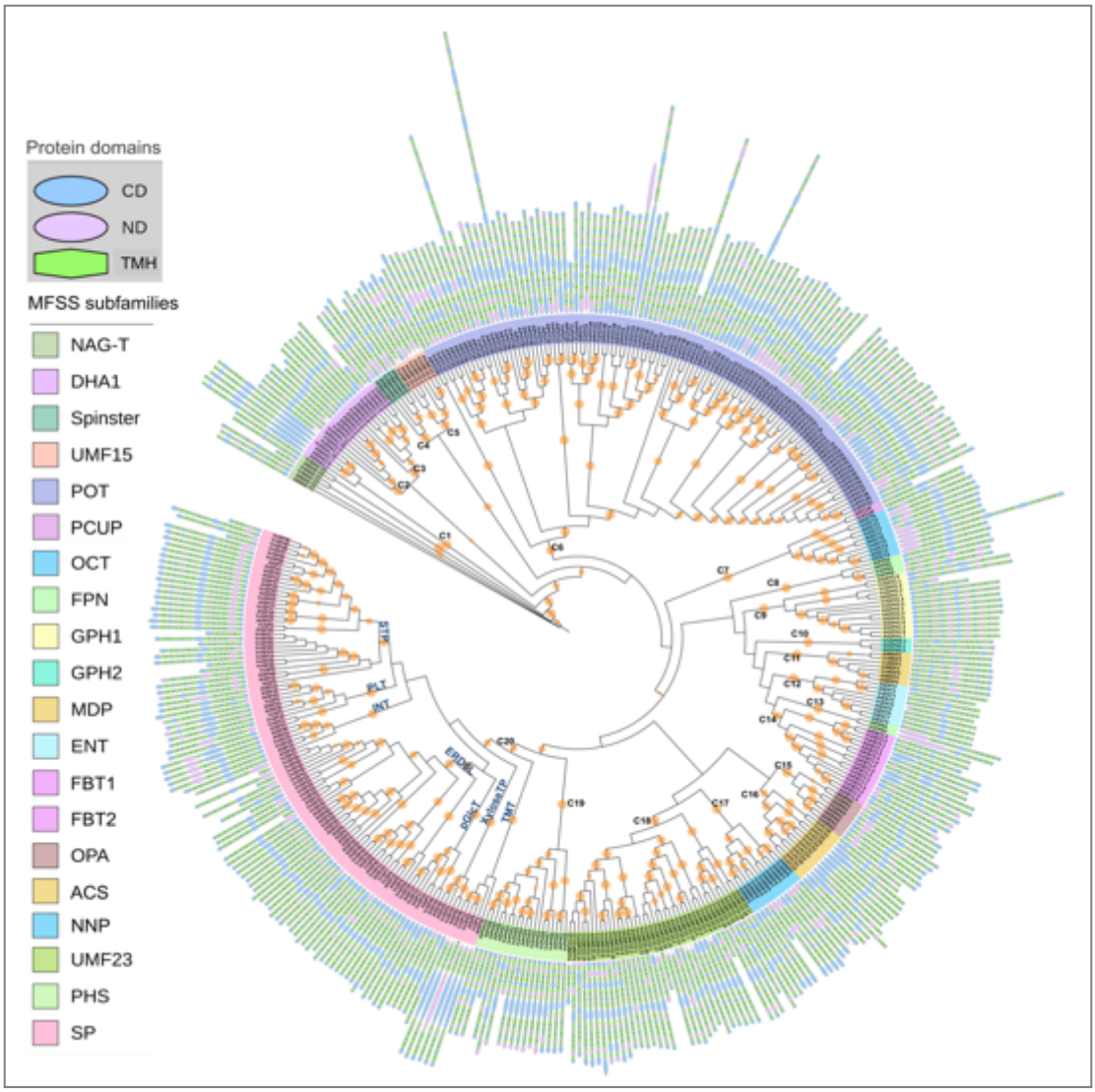


Figure 4.2 Phylogenetic tree of 416 MFSS transporters from *A.thaliana* and *V. vinifera*

The significant branching nodes (SH-like aLRT branch support value greater than 0.9) are denoted with an orange circle. Domain architecture of each protein is displayed at the tip of every leaf. CD – Cytoplasmic Domain, ND – Non-cytoplasmic Domain, TMH – Transmembrane helix. SP Family: STP – Sugar transport protein, PLT – Polyol transporter, INT – Inositol transporter, ERD6L - Sugar transporter ERD6-like, pGlcT – Plastidic glucose transporter, XyloseTP - D- xylose-proton symporter, TMT – Tonoplast monosaccharide transporter.

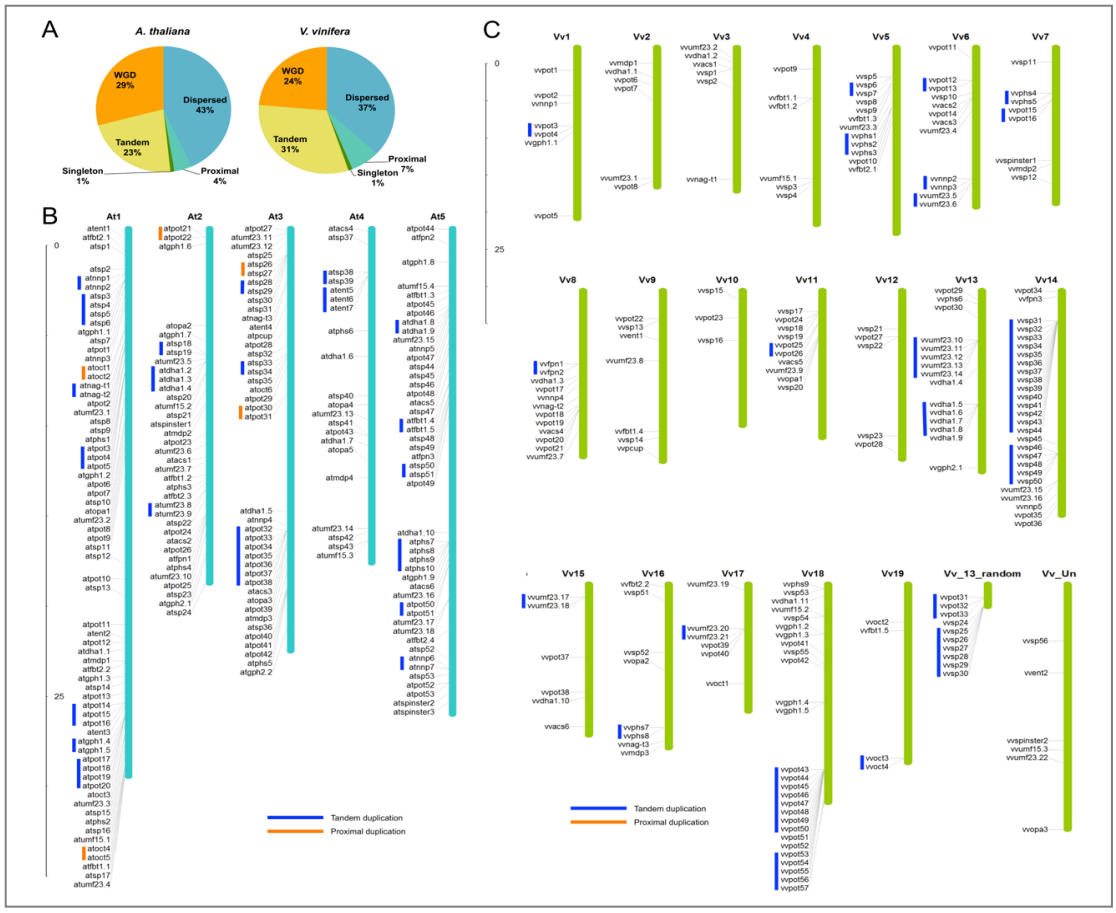


Figure 4.3 Gene duplication events and chromosomal map of MFSS genes

(A) Distribution of gene duplication types in MFS Superfamily of *A. thaliana* and *V. vinifera*. (B) Chromosomal map of MFSS genes in *A. thaliana*. (C) Chromosomal map of MFSS genes in *V. vinifera*. The vertical bars beside the gene names indicate the type of gene duplication.

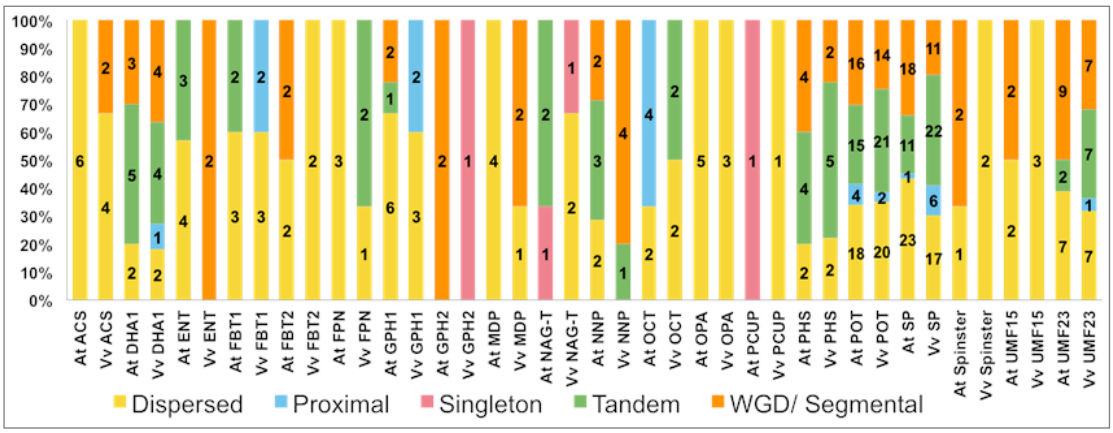


Figure 4.4 Gene duplication events in each MFSS subfamily

The numbers of transporters originating from different gene duplication events are shown for each MFSS subfamily on the percentage bar-plot. The numbers on the bar-plot indicate the number of MFSS transporters.

4.3.4 Transporter protein topology of MFSS families in Arabidopsis and grapes

The protein topologies were displayed in the phylogenetic tree as domain architecture of transmembrane, cytoplasmic, and non-cytoplasmic domains (**Figure 4.5**). In the SP family, Tonoplast Monosaccharide Transporters (TMT) and two *Arabidopsis* inositol transporters (INT) had a relatively large central hydrophilic domain. All the INT group proteins except AtSP23 (AtINT1) had a distinctly large extracellular domain. The cluster C1 in the DHA1 family had a long N-terminal hydrophilic loop. Most of the POT (NPF) transporters had an irregular distribution of large hydrophilic domains and transmembrane domains. In the FBT family, some transporters had equally large C-terminal, N-terminal, and central hydrophilic loops, while some had a distinct N-terminal cytoplasmic loop. The frequency distribution of the number of TMH and the protein lengths was visualized by plotting the Sparklines in Microsoft Excel ver. 14.7.1 (**Figure 4.5 A, B**). The number of TMH in MFSS transporters was highly conserved, between 9 and 12 TMH in both the plants. More than 70% of MFSS transporters had a large central cytoplasmic loop flanked with five to seven TMH (**Figure 4.2**, **Figure 4.5A**). The remaining transporters were smaller in size except for five *V. vinifera* transporters, VvOCT4, VvPOT9 (VvNPF1.4), VvPOT20 (VvNPF8.1), VvPOT39 (VvNPF2.6) and VvPOT42 (VvNPF6.1) that had up to 29 TMH (**Figure 4.2**). The frequency distribution of protein lengths of MFSS transporters in grapes was more irregular than that in *Arabidopsis*.

4.3.5 Expression of MFSS genes across plant tissues and their subcellular localizations

The expression of MFSS genes was evaluated using pre-analyzed microarray datasets. Five *Arabidopsis* microarray studies (GSE5630, GSE5631, GSE5632, GSE5633, and GSE5634) and one grape microarray study (GSE36128) covering various tissues at different developmental stages were selected. The *Arabidopsis* microarrays had 183 MFSS genes, and the grape microarray had 203 MFSS genes. The expression of a majority of the genes was not tissue-specific; about 70% of MFSS transporters (161

from *Arabidopsis* and 138 from grapes) were expressed in all tissue types in *Arabidopsis* and grapes (**Figure 4.6**). Only a few genes were tissue-specific, e.g. *AtPOT48* (*AtNPF7.1*), *AtPHS7* (*AtPHT1;6*) and *AtSP18* (*AtPMT1*/ *AtPLT1*) were specific to flowers, *AtPHS4* (*AtPHT1;4*) was specific to root. Similarly, in grape, *VvPOT33*, *VvSP50* (*VvHT11*) and *VvOCT3* were specific to stem, *VvPOT24* (*VvNPF1.2*) to berries and *VvNNP3* to floral tissues. Three genes (*AtGPH1.7*, *AtNNP2*, and *AtNNP6*) from *Arabidopsis* and eight (*VvSP43*, *VvPOT34*, *VvSP32*, *VvPOT38*, *VvPHS7*, *VvPOT47*, *VvPOT9*, and *VvUMF23.22*) from grape had very low expression values, below the threshold value of transcribed genes in the studied tissues.

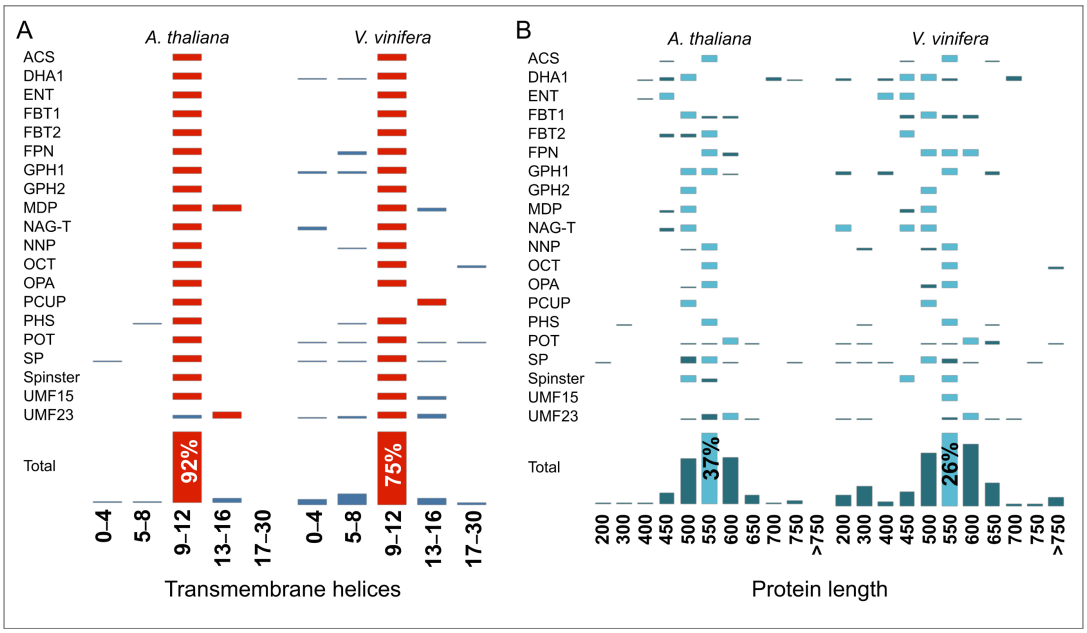


Figure 4.5 Protein topology overview

(A) Frequency distribution of the number of TMH in transporters of different MFSS subfamilies. The Red bar – Highest frequency. (B) Frequency distribution of protein lengths (number of amino acids) of transporters from different MFSS subfamilies. Light blue bar – Highest frequency.

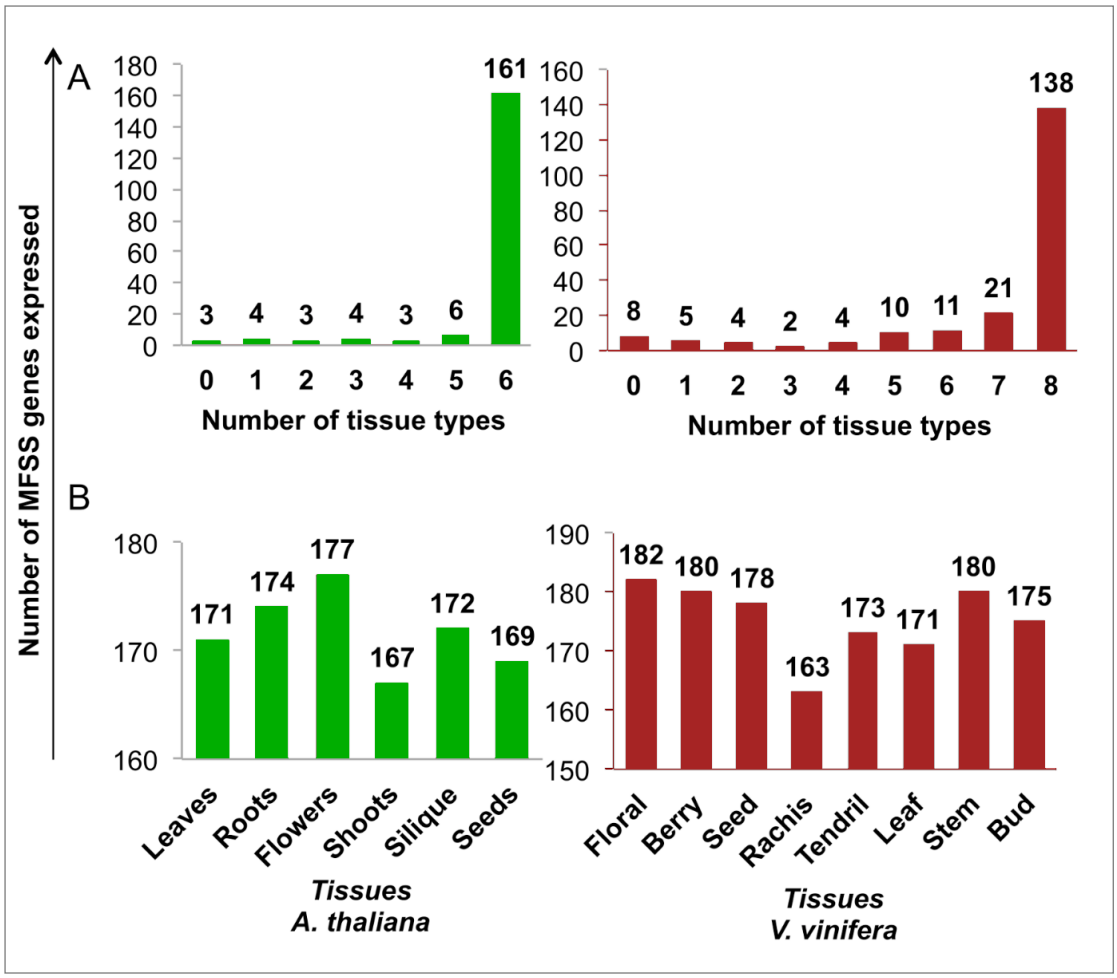


Figure 4.6 Expression of MFSS transporter genes in various tissues of *A. thaliana* and *V. vinifera*

(A) The number of MFSS transporters with multiple tissue specificity. (B) The number of MFSS transporter genes expressed in different tissue types.

Prediction of subcellular localization showed that more than 60% of the MFSS transporters were predicted as plasma membrane proteins, some were localized to the vacuole, chloroplast, Golgi bodies, and mitochondria; while about 20% lacked prediction (**Figure 4.7**). Interestingly, no MFSS transporter was localized to the nucleus, cytoplasm, endoplasmic reticulum, extracellular space or peroxisome. The GPH1, MDP, NAG-T, NNP, OPA, PCUP, PHS, POT and SP family proteins were predicted as plasma membrane-localized proteins with few exceptions; VvMDP2, VvPOT21 (VvNPF5.6), VvPOT23, AtSP13 (AtERDL5), AtSP14 (pGLT/ PLST2), AtSP17 (PLST3) and VvSP18 (VvpGlT) all had an unassigned subcellular localization. AtNNP5 (AtNRT2.7), VvSP23 (VvERD6-Like) and VvSP38 (VvERD6-Like) were

localized to the vacuole and AtSP52 (XYLL3) was the only sugar porter localized to the chloroplast. The OCT family proteins were localized to the vacuole except for AtOCT3 (AtOCT1) and VvOCT1, which were localized to the plasma membrane.

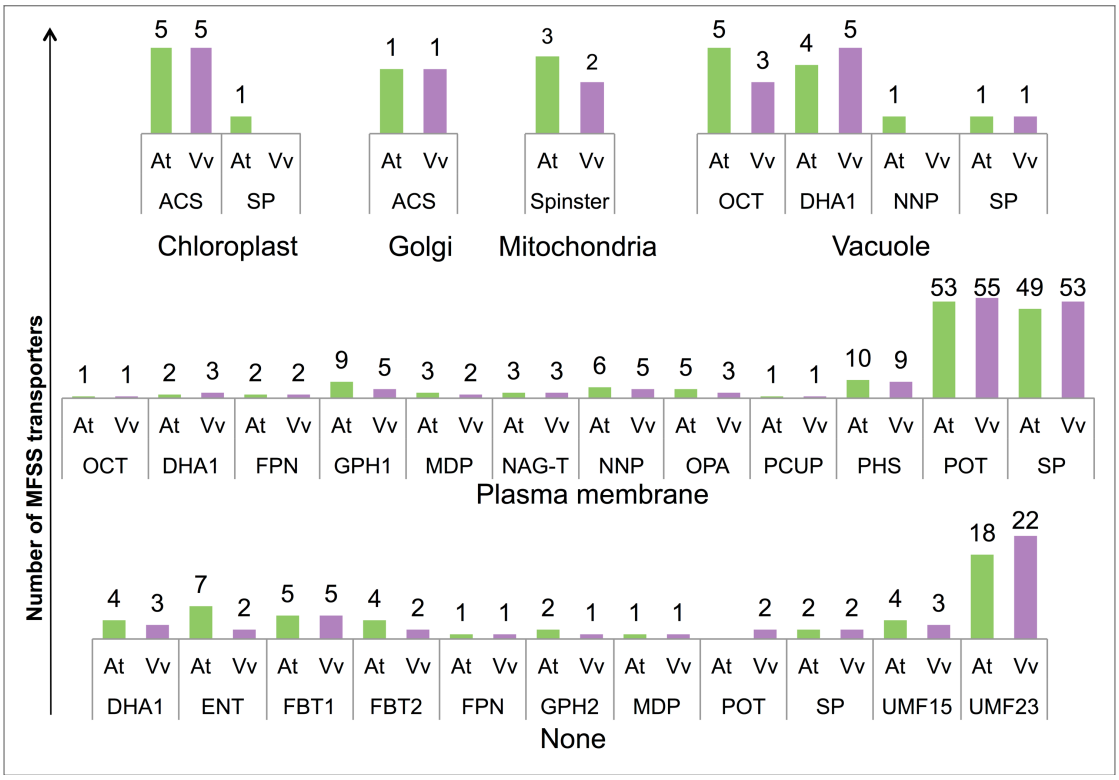


Figure 4.7 Subcellular localizations of predicted MFSS transporter proteins

The number of transporters predicted with different subcellular localizations is shown for each MFSS subfamily in *A. thaliana* and *V. vinifera*.

Likewise, the ACS family had chloroplast-localized transporters, except AtACS6 (AtPHT4;6) and VvACS1, which were localized to Golgi bodies, while the spinster proteins were mitochondria-localized. The DHA1 family had a combination of plasma-membrane-localized and vacuole-localized transporters and some with unidentified subcellular localizations. The transporters in FBT1, FBT2, GPH2, UMF15, and UMF23 families had no subcellular localization prediction. Further, the tissue specificity of these transporters was also explored using their expression values on microarrays (**Figure 4.8**). Surprisingly, the chloroplast-localized ACS transporters were significantly transcribed in green as well as non-green tissues like flowers and roots. Each tissue type had transporters from every MFSS subfamily expressed in them.

		ACS	DHA1	ENT	FBT1	FBT2	FPN	GPH1	GPH2	MDP	NAG-T	NNP	OCT	OPA	PCUP	PHS	POT	SP	Spinster	UMF15	UMF23
Plasma membrane	At Flower		2				2	8		3	2	3	1	5	1	5	48	45			
	At Leaf		2				2	7		3	2	4	1	5	1	4	47	42			
	At Root		2				2	6		3	2	4	1	5	1	6	48	43			
	At Seed		2				2	7		3	2	3	1	5	1	5	44	43			
	At Shoot		2				2	6		3	2	4		5	1	3	46	42			
	At Silique		2				2	7		3	2	3	1	5	1	5	45	44			
	Vv Berry Flesh		3				2	4		2	3	3		3	1	4	48	45			
	Vv Berry Pericarp		3				2	4		2	3	5	1	2	1	7	48	47			
	Vv Berry Skin		3				2	5		2	3	4	1	2	1	4	47	37			
	Vv Bud		3				2	4		2	3	3	1	3	1	5	45	47			
	Vv Flower		3				2	5		2	3	4	1	3	1	7	45	49			
	Vv Leaf		3				2	5		2	3	4	1	2	1	8	45	39			
	Vv Rachis		3				2	4		2	3	3	1	2	1	6	45	35			
	Vv Seed		2				2	4		2	3	4	1	3	1	7	45	46			
	Vv Stem		3				2	4		2	3	4	1	2	1	7	49	42			
	Vv Tendril		3				2	4		2	3	4	1	2	1	6	45	41			
Vacuole	At Flower		2									1	5					1			
	At Leaf		2									1	5					1			
	At Root		2									1	5					1			
	At Seed		2									1	5					1			
	At Shoot		2									1	5					1			
	At Silique		2									1	5					1			
	Vv Berry Flesh		3										2					1			
	Vv Berry Pericarp		3										3					1			
	Vv Berry Skin		4										2					1			
	Vv Bud		3										2					1			
	Vv Flower		5										2					1			
	Vv Leaf		5										2								
	Vv Rachis		5										2					1			
	Vv Seed		4										2					1			
	Vv Stem		5										3					1			
	Vv Tendril		5										2					1			
Chloroplast	At Flower	5																1			
	At Leaf	5																1			
	At Root	5																1			
	At Seed	5																1			
	At Shoot	5																1			
	At Silique	5																1			
	Vv Berry Flesh	5																			
	Vv Berry Pericarp	5																			
	Vv Berry Skin	5																			
	Vv Bud	5																			
	Vv Flower	5																			
	Vv Leaf	5																			
	Vv Rachis	5																			
	Vv Seed	5																			
	Vv Stem	5																			
	Vv Tendril	5																			
Golgi	At Flower	1																			
	At Leaf	1																			
	At Root	1																			
	At Seed	1																			
	At Shoot	1																			
	At Silique	1																			
	Vv Berry Flesh	1																			
	Vv Berry Pericarp	1																			
	Vv Berry Skin	1																			
	Vv Bud	1																			
	Vv Flower	1																			
	Vv Leaf	1																			
	Vv Rachis	1																			
	Vv Seed	1																			
	Vv Stem	1																			
	Vv Tendril	1																			
Mitochondrial	At Flower																		1		
	At Leaf																		1		
	At Root																		1		
	At Seed																		1		
	At Shoot																		1		
	At Silique																		1		
	Vv Berry Flesh																		2		
	Vv Berry Pericarp																		2		
	Vv Berry Skin																		2		
	Vv Bud																		2		
	Vv Flower																		2		
	Vv Leaf																		2		
	Vv Rachis																		2		
	Vv Seed																		2		
	Vv Stem																		2		
	Vv Tendril																		2		
NONE	At Flower		3	3	4	2	1		1									2		4	14
	At Leaf		3	3	4	2	1		1									2		4	13
	At Root		3	3	4	2	1		1									2		4	13
	At Seed		3	3	4	2	1		1									2		4	13
	At Shoot		3	3	4	2	1		1									2		4	13
	At Silique		3	3	4	2	1		1									2		4	14
	Vv Berry Flesh		2	2	5	2	1		1	1							2	2		3	19
	Vv Berry Pericarp		3	1	5	2	1		1	1							2	2		3	21
	Vv Berry Skin		3	2	5	2	1		1	1							2	2		3	21
	Vv Bud		3	2	5	2	1		1	1							2	2		3	20
	Vv Flower		3	1	5	2	1		1	1							2	2		3	20
	Vv Leaf		3	2	5	2	1		1	1							2	2		3	19
	Vv Rachis		3	1	5	2	1		1	1							2	1		3	20
	Vv Seed		3	2	5	2	1		1	1							2	2		3	21
	Vv Stem		3	2	5	2	1		1	1							2	2		3	21
	Vv Tendril		3	2	5	2	1		1	1							2	2		3	21

Figure 4.8 Tissue specificity of transporters from different MFSS subfamilies

The figure shows, for each subcellular localization, the number of transporters expressed per tissue type. Due to the expression of genes in multiple tissues the sum of genes in each family per subcellular localization exceeds the actual number in them

4.3.6 Differential expression of MFSS genes under abiotic and biotic stress conditions

The microarray experiments selected in the study (**Table 4.1**) were from four different microarray platforms, two *A. thaliana* (GPL12621, GPL198) and two *V. vinifera* (GPL17894, GPL1320). Altogether, 130 and 147 MFSS transporters were significantly differentially expressed in *Arabidopsis* and grapes, respectively (**Supplementary Table 7**). However, only eleven deMFSSTs had log2 fold change ≥ 2 (**Table 4.2**). The stress-induced expression fold change of MFSS transporter genes was smaller than that of other genes (**Figure 4.9**). The expression pattern of all the deMFSSTs was visualized using heat maps (**Figure 4.10**). The number of differentially expressed MFSS genes was highest in heat-stressed *Arabidopsis* (65) and *Botrytis cinerea* infected grape berries (57), 10 MFSS genes were differentially expressed in *B. cinerea* infected *Arabidopsis* leaves, and 57 MFSS genes in *B. cinerea* infected grape berries. The proportion of upregulated and downregulated genes was different for every stress condition studied. Among the studied conditions, the percentage of upregulated deMFSSTs was highest in salt-stressed *Arabidopsis* and grapes than in any other stress.

Table 4.1 Microarray datasets used in the present study

	GEO dataset	Stress	Plant	Treatment/ Pathogen	Tissue
A	GSE39956	Heat	*A. thaliana*	Heat stress, 4 h	Leaves
B	GSE53409	Heat	*V. vinifera*	Heat stress, 2 h	Green berries
C	GSE39956	Salt	*A. thaliana*	150mM NaCl, 16 h	Above ground parts
D	GSE31594	Salt	*V. vinifera*	120mM NaCl, 24 h	Shoot tips to 4th leaf
E	GSE3220	Biotrophic	*A. thaliana*	*Erysiphe cichoracearum*, 1 dpi	Leaves
F	GSE53824	Biotrophic	*V. vinifera*	*Erysiphe necator*, 11 dpi	Leaves
G	GSE5684	Necrotrophic	*A. thaliana*	*Botrytis cinerea*, 2 dpi	Leaves
H	GSE52586	Necrotrophic	*V. vinifera*	*Botrytis cinerea*, 7 dpi	Green berries

dpi – days post-inoculation

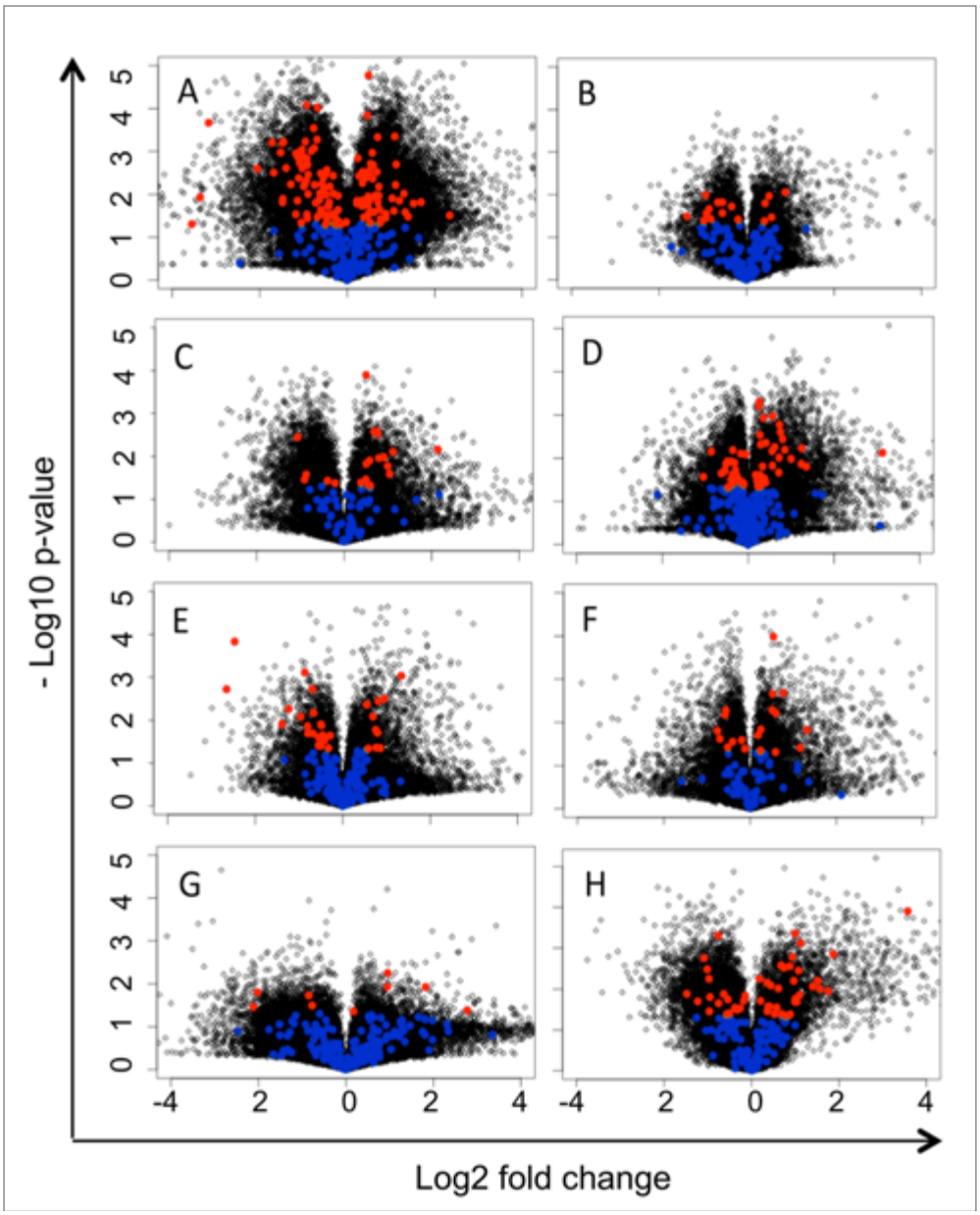


Figure 4.9 Volcano plots of significantly differentially expressed MFSS transporters in different stress conditions

Based on Student's t-test, the significantly differentially expressed MFSS transporter genes with p-value ≤ 0.05 (red) along with other MFSS transporter genes (blue) are shown on the background of all other genes (black) on an array. (A) Heat stress in *A. thaliana* leaves, (B) Heat stress in *V. vinifera* green berries, (C) Salt stress in *A. thaliana* leaves, (D) Salt stress in *V. vinifera* leaves, (E) *Erysiphe cichoracearum* infected *A. thaliana* leaves, (F) *Erysiphe necator* infected *V. vinifera* leaves, (G) *Botrytis cinerea* infected *A. thaliana* leaves, (H) *Botrytis cinerea* infected *V. vinifera* green berries.

Though the entire MFS Superfamily was not represented on microarrays, the number of deMFSSTs correlated well with the relative sizes of MFSS subfamilies. For instance, most of the deMFSSTs were from SP and POT families, each of which represented 27% of the superfamily. Other families represented less than 8% of the superfamily.

Table 4.2 Significantly differentially expressed MFSS transporters with p-value ≤ 0.05 and log2 fold change ≥ 2 or ≤ -2.

Plant	Stress	Gene	Previous gene name	Log2 fold change
Arabidopsis	Heat	*AtGPH1.5*	*AtSUC5*	-2.05
Arabidopsis	Heat	*AtNNP1*	*AtNRT2.1*	-3.17
Arabidopsis	Heat	*AtNNP3*	*AtNRT2.5*	-3.36
Arabidopsis	Heat	*AtPHS1*	*AtPHT1;8*	2.34
Grape	Salt	*VvPOT6*		2.14
Arabidopsis	Biotrophic	*AtPOT15*		-2.47
Arabidopsis	Biotrophic	*AtPOT29*		-2.65
Arabidopsis	Necrotrophic	*AtSP7*	*AtSTP1*	-2.12
Arabidopsis	Necrotrophic	*AtPOT24*		-2.02
Arabidopsis	Necrotrophic	*AtPHS3*	*AtPHT1;5*	2.77
Grape	Necrotrophic	*VvSP9*	*VvHT5*	3.57

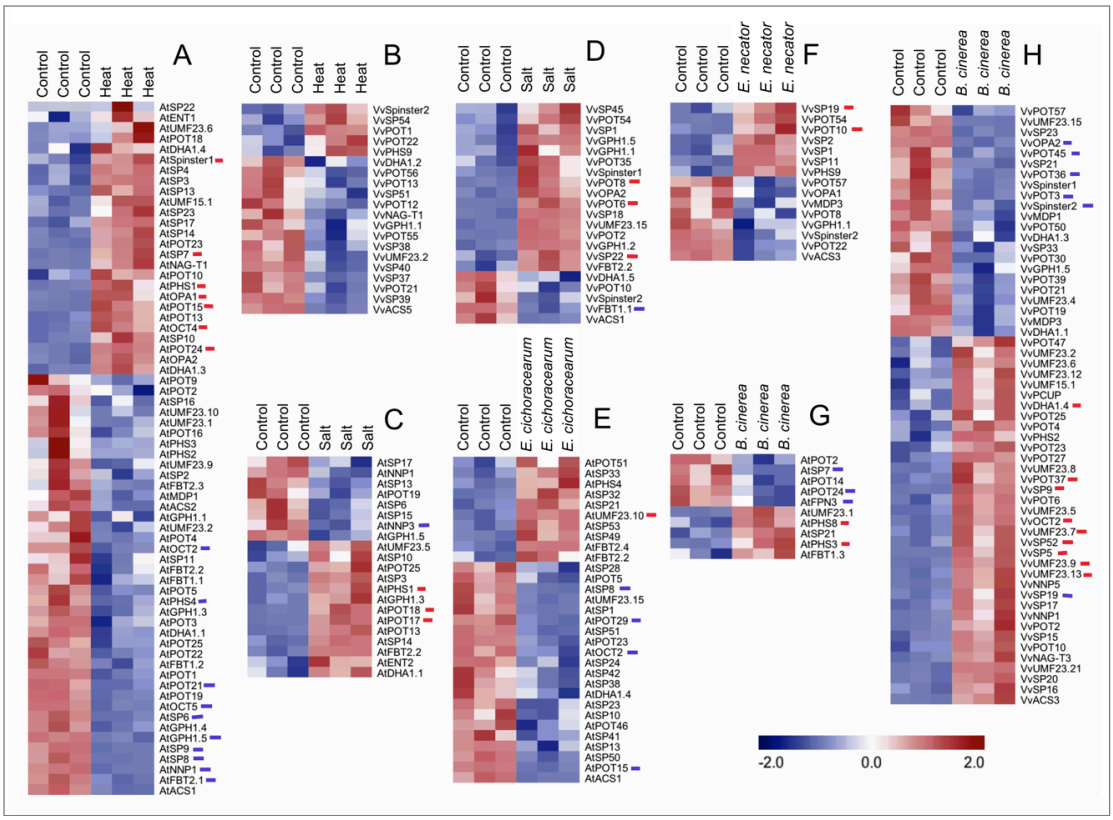


Figure 4.10 Expression patterns of significantly differentially regulated MFSS transporters (p-value ≤ 0.05 from Student's t-test) in response to abiotic and biotic stress conditions

The expression pattern of significantly differentially expressed MFSS transporter genes with p-value ≤ 0.05 (Student's t-test) was visualized using heat maps. Genes with fold change greater than 1.5 fold are indicated with red marks (upregulated) and blue marks (downregulated) (A) Heat stress in *A. thaliana* leaves, (B) Heat stress in *V. vinifera* green berries, (C) Salt stress in *A. thaliana* leaves, (D) Salt stress in *V. vinifera* leaves, (E) *Erysiphe cichoracearum* infected *A. thaliana* leaves, (F) *Erysiphe necator* infected *V. vinifera* leaves, (G) *Botrytis cinerea* infected *A. thaliana* leaves, (H) *Botrytis cinerea* infected *V. vinifera* green berries.

A mixed response of simultaneous upregulation as well as downregulation of transporters for the same type of substrate (i.e., same MFSS subfamily), was more prominent in SP and POT families (**Figure 4.10, Supplementary Table 8**). For example, in salt-stressed *Arabidopsis*, three SP transporters were upregulated, and another three were downregulated. In heat-stressed *Arabidopsis*, six sugar porters were upregulated and four were downregulated; moreover, eight POT transporters were upregulated and nine were downregulated. In *B. cinerea* infected grape berries, nine

POT transporters were upregulated and another nine were downregulated. The number of upregulated and downregulated POT transporters was almost equal in case of heat-stressed *Arabidopsis* and *B. cinerea* infected grape berries whereas, in salt stress, five POT transporters were upregulated and only one was downregulated grapevine, four POT transporters were upregulated and one was downregulated in *Arabidopsis*. Hence, the upregulation of POT transporters could be considered to be more prominent in salt-stressed *Arabidopsis* and grapevine leaf tissues.

4.3.7 Regulation of MFSS transporters during water and salt stress conditions

The transcriptome of salt stress experiment on TS110R grapevines in our study as detailed in Chapter 3, displayed the expression of 116 MFSS transporters with expression ≥ 1 FPKM (**Figure 4.11A**). Four MFSS transporters – VvPHS3, VvPOT27, VvUMF23.1, and VvUMF23.4, were significantly differentially expressed in salt-stressed TS110R grapevines. The expression of these genes was investigated in TSOR grapevines using qRT-PCR analysis (**Figure 4.11B**). All four transporters were highly upregulated in TSOR at 6 h of salt stress treatment and were gradually suppressed at later stages of stress. Whereas, in TS110R, two transporters were upregulated at 6 h and three on 7 d of salt stress. A previously reported microarray study (GSE31677) on water and salt-stressed grapevines displayed differences in the early and late stages of stress with upregulation of 41 MFSS transporters on 16 d of salt and water stress (**Figure 4.12**).

4.4 Discussion

4.4.1 Functional diversity of MFSS subfamilies across various organisms

The number of MFSS subfamilies in TCDB is far more than their numbers in individual organisms, and these might increase with gene annotation of newly sequenced organisms. TCDB currently has more than 80 MFSS subfamilies from different organisms; however, only 20 of them were present in *A. thaliana* and *V. vinifera.* Similarly, in yeast 17 MFSS subfamilies (Gaur et al., 2008) and in human 14 MFSS subfamilies have been reported (Quistgaard et al., 2016). The FPN/ Ferroportin family, known for the export of cellular iron to blood plasma in humans (Quistgaard et al.,

2016), is present in *A. thaliana* and *V. vinifera* but absent in yeast. Four MFSS subfamilies, SP, ACS, DHA1, and OCT, were common to all four of them suggesting their essential role in eukaryotic cells. However, SP and POT families were the largest in *Arabidopsis* and grape, while in yeast SP family was the largest followed by DHA1 and DHA2 families (Gaur et al., 2008) and OCT family is the largest in humans (Quistgaard et al., 2016). Though POT family is present in all, it is more expanded in *A. thaliana* and *V. vinifera* indicating their importance for nitrogen redistribution in plants (Léran et al., 2014). Likewise, the DHA families in yeast encode drug/ fungicide extruders that confer drug resistance to fungi (Conde et al., 2014; Gaur et al., 2008). Thus, the presence of organism-specific MFSS subfamilies or organism-specific expansion of MFSS subfamilies reflects their evolutionary significance with respect to the needs of the individual organism.

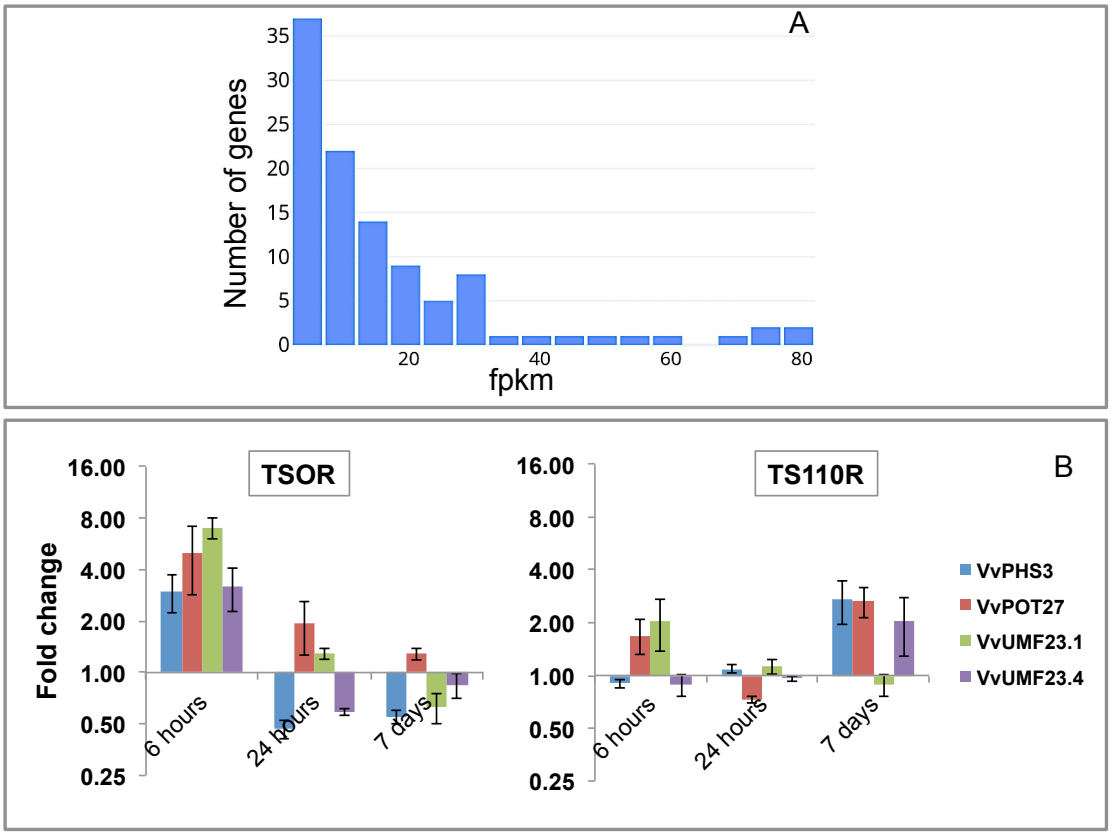


Figure 4.11 MFSS transporters in TSOR and TS110R grapevines

A – Histogram of expression values in FPKM of 116 MFSS transporters identified from transcriptome data of TS110R grapevines as studied in Chapter 3. B - Differentially expressed

MFSS transporters in TSOR and TS110R grapevines The y-axis is rescaled to logarithmic scale with base 2.

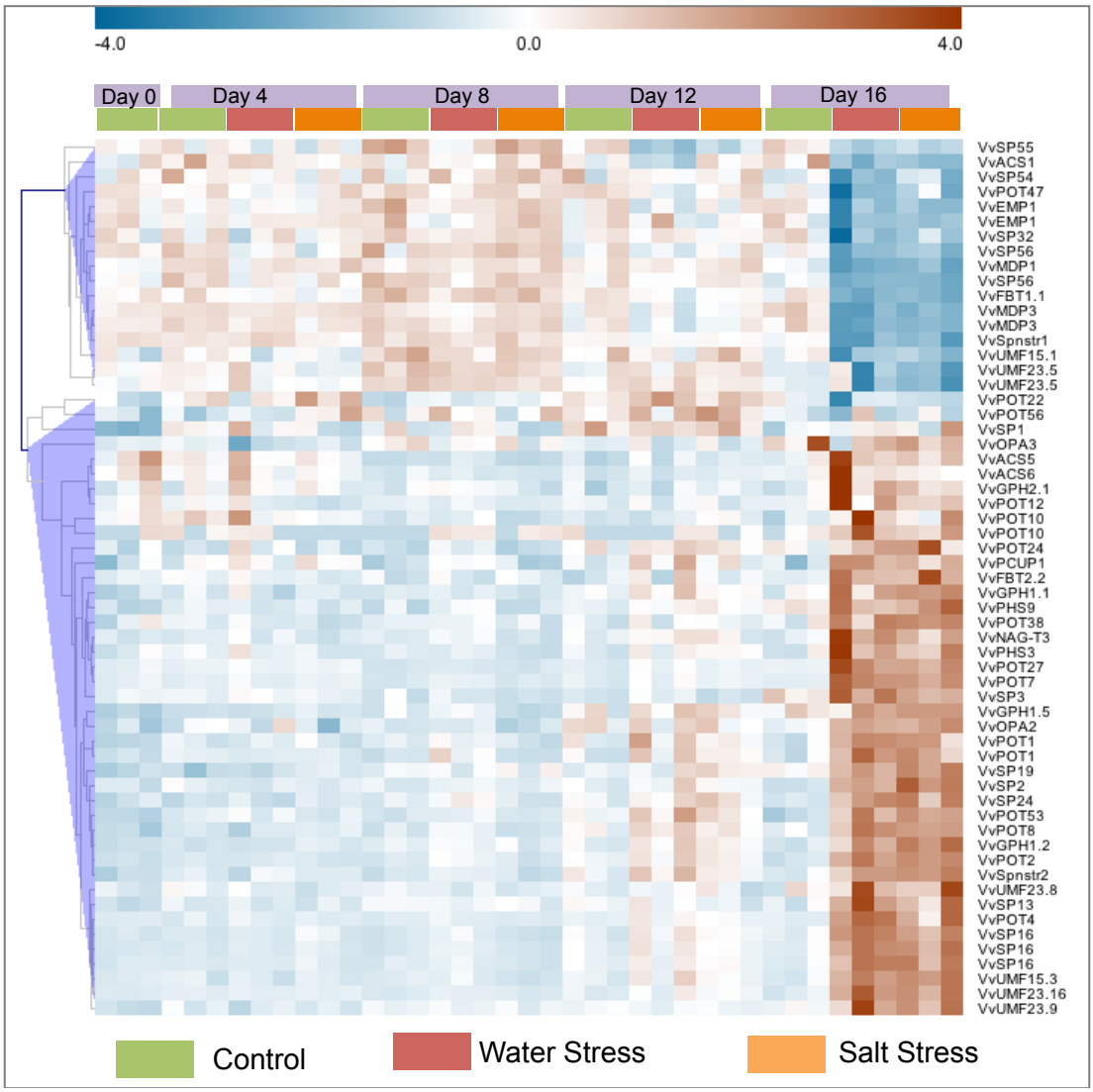


Figure 4.12 Expression pattern of MFSS transporters in response to water and salt stress

The heat map displays the expression values of MFSS transporters across individual control and stressed samples.

4.4.2 Varied nomenclatures of NNP and POT transporters

Plant nitrate transporters were earlier classified as low-affinity nitrate transporters (NRT1) and high-affinity nitrate transporters (NRT2) (Liu et al., 1999). TCID annotations of these NRT1 and NRT2 proteins from *Arabidopsis* identified them as POT and NNP family proteins, respectively. We identified 53 POT and 7 NNP proteins in *A. thaliana* and 57 POT and 5 NNP proteins in *V. vinifera*. This ratio of around 50

POT (NRT1) proteins and 4 NNP (NRT2) proteins is said to be conserved across angiosperms (von Wittgenstein et al., 2014). The POT and NNP are the only nitrate transporter (NRT) families in the MFS Superfamily. The POT/ NRT1/ PTR family proteins are also known to transport substrates like peptides, amino acids, dicarboxylates, glucosinolates, auxin, gibberellin, etc. (Komarova et al., 2008; Krouk et al., 2010; Li et al., 2015; Nour-Eldin et al., 2012; Tal et al., 2016). Therefore, another nomenclature study on NRT1 proteins renamed them as the NRT1/PTR family (NPF) transporters (Léran et al., 2014). A list of all NPF family and POT family transporters is given in the **Supplementary Table 7**.

4.4.3 Evolutionary ambiguity of NAG-T transporters

Phylogenetic clustering of proteins from two different organisms can reveal their evolutionary significance and functional relatedness. The substrate-specific family-wise clustering of all the predicted *A. thaliana* and *V. vinifera* MFSS transporters suggests their evolution from a common ancestral plant with preexisting substrate diversity. The unclustered NAG-T proteins indicate that they either evolved before all other MFSS families or they were introduced into plant genomes through horizontal gene transfer. In a previous study on NAG-T in yeast (Alvarez and Konopka, 2007), the plant Ngt1 homologues clustered closely with those from plant pathogenic fungi indicating a close similarity between fungal and plant Ngt1/ NAG-T homologues. Thus, the emergence of the NAG-T family in non-vascular plants and the presence of unclustered NAG-T transporters at the root of the phylogenetic tree of *A. thaliana* and *V. vinifera* suggests their probable origin through horizontal gene transfer from different pathogenic microbes. A cross-kingdom analysis of the NAG-T family is necessary to refine our understanding of the evolution of these transporters in plants, which might reveal the probable endosymbiont organism or pathogen responsible for its origin.

4.4.4 POT transporters are structurally more divergent and have poly-specificity to substrates

Closely clustered proteins with a significant branch support value in a phylogenetic tree are considered as homologous and structurally similar proteins. The POT and SP were the two largest families with many distinct sub-clusters in them. The SP family sub-clustering was exactly similar to the previously identified seven sub-groups STP, INT,

TMT, PLT/ PMT, pGlcT/ PLGT, XyloseTP/ VGT and ERD6-Like in *A. thaliana* and *V. vinifera* (Afoufa-Bastien et al., 2010; Johnson et al., 2006). Whereas, sub-clustering in the POT family in our research did not match with the previous study. The POT (NPF) family was earlier classified into eight NPF groups based on the clustering of sequences from 31 plants (Léran et al., 2014); however, only five of our POT family sub-clusters coincided with the five NPF groups- NPF1, NPF3, NPF4, NPF7, and NPF8. This suggests that sub-clusters of the SP family are more distinct than those in POT family indicating more structural variations in the POT transporters of both the plants.

Most of the POT transporters had an irregular distribution of large hydrophilic domains and transmembrane domains (**Figure 4.2**, **Figure 4.9**), which could be the reason for discrete distinction within the sub-clusters of the POT (NPF) family proteins. For instance, the glucosinolate transporters reported in *Arabidopsis*, AtPOT39 (GTR1/ AtNPF2.10) and AtPOT52 (GTR2 / AtNPF2.11) are said to have evolved from a tightly clustered low-affinity nitrate transporter AtPOT2, which has a weak affinity for glucosinolate transport (Nour-Eldin et al., 2012). However, the glucosinolate pathway is absent in grapes and the substrates transported by the two tightly clustered grape transporters VvPOT39 and VvPOT36 are not known. Thus, the multispecificity of POT transporters has helped both of these plants in evolving transporters with new substrate-specificity, which is mostly unexplored. Thus, the poly-specificity to substrates by structurally more divergent transporters indicates the late expansion of POT/ NPF family in comparison to the SP family.

4.4.5 Species specific SP and POT family expansion

The lengths of MFSS transporters was less uniform in *V. vinifera* than in *A. thaliana*, which could be due to the ancient nature of the *V. vinifera* genome having a large number of pseudogenes (Martin et al., 2010). The grapevine genome lineage has not undergone any genomic duplication after the formation of the palaeo-hexaploid genome, while the *Arabidopsis* genome lineage has undergone two whole genome duplication (WGD) events with many chromosomal rearrangements and gene losses (Jaillon et al., 2007). The percentage of WGD events was more evident in *A. thaliana* MFSS genes, while that of tandem and proximal duplications was more prominent in *V. vinifera* (**Figure 4.4**). Tandem duplicates are the paralogs that are adjacent to each

other on chromosomes, which result from the unequal crossing over of chromosomes (Cannon et al., 2004). While, proximal duplicates are the paralogs separated by less than 20 other genes (Wang et al., 2012), they are deduced to result from localized transposon activities or ancient tandem arrays interrupted by more recent gene insertions (Zhao et al., 1998). Thus, the relatively higher number of tandem duplications in grape SP and POT families indicates their species-specific expansion, probably during the domestication period after the formation of palaeo-hexaploid genome. The larger number of segmental duplications of the SP family in the *Arabidopsis* genome suggests their expansion through chromosomal or genome duplication events.

4.4.6 Structural features and their role in transporter protein stability

From the protein topology study, it was evident that the MFSS proteins had a central hydrophilic loop flanked by six to eight transmembrane helices. Twenty-six proteins with six or less than six TMH were identified in the present study. Such short proteins may form functional transporters after dimerization or trimerization with appropriate monomers. As reported previously, MFS transporter proteins have evolved from duplication and triplication of a single 'two transmembrane segment' (TMS) hairpin structure (Reddy et al., 2012). Furthermore, oligomerization of multiple TMS have also been reported to be functionally valid through several protein crystallization studies and protein interaction studies (Fan et al., 2015; Ludewig et al., 2003; Reinders et al., 2002; Safferling et al., 2003; Schulze et al., 2003; Sun et al., 2014; Tanabe et al., 2009; Veenhoff et al., 2001; Yuan et al., 2013). For example, phosphorylation induced dimerization of AtPOT1 (AtNPF6.3/AtNRT1.1) (Sun et al., 2014) reduces its nitrate affinity, while dimerization of shorter isoform of OsNRT1 shows higher nitrate affinity than its full-length isoform (Cubero et al., 2009). Thus, it can be said that the shorter (or truncated) transporters such as VvSP47, VvSP27, VvSP26, VvSP39, VvSP38, VvSP23, AtSP13, VvUMF23.22, VvUMF23.17, VvUMF23.18, VvUMF23.3, VvNNP2, VvGPH1.3, VvPOT51, VvPOT18, VvPOT19, VvPOT38, VvPOT8, VvDHA1.8, VvDHA1.6 and VvNAG-T1 (**Figure 4.2**) may show transporter activity after appropriate oligomerization. A distinctly large number of short MFSS transporters were present in the grape genome.

The presence of a large central cytoplasmic loop is the characteristic of MFSS transporters (Law et al., 2008). Structural studies have shown that the central large

cytoplasmic domain of MFSS proteins acts as a hinge during dimerization and appears like a cleft in the crystallized dimer of protein (Reinders et al., 2002; Sun et al., 2014). The protein topologies in the phylogenetic tree revealed that the lengths of these large cytoplasmic loops varied from family to family (**Figure 4.2**), and in some cases, they were extracellularly oriented. Several studies on MFSS protein structure have examined the role of these loops. A study on bacterial lactose permease marked the importance of its central hydrophilic loop for insertion into the plasma membrane (Weinglass and Kaback, 2000), while another study on sucrose transporters rejected this hypothesis (Reinders et al., 2002). The N-terminus of AtGPH1.6 (AtSUT2) determines the substrate affinity (Schulze et al., 2000), while truncation of the internal loop in a folate carrier does not affect its substrate affinity (Sharina et al., 2002). The extracellular domain of mammalian peptide transporters showed proteolytic activity (Beale et al., 2015) and the truncation of the extracellular plexins domain of INT transporters increased the *Vmax* of inositol uptake (Dotzauer et al., 2010).

These studies thus suggest that intracellular or extracellular large hydrophilic domains of MFSS transporters have diverse functions, such as insertion of the protein into the plasma membrane, interaction with other proteins, interaction with signaling molecules and regulation of substrate uptake rate; all of these factors may affect the substrate specificity of the transporter. Most of the POT (NPF) transporters had large intracellular or extracellular hydrophilic domains with a variable number of transmembrane domains (**Figure 4.2**, **Figure 4.5**), which could be the reason behind their poly-specificity to substrates (Lyons et al., 2014).

4.4.7 Stress-induced dynamics of the expression of MFSS transporters

Plants are complex multicellular sessile organisms and might require sudden metabolic fluxes to respond to changing environmental conditions. Microarray analysis gives an idea of the tissue-specific differential regulation of genes in response to a particular stress, where stress-induced upregulation of a gene is scored as upregulation of its phenotype. For example, abiotic stress-induced upregulation of redox enzymes suggests upregulation of antioxidative activity in the tissue. However, this is not applicable to transporter proteins. Upregulation of any solute transporter does not necessarily imply accumulation of that molecule in the tissue and *vice-á-versa*.

The MFS Superfamily in plants includes transporters of diverse small molecules like vitamins (folate), hormones (ABA and GA), nitrates, metal ions (phosphate, iron, cobalt, etc.) and several primary metabolites like monosaccharides, disaccharides, sugar-alcohols, amino-acids, nucleosides, di-peptides, tri-peptides, etc. These molecules may have different functions in different intracellular locations like cytoplasm, apoplast, and plastids, and the transporters might transport them across these locations within each cell. Therefore, it is not possible to conclude about the effect of any molecule by looking at its concentration in the tissue. Hence, to understand the effect of any transporter gene, multiple aspects have to be considered like its subcellular location, the orientation of substrate transport (influx or efflux) and its expression specificity to different cell types.

For example, increased chloride accumulation in shoots and roots in knockdown lines of a chloride efflux transporter AtPOT37 (AtNPF2.5) of root cortical cells has been reported (Li et al., 2017). However, downregulation of a chloride efflux transporter *AtPOT36* (*AtNPF2.4*) in root stele tissue was found in response to salt stress and ABA treatment, and its knockdown decreased chloride accumulation in shoots (Li et al., 2015). This indicates that AtPOT37 (AtNPF2.5) transports chloride ions away from the root symplast of cortical cells and prevents its symplastic transport to stele cells, and thus prevents its efflux into xylem from stele cells through AtPOT36 (AtNPF2.4). Therefore, simultaneous upregulation and downregulation of transporters of a family indicate the complexity of systemic trafficking of molecules from plastids, cytoplasm, symplast, apoplast, phloem, and xylem. The cumulative effect of these transporters needs to be considered to understand the phenotype or the metabolic flux. Stress-induced differential expression patterns of genes changes over a period of time depending on a multitude of factors like tissue type, plant type, plant growth phase (vegetative or reproductive), time of sampling (environmental conditions), stress level, etc.

The remobilization of inorganic phosphate (Pi) was evident from the expression modulation of Pi transporters during stress conditions. The two plasma membrane localized Pi – H+ symporters from the PHS (PHT1) family (Nussaume et al., 2011), *AtPHS4* (*AtPHT1;4*) and *AtPHS1* (*AtPHT1;8*), known for uptake of Pi from the rhizosphere and for root to shoot transport, respectively (Lapis-Gaza et al., 2014), had

contrasting expression patterns in the heat-stressed leaves. Two other plastidial Pi transporters (Guo et al., 2008), *AtACS1* (*AtPHT4;1*) and *AtACS2* (*AtPHT4;2*), were downregulated in heat-stressed leaves, suggesting inhibition of Pi uptake into chloroplasts; in addition, *AtPHS3* (*AtPHT1;5*) and *AtPHS8* (*AtPHT1;1*) were upregulated in *B. cinerea* infected leaves. However, in leaf, the cell-specificity of these transporters may be different, and thus it is difficult to comment on the direction of Pi translocation. The complete understanding of Pi remobilization is incomplete without knowing the expression of the other plastidial Pi transporter PHT2, which is also a Pi:H+ symporter (TC# 2.A.20) but it is not included in the MFS Superfamily in TCDB.

In eukaryotes, folate metabolism is compartmentalized between cytoplasm and cell organelles with the highest concentration in mitochondria (Gambonnet et al., 2001; Jabrin et al., 2003) and thus requires organelle-specific transporters for folate redistribution inside cells. A knock-down of *AtFBT1.2* (*AtFOLT1*), a chloroplast localized transporter, causes accumulation of folate in the chloroplast (Klaus et al., 2005), and therefore downregulation of this transporter and three other folate transporters suggests probable accumulation of folate in chloroplasts during heat stress.

The upregulation of POT (NPF/ NRT1) transporters was prominent in salt-stressed *Arabidopsis* and grapevine leaves. These salinity induced plasma membrane-localized transporters, AtPOT25 (AtNPF5.1), AtPOT18 (AtNPF5.13), AtPOT17 (AtNPF5.14) and AtPOT13 (AtNPF3.1), might be involved in nitrate uptake when expressed in leaf laminar cells. One of them, AtPOT13 (AtNPF3), is known for GA uptake in the root endodermal cells in response to ABA and NaCl treatment (Tal et al., 2016). Moreover, the combined application of nitrogen and GA_3 has been shown to alleviate the adverse effects of salt stress in *Brassica napus* (Siddiqui et al., 2008). Therefore, AtPOT13 (AtNPF3) can be considered as a salt stress-induced nitrate or GA_3 uptake transporter in leaves. A similar function may be associated with the upregulation of transporters from POT (NPF/ NRT1) family in salt-stressed grapevine leaves. However, no such mutant studies are available for grapevines.

The upregulation of SP transporters was prominent in salt-stressed grapevine leaves, which may be responsible for glucose remobilization during stress since these were the plastidial glucose transporters (Zeng et al., 2013). Their closest *Arabidopsis* homolog, AtSP1 (PLST1), is predicted to be a chloroplast localized glucose exporter

(http://www.uniprot.org/uniprot/Q0WVE9). Therefore, it is possible that salt-stress induces glucose remobilization to the cytoplasm for instant energy to trigger stress responses. Derivatives of inositol have been identified as molecules that confer tolerance to various types of abiotic stress in plants (Conde et al., 2014; Nelson et al., 1999; Sambe et al., 2015); therefore upregulation of a plasma membrane-localized inositol:H+ symporter *AtSP10* (*AtINT2*) (Schneider et al., 2007) in heat and salt stresses implies the uptake of inositol and its derivatives into the cytoplasm during these stress conditions in *Arabidopsis*.

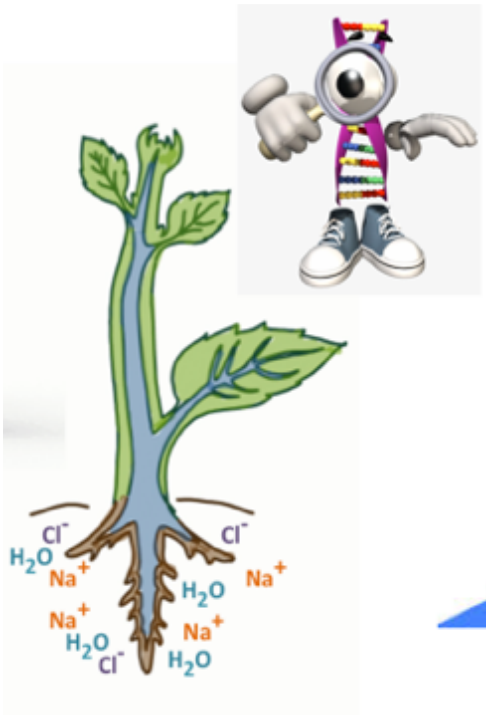
Every stressed plant displays chlorosis. It has been observed that the lack of *AtFPN1* (*AtIREG1*), a plasma membrane-localized iron exporter in vascular cells, or overexpression of *AtFPN3* (*AtIREG3/MAR1*), a chloroplast localized iron and cobalt transporter, causes chlorosis in *Arabidopsis* by decreasing the iron and cobalt reserves in the leaves (Conte et al., 2009; Morrissey et al., 2009). Thus, downregulation of *AtFPN1* (*AtIREG1*) in heat-stressed *Arabidopsis* and upregulation of *AtFPN3* (*AtIREG3/MAR1*) in *B. cinerea* infected *Arabidopsis* indicates the probable onset of chlorosis under these stress conditions.

4.5 Conclusions

This study provides comprehensive identification and classification of MFSS transporters in *Arabidopsis* and grape genomes. The phylogenetic and gene duplication analyses revealed the evolutionary mechanisms contributing to the expansion of MFSS families. The transmembrane topology displayed with the phylogenetic tree provided insights into conserved and family-specific features of the topology. The microarray studies revealed that these transporters were expressed in all tissue types with very little tissue specificity and were mostly plasma-membrane localized transporters. Furthermore, expression modulation in response to biotic and abiotic stress conditions revealed that the percentage of upregulated transporters was higher in salt-stressed plants than in other stress conditions.

Chapter 5

Summary and Future Prospects



Chapter 5 Summary and Future prospects

5.1 Summary

Grapevines are perennial woody plants cultivated across the world, mainly for wine production and for the consumption of fresh grapes or processed raisins. However, viticulture has been facing various environmental threats like salinity stress, drought, and high temperature. Soil salinity is becoming a significant problem for the viticulture industry because of poor vine growth and severe foliar damage, leading to a drastic reduction in the productive lifespan of grapevines. Salinity also affects fruitfulness and berry maturation in grapevines leading to a decrease in berry quality and yield. Thompson Seedless is a popularly cultivated table grape variety in India. However, it is highly susceptible to salt stress when grown on its own roots. Therefore, grafting of these vines onto salt tolerant rootstocks was introduced in India. Currently, Dogridge and 110R rootstocks are being employed to combat water stress, and soil and water salinity problems in the country. Moreover, 110R was recently identified as the most potent salt tolerant rootstock. Therefore, in the present study, we investigated the differences in response to salinity between own-rooted Thompson Seedless (TSOR), and 110R grafted Thompson Seedless (TS110R) grapevines to identify salt tolerance related molecular adaptations in TS110R vines.

The salinity-stress experiment was conducted in collaboration with the National Research Centre for Grapes (NRCG), Pune. Sixteen-month old potted grapevines were subjected to prolonged salt stress by treating them with 150 mM NaCl solution. The salt stress-induced growth retardation was observed in both the vines. However, it was more prominent in TSOR vines. The shoot growth of TS110R was unaffected until ten days of stress, while that of TSOR was aborted within four days, indicating the ability of TS110R vines to tolerate the stress for a relatively long period. A sudden and consistent rise of sodium and chloride ions into the shoots was observed in TSOR, while TS110R suppressed it till fifteen days; suggesting exclusion of ions by the rootstock, which could be its primary mechanism of salt tolerance. The molecular response to salt stress was investigated by comparing the proteomes and transcriptomes of salt-stressed vines against that of control vines using non-targeted proteomics and RNA sequencing approaches, respectively. Furthermore, a high-throughput *in-silico* analysis of Major

Facilitator Superfamily, a transporter protein superfamily, was carried out to identify novel transporters involved in salt stress response in plants.

5.1.1 Salinity induced proteomic changes in TSOR and TS110R grapevines

Differential expression of proteins in response to salt stress was determined using untargeted, label-free shotgun proteomics approach. The gene ontology enrichment analysis and KEGG pathway analysis of significantly differentially expressed proteins predicted several similarities and dissimilarities in salinity response of TSOR and TS110R grapevines. The comparison between unstressed TSOR and TS110R vines identified significant over-representation of proteins involved in the synthesis of nitrogenous compounds as well as in oxidation-reduction processes. These biological processes are usually upregulated in response to stress conditions; hence, their abundance in unstressed TS110R vines was predicted to be responsible for tolerating the grafting induced oxidative stress. The comparison between stressed and unstressed vines revealed that TSOR vines were subjected to higher oxidative stress than TS110R vines. A redox signaling through regulation of methylglyoxal and glutathione levels was predicted in TSOR vines which was absent in TS110R.

Similarly, the upregulation of photorespiration along with chloroplastic glutamine synthases in TSOR vines indicated the occurrence of severe photo-oxidative stress in them. Whereas in TS110R, these proteins were unaffected or progressively decreased with increasing duration of stress, implying efficient acclimation to stress. This could be due to the preconditioning of TS110R vines to grafting induced upregulation of proteins being involved in the synthesis of nitrogenous compounds and oxidation-reduction processes. These processes were readily available for the recycling of ammonia and detoxification of redox stress, respectively in salt-stressed TS110R vines. This was further supported by early upregulation of translation process in TS110R, which was upregulated in TSOR as well but at the mid-stage of stress.

The salt-treated TSOR vines displayed increased CO_2 assimilation rate along with increased photosynthesis-related proteins in response to salt stress, which were largely unaffected in TS110R vines. However, despite increased photoassimilation, TSOR vines displayed early retardation of growth as compared to that of TS110R vines.

This could be due to the channeling of photosynthates towards stress tolerance in TSOR vines. Additionally, the difference in stomatal behavior of TS110R and TSOR vines was evident from the differences in transpiration rate and stomatal conductance. Salinity induced an immediate decrease in transpiration rate and water conductance in TS110R vines, which is a characteristic of isohydric behavior, suggested that grafting on 110R rootstock had transformed the stomatal behavior of Thompson Seedless vines. The differential regulation of proteins involved in stomatal regulation, in the two vines, further supported this hypothesis.

Overall, the results indicated that TSOR vines responded fervently to stress, while TS110R vines adopted a preventive approach to oxidative stress. The findings of this study contribute to the knowledge of salinity response in woody and grafted plants and open the scope for further studies on salt stress-specific differences induced by grafting

5.1.2 Transcriptomic changes in salt-stressed TS110R grapevines

The present study focused on the transcriptome of salt-tolerant TS110R vines to understand the transcript expression changes occurring in parallel to the changes in protein expression. RNA sequencing was used for studying the transcriptomes of control and stressed vines at three time points covering early (6 h), mid (24 h) and late (7 d) stages of stress. The results of statistical analysis and bioinformatic functional categorization enabled the identification of significantly altered biological functions in response to salt stress. A massive enrichment of transcription factors indicated the onset of adaptation to stress through regulation of stress-specific gene expression. Several abiotic stress-related genes such as Dehydrins and LEAs were upregulated in stressed plants. Temporal differences were observed in stress-induced hormonal signaling. The induction of ABA synthesis and signaling and inhibition of GA and auxin signaling was evinced from differentially expressed genes (DEGs) at 6 h, which indicated that the early stage of stress represented the growth inhibition or quiescent phase of salt stress.

Similarly, the late-stage represented the growth recovery phase of salt stress, wherein genes involved in degradation or suppression of ABA signaling were upregulated; while the GA degrading enzymes were downregulated. The extracellular genes involved in elongation and modification of cell wall also displayed temporal

regulation. The cell wall lignification was upregulated during the quiescent phase as revealed from increased expression of extracellular peroxidases while cell wall elongation was prominent at the mid-stage, which was followed by elongation of the cell wall at growth recovery phase. Similarly, photosynthesis also displayed temporal regulation with downregulation at the early stage, which was upregulated at the late phase. The findings of this study add to the knowledge of temporal regulation of salinity response of grapevines and open the scope for further studies on their control though transcription regulation.

5.1.3 Comparison of transcriptome and proteome results

The biological processes enriched from the DEGs were different from those represented by the differentially expressed proteins (DEPs), which indicated that the number of transcripts and proteins of a gene are not always equally abundant in a cell. The transcription factors were highly over-represented in DEGs but not in DEPs, which could be due to the technical limitations of the proteomics technology in detecting the low abundant proteins. Photosynthesis was the only GO term significantly enriched in both DEGs and DEPs; however, the genes displayed contrasting expression patterns. While the transcriptome exhibited a steady increase in expression of the photosynthesis-related genes; the respective proteins were upregulated at an early stage and downregulated at a late stage. This indicated that the regulation of these genes at transcriptome and proteome levels might have feedback control.

5.1.4 Major facilitator superfamily transporters involved in salt stress response

We also performed phylogenetic and microarray analysis of the Major Facilitator Superfamily (MFS) proteins in *A. thaliana* and *V. vinifera*, highlighting their substrate diversity and physiological significance. MFS is the largest superfamily of secondary transporters across all types of organisms, from single-celled prokaryotes to multicellular eukaryotes, which transports diverse molecules like sugars, sugar-alcohols, vitamins, amino acids, oligopeptides, hormones, etc. across cell membranes. Homology-based characterization and Transporter Classification Database-based annotation of this superfamily proteins enabled identification of several known as well as novel transporter families in plants. Expression analysis of these transporters in 13 microarray experiments, along with extensive literature mining on functional studies in

Arabidopsis, helped us to elaborate on the functional significance of these transporters under various stress conditions.

We identified 213 MFSS genes in *A. thaliana* and 203 in *V. vinifera*, which were classified into six families and 20 subfamilies under the MFS Superfamily. The Sugar Porter (SP) and the Proton dependent Oligopeptide Transporter (POT) families were the largest of all, with more than 50 genes in them. The analysis of secondary protein structure revealed the transmembrane topology of these proteins and identified the similarities and differences among the transporters of different MFSS subfamilies. The majority of these proteins were plasma membrane-localized with few exceptions and had muti-tissue specificity. The percentage of upregulated MFSS genes was relatively higher in response to salt stress than in other conditions in the studies microarrays. The expression of transporters of sugars, nitrates, oligopeptides, and phosphates along with some transporters of unknown substrates like UMF23 transporters was induced in response to salt stress in the studies using *Arabidopsis* and grape microarrays. Our transcriptomic analysis of TS110R grapevines also identified the expression of 116 MFSS genes, of which the expression of four genes was altered in response to salt stress, which included a phosphate and an oligopeptide transporter, along with two UMF23 transporters. These four genes displayed different expression patterns in the two grapevines. Overall, this study adds to the knowledge of functional and structural diversity and evolution of MFSS transporters in *Arabidopsis* and *Vitis* and opens the scope for detailed physiological and functional studies on these transporter proteins.

5.2 Future prospects

The present study provides an in-depth insight into the molecular changes occurring within the salt-stressed grapevines. The findings from this study have predicted several mechanisms of salinity tolerance in grapevines, which can be investigated in detail in the future.

5.2.1 Transcriptomic study of salinity stress in 110R roots

The 110R rootstock is known for its ability to exclude salts, which was observed in our study as well. It is, therefore, necessary to conduct transcriptomic research on the 110R roots in comparison with Thompson Seedless roots to unravel the molecular mechanisms of salt exclusion in them.

5.2.2 Investigation of root to shoot signaling of stress

The 110R grafted grapevines did not uptake salts till 15 days, and yet they displayed salt stress response at the molecular level in leaves. This suggests the presence of root to shoot signaling of salt stress in them. Therefore, a comparative study on the xylem sap of grafted and non-grafted grapevines is needed to identify the rootstock-specific chemical or molecular signals transported in response to salt stress.

5.2.3 Investigation of grafting induced change in stomatal behavior

The physiological observations in the present study revealed that the water conductance and transpiration rate of 110R grafted Thompson Seedless grapevines significantly dropped in response to salt stress, which is contrasting to the normal anisohydric behavior of own-rooted Thompson Seedless vines. This indicated a grafting induced modification of stomatal behavior in grapevines. However, this finding needs further validation through a thorough investigation of physiological parameters.

5.2.4 Prediction of substrates transported by UMF23 transporter

The previously studied salt stress in grapevines, as well as our transcriptomic study, identified upregulation of Unknown MFS 23 (UMF23) family transporters in response to salt stress. However, their substrate specificity is not known yet. Therefore, biophysical studies such as expression in *Xenopus* oocytes need to be conducted for these transporters to identify their substrate specificity.

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Supplementary data

The supplementary data is being provided in a CD.

- Supplementary table 1: The list of primers used for qRT-PCR analysis
- Supplementary table 2: The list of total proteins identified along with corresponding protein group and the number of peptides detected
- Supplementary table 3: The list of differentially expressed proteins across three time points 6 h (6H), 48 h (48H) and 7 d (7D) in TSOR and TS110R vines. The bold red values are the upregulated DEPs (fold change ≥ 1.5, p-value ≤ 0.05, q-value ≤ 0.05) and the bold blue values are the downregulated DEPs (fold change ≤ 0.7, p-value ≤ 0.05, q-value ≤ 0.05)
- Supplementary table 4: The list of differentially expressed genes across three time points 6 h (6H), 48 h (24H) and 7 d (7D) in TS110R vines. The bold red values are the upregulated DEGs (log2 fold change ≥ 1.3 in both GTT and Cufflinks analyses) and the bold blue values are the downregulated DEGs (log2 fold change ≤ -1.3 in both GTT and Cufflinks analyses)
- Supplementary table 5: Expression pattern of transcription factors clustered under the term Transcription factor activity across three time points 6 h (6H), 48 h (24H) and 7 d (7D) in TS110R vines. The bold red values are the upregulated DEGs (log2 fold change ≥ 1.3 in both GTT and Cufflinks analyses) and the bold blue values are the downregulated DEGs (log2 fold change ≤ -1.3 in both GTT and Cufflinks analyses).
- Supplementary table 6: Accession numbers of MFSS transporters and their alternative gene names
- Supplementary table 7: List of significantly differentially expressed MFSS transporters (p-value ≤ 0.05) displayed in the heat map (Figure 4.10)
- Supplementary table 8: POT family transporters with corresponding nomenclature in NPF family

Curriculum Vitae

Curriculum Vitae

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Degrees Obtained	Board/University	Year
M.Sc. Biotechnology	University of Pune	2012
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Research Experience

Research experience at CSIR-NCL as JRF (2 years) and SRF (till date) on "**Functional Analysis of Salt Stress Response in Grapevines**" project. Technical expertise achieved in molecular biology (Protein extraction, label free shotgun proteomics and statistical analyses), Transcriptomic analysis (quality check of raw fastq files, transcript quantitation and statistical analyses), and Bioinformatics (Gene family identification, annotation of genes, meta-analysis of microarray data sets).

Specialized Skills

- **Proteomics**
  - Protein extraction from plant tissues
  - Data acquisition on mass spectrometer TripleTOF, (ABSCIEX) and Synapt HDMS (Waters)
  - Data analysis using different softwares (PLGS, ProgenesisQIP, Protein Pilot)
- **Transcriptomics**
  - RNA extraction
  - RNAseq data analysis using tools like Cufflinks and Trinity
  - Gene expression profiling- qPCR analysis
- **Bioinformatics**
  - Using R tools, Perl and python scripts for handling and analyzing RNA and protein sequences
  - Local BLAST installation and execution on P.C.
  - Gene Ontology analysis – Cytoscape, BiNGO, ClueGO
- **Phylogenetic study**
  - Evolutionary analysis of a gene families - MCScanX
  - Phylogeny – PhyML, RaXML, iTOL
- **Training students**
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Publications

- **Patil, Sucheta S.**, Ramya Prashant, Narendra Y. Kadoo, Anuradha Upadhyay, and Vidya S. Gupta. "Global study of MFS superfamily transporters in arabidopsis and grapes reveals their functional diversity in plants." Plant Gene (2019): 100179 (https://doi.org/10.1016/j.plgene.2019.100179)
- **Patil Sucheta**, Kadoo Narendra, Upadhyay Anuradha, Shinde Manisha, Prashant Ramya, Gupta Vidya. “Comparative Proteomics Unravels the Differences in Salt Stress Response of Own Rooted and 110R Grafted Thompson Seedless Grapevines.” Journal of Proteome Research (2019) (https://doi/abs/10.1021/acs.jproteome.9b00420)

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Posters presented

1. Patil Sucheta, Prashant Ramya, Kadoo Narendra, Upadhyay Anuradha and Gupta Vidya (2014) Genomic Analysis of a Transporter Protein Superfamily presented at CSIR-National Chemical Laboratory, Pune on the "National Science Day" 2014
2. Patil Sucheta, Manisha Shinde, Prashant Ramya, Jadhav Uma, Kadoo Narendra, Upadhyay Anuradha and Gupta Vidya (2016) Functional Analysis of Salinity Stress Response in Grapevines presented at CSIR-National Chemical Laboratory, Pune on the "National Science Day" 2016
3. Patil Sucheta, Prashant Ramya, Kadoo Narendra, Gupta Vidya (2017) Genome Wide Study of Major Facilitator Superfamily Transporters in *Arabidopsis thaliana* and *Vitis vinifera*. "Accelerating Biology 2017: Delivering Precision", conducted by C DAC, Pune from January 17-19, 2017 [Poster presentation]
4. Patil Sucheta, Prashant Ramya, Kadoo Narendra, Gupta Vidya (2017) Exploring the behaviour of transporter proteins in response to abiotic stress

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5. Patil Sucheta, Manisha Shinde, Kadoo Narendra, Upadhyay Anuradha and Gupta Vidya (2018) Integration of Transcriptomic and Proteomic Analyses for Identification of Molecular Responses in Salt Stressed Grapevines. "International Conference on Proteomics for Cell Biology and Molecular Medicine", conducted by Proteomics Society of India from Dec 12-14, 2018

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Plant Gene 18 (2019) 100179

Contents lists available at ScienceDirect

Plant Gene

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

Global study of MFS superfamily transporters in arabidopsis and grapes reveals their functional diversity in plants

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ARTICLE INFO

Keywords:
Major facilitator superfamily
Transporter family
Phylogeny
Transmembrane topology
Gene duplication
Biotic and abiotic stress

ABSTRACT

The Major Facilitator Superfamily (MFS) is the largest superfamily of secondary transporters present in all organisms, from prokaryotes to higher eukaryotes, that facilitates transport of diverse molecules like sugars, vitamins, amino-acids, hormones, etc. across cell membranes. The superfamily was further expanded to MFS Superfamily (MFSS) to integrate MFS with nine more families. The present study revealed their land plant specific diversity through identification across six species from unicellular alga to higher flowering plants. We identified 71, 131, 254, 260, 213 and 203 MFSS transporters in *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa* (var. Japonica), *Arabidopsis thaliana* and *Vitis vinifera*, respectively and classified them into MFSS families and subfamilies based on their transporter classification identifiers (TCIDs). Detailed analysis of 20 land plant specific subfamilies was conducted in *A. thaliana* and *V. vinifera*. Phylogenetic and gene duplication studies revealed the expansion of sugar porter and proton dependent oligopeptide transporter families in *Arabidopsis* and grape. The subcellular localization of the majority of the transporters was predicted to be in the plasma membrane. Furthermore, the microarray expression analysis of MFSS transporters from *Arabidopsis* and grapes revealed their multi-tissue-specificity and differential regulation under biotic and abiotic stress conditions. Studies of the transmembrane topology highlighted the presence of central cytoplasmic loop along with family specific topological variations that were evident from phylogenetic analysis. Overall, this study adds to the knowledge of functional and structural diversity and evolution of MFSS transporters in plants and opens the scope for detailed physiological and functional studies on these proteins.

1. Introduction

In living cells, transporter proteins play important roles like uptake of nutrients, maintenance of membrane potential, extrusion of toxic compounds, etc. The studies on microbial transporter proteins have shown that about one-third of the proteins of a cell are embedded in biological membranes, and one-third of them function to catalyze the transport of molecules across the membranes (Paulsen et al., 1998). For example, the *E. coli* genome encodes about 300 transporter proteins that constitute 7% of the proteome. Similarly, in *Arabidopsis thaliana*, 2.5% of the proteome consists of transporter proteins (Arabidopsis Genome Initiative, 2000; Blattner et al., 1997), while in *Vitis vinifera*, 7% of the predicted genes are identified as transporter proteins (Grimplet et al., 2012). Thus, a small percentage of every genome encodes a repertoire of diverse transporter proteins that catalyze the transport of various substrates based on extra- and intra-cellular cues.

Transporter proteins are of two types, channel proteins and carrier proteins. Channel proteins transport a solute down its electrochemical gradient, while the carrier proteins actively translocate substrates against the concentration gradient (Busch and Saier, 2002; Saier, 1999). Based on the source of energy utilized by the carrier proteins, they are classified as primary active transporters and secondary active transporters. The primary active transporters utilize the energy of ATP hydrolysis, photon absorption, substrate decarboxylation or methyl

Abbreviations: MFS, Major Facilitator Superfamily; MFSS, MFS Superfamily; MFSST, MFS Superfamily Transporter; TCDB, Transporter Classification Database; TCID, Transporter Classification Identifier; TMH, Transmembrane helices

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https://doi.org/10.1016/j.plgene.2019.100179
Received 31 January 2019; Received in revised form 1 March 2019; Accepted 21 March 2019
Available online 22 March 2019

Journal of proteome research
Cite This: J. Proteome Res. XXXX, XXX, XXX−XXX
Article
pubs.acs.org/jpr

Comparative Proteomics Unravels the Differences in Salt Stress Response of Own-Rooted and 110R-Grafted Thompson Seedless Grapevines

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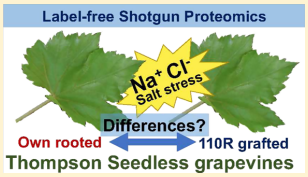
Supporting Information

ABSTRACT: Thompson Seedless, a commonly grown table grape variety, is sensitive to salinity when grown on its own roots, and therefore, it is frequently grafted onto salinity-tolerant wild grapevine rootstocks. Rising soil salinity is a growing concern in irrigated agricultural systems. The accumulation of salts near the root zone severely hampers plant growth, leading to a decrease in the productive lifespan of grapevine and causing heavy yield losses to the farmer. In the present study, we investigated the differences in response to salinity between own-rooted Thompson Seedless (TSOR) and 110R-grafted Thompson Seedless (TS110R) grapevines, wherein 110R is reported to be a salt-tolerant rootstock. The grapevines were subjected to salt stress by treating them with a 150 mM NaCl solution. The stress-induced changes in protein abundance were investigated using a label-free shotgun proteomics approach at three time-points viz. 6 h, 48 h, and 7 days of salt treatment. A total of 2793 proteins were identified, of which 246 were differentially abundant at various time-points in TSOR and TS110R vines. The abundance of proteins involved in several biological processes such as photosynthesis, amino acid metabolism, translation, chlorophyll biosynthesis, and generation of precursor metabolites was significantly affected by salt stress in both the vines but at different stages of stress. The results revealed that TSOR vines responded fervently to salt stress, while TS110R vines adopted a preventive approach. The findings of this study add to the knowledge of salinity response in woody and grafted plants and hence open the scope for further studies on salt stress-specific differences induced by grafting.



KEYWORDS: *salt stress, label-free proteomics, gene ontology, Vitis vinifera, 110R rootstock, grafting*

INTRODUCTION

Soil salinity is one of the major environmental factors limiting crop productivity with 20−50% yield losses. Soil salinization is caused by several factors such as high evaporation of soil water, insufficient rains, use of poor quality water for irrigation, and indiscriminate use of fertilizers. A strong link has been established between excess irrigation and soil salinization.[1] Additionally, lack of leaching of salts from the soil due to little or no precipitation makes soil salinity more adverse and irreversible. This is a major problem in arid regions, such as Pakistan,[2] northwestern China,[3] and northwestern parts of India.[4−7] Australia is also highly affected by salinity mainly due to the high groundwater table, leading to salinization of surface soils.[8,9] Salinization of soils is increasing rapidly worldwide[10] and has been estimated to reach 50% of the arable land by the year 2050.[11]

Developing salt-tolerant crops is thus essential for practicing sustainable agriculture under saline conditions. Several physiological and molecular studies of salinity stress in plants have revealed various mechanisms of salt stress tolerance. Some of the well-known mechanisms include accumulation of compatible osmolytes to maintain turgor, maintenance of ionic homeostasis, increased water use efficiency (WUE), enhanced photosynthesis, and alleviation of oxidative stress through the antioxidant metabolism.[12] These physiological and metabolic changes are a result of the complex regulation of several enzymes, signaling pathways, and structural molecules. Additionally, several sodium, potassium, and chloride transporters are known for the ionic homeostasis in salt-stressed plants.[13−15] Some transporters from the major facilitator superfamily such as sugar, nitrate, and oligopeptide transporters are also involved in the regulation of salt stress in plants.[16−19] In grapevines, salinity tolerance of certain species is associated with the transporters in roots that prevent the transport of salts to shoots.[15]

Grape (*Vitis vinifera* L.) is a perennial woody vine cultivated across the world mainly for wine production and consumption of fresh grapes or processed raisins. Viticulture has been facing various environmental threats among which drought and soil

Received: June 25, 2019
Published: December 6, 2019

DOI: 10.1021/acs.jproteome.9b00420
J. Proteome Res. XXXX, XXX, XXX−XXX