Biochemical and Functional Study of a Putative Lambda Class Glutathione-S-Transferase Gene in the Wild Soybean

CHAN, Ching

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biology

The Chinese University of Hong Kong

March 2014

Abstract of thesis entitled:

Biochemical and functional study of a putative Lambda class glutathione-S-transferase gene in the wild soybean

Submitted by **CHAN Ching**

for the degree of Doctor of Philosophy in Biology

at The Chinese University of Hong Kong

In a survey of candidate genes located in the salinity tolerance locus of soybean, we identified a putative glutathione-S-transferase (GST) gene (*GmGSTL1*) which was up-regulated in response to salt treatment. Phylogenetic analyses revealed that this putative GST belongs to the Lambda class, a plant-specific group with unknown functions. We expressed *GmGSTL1* in heterologous systems, including tobacco BY-2 cells and *Arabidopsis thaliana*, to test its ability to protect cell/plant against salinity stress. Compare to the wild type control, we observed a marked reduction of ROS accumulation in transgenic cells under salt treatment, and their survival rate was also improved. Similarly, expression of *GmGST1* in transgenic *A. thaliana* also alleviated stress symptoms under salt treatment. To further address the possible protective mechanisms of GmGSTL1, we identified two candidate flavonoid interactants (quercetin and kaemferol) of the GmGSTL1 protein from soybean leaf extract. Exogenous application of quercetin could reduce salinity-induced ROS accumulation in BY-2 cells and leaf chlorosis in *A. thaliana*.

摘要

我們在大豆的耐鹽候選基因中進行了篩選和調查，確定了一個穀胱甘肽-S -轉移酶（Glutathion-S-transferase ）基因（ GmGSTL1 ）具抗鹽特性，其表達量也跟隨鹽處理上調。系統發育分析表明，GmGSTL1 屬於 LAMBDA 類，文獻對這類蛋白功能的記載甚少。我們在異源系統，包括煙草 BY- 2 細胞和擬南芥，測試其保護細胞/植物對鹽脅迫的功能。結果表示 GmGSTL1 轉基因細胞的活性氧積累比對照顯著降低，存活率也有所改善。同樣，轉基因擬南芥在鹽處理壓力下的症狀也得以緩解。為了進一步剖析 GmGSTL1 的保護機制，我們在大豆葉片中提取多元酚，並發現兩個候選黃酮（槲皮素，山奈酚）與 GmGSTL1 起相互作用。槲皮素的外源性應用同樣可以緩解細胞/植物在鹽脅迫下的症狀，表示槲皮素在功能上與 GmGSTL1 相約。

Thesis/Assessment Committee

Professor FUNG, Ming Chiu (Chair)

Professor LAM, Hon Ming (Thesis Supervisor)

Professor NGAI, Sai Ming (Committee Member)

Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualifications.

Signed …………………………………..

CHAN Ching

Acknowledgement

I feel so grateful to have the substantial number of people who have helped and encouraged me to achieve this difficult task. I sincerely appreciate all of their support.

I am especially grateful to my principal supervisor, Prof. LAM Hon-Ming, for introducing me to the fascinating world of plant biotechnology. He has always been immense source of support and inspiration. From time to time, I was challenged with questions that refined my ideas and finally sharpened my focus. No doubt, I have avoided numerous pitfalls along the path towards my dissertation with his advice. I was greatly indebted to him, especially for his patience in proofreading the manuscript.

My gratitude is extended to my thesis committee members, Prof. Fung Ming-Chiu and Prof. Ngai Sai-Ming, for their questions and comments during the qualifying exam and graduate seminars.

Thanks Dr. Cheung Ming-Yan and Dr. Wang Hongmei for their discussion and advice on various experimental designs and invaluable suggestions. Special thanks go to my fellow peers, Ms. Tong Suk-Wah, Ms. Wong Fuk-Ling, Dr. Wong Chi-Fai, Ms. Qi Xinpeng and all other members of SC298 and EG02, for their encouragement, help and warm friendship. Working in the lab is always happy and enjoyable.

Above all, I would like to express my love to my parents and my brother, to whom I owe a great deal for everything. Without their support, I could not have had the courage to face the many difficulties in life and go on with my PhD studies.

Table of contents

Abstract -- i

摘要 -- ii

Declaration --- iii

Acknowledgements --- iv

Table of contents --- v

List of tables -- x

List of figures --- xi

Abbreviations -- xiii

Chapter 1 Introduction

1.1 Salinity stress -- 1

1.2 Diversity of salinity stress tolerance in plant species --------------------------- 1

1.3 Salinity stress determinants --- 3

1.3.1 Ion homeostasis --- 3

1.3.2 Osmolyte biosynthesis -- 5

1.3.3 Free radical scavenging -- 6

1.3.4 Water transport -- 7

1.3.5 Transducer of long distance response --- 7

1.3.6 Transcription factors --- 8

1.4 Reactive oxygen species and Glutathione-S-transferases -------------------- 10

1.4.1 Reactive oxygen species (ROS) -- 10

1.4.2 Enzymatic and non-enzymatic ROS scavenging mechanisms --------------- 11

1.4.3 Glutathione-S-transferases -- 12

1.4.4 Classification of Glutathione-S-transferase ------------------------------------ 13

1.4.5 GST function -- 15

1.5 Survey of candidate salt tolerant genes -- 15

1.6 Hypothesis and objectives of the study --- 16

Chapter 2 Materials and Methods

2.1 General chemicals and materials -- 18

2.1.1 Vectors and plasmids -- 18

2.1.2 Bacterial strains --- 20

2.1.3 Primers -- 21

2.1.4 Cell lines -- 26

2.1.5 Plant materials -- 28

2.2 Molecular biology -- 29

2.2.1 RNA extraction -- 29

2.2.2 Reverse transcription -- 30

2.2.3 Generation of DIG-labelled probe for Northern Blot ------------------------ 30

2.2.4 Expression analysis by Northern Blot --- 31

2.2.5 Expression analysis by Quantitative Polymerase Chain Reaction ----------- 32

2.2.6 Cloning of candidate genes -- 33

2.3 Establishment of transgenic models --- 33

2.3.1 Establishment of transgenic BY-2 cell --- 33

2.3.2 Establishment of transgenic *Arabidopsis thaliana* --------------------------- 34

2.4 BY-2 cell salt treatment and survival analysis --------------------------------- 35

2.4.1 Cell viability assay -- 35

2.4.2 ROS detection by H_2DCFDA staining --- 36

2.5 A. thaliana salt treatment and physiological analysis ------------------------ 36

2.5.1 Salt treatment --- 36

2.5.2 Chlorophyll content measurement --- 36

2.5.3 Histochemical staining -- 37

2.6 Phylogenetic analysis -- 37

2.7 Protein purification and enzyme activity assay -------------------------------- 38

2.7.1 Sub-cloning of GmGSTL1 --- 38

2.7.2 Protein expression and purification --- 38

2.7.3 GST enzyme activity assay --- 38

2.8 Ligand identification -- 39

2.8.1 Total phenolic compound extraction and purification ------------------------ 39

2.8.2 Ligand identification --- 40

2.8.3 High Performance Liquid Chromatography (HPLC) ------------------------- 40

2.8.4 Fourier Transform Mass Spectroscopy (FTMS) ------------------------------ 41

2.8.5 Ligand quantification --- 41

2.9 Statistic analysis --- 42

Chapter 3 Results

3.1 Expression profiling of gene candidates --------------------------------------- 43

3.2 Functional screening using transgenic tobacco BY-2 cells ------------------ 46

3.3 Phylogenetic analysis of Glyma03g33340 (GmGSTL1) ---------------------- 48

3.4 Expression analysis of *GmGSTL1* --- 53

3.4.1 *GmGSTL1* was mainly expressed in soybean leaf ---------------------------- 53

3.4.2 *GmGSTL1* was induced in leaf under salinity stress -------------------------- 55

3.5 Functional analysis --- 57

3.5.1 Over-expression of *GmGSTL1* in BY-2 cells reduced ROS accumulation under salinity stress -- 57

3.5.2 Over-expression of *GmGSTL1* in BY-2 cells enhanced cell survival under salinity stress --- 61

3.5.3 Over-expression of *GmGSTL1* in *A. thaliana* reduced salinity-induced leaf chlorosis -- 63
3.5.4 Over-expression of *GmGSTL1* in *A. thaliana* reduced salinity-induced ROS accumulation --- 66
3.6 Enzyme activity assay --- 69
3.6.1 Protein expression and purification --- 69
3.6.2 GmGSTL1 exhibited GSH dependent GST activity -------------------------- 70
3.7 Ligand identification --- 74
3.7.1 Optimization of HPLC gradient for analysis ------------------------------------ 75
3.7.2 GmGSTL1 interact with polyphenolic metabolites --------------------------- 79
3.8 Complementation assay --- 84
3.8.1 External supplementation of quercetin reduced ROS accumulation in BY-2 cells under salinity stress --- 84
3.8.2 External supplementation of quercetin enhanced plant survival under salinity stress --- 87

Chapter 4 Discussion and Conclusion
4.1 Glyma03g33340 encode a putative Lambda class glutathione-S-transferase -- 90
4.2 GmGSTL1 expression is mainly in soybean leaf and its expression is induced under salinity stress -- 91
4.3 Over-expression of GmGSTL1 alleviate salinity stress induced symptoms in transgenic cells and plants --- 92
4.4 GmGSTL1 encodes a functional enzyme exhibiting GSH dependent GST activity --- 92
4.5 GmGSTL1 interacts with polyphenolic metabolites ------------------------- 93

4.6 External supplementation of quercetin lowers salinity-induced symptoms in both transgenic cells and plants -- 95

4.7 Conclusion -- 95

References -- 97

Appendix - Buffer and medium formulation --------------------------------------- 122

List of tables

Table 2.1 Vectors and plasmids used in the study ------------------------------------ P.18

Table 2.2 Bacterial strains used in the study --- P.20

Table 2.3 Primers used in the study -- P.21

Table 2.4 Tobacco BY-2 cell lines used in the study -------------------------------- P.26

Table 2.5 Plant materials used in the study --- P.28

Table 3.1 Authentic standards used in FTMS experiment ------------------------- P.75

List of figures

Fig. 1.3 Phylogenetic relationship of GST classes --------------------------------- P.14

Fig. 3.1 Expression profile of candidate genes under 0.9% NaCl treatment ----- P.45

Fig. 3.2 Functional screening using transgenic tobacco BY-2 cells -------------- P.47

Fig. 3.3 Phylogenetic analysis of different classes of GSTs ----------------------- P.50

Fig. 3.4 Sequence alignment of GmGSTL1 and its homologues in *A. thaliana*, *Medicago*, maize, rice, and wheat --- P.51

Fig. 3.5 Differential expression of *GmGSTL1* in leaf and root ------------------- P.54

Fig. 3.6 Expression of *GmGSTL1* was induced in soybean leaf under 0.9% NaCl treatment --- P.56

Fig. 3.7 Expression of *GmGSTL1* in tobacco BY-2 cell lines and transgenic *A. thaliana* -- P.58

Fig. 3.8 ROS accumulation was observed in a sub-population of cells --------- P.59

Fig. 3.9 Gain-of-function study using transgenic tobacco BY-2 cells ----------- P.60

Fig. 3.10 Ectopic expression of *GmGSTL1* in BY-2 cells enhanced cell survival under salinity stress -- P.62

Fig. 3.11 Growth performance of *GmGST1* transgenic lines under salt stress ----- P.64

Fig. 3.12 Histological staining with diaminobenzidine tetrahydrochloride (DAB) for ROS --- P.67

Fig. 3.13 Recombinant GmGSTL1 expression and characterization --------------- P.71

Fig. 3.14 GmGSTL1 protein purification and enzyme assay ------------------------ P.73

Fig. 3.15 Liquid chromatography profile and spectrum of available authentic standards -- P.77

Fig. 3.16 GmGSTL1 binds to polyphenolic metabolites ----------------------------- P.80

Fig. 3.17 Standard curves and ligand quantification ---------------------------------- P.82

Fig. 3.18 Application of quercetin reduced salinity induced ROS accumulation in BY-2 cells -- P.85

Fig. 3.19 Application of quercetin reduced salinity induced leaf chlorosis and ROS accumulation -- P.88

Abbreviations

A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
ABA	Abscisic acid
ANOVA	Analysis of variance
AQP	Aquaporin
ATP	Adenosine triphosphate
BY-2	Bright yellow-2
CDNB	1-chloro-2,4-dinitrobenzene
Col-0	Columbia-0 ecotype
DAB	Diaminobenzidine tetrahydrochloride
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECe	Electron conductivity
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FTMS	Fourier Transform Mass Spectroscopy
GSH	Glutathione
GST	Glutathione-S-transferase
GuHCl	Guanidinium chloride
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HPLC	High Performance Liquid Chromatography
i.d.	Internal dimension
kDa	Kilo dalton

LB	Lysogeny broth
MALDI-TOF/ TOF MS	Matrix assisted laser desorption ionization - Time of flight/ Time of flight Mass Spectroscopy
MS	Murashige and Skoog
NaCl	Sodium chloride
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Reverse transcription
SSR	Short sequence repeat
v/v	Volume to volume ratio
WT	Wild type

Chapter 1 Introduction

1.1 Salinity stress

Salinity is among the most detrimental abiotic stress that constantly persists in nature and significantly impairs crop yield. Over 800 million hectare of land is considered salt affected, accounting for 20% irrigated land worldwide [1, 2]. Natural salinity could be resulted from rainwater, release of soluble salt from parental rocks and accumulation overtime in arid zones [3, 4]. Soluble salts consisted of chlorides, sulfates and carbonates of sodium, calcium and magnesium, while sodium chloride was the most abundant and soluble compound [4], contributing to a major part for the formation of saline land. Soil salinity, defined by electron conductivity (ECe), an equivalence of approximately 40 mM NaCl, will be classified as saline land. Besides naturally occurring saline land, secondary salinity was also resulted from land clearing and excessive irrigation [4].

1.2 Diversity of salinity stress tolerance in plant species

Due to the natural occurrence of salinity and widespread of salt by rainwater, plant evolved various mechanisms to regulate and develop adaptive advantages. Halophytes are group of plants able to survive extreme salinity, for up to 450

mM NaCl [5], while rice [6] and *Arabidopsis thaliana* [7] are salt sensitive, and their growth will be hindered under 100 mM NaCl [4]. Wheat, barley [8] and legume plant [9] are moderately tolerant and variation in degree of tolerance also exists within these species [4].

Although plants possess intrinsic mechanism to elicit response and adapt to stressful environment, some get the ability lost during domestication and human selection [10]. Soybean germplasms display various degree of tolerance to salinity stress [11]. The salt tolerance capacity could also deviate between geological locations [11], underlying specific variance in development or response pathways. It is, therefore, important to understand the cascade mechanism and identify salt tolerant genes involved, so as to enhance plant survival on marginal lands.

Much effort has been devoted into the study of osmotic and ionic component of salinity stress, from stress sensor, signal transduction to regulator and effecter gene expression [4, 12-14]. However, a consolidated molecular mechanism for control of ion accumulation and osmotic stress tolerance, and the sensor for salinity stress still remained to be established. Early genetic studies attempted to

locate major QTL for stress tolerance, however, most of the outcome suffered from low marker resolution, and hence, too many gene candidates in the target region for functional analysis [15].

1.3 Salinity stress determinants

Plant adaptation to salinity stress involved complex regulation of overlapping pathways. Earlier studies emphasized on hormone regulation, signal transduction and physiological consequences [16, 17]; while recent studies focused on functional genetics and downstream biochemical reactions [18-21]. Overall, major salinity stress determinants could be generally categorized into the following sections.

1.3.1 Ion homeostasis

In hyper-saline environment, perturbation of ion homeostasis in plant was not only confined to Na^+ and Cl^-, but also ions with similar chemical property and hence affinity to transporter proteins, such as K^+. Despite the fact that K^+ serves as essential enzymes cofactors, while Na^+ does not, plants do not evolve transport system that completely exclude Na^+. Many plasma membrane ion channels exhibit dual affinity to both Na^+ and K^+ [22-24]. The

single genetic locus, *SOS3*, encoding a signal transduction intermediate, was found to modulate the affinity of K^+ uptake [25, 26]. Transport proteins are usually energy-dependent transmembrane proteins which mediate ion influx/efflux by the utility of ATP, including H^+ translocating ATPase, pyrophosphatase, Ca^{2+}-ATPase, secondary transporters and ion channels [22, 27-29]. While transport determinants mediating ion homeostasis in plant resemble that in yeast [30], the identification of structure and/or function of candidate transport proteins were frequently performed by the functional complementation of transport-deficient yeast mutant [31, 32].

Vacuolar compartmentation is the second line of defense to maintain cytosolic ion homeostasis when external salt concentration exceeds the exclusion capacity of plasma membrane ion transporters. In this scenario, Na^+ and Cl^- will be transported into the vacuole of the plant cells, mediated by another group of energy-dependent ion transporters. The NHX1 in yeast and the plant NHE-like protein belongs to this group of endomembrane Na^+/H^+ anti-porter [28, 33-35].

Hyper-salinity also resulted in the accumulation of cytosolic Ca^{2+}. Such

response was considered adaptive, because external supplementation of Ca^{2+} reduced NaCl toxicity by increasing K^+/Na^+ selectivity [26, 36]. High external salinity also induced Ca^{2+} uptake from the apoplast and Ca^{2+} release from intracellular compartment [37, 38]. The resulting increase in cytosolic Ca^{2+} in turn induce further stress sensing signature leading to adaptation [12, 37, 39].

1.3.2 Osmolyte biosynthesis

One universal response to external change in osmotic pressure is the synthesis and accumulation of “compatible solutes”, a collective term for hydrophilic sugars, or their derivatives, which adjust internal osmotic potential and regulate water influx. The osmolytes are usually products or intermediates of various metabolic processes, including sucrose, fructose, inositols, trehalose, etc., and their accumulation will not inhibit normal metabolic reactions [40-42]. The osmolytes are typically hydrophilic and can replace hydration water molecules on protein surface and hence stabilize and protect protein molecules or protein complexes [43, 44]. Some are believed to serve as low-molecular-weight chaperones and hence function as “osmoprotectants” [4].

However, beyond the biophysical characteristic of "osmoprotectants", biochemical and molecular analyses lead to discrepancy in the role of metabolite for osmotic stress tolerance. One typical example for osmotic stress induced metabolic pathway is proline synthesis [43]. In plant, osmolyte biosynthesis is associated with induced expression of the associated enzyme for the synthesis of the osmolyte, following stress [45-47]. But in *sos1* mutant of *A. thaliana*, proline accumulated 2-fold higher than in wild type plant under salt stress, but the mutant was not salt tolerant [26, 48]. Therefore, it had been postulated that the function of "osmoprotectant" itself might be limited, but the activation of the specific metabolic pathway that was critical leading to stress tolerant [49-51]. And there might be more than one function for osmolyte accumulation, for example scavenging of reactive oxygen species [52-54].

1.3.3 Free radical scavenging

Photosynthetic organisms and aerobic life depends on molecular oxygen [55] and hence inevitably encounters reactive oxygen species (ROS) as a by-product of metabolic processes. The major sites of cellular compartment

for ROS accumulation are chloroplast, mitochondria and peroxisomes [56, 57]. Under normal circumstances, ROS accumulation is well balanced by scavenging mechanisms [58]. But under abiotic and/or biotic stresses, over-production of ROS could lead to toxicity and damage to cytosolic protein, lipid and DNA. Anti-oxidative defense could be generally classified into enzymatic and non-enzymatic mechanisms and will be further elaborated in section 1.4.

1.3.4 Water transport

Water mobility under stress was modulated by membrane protein, aquaporins (AQP) [59, 60]. In maize, salinity reduced water permeability in root cortex for about 5-fold [61]. The reduction of osmotic water permeability could be accounted by the controlled opening of water channels, or the change in number of the water channels itself. Regulation of AQP amount and activity is obviously critical for water transport under stress. In spinach, low water potential was found to associate with reduced phosphorylation of AQP through a membrane bound Ca^{2+} dependent protein kinase [45, 62].

1.3.5 Transducer of long distance response

Long distance signal transduction and coordination between different tissues would be vital for plant survival under abiotic stress. Communication along the plant body could be achieved by classical growth regulators, such as ABA, auxin, ethylene, and cytokinin [63-68], as well as crosstalk regulators of source-sink relationships [69-71]. Tropism, the growth or turning movement of plant in response to environmental stimuli, was conveyed by auxin transport and cytokinin conjugate to inositols [72, 73]. Mutant in *ABI1* altered ABA-perception in *A. thaliana* and reduced *P5CS* expression and hence proline accumulation [74]. Likewise, root Na^+ uptake elicit metabolic connection in the leaf, such as changes in photosynthetic capacity and movement of glutathione and its conjugates into the apoplast [75-78].

1.3.6 Transcription factors

Transcription factors plays important role in modulating gene expression under a range of environment stresses. Genome-wide transcriptome studies identified hundreds of genes candidates encoding transcription factors that are differentially expressed in response to stresses [79]. Using genetic and molecular approaches, different classes of transcription factors were shown to be involved in protection mechanisms in plant, including the WRKY family

[80], MYB family [81], ABF and bZIP family [82], NAC transcription factors [83], AREB/ABF [84], C2H2-type zinc finger transcription factors [85], DREBs [86] and etc.

The regulatory function mediated by transcription factors consisted of complex and overlapping cascades. The same transcription factor could be induced by multiple stimuli and the induction of a single transcription factor could modulate multiple downstream signaling pathways. Cross-talk between different transcription factors further complicated stress responsive gene network [80, 87-90]. Schematic visualization was usually represented by Venn diagrams [90]. For instance, the WRKY transcription factor, one of the largest family of transcription regulators in plant, plays important role in abiotic stress tolerance via a number of other important protein families, including MAP kinase, MAP kinase kinases, 14-3-3 proteins and calmodulin [80]. Over-expression of WRKY family members were shown to confer abiotic and/or biotic stress tolerance in rice [91, 92], *A. thaliana* [93, 94] and soybean [95]. Another example is the CBF3/DREB1,which response rapidly to low temperature [96]. The circadian clock pathway, on the other hand, was also regulated by CBF3/DREB1a, linking the possible interaction between

the two pathways [79].

1.4 Reactive oxygen species and Glutathione-S-transferases

Reactive oxygen species (ROS) are highly reactive and toxic chemicals which causes damage to cytosolic protein, lipid, carbohydrate and DNA. Various abiotic stresses could lead to overproduction of ROS, predominately in the chloroplast and mitochondria [97, 98]. Under normal situation, plants possess intrinsic mechanisms to maintain ROS homeostasis by enzymatic and nonenzymatic means [98]. The glutathione (GSH) redox cycle, mediated by glutathione-S- transferase (GST) and Mn-dependent superoxide dismutase (MnSOD) is crucial for peroxide scavenging [97]. For instance, ROS itself lacks specificity. Localized stress resulted production of ROS need to be perceived by specific receiver for specific downstream signaling, selectively regulated gene expression, and subsequent scavenger/ transporter delivery to the right compartment.

1.4.1 Reactive oxygen species

Reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals, alkoxy radicals, hydrogen peroxide and singlet oxygen, are

continuously produced in "energy factories" [99]. In plant, the chloroplast photosystem I & II and the mitochondria complex I & III of electron transport chain are prime sites for the generation of ROS [19, 97]. Abiotic stresses, such as salt, drought, heavy metals could lead to the over-production of ROS and lead to oxidative damage to membrane protein, lipid, DNA and other cytosolic components [98]. Leaf chlorosis, which is the first convenient visible indicator of senescence-associated programmed cell death, is a well-known consequence of ROS accumulation [100]. The up-regulation of antioxidant genes and enhanced antioxidative enzyme activity, such as GST, in various organisms dictate an evolutionarily conserved response to oxidative stress [101].

1.4.2 Enzymatic and non-enzymatic ROS scavenging mechanisms

Enzymatic ROS scavenging mechanisms involved superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, glutathione-S -transferase, glutathione peroxidase, dehydroascorbate reductase and etc [98]. Reports showed that over-expression of these enzymes and the associated increase in enzyme activity conferred abiotic stress tolerance in various plant models, including tobacco [102, 103], rice [104-106], *A. thaliana* [107-109]

and wheat [110, 111].

Non-enzymetic ROS scavenging was mediated by low-molecular-weight antioxidants including GSH, ascorbate, tocopherols, proline, betaine and others [19, 112]. In addition to scavenging of ROS, they also play important roles as enzyme cofactors, induction of acclimation processes and execution of programmed cell death [19]. GSH tri-peptide present in essentially all cell compartments [113] in reduced form [58]. The ratio of reduced GSH to GSSH, its oxidized form, is critical for plant perception to cellular oxidative stress [114, 115] and hence downstream signaling pathways to restore redox balance [112]. Under abiotic stresses, total GSH content was increased in sunflower seedling [116], tomato [117], wheat [118], and groundnut cell lines [119]. GSH also plays important role in the regeneration of ascorbate in the ascorbate-GSH cycle [19].

1.4.3 Glutathione-S-transferases

Glutathione-S-transferases (GSTs) are ancient and highly diverse gene family essential for enzymatic detoxification, cell signaling and other cellular processes in microbes, animals and plants [98, 101, 120]. They are dimmeric

soluble proteins with common transferase activity which catalyze the nucleophilic attack by reduced glutathione to electrophilic center, such as 1-chloro-2,4-dinitrobenzene (CDNB); as well as the peroxidase activity towards hydroperoxide [121]. GSTs protect cellular integrity by metabolizing carcinogens, insecticides, herbicides, by-products of oxidative stress, and hence are of great interest to toxicologists [122-124]. In plant, animals and microbes, exposure to oxidative damage lead to the up-regulation of GSTs, indicating the evolutionarily conserved defense strategy of GSTs against oxidative stress [125-127].

1.4.4 Classification of Glutathione-S-transferase

GSTs are classified into multi-gene families which are structurally distinct and have separate evolutionary origin [120]. In *A. thaliana*, there are so far 54 GSTs documented [99]. Previously, classification of plant GSTs were based on common structure and function: type I GSTs were with 3 exons and encode herbicide detoxification activity; type II were with 10 exons; type III GSTs were with 2 exons and were auxin responsive [128]. However, with increasing discovery of new members in various plant models, this nomenclature became inappropriate. Therefore, classification system based

on animal GSTs were extended to plant [121]. Fig. 1.3 summarized phylogenetic relationship of GSTs.

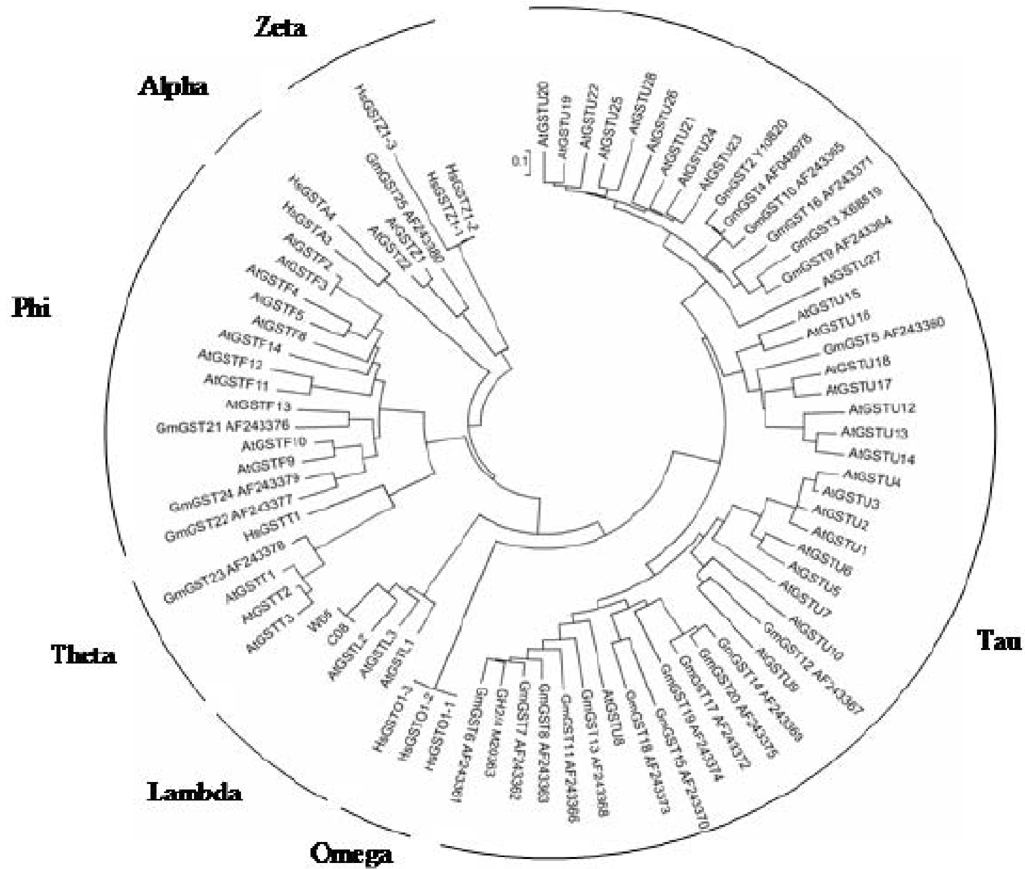


Fig. 1.3 Phylogenetic relationship of GST classes. The Tau (U) and Phi (F) class GST are plant-specific and most well studied. GSTF is auxin responsive and participate in pro-anthocyanidin transportation pathway. GSTU function as xenobiotics conjugating and scavenging. The Theta (T) and Zeta (Z) class share close homologs with the mammalian system. The Lambda (L) class is plant specific and the function is largely unknown.

1.4.5 GST function

The Phi (F) and Tau (U) classes GST are believed to be plant-specific [121], with the former being auxin responsive [129] and participating in pro-anthocyanidin transportation pathway [130]; while the latter is well known for the xenobiotics conjugating property [129]. The Theta (T) and Zeta (Z) classes share close homologs with the mammalian system and are involved in hydroperoxide reduction and tyrosine catabolism respectively [131, 132]. Despite structural and functional heterogeneity, the fundamental catalytic property is to catalyze nucleophilic substitution or addition of reduced glutathione (GSH) on electrophilic center of various non-polar compounds [101]. There were reports showing that over-expression of enzymes with general GST activity improved abiotic stress resistance in various plant models [133-135]. However, unlike GSTU catalyzed GSH conjugation of exogenous xenobiotics, there were little evidence for GST/GSH conjugation to secondary metabolites [99], and hence, the endogenous function of GST individual subfamily in relation to stress tolerance mechanism remained obscure.

1.5 Survey of candidate salt tolerant genes

Previous QTL analysis for salinity tolerant locus identified RFLP marker, Sat_091 and Satt237, associated with tolerance phenotype in breeding descendants [136]. Similar result was also obtained in early SSR marker analysis in our lab (Unpublished data). Therefore, a gene cluster close to Sat_091 was selected for initial screening in this study. Based on expression analysis and preliminary gain-of-function test in transgenic cells, one gene candidate, Glyma03g33340, was selected for in depth characterization.

1.6 Hypothesis and objectives of the study

Initial screening result showed that Glyma03g33340 was induced in soybean and over-expression of the gene in tobacco BY-2 cells significantly increased cell survival rate under salinity stress. Phylogenetic analysis showed that Glyma03g33340 encode a putative glutathione-S-transferase gene which belong to the plant-specific class Lambda. The function of this class of GST is largely unknown. Therefore, we hypothesized that GmGSTL1 might play a role in enhancing salt tolerance in plant. To further address the molecular mechanism for the gene function, we aimed:

(1) To confirm the putative function of the gene candidate in relation to ROS scavenging, which is the common property of GSTs;

(2) To address the function by measuring the conventional GSH dependent GST activity; and

(3) To identify other putative function by resolving potential molecular interactants.

Chapter 2 Materials and Methods

2.1 General chemicals and materials

2.1.1 Vectors and plasmids

The initial screening process consisted of 21 gene candidates, denoted by salt00, salt01, …, salt21. The exact gene loci were not revealed as other experiments were still in progress. The target gene, which forms the focus of this report, was salt00 (Phytozome locus Glyma03g33340) which was later renamed as GmGSTL1 in the publication.

Table 2.1 Vectors and plasmids used in the study

Vectors/ plasmids	Insert locus	Purpose	Source
V7	n/a	Binary vector for plant transformation	Lab stock
V7-salt00	Salt00/GmGSTL1	For transgenic over-expressors	Constructed in this study
V7-salt02	Salt02	For transgenic over-expressors	Constructed in this study

V7-salt03	Salt03	For transgenic over-expressors	Constructed in this study
V7-salt05	Salt05	For transgenic over-expressors	Constructed in this study
V7-salt13	Salt13	For transgenic over-expressors	Constructed in this study
pET	n/a	Recombinant protein expression vector	Lab stock
pET-GmGST	GmGSTL1	Expression of His-tagged GmGSTL1	Constructed in this study

2.1.2 Bacterial strains

Table 2.2 Bacterial strains used in the study

Bacterial strains	Selection media	Purpose	Source
E. coli DH5α	LB + 50 mg/L kanamycin or 100 mg/L ampicillin	General cloning and plasmid amplification	Lab stock
E. coli DE3	LB + 100 mg/L ampicillin	Recombinant protein expression	Lab stock
A. tumefaciens LBA4404	YEP + 50 mg/L kanamycin +50 mg/L rifampicin +25 mg/L streptomycin	Transformation of BY-2 cells	Lab stock
A. tumefaciens GV3101	YEP + 50 mg/L kanamycin +50 mg/L rifampicin +25 mg/L gentamycin	Transformation of *A. thaliana*	Lab stock

2.1.3 Primers

Table 2.3 Primers used in the study

Primers	Sequences	Purpose
HMOL7721	GGGGATCCATGGCAACTCCGAGTGTGTTA	Cloning of Salt00
HMOL7722	GGCTCGAGTTAAGCCAAAAACTTTTTCTTGAAA	
HMOL7435	AAAAGCTTATGGCAGCAATAATGTTGAC	Cloning of Salt02
HMOL7436	AAGGATCCCTACAGCCCAAGTTTAGATA	
HMOL7437	AATCTAGAATGGGTTGGATTCCCTGTTC	Cloning of Salt03
HMOL7438	AACTCGAGTCATATCCCCCTCCTGGTTC	
HMOL7441	AATCTAGAATGGCGGCCATAACCCGCC	Cloning of Salt05
HMOL7442	AACTCGAGTTATGCTGCAGCAGATTGCTTC	

HMOL7455	AATCTAGAATGGCCGACGGTCCGGCTAG	Cloning of Salt13
HMOL7456	AACTCGAGCTACTCCGGGCCTTGCATTG	
HMOL7435	AAAAGCTTATGGCAGCAATAATGTTGAC	Northern Blot Probe, Salt02
HMOL7436	AAGGATCCCTACAGCCCAAGTTTAGATA	
HMOL7441	AATCTAGAATGGCGGCCATAACCCGCC	Northern Blot Probe, Salt05
HMOL7442	AACTCGAGTTATGCTGCAGCAGATTGCTTC	
HMOL7445	AATCTAGAATGCAAATGGATATTGTTTATAC	Northern Blot Probe, Salt07
HMOL7446	AACTCGAGTCACTTGATTGGTGCAAGAATG	
HMOL7455	AATCTAGAATGGCCGACGGTCCGGCTAG	Northern Blot Probe, Salt13
HMOL7456	AACTCGAGCTACTCCGGGCCTTGCATTG	
HMOL7461	AATCTAGAATGGGGAAGACACATGGAATG	Northern Blot Probe, Salt16

HMOL7462	AACTCGAGTTAAGACCTAGGCTTCTCCTTC	
HMOL7463	AATCTAGAATGGAAGAAGCCTTTTGCTGG	Northern Blot Probe, Salt17
HMOL7464	AACTCGAGTTAAGACCTAGGCTTCTCCTTC	
HMOL7467	AAAAGCTTATGGGGAGGAAGGGGAATTG	Northern Blot Probe, Salt19
HMOL7468	AACTCGAGTCAACTGTCCACACCCTTGTC	
HMOL7469	AAAAGCTTATGGGCTCACTAGAAAGATC	Northern Blot Probe, Salt20
HMOL7470	AACTCGAGCTTTCCCCTTTGCCTTGGAC	
HMOL8062	GCCCTCCTGCTTTAACTTCC	qPCR, Salt00
HMOL8063	TGTGCAAAAGGGCACAGATA	
HMOL7845	CCCCAGGAAAGTTGAAAAGGA	qPCR, Salt03
HMOL7846	TTGCCAACTCACGGAACGCA	

HMOL8095	TCAGAAGTCATGCCCAGAAGT	qPCR, Salt08
HMOL8096	TTTTTGGATGCCTTTTGTGG	
HMOL7873	CCGAAAGATAGAAGTCCAAAGGTT	qPCR, Salt18
HMOL7874	TGAGATTCCAATTCCTGGACCT	
HMOL2976	CTCAGGTGATTTCATCTTTG	qPCR, Soybean *Tubulin*
HMOL2977	GAATTCAGTCACATCCAC	
HMOL7829	GGTAACATTGTGCTCAGTGGTGG	qPCR, *Arabidopsis Actin*
HMOL7830	AACGACCTTAATCTTCATGCTGC	
HMOL6911	CTGGCCGTGACCTAACTGAT	qPCR, tobacco *Actin*
HMOL6912	GCAAGCTCCTCCTTCATGTC	
HMOL8055	CCGGATCCATGGCAACTCCGAGTGTGTTA	Salt00 sub-cloning into pET

HMOL8056	GGCTCGAGTTATTAAGCCAAAAACTTTTTCTTG

2.1.4 Cell lines

Tobacco BY-2 WT and empty vector transformant were from lab stock and used as control for experiments. V7-salt00, 02, 03, 05, 13 denoted overexpression cell line for respective gene candidate for initial screening purpose. GmGST_b1 and _b2 denoted independent transformant of GmGSTL1 (same as salt00) used for further experiments.

Table 2.4 Tobacco BY-2 cell lines used in the study

BY-2 cell lines	Overexpressing locus	Source
WT	tobacco ecotype for transformation	Lab stock
V7	Empty vector transformant	Lab stock
V7-salt00	Salt00	Constructed in this study
V7-salt02	Salt02	Constructed in this study
V7-salt03	Salt03	Constructed in this study
V7-salt05	Salt05	Constructed in this study
V7-salt13	Salt13	Constructed in this study

GmGST_b1	GmGSTL1 (Glyma03g33340)	Constructed in this study
GmGST_b2	GmGSTL1 (Glyma03g33340)	Constructed in this study

2.1.5 Plant materials

Soybean accession W05 and C08 were used for initial expression analysis. *A. thaliana* WT and V7 empty vector transformant were obtained from lab stock. GmGST_a1 and _a2 denoted independent transformant of GmGSTL1 used for further experiments.

Table 2.5 Plant materials used in the study

Plant materials	Overexpressing locus	Source
W05	soybean accession	Lab stock
C08	soybean accession	Lab stock
A. thaliana WT	*A. thaliana* ecotype Col-0	Lab stock
V7	*A. thaliana* empty vector transformant	Lab stock
GmGST_a1	GmGSTL1 (Glyma03g33340)	Constructed in this study
GmGST_a2	GmGSTL1 (Glyma03g33340)	Constructed in this study

2.2 Molecular biology

2.2.1 RNA extraction

Extraction of total RNA was performed according to standard procedures as described [137, 138]. Briefly, plant materials were grounded in liquid nitrogen with mortar and pestle. Phenol/chloroform/isoamylalcohol (25:24:1) and extraction buffer was added in 1:1 (v/v) ratio sequentially. The homogenate was centrifuged at 8,228 x *g* for 5 minutes at 4℃. The upper aqueous layer was transferred into a new tube and a second extraction with phenol/chloroform/isoamylalcohol (25:24:1) was performed in 1:1 (v/v) ratio. The solution was again centrifuged at 8,228 x *g* for 5 minutes at 4 ℃. The upper aqueous layer was transferred into a new tube, and residual phenol was cleaned up by adding 1 volume of chloroform/isoamylalcohol (24:1). The solution was centrifuged at 8,228 x *g* for 5 minutes at 4℃ and the upper aqueous layer was transferred into a new tube. To precipitate the RNA, 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volume of absolute ethanol was added sequentially. The mixture was kept in -80℃ for 20 minutes and centrifuged at 8,228 x *g* for 15 minutes at 4℃. The pellet was resuspended in 3M sodium acetate (pH 5.6) and centrifuged at 18,500 x *g* for 15 minutes at 4℃. The pellet was resuspended in 0.3M sodium acetate (pH 5.6) and absolute ethanol (2:5, v/v ratio) and kept at -80℃ for 20 minutes to precipitate the RNA. After centrifugation, the pellet was air dried and

resuspended in DEPC-treated water.

2.2.2 Reverse transcription

Reverse transcription for cDNA synthesis was performed as previously described [137, 138] using Invitrogen SuperScript III Reverse Transcriptase. Briefly, 1 μg total RNA was used as template and subjected to DNaseI treatment. And then 1 μl of 50 μM oligo(dT), 1 μl of 10 mM dNTP and DEPC-treated water was added to give a 13 μl reaction mixture. The mixture was denatured at 65℃ and chilled on ice for 1 minute. Next, 2μl of 10x RT buffer, 4μl of 25 mM MgCl2, 2μl of 0.1 M DTT, 1μl of RNaseOUT (40U/μl) and 1μl of SuperScript III RT (200U/μl) were added sequencially. The mixture was incubated at 50℃ for 2 hours and inactivated at 70℃ for 15 minutes.

2.2.3 Generation of DIG-labelled probe for Northern Blot

To generate DIG-labelled probe for Northern Blot analysis, two rounds of PCR were carried out for each candidate gene. The first round of PCR generated full length amplicon as template for the next PCR, with primer pairs as listed in Table. 2.3. The second round of PCR generated anti-sense single-stranded DNA probe by the addition of reverse primer only.

The first round of PCR was performed by amplifying 1 µl of the cDNA as template with the following components: 2 µl of 10x PCR buffer, 0.4 µl 10 mM dNTP, 0.6 µl of 50 mM MgCl2, 0.4 µl of primer mix, 0.1 µl of Platinum Taq DNA Polymerase (Invitrogen) and autoclaved milli-Q water to 20 µl. The reaction was incubated in a thermal cycler with the following profile: 94 for 2 minutes; 35 cycles of denaturation, 94℃ for 30 seconds, annealing, 55℃ for 30 seconds, extension, 72℃ for 2 minutes; and a final extension of 7 minutes at 72℃.

The second round of PCR was performed by amplifying 5 µl of the PCR product from round one with the following components: 5 µl of 10x PCR buffer, 1 µl of DIG-labelled dNTP (Roche), 1.5 µl of 50 mM MgCl2, 1 µl of reverse primer, 0.2 µl of Platinum Taq DNA Polymerase (Invitrogen) and autoclaved milli-Q water to 50 µl. The reaction was incubated in a thermal cycler with the following profile: 94 for 2 minutes; 40 cycles of denaturation, 94℃ for 30 seconds, annealing, 55℃ for 30 seconds, extension, 72℃ for 2 minutes; and a final extension of 7 minutes at 72℃.

2.2.4 Expression analysis by Northern Blot

Northern Blot was performed as previously described [137, 138]. Briefly, 10 µg of total RNA was separated on denaturing agarose gel. Loading control was revealed by

ethidium bromide staining. The RNA was then transferred onto nylon membrane by capillary action, soaked in 10x SSC buffer for 16 hours.

Blotted RNA was UV-crosslinked to the nylon membrane. The membrane was rinsed in DEPC-treated water and pre-hybridized at 42℃ for 2 hours. Hybridization was then carried out with 25 ng/ml DNA probe at 42℃ for 16 hours. After hybridization, the membrane was washed twice with low stringency at room temperature for 15 minutes, followed by high stringency wash at 68℃ for 15 minutes. Then, the membrane was blocked with 1% blocking reagent for 2 hours at room temperature, followed by incubation with anti-DIG antibody (1:10,000 in blocking solution) for 30 minutes at room temperature. The membrane was washed with 1x maleic acid buffer and detection buffer before incubation with CSPD substrate. Hybrid signals were then detected by X-ray film.

2.2.5 Expression analysis by Quantitative Polymerase Chain Reaction

For gene candidates with low expression, quantitative PCR was employed according to previously described [137, 138]. Briefly, amplification was carried out using 50-fold diluted cDNA as template. 3 μl was used for each reaction. The reaction consisted of the following components: 10 μl of 2x SYBR Green supermix

(Bio-Rad), 0.3 µl of 10 µM primer pair (Table. 2.3) and 6.7 µl of autoclaved milli-Q water. Real-time detection was carried out using Bio-Rad iQ5 Real-Time PCR detection system with standard 2-step thermo cycle. Soybean tubulin expression was used as internal control for calculating relative gene expression using the ΔΔCT method [139].

2.2.6 Cloning of candidate genes

The full length cDNA of gene candidates were amplified by primer pairs listed in Table. 2.3 using high fidelity *pfu* DNA polymerase (Promega). The full length amplicon was purified by spin columns from Qiagen Gel Extraction Kit with homemade buffers. Purified PCR fragment was digested with the respective restriction enzyme(s), followed by clean up with spin columns and ligation into V7 binary vector [140] and transformation into competent *E. coli* strain DH5α. Positive clones were selected by PCR checking of kanamycin resistant colonies.

2.3 Establishment of transgenic models

2.3.1 Establishment of transgenic BY-2 cell

BY-2 transgenic cell lines were constructed as previously described [137, 138]. Briefly, constructs bearing the full-length *GmGSTL1* under cauliflower mosaic virus

35S constitutive promoter were transformed into *Agrobacterium tumefaciens* LBA4404. An overnight culture was inoculated from positive clones. For each transformation, 5 ml BY-2 cells (three-day freshly sub-cultured cells) was mixed with 150 µl of *A. tumefaciens* bacteria culture. Acetosyringone was added to a final concentration of 100 µM. The cell mixture was pipetted through the tips 20 times to introduce lesions in the BY-2 cells to increase efficiency of transformation. The culture was kept in dark for 3 days at 28℃. To minimize recurrent of bacteria growth, the mixture was washed with Murashige and Skoog (MS) medium supplemented with 500µg/ml cefotaxime. The remaining BY-2 cells were plated on MS medium supplemented with 500µg/ml cefotaxime, 50 µg/ml kanamycin and kept in dark for 4 weeks at 28℃. Transgenic BY-2 cell lines stably expressing *GmGSTL1* were used in subsequent experiments.

2.3.2 Establishment of transgenic *Arabidopsis thaliana*

A . thaliana transgenic lines were constructed by vacuum infiltration as previously described [137, 138]. Briefly, constructs bearing the full-length *GmGSTL1* under cauliflower mosaic virus 35S constitutive promoter were transformed into *A. tumefaciens* GV3101. A 5 ml overnight culture was inoculated from positive clones. The starter culture was expanded to 500 ml and continuously grew until OD600 =

0.8. The cells were pelleted by centrifugation and resuspended in MS medium supplemented with 0.05% Silwet L-77. The above-ground part of plants were dipped into the bacteria suspension and vacuum suction was applied for the whole entity for 10 minutes. The plants were then grown normally and seeds were harvested for screening. Transformants from different plants were treated as independent. Single-insertion (T2 3:1 segregation ratio) homozygous (T3 no segregation) *A. thaliana* lines were used in subsequent experiments.

2.4 BY-2 cell culture, salt treatment and survival analysis

2.4.1 Cell viability assay

Three-day freshly sub-cultured BY-2 cells were used. For viability assay, NaCl was added to the cultures to give a final concentration of 100 mM. The cultures were maintained at 28℃ for 24 hours. For visualization and counting, trypan blue was mixed with cell aliquots in 1:1 ratio and at least 10 random views were captured for each experiment. The survival rate was calculated as the percentage of live cells versus the total number of cells. The experiment was repeated twice with independent biological preparation to check trend consistency.

2.4.2 ROS detection by H_2DCFDA staining

Three-day freshly sub-cultured BY-2 cells were used. For ROS detection, cells were pre-stained with 10 µM H_2DCFDA for 30 minutes, followed by washing with fresh MS medium. NaCl was then added to give a final concentration of 100 mM. Fluorescence signals were captured using a confocal microscope (Olympus FV1000) or a microplate reader (Synergy H1; Excitation: 485 nm; Emission: 528 nm) [141].

2.5 *A. thaliana* plant culture, salt treatment and physiological analysis

2.5.1 Salt treatment

Seeds were germinated on MS medium for 10 days until root length reached approximately 10 mm. The seedlings were transferred onto fresh MS medium (control) or medium with NaCl with or without quercetin supplement at various concentrations. Plants were then maintained under standard growth conditions for 14 days.

2.5.2 Chlorophyll content measurement

Chlorophyll was extracted from 0.4 g pooled plant samples by direct immersion into 100% dimethyl formamide, followed by incubation at 4 ℃ overnight. The absorbances at 603 nm, 647 nm and 664 nm were measured by a spectrophotometer

(Synergy H1). The chlorophyll content was calculated according to Morgan [142]. The experiment was repeated twice with independent biological preparation to check trend consistency.

2.5.3 Histochemical staining

Histological staining for ROS was performed based on Jambunathan [161]. Briefly, plant materials were immersed in DAB solution (1 mg/ml in 50 mM Tris-acetate, pH 5.0) for 24 h. The samples were then boiled in 96% ethanol and fixed in fixer solution (ethanol: lactic acid: glycerol = 3:1:1) prior to imaging.

2.6 Phylogenetic analysis

For phylogenetic analyses, the sequences of known GST members from soybean, *A. thaliana* and mammalian GSTs were retrieved from Phytozome and NCBI respectively. Multiple sequence alignments were performed by ClustalW algorithm using MEGA (version 4.0). A phylogenetic tree was constructed using a neighbour-joining method (MEGA version 4.0) with default settings. Each protein was named with the abbreviation of the species followed by the Phytozome loci tag or GenBank accession number.

2.7 Protein purification and enzyme activity assay

2.7.1 Sub-cloning of GmGSTL1

The full-length sequence of *GmGSTL1* was PCR amplified from its V7 construct by primer pairs (Table. 2.3). The purified PCR fragment was fused to a poly-histidine tag in the pET-32a(+) vector (Novagen, Cat. No. 69017-3).

2.7.2 Protein expression and purification

The recombinant GmGSTL1-His fusion protein was expressed in *E. coli* strain DE3. Whole cell lysate was analyzed by SDS-PAGE, and the target band was excised for matrix-assisted laser-desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis to confirm correct expression of target protein. Then, the recombinant protein was purified by HisTrap affinity column according to the manufacturer's protocol (GE Healthcare, 71-5027-68 AH).

2.7.3 GST enzyme activity assay

For enzyme assays, a buffer exchange was performed according to the instructions for HisTrap desalting column (GE Healthcare, 71-7154-00 AK). The protein was eluted with Dulbecco's Phosphate Buffered Saline (pH 7.0). GST activity was determined by measuring the absorbance of the CDNB conjugate according to the

Glutathione S-transferse Assay Kit (Sigma, CS0410). Absorbance at 340 nm was monitored for 20 minutes at 50 s intervals. Specific activity was calculated according to the formula: $[(\Delta A340)/\text{min} \times V(\text{ml}) \times \text{dil}] / [\varepsilon \text{mM} \times V\text{enz}(\text{ml})] = \mu\text{mol/ml/min}$; where dil = the dilution factor of the original sample; ε = the extinction coefficient for the CDNB conjugate at 340 nm.

2.8 Ligand identification

2.8.1 Total phenolic compound extraction and purification

The identification of novel ligands binding to GmGSTL1 was performed according to Dixon, et al. [129] with the following modifications. Total phenolic compounds were purified from soybean leaves. Samples were ground and then extracted in 80% methanol overnight with continuous shaking at ambient temperature. Large particles were removed by centrifugation at 5,000 x *g* for 10 minutes. Hexane was added in 1:1 ratio to remove lipid components. The aqueous layer was retained and applied to an affinity column embedded with HP-20 resin. The column was washed with milli-Q water, followed by the elution of bound phenolic compounds with absolute methanol. Extracted fractions were pooled and concentrated by Speedvac centrifugation.

2.8.2 Ligand identification

The recombinant protein was first immobilized onto a HisTrap column in the equilibration buffer (50 mM Na_2HPO4, pH 7.4, 0.3 M NaCl, 1 mM PMSF, 6 M GuHCl, 10 mM imidazole) and gradually flushed with the washing buffer (50 mM Na_2HPO4, pH 7.4, 0.5 M NaCl, 10 mM imidazole) to remove GuHCl. The total phenolic extract was then loaded onto the column in washing buffer, followed by elution with 50 mM imidazole. Eluted fractions were pooled and concentrated for subsequent analyses.

2.8.3 High Performance Liquid Chromatography (HPLC)

HPLC was optimized using the Waters 2690 separation module system with a Waters 996 photodiode array detector. Chromatographic separation was carried out using a 150 x 2.1 mm i.d., 5 µm Symmetry Shield C18 steel column with a 10 x 2.1 mm i.d., 3.5 µm Symmetry C18 guard column, operated at ambient temperature. The mobile phase consisted of 1% formic acid in water (eluant A) and 1% formic acid in acetonitrile (eluant B) with a linear gradient elution at a flow rate of 0.1 ml/min. The elution program was as followed: 8-10% B (0-10 min); 10-20% B (10-35 min); 20-20% B (35-50 min); 20-45% B (50-60 min); and 45-80% B (60-80 min). The injection volume for all samples was 10 µl.

2.8.4 Fourier Transform Mass Spectroscopy (FTMS)

The FTMS analysis was performed with the same HPLC profile as described above, interfaced with a Bruker Daltonik mass spectrometer equipped with an ESI source. NH_4HCO_3 was used as the ion carrier instead of formic acid, as MS in the negative mode was reported to be more favorable for phenolic compound analyses [144]. Data acquisition and processing were performed using DataAnalysis Version 4.0 SP1 (Bruker Daltonik). The mass spectra were recorded in the range of m/z 100-1000. Optimization of ionization conditions was based on the intensity of the mass signals of deprotonated molecules, and was performed using the ESI tuning mix (Agilent Technologies, G2421-60001) for negative MS experiments. Mass parameters were optimized as follows: capillary voltage, 4.0 kV; spray shield, 3.5 kV; neb gas flow, 2.0 L/min; dry gas flow, 4.0 L/min; dry temperature, 200℃. Standards and purified phenolic extracts described above were reconstituted in methanol/water (50:50, v/v). The standards (10 mg/ml) and sample extracts were filtered by a syringe filter with a 0.2 µm PVDF membrane (Gelman Laboratory), and 10 µl was injected into the HPLC column for analysis.

2.8.5 Ligand quantification

Standard curve for candidate ligands were generated for ligand quantification.

Authentic standards were diluted in 10-fold series and subjected to FTMS analysis. Three injection replicates were performed for each concentration. And the mean peak area (log value) was plotted against Standard concentration (-log value) to give the standard curve. The amount of ligand retrieved by the recombinant protein was estimated by converting mean area of the EIC according to the formula generated by the standard curves.

2.9 Statistical analysis

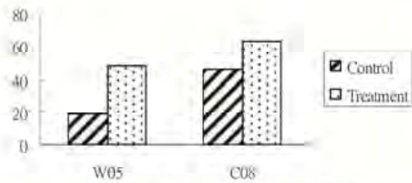
Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) (version 16.0). Mean difference was compared using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Samples with significance difference ($P<0.05$ or $P<0.01$) were indicated.

Chapter 3 Results

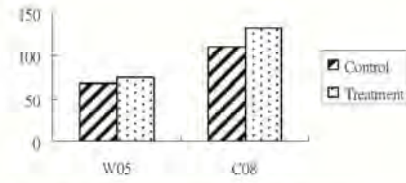
3.1 Expression profiling of gene candidates

To identify prominent gene(s) for functionally test, we first studied the expression profile of gene candidates under NaCl treatment. Two soybean accessions, W05 and C08, were subjected to 0.9% NaCl treatment and leaf samples were harvested 3 days after treatment. Northern blot was employed for expression analysis. For those genes with relatively lower endogenous expression, qPCR was employed. Most genes were differentially expressed in both soybean accessions (Fig. 3.1), indicating general responsiveness to salinity stress. Among these gene candidates, Salt00, encoding a glutathione-S -transferase (Phytozome locus Glyma03g33340), was about 7-fold up-regulated in W05.

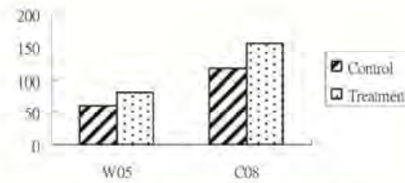
Salt 02



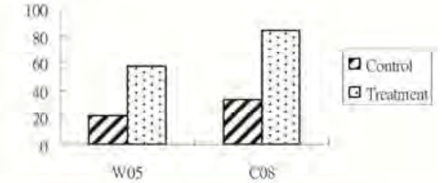
Salt 05



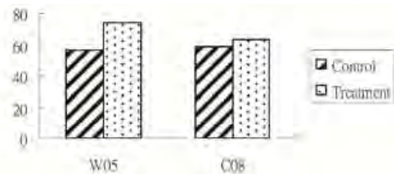
Salt 07



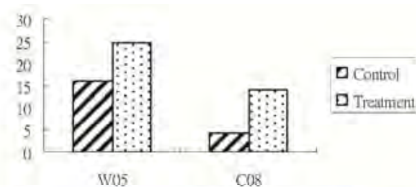
Salt 13



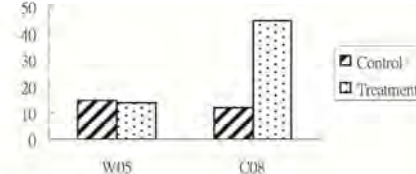
Salt 16



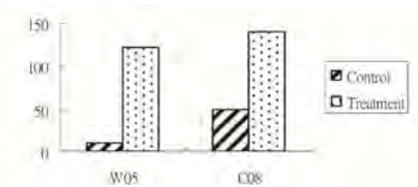
Salt 17



Salt 19



Salt 20



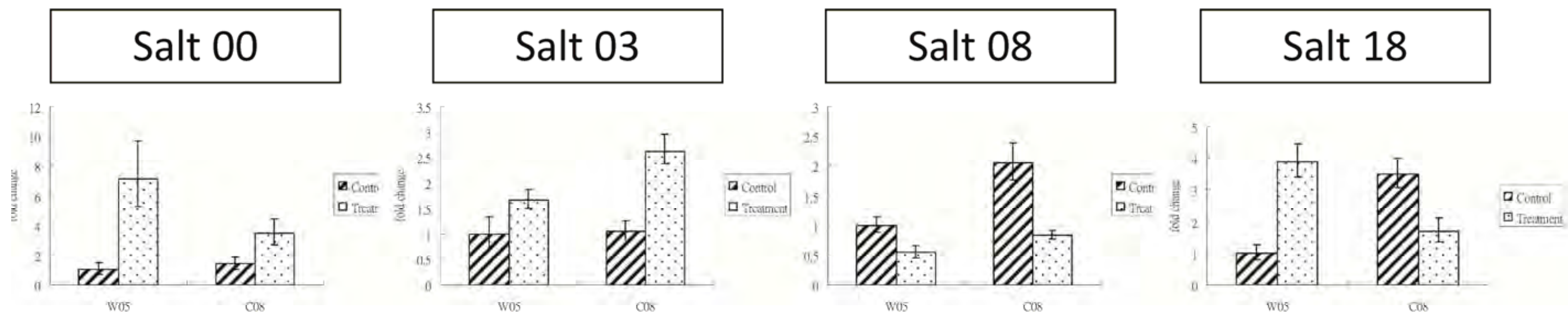


Fig. 3.1 Expression profile of candidate genes under 0.9% NaCl treatment. Salt 00, 02, 03, 05, 07, 08, 13, 16, 17, 18, 19, 20 denote gene candidates for initial screening. Most genes were responsive to salt treatment. Upper two pannels: Northern blot analysis. RNA loading was revealed by ethidium bromide staining. Specific gene expression was quantified by measurement of band intensity using ImageJ (ver. 1.47). Third pannel: qPCR analysis. Relative expression was calculated by the ΔΔCT method (Livak and Schmittgen 2001), using expression of the soybean *tubulin* gene for normalization. The expression of W05 untreated sample was set to 1 for comparison. W05 and C05 were soybean accessions.

3.2 Functional screening using transgenic tobacco BY-2 cells

We obtained cDNA clone of 5 genes to performed gain-of-function test in the tobacco BY-2 cells. All cDNA clones alleviated salinity stress in BY-2 cells under specific context, as indicated by reduced dead rate under stress (Fig. 3.2). This underlined the importance of the gene cluster in salinity stress tolerance determinant. Transgenic line of Salt00 (Phytozome locus Glyma03g33340) displayed significant higher survival rate over a range of salt concentration and its function was further characterized in this study.

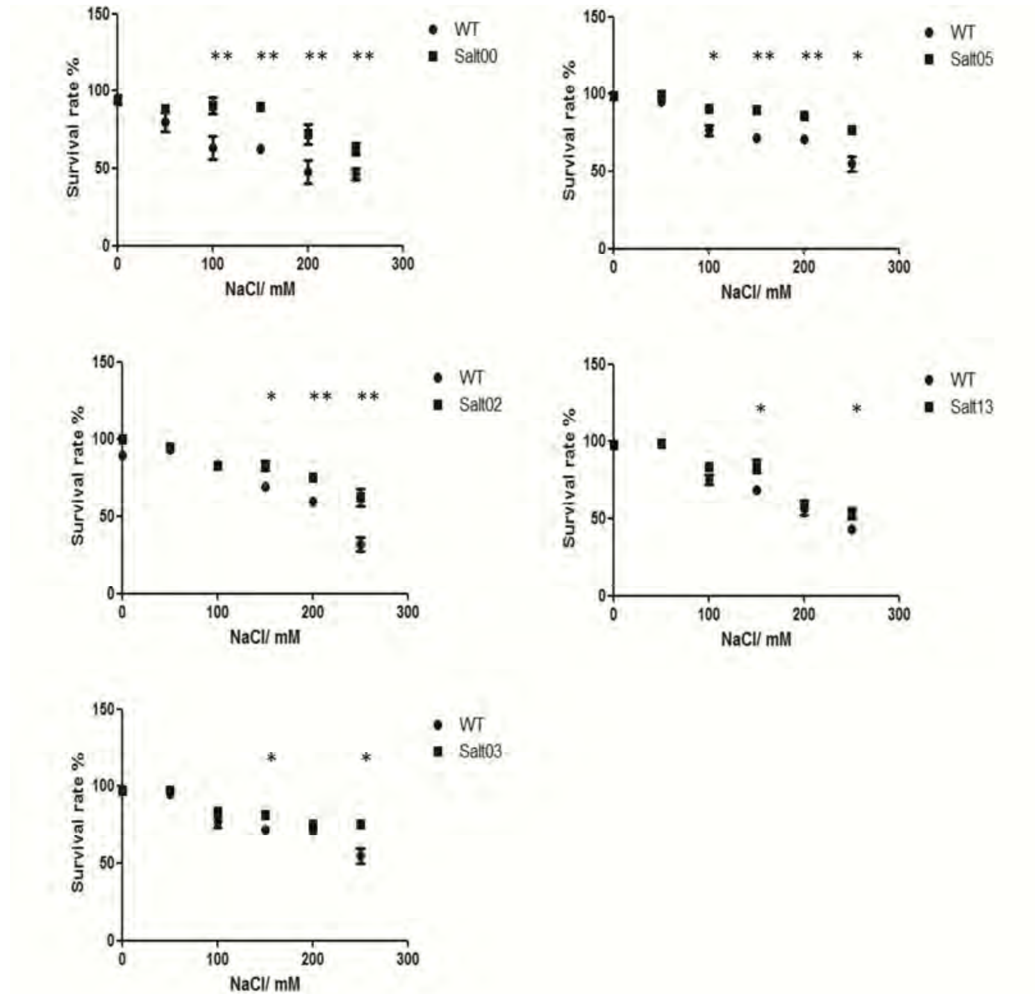


Fig. 3.2 Functional screening using transgenic tobacco BY-2 cells. BY-2 cells were subjected to NaCl treatment at different concentration. Salt00, Salt02, Salt03, Salt05, Salt13 denoted BY-2 cell lines overexpressing the respective candidate gene. Survival rates were monitored by trypan blue staining. Values represented mean % survival of at least 10 random views of a total cell counts more than 400. Error bar: standard error. * and **Denoted significant mean difference at $p<0.05$ and $p<0.01$ respectively, when compared to WT, using one-way ANOVA followed by the post hoc Tukey's test.

3.3 Phylogenetic analysis of Glyma03g33340

Glutathione-S-transferase (GST) constitute a very diverse group of proteins. Despite their common glutathione (GSH) dependent transferase activity, the structures and functions are highly heterogeneous between classes. Therefore, phylogenetic classification was essential for inferring possible direction of subsequent experiments.

Sequences for known GST members from *A. thaliana* (At) and mammal (Hs) were retrieved from the Phytozome and NCBI database. Phylogenetic tree was constructed using MEGA (version 4.0) by neighbor-joining method. Glyma03g33340 was found to belong to the lambda class (Fig. 3.3), and was then named GmGSTL1, which showed 79.1%, 76.2% and 72.8% sequence identity with the AtGSTL3, AtGSTL1 and AtGSTL2 proteins from *A. thaliana*, respectively. The N-terminal consisted of TRX domain [120] which harbors the GSH binding consensus (G-site) and the well conserved active site cysteine (Fig. 3.4). The C-ternimal consisted of the typical alpha helical domain of Class Lambda Glutathione S-transferases (Fig. 3.4) with hydrophobic substrate binding pocket (H-site) which is for endogenous and xenobiotic alkylating agents, including carcinogens, therapeutic drugs, environmental toxins, and

products of oxidative stress [99, 121].

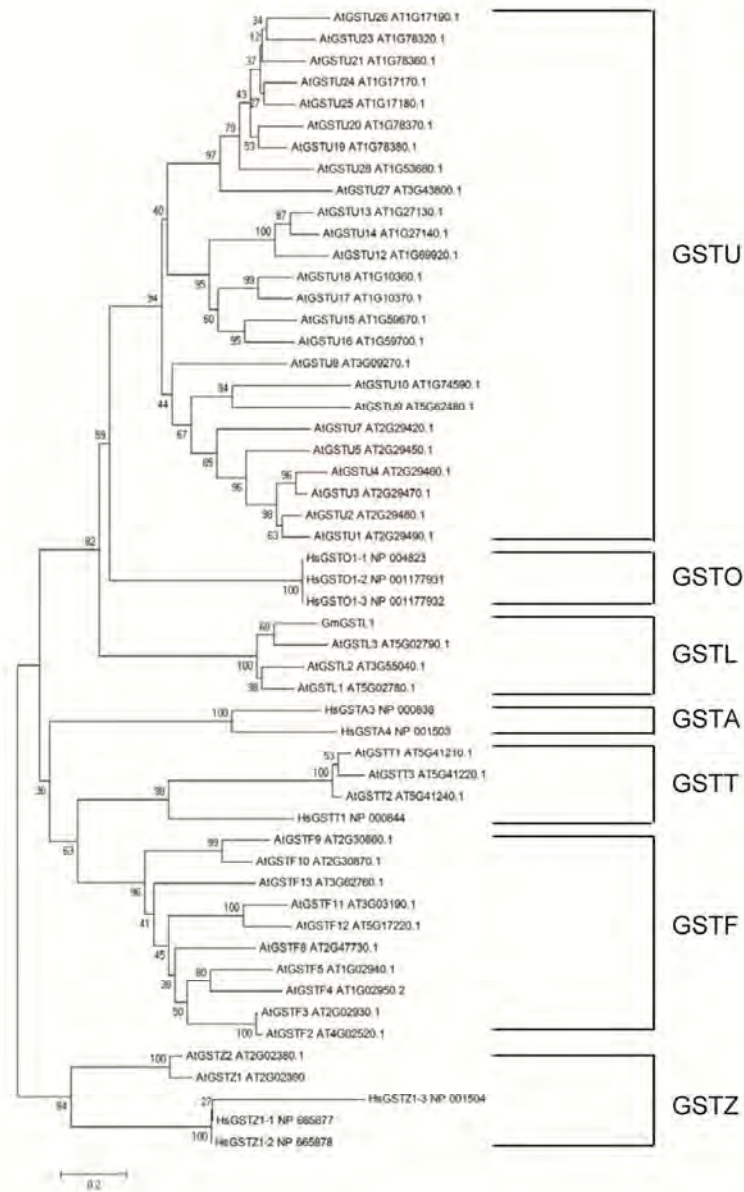


Fig. 3.3 Phylogenetic analysis of different classes of GSTs. Known GST members from *Arabidopsis thaliana* (At) and mammal (Hs) were retrieved from the Phytozome and NCBI database. Phylogenetic tree was constructed using MEGA (version 4.0) by neighbor-joining method with default setting. GmGSTL1 belongs to the GSTL class.

```
                                *

                           10        20        30                  50        60        70        80
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GmGSTL             -----MATPSVLEVRTPALTSISEPLPFFDGTTRLYICYLCPFAQRAWITRNCKGLQDKIELVPIDLKNRPAWYKEKVYP
AT5G02790.1        ----MAPSFIFVEDRPAPLDATSDPPSLFDGTTRLYTSYVCPFAQRVWITRNFKGLQEKIKLVPLDLGNRPAWYKEKVYP
Medtr7g100320.1    -----MAT-TVKEIRTPPLTSNSDPPPIFNGDTRLYISYVCPYAQRAWIARNYKGLQDKIKLVSINLQDRPAWYKEKVYP
GRMZM2G162486_T01  -MAAAAPASSVKEVLPSPLTSASEPPPLFDGTTRLYVAYLCPFAQRAWIARNYKGLQDKIKIVAIDLADRPAWYKEKVYP
LOC_Os03g17480.1   MAAAAAPRSSGKEALPAALGSASEPPRLFDGTTRLYICYFCPFAQRAWIIRNFKGLQDKIELVGIDLQDKPAWYKEKVYE
Y17386             MAAAAAIASSTKEVLPPALGAVSEPPPLFDGTTRLYICYICPFAQRAWVTRNCKGLQEEIKLVAINLEDKPAWYKEKVYP

                           90       100       110       120       130       140       150       160
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GmGSTL             TNKVPSLEHNSKVLGESLDLIRYIDANFEGAPLFPTDPAKREFGEQLISHVDTFTSGIYPTFKG--DPIQQTSAAFDYLE
AT5G02790.1        ENKVPALEHNGKIIGESLDLIKYLDNTFEGPSLYPEDHAKREFGDELLKYTDTFVKTMYVSLKG--DPSKETAPVLDYLE
Medtr7g100320.1    ENKVPSLEHNGKVLGESLDLISYIDVNFEGLSLVPSDPAKKEFGDQLISHVDTFTKDLYSSLKG--DPIKQAGPAFDYLE
GRMZM2G162486_T01  ENKVPSLEHDNQVKGESLDLVKYIDSNFEGPSLLPEDHAKQQFAEELLGYTDAFNKAFYSCLVDREDVSEEAVAALDKIE
LOC_Os03g17480.1   QGTVPSLEHNGKIMGESLDLIKYIDSHFEGPALLPEDPEKRQFADELIAYANAFTKALYSPLISKADLSAETVAALDKIE
Y17386             QGTVPSLEHDGRVTGESLDLIKYIDTNFQGPALLPQDPAKRQFADELIAYADAFTKALYSPLISQVAMSDEAVAALDKIE

                          170       180       190       200       210       220       230       240
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GmGSTL             NALGKFDDGPFFLGQFSLADIAYVSFLERFQIVFSEIFKHDITAGRPKLATWIQEGNK---------IDGYKQTKVDREE
AT5G02790.1        NALYKFDDGPFFLGQLSLVDIAYIPFIERFQTVLNELFKCDITAERPKLSAWIEEINK---------SDGYAQTKMDPKE
Medtr7g100320.1    NALGKFDDGPFFLGQFSWVDIAYVPFVERFHIVFSEVFKHDITEGRPKLAAWIEVQLKQLSLKLQFSIIAFIYELITNQG
GRMZM2G162486_T01  DALGKFNDGPFFLGQFSLVDVAYVPFIERFQILYSNIKNYDVTKGRPNLQKFIEEVNK---------IDAYTQTKLDPQF
LOC_Os03g17480.1   AALSKFGDGPFFLGQFSLVDIAYVTIIERIQIYYSHIRKYEITNGRPNLEKFIEEINR---------IEAYTQTKNDPLY
Y17386             AALSKFSDGPFFLGQFSLVDIAYVTILERVQIYYSNLRNYEIAKDRPNLERYTEEMNK---------IEAYKQTKNVPLA

                          250
                   ....|....|....
GmGSTL             YLEAFKKKFLVL--
AT5G02790.1        IVEVFKKKFM----
Medtr7g100320.1    HQSKLEENFICSEN
GRMZM2G162486_T01  LLEQTKKRLGIA--
LOC_Os03g17480.1   LLDLAKTHLKVA--
Y17386             LLDAAKRHLKIA--
```

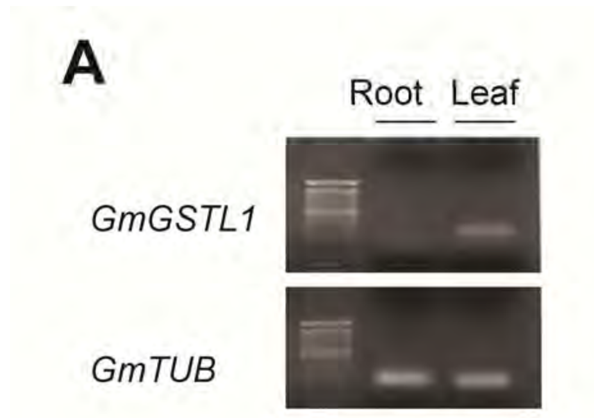
Fig. 3.4 Sequence alignment of GmGSTL1 and its homologues in *A. thaliana*, *Medicago*, maize, rice, and wheat. The N-terminal domain consisted of TRX domain (solid arrow) which contained the GSH binding motif and the active site cysteine (asterisk). The C-terminal domain consisted of typical alpha helical domain of class Lambda GST (dashed arrow) with hydrophobic substrate binding pocket which is for substrate binding. Shaded region represented conserved residues.

3.4 Expression analysis of *GmGSTL1*

The first screening attempt for candidate gene expression was perform using soybean leaf samples under a specific salt treatment time point. To further elucidate the tissue specific expression and salinity-induced changes of *GmGSTL1*, root and leaf samples were harvested at various time points for expression profiling.

3.4.1 *GmGSTL1* was mainly expressed in soybean leaf

Gene expression was monitored by both reverse transcription PCR and qPCR. Under normal conditions, *GmGSTL1* expression was about 10-fold higher in leaf samples than root samples (Fig. 3.5).



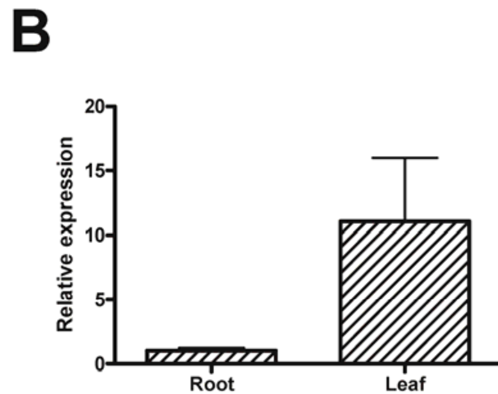


Fig. 3.5 Differential expression of *GmGSTL1* in leaf and root. (A) The expression of *GmGSTL1* was monitored by RT-PCR and visualized by ethidium bromide staining after 30 PCR cycles for *GmGSTL1* and 25 cycles for the soybean *tubulin* gene (*GmTUB*), respectively. (B) Relative expression was calculated by the ΔΔCT method (Livak and Schmittgen 2001), using expression of the soybean *tubulin* gene for normalization. The expression of *GmGSTL1* in untreated sample was set to 1 for comparison. *GmGSTL1* was predominantly expressed in the leaf.

3.4.2 *GmGSTL1* was induced in the leaf under salinity stress

Under 0.9% NaCl treatment, expression of *GmGSTL1* was induced in leaf samples to about 2-fold after 30 minutes, and up to 7-fold after 24 hours treatment (Fig. 3.6A,B). On the other hand, expression of *GmGSTL1* in root samples was reduced over time (Fig. 3.6C).

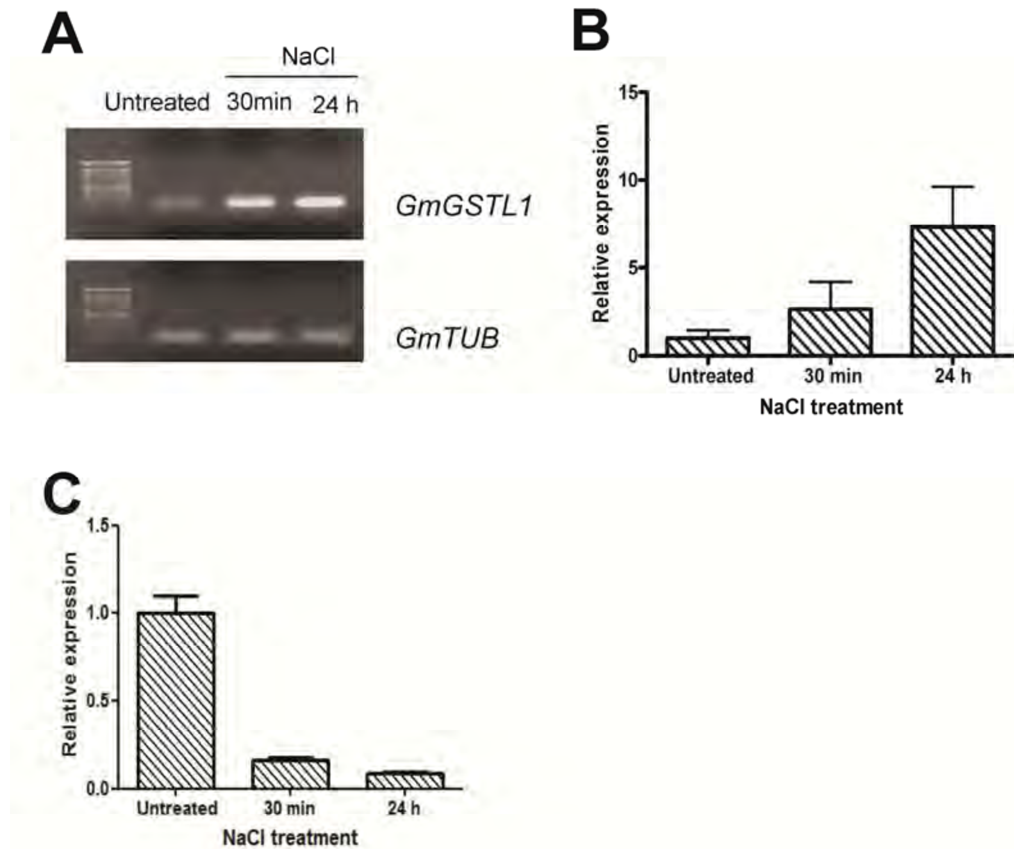


Fig. 3.6 Expression of *GmGSTL1* was induced in soybean leaf under 0.9% NaCl treatment. (A) The expression of *GmGSTL1* was monitored by RT-PCR and visualized by ethidium bromide staining after 30 PCR cycles for *GmGSTL1* and 25 cycles for the soybean *tubulin* gene (*GmTUB*), respectively. (B) Relative expression was calculated by the ΔΔCT method (Livak and Schmittgen 2001), using expression of the soybean *tubulin* gene for normalization. The expression of *GmGSTL1* in untreated sample was set to 1 for comparison. (C) In root samples, the expression of *GmGSTL1* was reduced under salt stress.

3.5 Functional analysis

The function of *GmGSTL1* was assessed by heterologous expression in both BY-2 cell and *A. thaliana*. Transgenic lines stably expressing the transgene were selected for subsequent experiments. Fig. 3.7 summarized transgene expression in BY-2 cell lines and *A. thaliana* respectively.

3.5.1 Over-expression of *GmGSTL1* in BY-2 cells reduced ROS accumulation under salinity stress

Salinity-induced ROS accumulation could be revealed by monitoring the increase in fluorescence intensity of H_2DCFDA staining. Confocal microscope imaging revealed that ROS accumulation was not homogenous in the cell population (Fig. 3.8). Only a sub-population of cells were vulnerable to ROS accumulation, probably because the culture contained mixed population at various cell stages. Therefore, measurements in microplate format was adopted [141]. Under 100 mM NaCl treatment, wild type (WT) and empty vector transgenic control (V7) cell lines exhibited rapid increase in fluorescence signal, indicating an increase in ROS under stress; while GmGSTL1 transformants (GmGST_b1 and GmGST_b2) contained much lower ROS (Fig. 3.9).

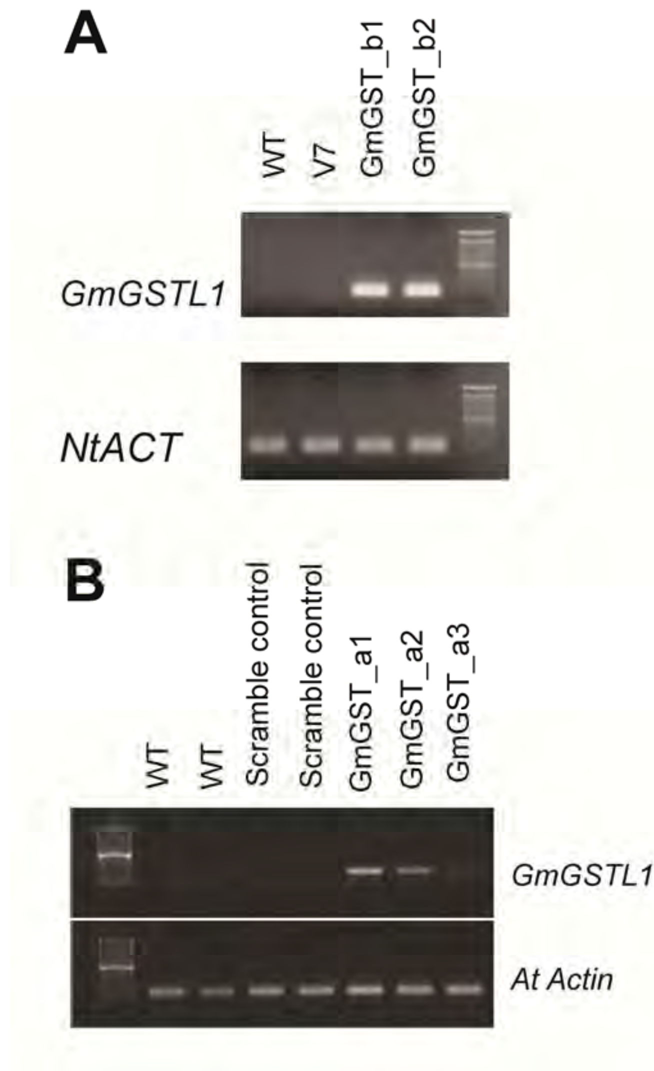


Fig. 3.7 Expression of *GmGSTL1* in tobacco BY-2 cell lines and transgenic *A. thaliana* (A) The expression of *GmGSTL1* was monitored by RT-PCR and visualized by ethidium bromide staining after 25 PCR cycles for both the *GmGSTL1,* the tobacco *actin* gene (*NtACT*) and (B) *Arabidopsis actin* gene (*AtACT*). No signal of *GmGSTL1* was detected in the untransformed wild type (WT, Col-0) or other controls.

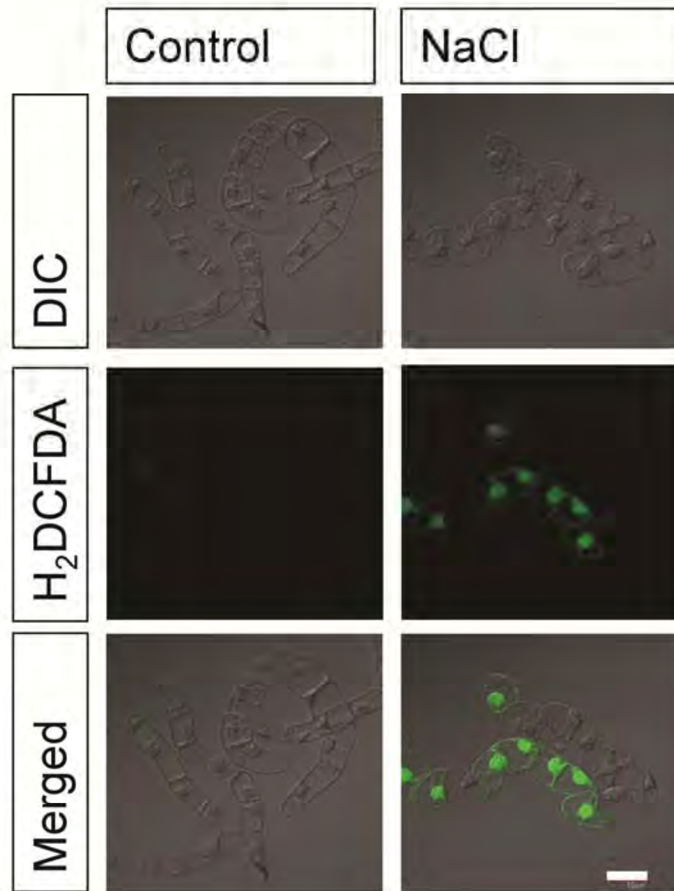


Fig. 3.8 ROS accumulation was observed in a sub-population of cells. Three-day culture was subjected to 100mM NaCl treatment for 1 hour. H_2DCFDA staining was visualized by confocal microscopy. It was observed that ROS accumulation was not homogenous but only occured in a sub-population of cells. Scale bar = 50 µm

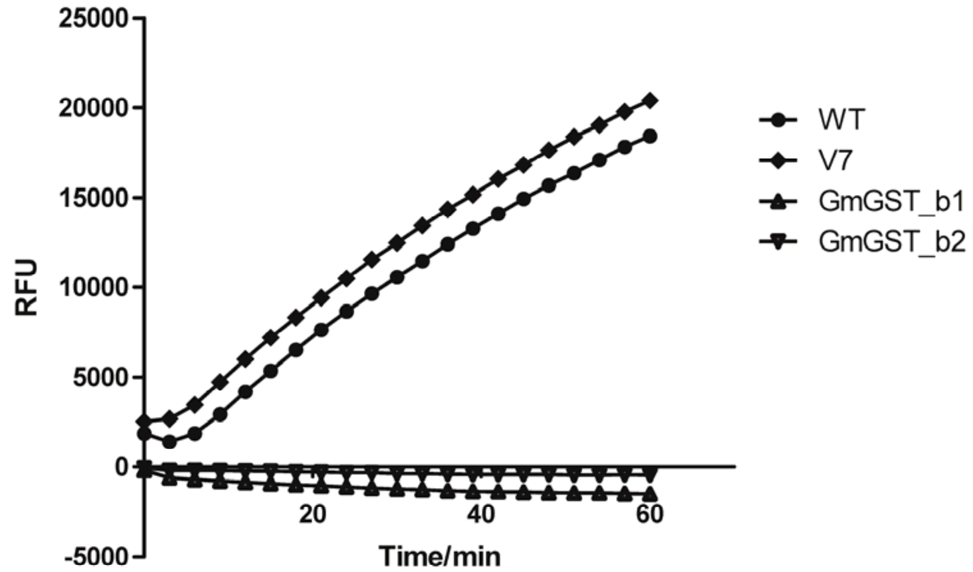


Fig. 3.9 Gain-of-function study using transgenic tobacco BY-2 cells. Ectopic expression of *GmGSTL1* in BY-2 cells reduced ROS accumulation under salinity stress. Three-day-old culture was subjected to 100 mM NaCl treatment for 1 h. H_2DCFDA fluorescence was quantified in microplates. The increase in Relative Fluorescence Unit (RFU) was much more profound in control cell lines (WT, wild type and V7, empty-vector transgenic control) than two independent *GmGSTL1* transformants (GmGST_b1 and GmGST_b2). Error bar: standard error.

3.5.2 Over-expression of *GmGSTL1* in BY-2 cells enhanced cell survival under salinity stress

Salinity-induced cell death was assessed using conventional trypan blue staining. Viable cells with intact cell membrane would exclude the dye and appear colourless under microscope, while dead cells would be stained blue. After the application of 100 mM NaCl for 1 day, cell death was observed. The survival rates in the WT and V7 lines were 65±3% and 62±1% respectively, which were significantly lower than those of the transgenic lines, 80±5% and 84±6% for GmGST_b1 and GmGST_b2, respectively (Fig. 10).

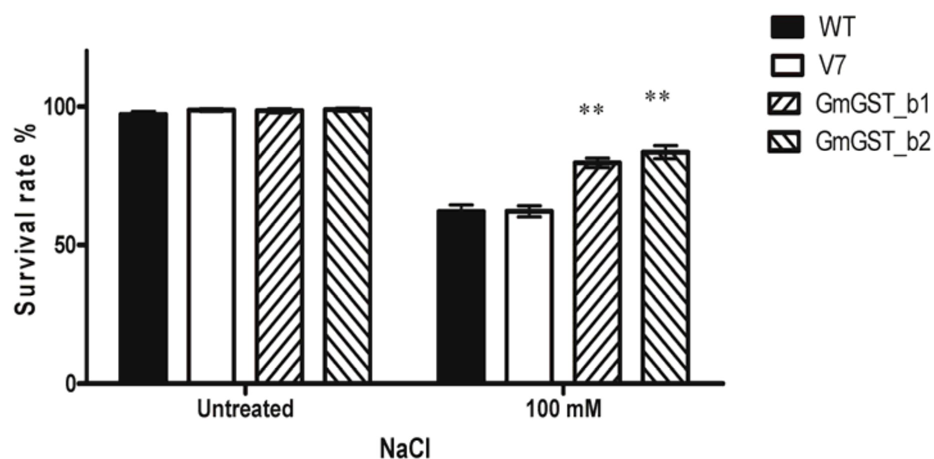
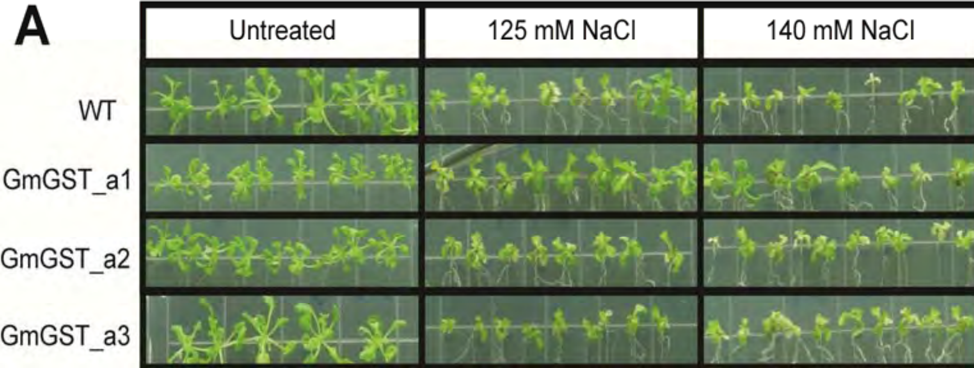


Fig. 3.10 Ectopic expression of *GmGSTL1* in BY-2 cells enhanced cell survival under salinity stress. Three-day-old culture was subjected to 100 mM NaCl treatment for 24 h. Survival rates were monitored by trypan blue staining. Values represented mean % survival of at least 10 random views of a total cell counts more than 400. Error bar: standard error. **Denoted significant mean difference at $p<0.01$ when compared to WT, using one-way ANOVA followed by the post hoc Tukey's test. WT: wild type BY-2 cells; V7: empty-vector transgenic control; GmGST_b1 and GmGST_b2: two independent *GmGSTL1* transformants.

3.5.3 Over-expression of *GmGSTL1* in *A. thaliana* reduced salinity-induced leaf chlorosis

To provide further evidence for the protective function of *GmGSTL1 in planta*, transgenic *A. thaliana* ectopically expressing *GmGST1* was constructed. No abnormal phenotypes were observed in the transgenic plants under normal growth conditions. Under NaCl treatment, growth retardation was observed (Fig. 3.11A). Severe leaf chlorosis was observed and quantitative measurements confirmed that the chlorophyll content in the wild type (WT) plant was reduced (Fig. 3.11B) On the other hand, the *GmGSTL1* transgenic lines exhibited enhanced tolerance and higher chlorophyll contents than WT in general. Similar patterns of results were obtained when the NaCl concentration was raised to 140 mM (Fig. 3.11B).


A
Untreated
125 mM NaCl
140 mM NaCl
WT
GmGST_a1
GmGST_a2
GmGST_a3

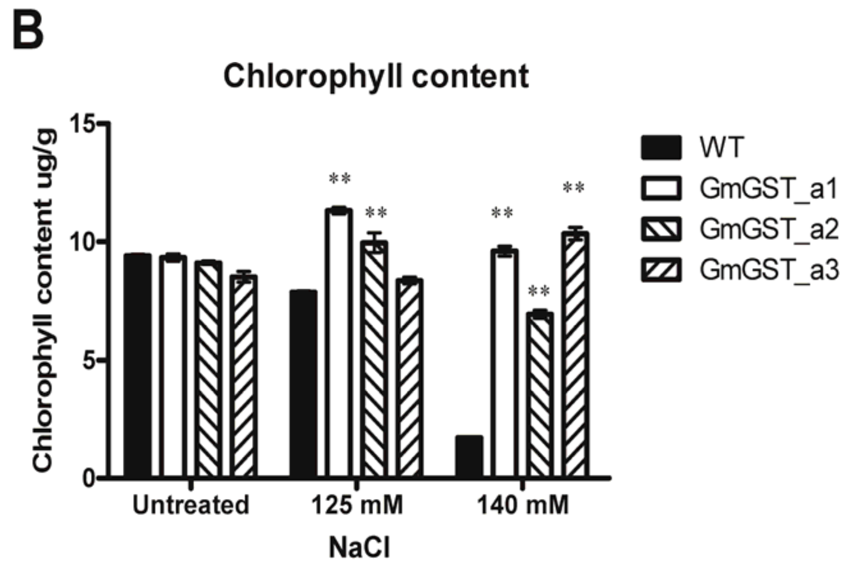
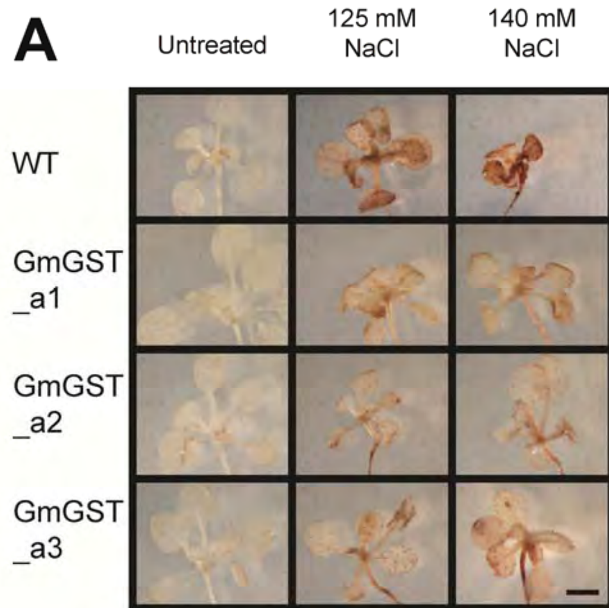

B
Chlorophyll content
Chlorophyll content ug/g
15
10
5
0
Untreated
125 mM
140 mM
NaCl
**
WT
GmGST_a1
GmGST_a2
GmGST_a3

Fig. 3.11 Growth performance of *GmGST1* transgenic lines under salt stress. Ten-day seedlings were subjected to NaCl treatment for 14 days. (A) Growth phenotypes under salt treatment. (B) The extent of leaf chlorosis was revealed by determining the chlorophyll content. GmGST_a1, GmGST_a2, and GmGST_a3 were independent *GmGSTL1* transgenic lines. WT: untransformed wild type Col-0. N=3 (each data point represented a pool sample of at least 4 individual plants). Error bar: standard error. ** denotes significant difference at $p<0.01$ level when compared to the WT control, according to one-way ANOVA analysis followed by the post hoc Tukey's test.

3.5.4 Over-expression of *GmGSTL1* in *A. thaliana* reduced salinity-induced ROS accumulation

To further elucidate the protective function of *GmGSTL1*, We included the histological staining of ROS using diaminobenzidine tetrahydrochloride (DAB) as another marker (Jambunathan, 2010) (Fig. 3.12A). The results were consistent with that of the chlorophyll determination. Quantitative measurement of the DAB staining using ImageJ (ver. 1.47) also showed that the *GmGSTL1* transgenic lines accumulated less ROS (Fig. 3.12B).


A
Untreated
125 mM NaCl
140 mM NaCl
WT
GmGST _a1
GmGST _a2
GmGST _a3

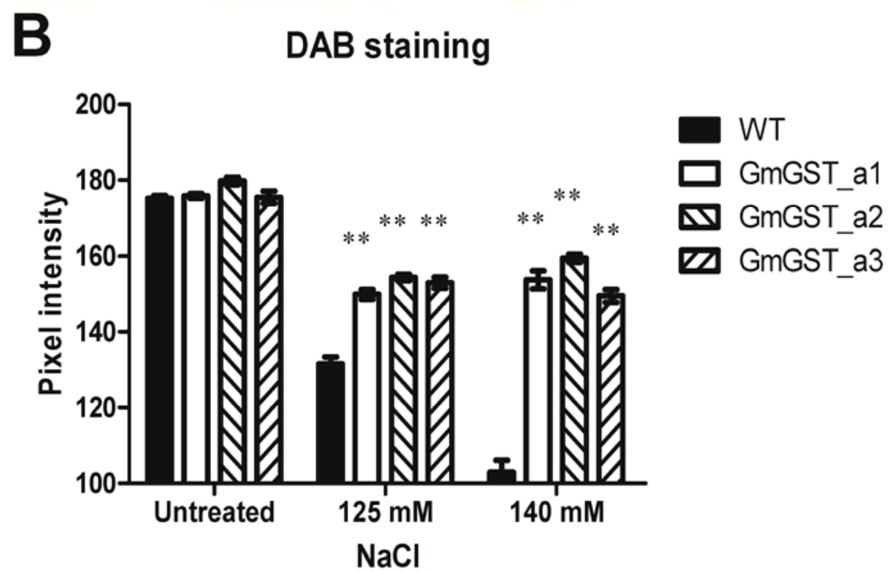

B
DAB staining
200
180
160
140
120
100
Pixel intensity
Untreated
125 mM
140 mM
NaCl
WT
GmGST_a1
GmGST_a2
GmGST_a3

Fig. 3.12 (A) Histological staining with diaminobenzidine tetrahydrochloride (DAB) for ROS. Scale bar = 2 mm. WT: untransformed wild type Col-0; GmGST_a1, GmGST_a2, GmGST_a3: three independent *GmGSTL1* transgenic lines. (B) Staining signals were quantified by measuring the image pixel intensity using ImageJ (ver. 1.47). N≥10. Error bar: standard error. ** denotes significant difference at $p<0.01$ level when compared to the WT control, according to one-way ANOVA analysis followed by the post hoc Tukey's test.

3.6 Enzyme activity assay

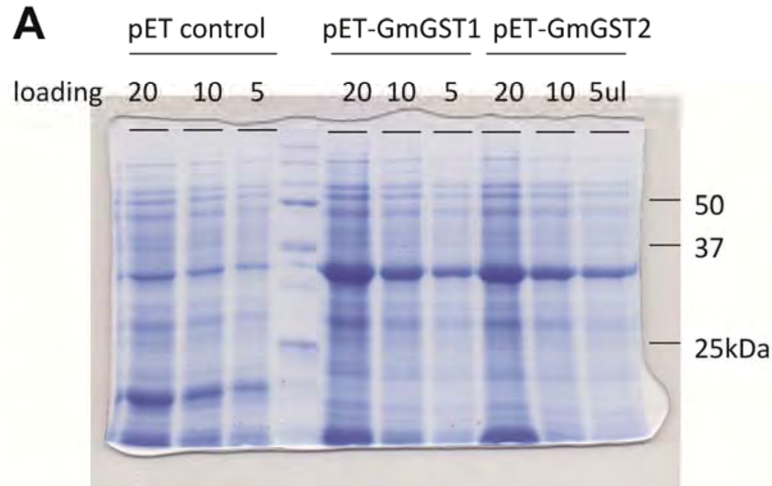
Given *GmGSTL1* provided functional protection to plant, demonstrated by improved survival and reduced ROS accumulation, the most straight forward postulation would be that protection was accounted by an associate increase in GST enzyme activity in transgenic over-expressors. Therefore, we intended to express the protein *in vitro* and assay for the enzyme activity of GmGSTL1 protein.

3.6.1 Protein expression and purification

His-tagged GmGSTL1 recombinant protein was expressed in *Escherichia coli* as described in methods and materials. Using matrix-assisted laser-desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis, 13 trypsin-digested peptides were obtained from the target protein band on the PAGE gel (Fig. 3.13A). The fragments were matched to the hypothesized sequence with an overall score of 244, confirming the correct expression of the target (Fig. 3.13B). Purification was performed using HisTrap affinity column and HisTrap desalting column (Fig. 3.14A). The whole process was carried out in the cold room to retain maximum possible enzyme activity of the recombinant protein.

3.6.2 GmGSTL1 exhibited GSH dependent GST activity

The specific GSH-dependent GST activity was determined by measuring the absorbance of the CDNB conjugate. The specific enzyme activity of GmGSTL1 was found to be 63.52±6.12 nmol/mg/min (Fig. 3.14B), which was in the same order of magnitude as the reported soybean homologue but up to three orders of magnitude lower than some other reported GSTs [145]. The relatively low GSH-dependent GST activity of the Lambda class GSTs suggested that they may play a functional role different from other high-activity GSTs.



B

Gel Idx/Pos	176/H7	Instr./Gel Origin	Ab347000116/MSMS28092011	Process Status	Analysis Succeeded
Plate [#] Name	[1] 4792	Instrument Sample Name		Spectra	7

Rank	Protein Name	Accession No.	Protein MW	Protein PI	Pep. Count	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Best Ion Score	Best Ion C. I. %	Result Type
1	Glyma03g33340.4	Glyma03g33340.4	26805.8	5.73	13	244	100	124	100	69	99.999	Mascot

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
934.4893	934.5119	0.0226	24	64	70	NRPAWYK					Mascot
994.5428	994.5672	0.0244	25	193	201	HDITAGRPK					Mascot
1114.6466	1114.6787	0.0321	29	88	97	VLGESLDLIR	35	97.44			Mascot
1114.6466	1114.6787	0.0321	29	88	97	VLGESLDLIR					Mascot
1257.6877	1257.7218	0.0341	27	183	192	FQIVFSEIFK					Mascot
1398.6899	1398.733	0.0431	31	220	230	VDREEYLEAFK	20	24.092			Mascot
1398.6899	1398.733	0.0431	31	220	230	VDREEYLEAFK					Mascot
1580.9258	1580.9723	0.0465	29	50	63	GLQDKIELVPIDLK					Mascot
1712.8966	1712.9403	0.0437	26	73	87	VYPTNKVPSLEHNGK					Mascot
1735.9014	1735.9532	0.0518	30	202	216	LATWIQEGNKIDGYK					Mascot
2122.0603	2122.1272	0.0669	32	98	116	YIDANFEGAPLFPTDPAKR	69	99.999			Mascot
2122.0603	2122.1272	0.0669	32	98	116	YIDANFEGAPLFPTDPAKR					Mascot
2138.04	2138.1235	0.0835	39	139	158	GDPIGQTSAAFDYLENALGK					Mascot
2233.2126	2233.2808	0.0682	31	183	201	FQIVFSEIFKHDITAGRPK					Mascot

Fig. 3.13 Recombinant GmGSTL1 expression and characterization. (A) Whole cell lysate was separated on SDS-PAGE gel. The target band (~32kDa) was excised and subjected to MALDI-TOF/TOF MS analysis. (B) 13 peptide counts were matched to Glyma03g33340 with an overall score of 244, confirming correct expression of target protein. pET-GmGST1 and 2 represented independent bacterial preparations.

B

GST specific activity (nmol/mg/min)		
GmGSTL1	BSA	Blank
63.52±6.12	0	0.10±3.34

Fig. 3.14 GmGSTL1 protein purification and enzyme assay. (A) Coomassie blue staining showing the purification of the recombinant protein. The predicted molecular weight of the His-tagged GmGSTL1 was about 32 kDa. (B) Specific activity of GmGSTL1. Specific enzyme activity was determined and calculated according to the manufacturer's manual (Sigma, Cat. No. CS0410). The unit was converted to published values for comparison (McGonigle et al. 2000).

3.7 Ligand identification

In wheat, GSTL was found to interact with phenolic anti-oxidant [143]. *In vitro* experiments showed that TaGSTL1 could mediate the GSH-dependent reduction of derivatives to regenerate active quercetin, which acts as a proton donor to oxidative species [143]. We, therefore, extracted the total polyphenolic compounds from soybean leaves and searched for potential ligands that might bind to GmGSTL1. Owing to limited standards available, we only targeted the well-known phenolic anti-oxidants, including chlorogenic acid, rutin, quercetin, and kaemferol, and also those flavonoids most abundant in soybean, including daidzin, glycitin, genistin, glycitein, and daidzein. Details of authentic standards used in the experiment were summarized in Table 3.1.

Table 3.1 Authentic standards used in FTMS experiment

No.	Chemical Name	Chemical Formula	Calculated m/z [M-H]-	Measured m/z [M-H]-	Error (ppm)	Retention time (min)
1	GSH	$C_{10}H_{17}N_3O_6S$	306.075432	306.077290	6.07	1.94
2	Chlorogenic acid	$C_{16}H_{18}O_9$	353.086709	353.087630	2.61	1.97
3	Daidzin	$C_{21}H_{20}O_9$	415.102359	415.105220	6.89	19.59
4	Glycitin	$C_{22}H_{22}O_{10}$	445.112923	445.113200	0.62	21.67
5	Genistin	$C_{21}H_{20}O_{10}$	431.097273	431.100390	7.23	30.54
6	Rutin	$C_{27}H_{30}O_{16}$	609.145011	609.144500	-0.84	30.98
7	Glycitein	$C_{16}H_{12}O_5$	283.060100	283.061470	4.84	46.02
8	Daidzein	$C_{15}H_{10}O_4$	253.049535	253.050970	5.67	49.09
9	Quercetin	$C_{15}H_{10}O_7$	301.034279	301.036220	6.45	59.11
10	Kaemferol	$C_{15}H_{10}O_6$	285.039364	285.040990	5.70	64.08

3.7.1 Optimization of HPLC gradient for analysis

A number of solvent gradients and flow rate combinations were tested to resolve the available standards. Eventually, the HPLC profile was modified from Chang and Wong [144]. The retention time as well as the first dimension mass spectrum provided dual evidence for the true identity of target compounds. The resolved extracted ion chromatogram (EIC) and mass spectrum were summarized in Fig. 15.

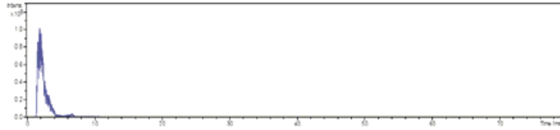
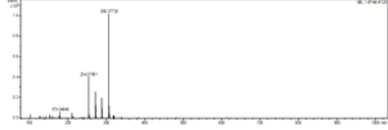
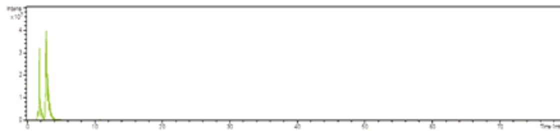
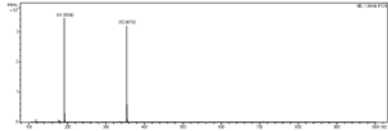
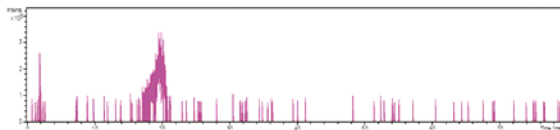
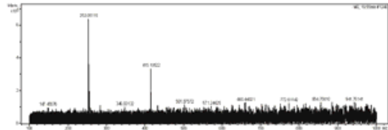
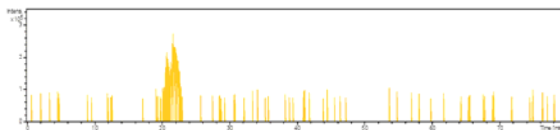
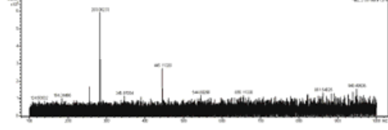
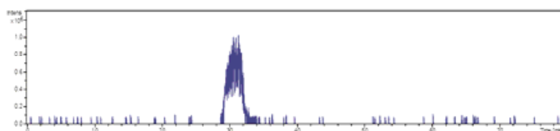
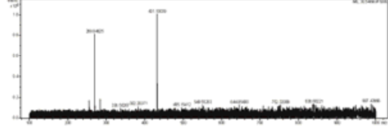
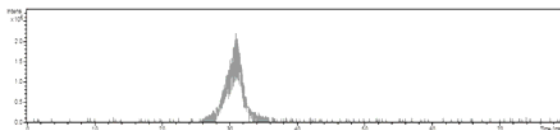
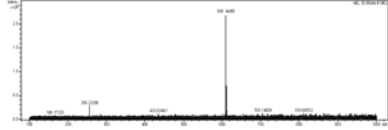
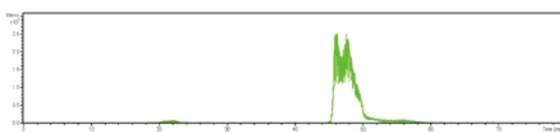
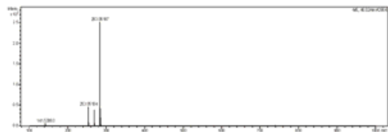
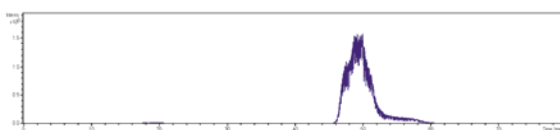
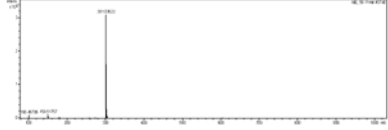
	Standards	
	EIC	Spectrum
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

Fig. 3.15 Liquid chromatography profile and spectrum of available authentic standards. 1.GSH, 2.Chlorogenic acid, 3.Daidzin, 4.Glycitin, 5.Genistin, 6.Rutin, 7.Glycitein, 8.Daidzein, 9.Quercetin, 10.Kaemferol. EIC: extracted ion chromatogram. Y-axis: signal intensity of ion with the given mass to charge ratio. X-axis: retention time. Spectrum: ion spectrum of the EIC peak.

3.7.2 GmGSTL1 interact with polyphenolic metabolites

Total phenolic compounds were extracted by solvent extraction followed by affinity column as described in section 2.8.1. Fraction recovered by HisTrap column for the recombinant protein was subjected to FTMS analysis. However, we did not resolve expected flavonoids which are abundant in soybean seed. Instead, two candidate flavonoids, namely kaemferol (EIC 285.041±0.001, retention time 63.82) and quercetin (EIC 301.036±0.001, retention time 60.50), were identified in the GmGSTL1-bound fraction (Fig. 3.16). Estimation of the ligand amounts was also performed using standard curves (Fig. 3.17).

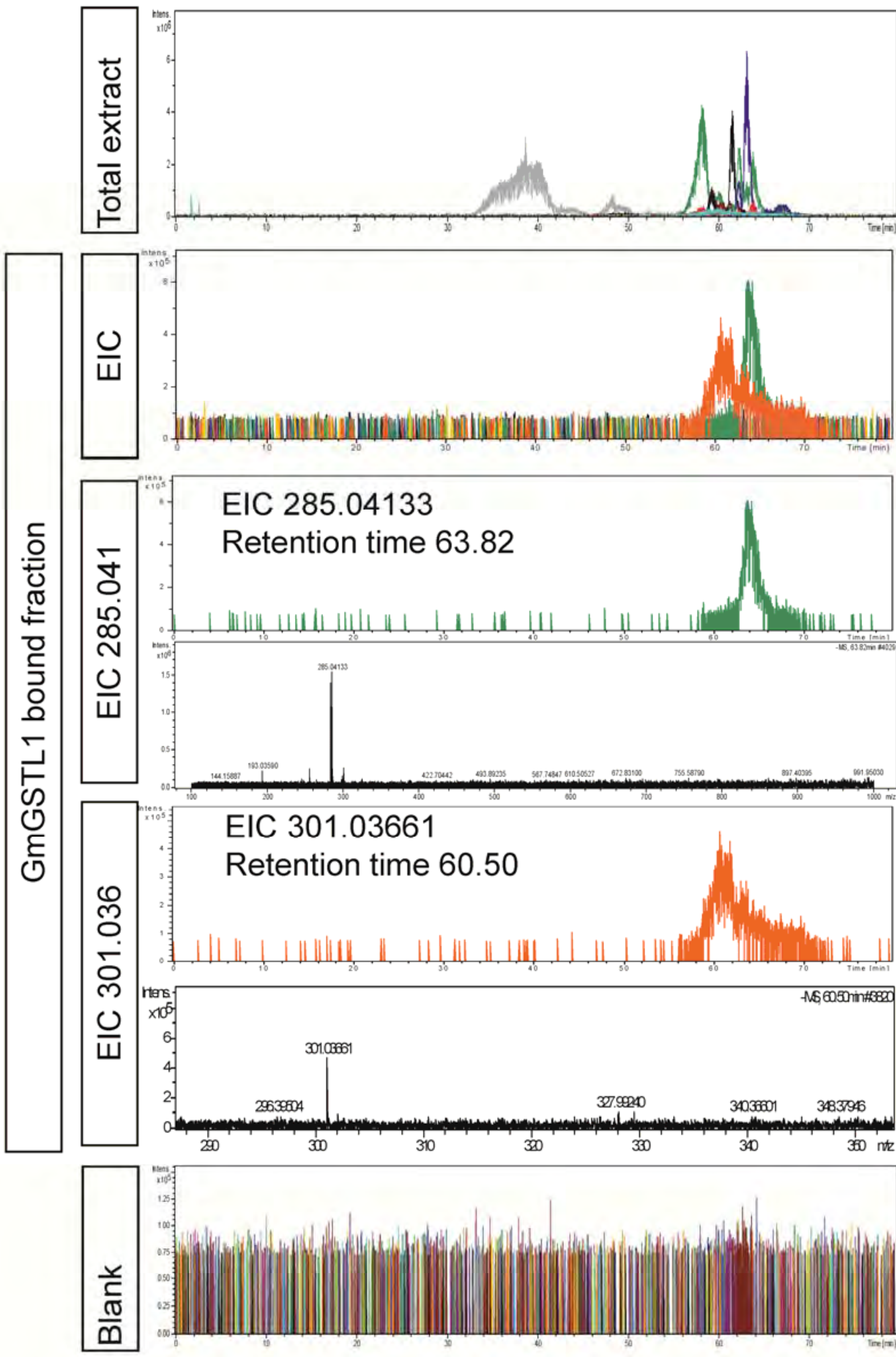
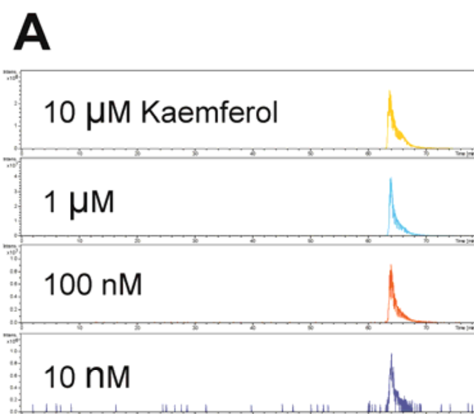
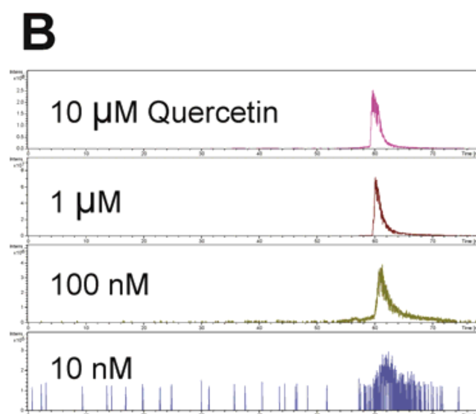

Total extract
GmGSTL1 bound fraction
EIC
EIC 285.041
EIC 285.04133
Retention time 63.82
EIC 301.036
EIC 301.03661
Retention time 60.50
Blank

Fig. 3.16 GmGSTL1 binds to polyphenolic metabolites. Total phenolic compound was extracted from soybean leaflet by solvent extraction followed by purification using affinity column embedded with HP-20 resin. Recombinant protein of GmGSTL1 was immobilized on His-Trap column and flushed with total phenolic extracts. Fractions of eluted ligands were pooled, concentrated and subjected to FTMS analysis. Two candidate flavonoids, namely kaemferol (EIC 285.041±0.001, retention time 63.82) and quercetin (EIC 301.036±0.001, retention time 60.50), were identified in the GmGSTL1-bound fraction. EIC: extracted ion chromatogram.

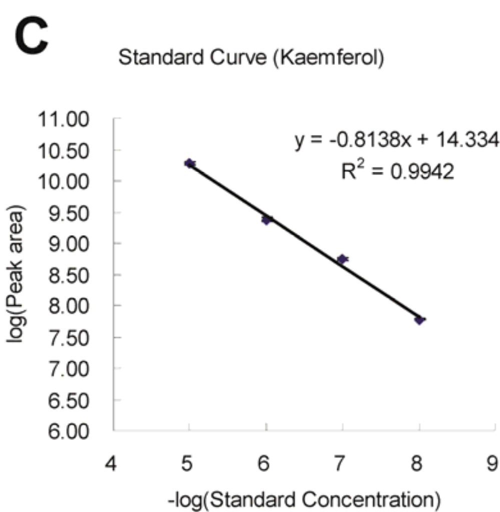
A



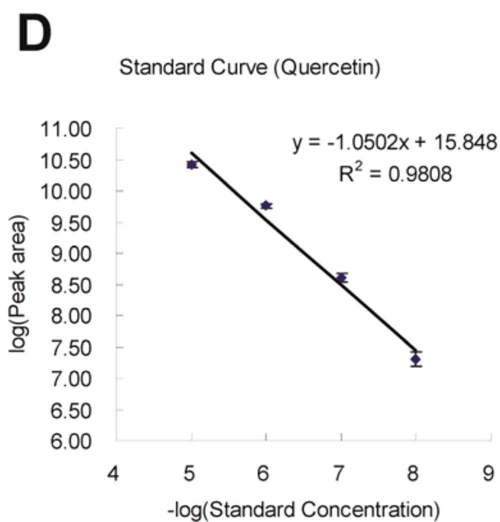
B



C



D



E

Ligand EIC In samples	Peak area	log Area	Concentration (nM)	Concentration Average	Concentration SD	Ligand amount (μg) per 16 mg bait protein
Kaemferol	141,503,545	8.15	25.23	28.11	3.10	8.01±0.85
	168,980,809	8.23	31.38			
	152,748,133	8.18	27.72			
Quercetin	79400586.67	7.88	25.68	30.37	4.07	9.14±1.22
	98040858.33	7.99	33.03			
	96077114.33	7.98	32.40			

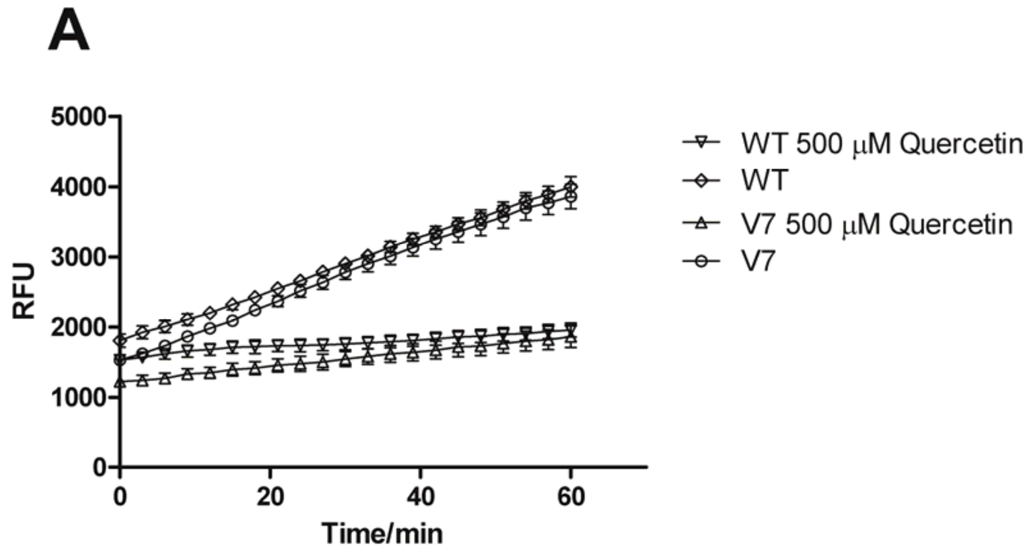
Fig. 3.17 Standard curves and ligand quantification. (A,B) Authentic standards were diluted in 10-fold series and subjected to FTMS analysis. (C,D) Standard curves were generated by plotting log values of peak area versus –log value of standard concentrations. Error bar: standard error of injection triplicates. (E) Calculation of ligand amounts.

3.8 Complementation assay

Quercetin has one more -OH group and has ~4-fold higher anti-oxidation activity (based on TEAC value) than kaemferol [146]. Therefore, we focused our further tests on quercetin and studied its possible protection roles against salinity stress in plants. Interestingly, supplementation of exogenous quercetin could functionally improve plant tolerance toward salinity stress in a way similar to the ectopic expression of the *GmGSTL1* transgene.

3.8.1 External supplementation of quercetin reduced ROS accumulation in BY-2 cells under salinity stress

Kinetics of H_2DCFDA fluorescence accumulation in the untransformed wild type cells (WT) and empty vector transformation control (V7) were again monitored same as section 3.5.1. Addition of quercetin reduced H_2DCFDA fluorescence accumulation in WT and V7 during the 1 hour kinetic measurement (Fig. 3.18A). Reducing quercetin supplementation resulted in higher ROS accumulation (Fig. 3.18B).


A
WT 500 μM Quercetin
WT
V7 500 μM Quercetin
V7
RFU
5000
4000
3000
2000
1000
0
0
20
40
60
Time/min

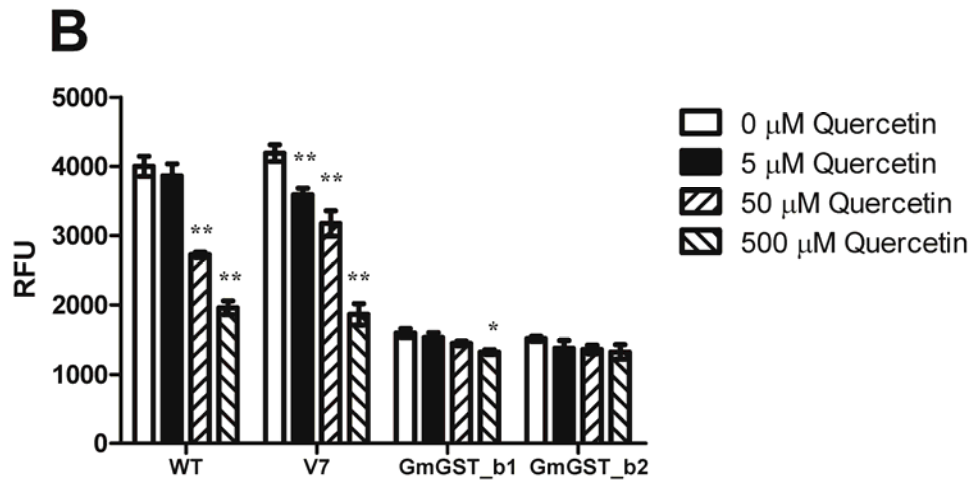
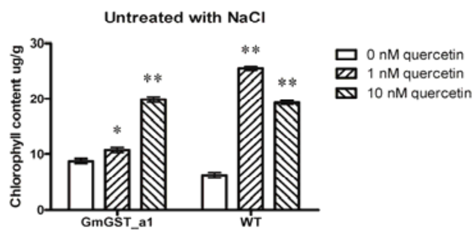

B
0 μM Quercetin
5 μM Quercetin
50 μM Quercetin
500 μM Quercetin
RFU
5000
4000
3000
2000
1000
0
**
*
WT
V7
GmGST_b1
GmGST_b2

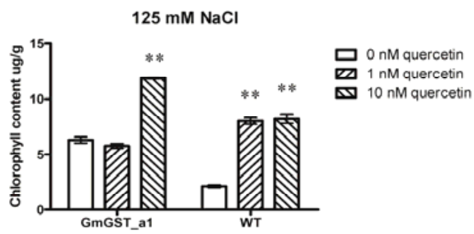
Fig. 3.18 Application of quercetin reduced salinity induced ROS accumulation in BY-2 cells. Three-day-old culture was subjected to 100 mM NaCl treatment for 1 h. H_2DCFDA fluorescence was quantified in microplates. (A) Kinetics of H_2DCFDA fluorescence accumulation in the untransformed wild type cells (WT) and empty vector transformation control (V7) with or without 500 μM quercetin. (B) Endpoint measurement after 60 min in the presence of different final concentration of quercetin. Error bar: standard error. N=4. * and ** denoted significant mean difference at $p<0.05$ and $p<0.01$ respectively when compared to the control without quercetin supplementation, using one-way ANOVA followed by the post hoc Tukey's test.

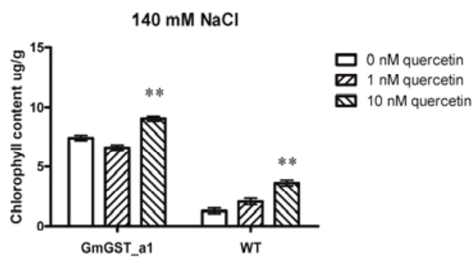
3.8.2 External supplementation of quercetin enhanced plant survival under salinity stress

A. thaliana seedlings were again subjected to NaCl treatment, and chlorophyll content was measured. Application of quercetin significantly improved plant survival under salt treatment and the measured chlorophyll content was significantly higher than the respective control group (with 0 mM quercetin) (Fig. 3.19A). ROS accumulation by DAB staining was also employed as an alternative marker. The result was consistent with chlorophyll measurement (Fig. 3.19B).

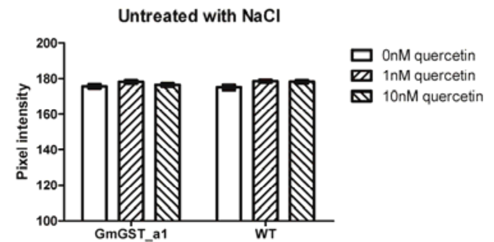
A

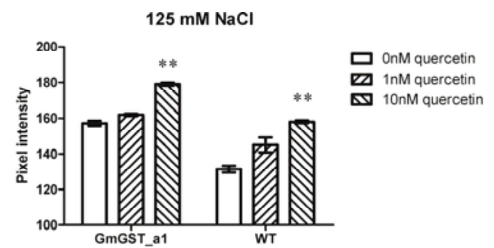






B





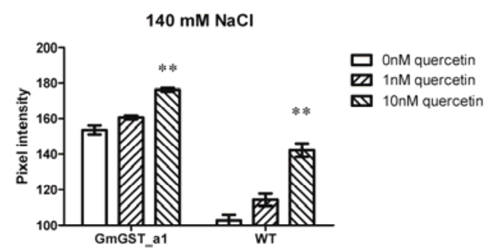


Fig. 3.19 Application of quercetin reduced salinity induced leaf chlorosis and ROS accumulation. (A) Chlorophyll content and (B) ROS accumulation were measured when 10-day-old seedlings were subjected to NaCl treatment without or with 1 nM and 10 nM quercetin supplements for 14 days. WT: untransformed wild type Col-0. GmGST_a1: GmGSTL1 transgenic line. Error bar: standard error. * and ** denoted significant difference at $p<0.05$ and $p<0.01$ respectively, when compared to the control without quercetin supplementation, using one-way ANOVA followed by the post hoc Tukey's test.

Chapter 4 Discussion and Conclusion

The anti-oxidative characteristics of GST family have been extensively reviewed [98, 99, 147]; however, the molecular nature of the transferase activity in relation to plant stress tolerance is largely untouched. In this study, we reported a salinity stress-induced Lambda class GST in wild soybean, which is tolerant to salt stress, and provided detailed evidence for its physiological role and further extended its endogenous function through the identification of molecular interactant, which function in a way similar to GmGSTL1 over-expression, *in planta*.

4.1 Glyma03g33340 encode a putative Lambda class glutathione-S-transferase

Glutathione-S-transferases (GSTs) are ancient and highly diverse gene family. Despite the common function for catalyzing GSH dependent transferase activity, the structure and function are highly diverse between classes [129]. The F and U classes are the biggest and most studied plant-specific classes, while T and Z class are close to mammalian GSTs [121]. The function of L class GST is largely unknown. Phylogenetic analysis showed that Glyma03g33304 belong to the L class (Fig 3.3) and study its function will give putative insight to the function of L class of GST enzyme. By multiple alignment with close homologs of other plant species, including *A. thaliana*, *Medicago*, maize, rice and wheat,

the conventional conserved domain for GST activity could be identified. The N-terminal consisted of TRX domain [120] which harbors the GSH binding consensus (G-site) and the well conserved active site cysteine (Fig. 3.4). The C-ternimal consisted of the typical alpha helical domain of Class Lambda Glutathione S-transferases (Fig. 3.4) with hydrophobic substrate binding pocket (H-site) which is for endogenous and xenobiotic alkylating agents, including carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress [99, 121].

4.2 *GmGSTL1* expression is mainly in soybean leaf and its expression is induced under salinity stress

To elucidate the tissue specific expression and salinity-induced changes, root and leaf samples were harvested at various time points for expression profiling. Under normal conditions, *GmGSTL1* expression was about 10-fold higher in leaf samples than root samples (Fig. 3.5). Under 0.9% NaCl treatment, expression of *GmGSTL1* was induced in leaf samples to about 2-fold after 30 minutes, and up to 7-fold after 24 hours treatment (Fig. 3.6A,B). On the other hand, expression of *GmGSTL1* in root samples was reduced over time (Fig. 3.6C).

4.3 Over-expression of *GmGSTL1* alleviate salinity stress induced symptoms in transgenic cells and plants

The functional role of *GmGSTL1* was double confirmed by heterologous system, including the tobacco cell model and *A. thaliana* plant model. In both cases, overexpression of *GmGSTL1* conferred functionally protection and enhanced survival of transformant under salt treatment. In the BY-2 cells, over-expression of *GmGSTL1* reduced ROS accumulation, as revealed by reduced fluorescence (Fig. 3.9) and enhanced cell survival (Fig. 3.10) under salt treatment. In plant, over-expression of *GmGSTL1* reduced leaf chlorosis (Fig. 3.11) and ROS accumulation, as revealed by DAB staining (Fig. 3.12) under salt treatment.

4.4 *GmGSTL1* encodes a functional enzyme exhibiting GSH dependent GST activity

GmGSTL1 was one gene candidate in the salt tolerant QTL that was up-regulated in response to salinity stress. Altered transcript profile ordinarily followed or paralleled by increased enzyme activity [148]. An accompanied increase in enzyme activity, however, might not provide direct explanation in these cases, as the specificity GST activity of GmGSTL1 was up to three order of magnitude lower (Fig. 3.14) than some other reported GmGSTs [145]. We

then hypothesize the alternative mechanism, through which GmGSTL1 acted indirectly on a substrate which provided the anti-oxidative capacity.

4.5 GmGSTL1 interacts with polyphenolic metabolites

The relatively low GSH-dependent GST activity of the Lambda class GSTs suggests that they may play a functional role different from other high-activity GSTs. In plant, GSTs were found to interact with secondary metabolites [143, 149, 150]. For example, rutin and kaempferol-3-O-rutinoside, not previously described as natural products in wheat, were identified as putative ligands to TaGSTL1 [143]. Instead of directly acting on ROS, GSTLs were proposed to help maintaining the antioxidant flavonol pool by catalyzing the conversion of quinone intermediates to its active antioxidative form. *In vitro* experiments showed that TaGSTL1 could mediate GSH dependent reduction of derivatives to regenerate active quercetin which act as proton donor to oxidative species. The oxidized quercetin derivatives then spontaneously react with water and GSH to form adduct, which is recycled as substrate for GSTL enzyme activity [143].

Therefore, we extract total polyphenolic compounds from soybean leaflets and

looked for potential ligands that might bind to GmGSTL1. Owing to limited standards available, we attempted to target for well known phenolic antioxidants, including chlorogenic acid, rutin, quercetin, kaemferol, and also those flavonoids mostly abundant in soybean seeds, including daidzin, glycitin, genistin, glycitein and daidzein (Fig. 3.15). From FTMS data, we did not resolve expected flavonoids which are abundant in soybean seed. Instead, two candidate flavonoids, namely kaemferol (EIC 285.039364) and quercetin (EIC 301.034279), were identified in GmGSTL1 bound fraction (Fig. 3.16).

The role of flavonoids as in vivo anti-oxidant had been under debate. On the one hand, polyphenolic compound, including phenolic acids, phenolic diterpenes, falvonoids, and volatile oils were considered natural anti-oxidant from spice, herbs and various plant sources [151]. On the other hand, in 2012, the USDA Nutrient Data Laboratory removed the USDA ORAC Database for Selected Foods from its website, quoting, “the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health” [152]. Nevertheless, the functional role of polyphenols against oxidative stress had been well documented in different models [153-157]. Furthermore, despite the precision of any chemical test in

determining the actual bioactivity of polyphenols, the capacity in absorbing a particular free radical should be obvious from pure chemistry *per se*. Since polyphenols present ubiquitously in plant [158] and protect plant tissues against UV radiation [159], herbivore ingestion, fugal and virus infection [160], it is not surprised to postulate that the ubiquitous existence also provide anti-oxidative cushion for the ubiquitously produced, short ranged ROS damage [98], hence, plays important role in plant survival under abiotic stress.

4.6 External supplementation of quercetin lowers salinity-induced symptoms in both transgenic cells and plants

Since quercetin was shown to interact with GmGSTL1, we attempt to test whether bound ligand possess any functional role for stress tolerance. It was observed that the application of quercetin reduced ROS accumulation in BY-2 cells (Fig. 3.18), delayed salinity induced leaf chlorosis (3.19A) and ROS accumulation in plant (Fig. 3.19B).

4.7 Conclusion

In this study, we report the identification of a putative Lambda class GST, we name the gene *GmGSTL1*, using soybean varieties which display differential

salt stress tolerance capability. Two candidate flavonoids, namely quercetin and kaemferol, from soybean leaf extract were found to bind to the GmGSTL1 protein. Either overexpression of gene transcript or external supplementation of binding ligand could functionally improve plant tolerance to salinity stress. Overall, we present molecular and physiological evidence for salinity stress tolerance, mediated by Lambda class GST through the interaction with polyphenolic metabolites in plant.

References

1. Qadir M, Tubeileh A, Akhtar J, Larbi A, Minhas PS, Khan MA: **Productivity enhancement of salt-affected environments through crop diversification**. *Land Degradation & Development* 2008, **19**:429-453.
2. Owens S: **Salt of the Earth**. *EMBO reports* 2001, **2**:877-879.
3. Rengasamy P: **Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview**. *Australian Journal of Experimental Agriculture* 2002, **42**(3):351-361.
4. Munns R, Tester M: **Mechanisms of salinity tolerance**. In: *Annual Review of Plant Biology.* vol. 59; 2008: 651-681.
5. Garthwaite AJ, von Bothmer R, Colmer TD: **Salt tolerance in wild Hordeum species is associated with restricted entry of Na+ and Cl- into the shoots**. *Journal of Experimental Botany* 2005, **56**(419):2365-2378.
6. Aslam M, Qureshi RH, Ahmed N: **A rapid screening technique for salt tolerance in rice (Oryza sativa L)**. *Plant and Soil* 1993, **150**(1):99-107.
7. Cramer GR: **Response of abscisic acid mutants of Arabidopsis to salinity**. *Functional Plant Biology* 2002, **29**(5):561-567.
8. Colmer TD, Munns R, Flowers TJ: **Improving salt tolerance of wheat and barley: future prospects**. *Australian Journal of Experimental Agriculture*

2005, **45**(11):1425-1443.

9. Kapulnik Y, Teuber LR, Phillips DA: **Lucerne (Medicago-sativa L) selected for vigor in a nonsaline environment maintained growth under salt stress**. *Australian Journal of Agricultural Research* 1989, **40**(6):1253-1259.

10. Lam HM, Xu X, Liu X, Chen WB, Yang GH, Wong FL, Li MW, He WM, Qin N, Wang B *et al*: **Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection**. *Nature Genetics* 2010, **42**(12):1053-U1041.

11. Lam H-M, Chang R, Shao G, Liu Z: **Researches on Tolerance to Stresses in Chinese Soybean**: China Agriculture Press, Beijing; 2009.

12. Phang TH, Shao GH, Lam HM: **Salt tolerance in soybean**. *Journal of Integrative Plant Biology* 2008, **50**(10):1196-1212.

13. Turkan I, Demiral T: **Recent developments in understanding salinity tolerance**. *Environ Exp Bot* 2009, **67**(1):2-9.

14. Yamaguchi T, Blumwald E: **Developing salt-tolerant crop plants: challenges and opportunities**. *Trends Plant Sci* 2005, **10**(12):615-620.

15. Chan C, Qi X, Li M-W, Wong F-L, Lam H-M: **Recent Developments of Genomic Research in Soybean**. *Journal of Genetics and Genomics* 2012, **39**(7):317-324.

16. Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ: **Plant cellular and molecular responses to high salinity**. *Annu Rev Plant Physiol Plant Molec Biol* 2000, **51**:463-499.

17. Zhu J-K: **Salt and drought stress signal transduction in plants**. *Annual Review of Plant Biology* 2002, **53**:247-273.

18. Horie T, Hauser F, Schroeder JI: **HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants**. *Trends Plant Sci* 2009, **14**(12):660-668.

19. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R: **Reactive oxygen species homeostasis and signalling during drought and salinity stresses**. *Plant Cell Environ* 2010, **33**(4):453-467.

20. Phang T-H, Shao G, Lam H-M: **Salt tolerance in soybean**. *Journal of Integrative Plant Biology* 2008, **50**(10):1196-1212.

21. Pitzschke A, Schikora A, Hirt H: **MAPK cascade signalling networks in plant defence**. *Curr Opin Plant Biol* 2009, **12**(4):421-426.

22. Blumwald E, Aharon GS, Apse MP: **Sodium transport in plant cells**. *Biochimica Et Biophysica Acta-Biomembranes* 2000, **1465**(1-2):140-151.

23. Schachtman D, Liu WH: **Molecular pieces to the puzzle of the interaction between potassium and sodium uptake in plants**. *Trends Plant Sci* 1999,

4(7):281-287.

24. Chrispeels MJ, Crawford NM, Schroeder JI: **Proteins for transport of water and mineral nutrients across the membranes of plant cells**. *Plant Cell* 1999, **11**(4):661-675.

25. Liu JP, Zhu JK: **A calcium sensor homolog required for plant salt tolerance**. *Science* 1998, **280**(5371):1943-1945.

26. Liu JP, Zhu JK: **An Arabidopsis mutant that requires increased calcium for potassium nutrition and salt tolerance**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(26):14960-14964.

27. Binzel M, Ratajczak R: **Function of membrane transport systems under salinity: Tonoplast**. *Salinity: Environment - Plants - Molecules* 2002:423-449.

28. Sze H, Li XH, Palmgren MG: **Energization of plant cell membranes by H+-pumping ATPases: Regulation and biosynthesis**. *Plant Cell* 1999, **11**(4):677-689.

29. Niu XM, Bressan RA, Hasegawa PM, Pardo JM: **Ion homeostasis in NaCl stress environments**. *Plant Physiology* 1995, **109**(3):735-742.

30. Serrano R, Culianz-Macia FA, Moreno V: **Genetic engineering of salt and**

drought tolerance with yeast regulatory genes. *Scientia Horticulturae* 1999, **78**(1-4):261-269.

31. Miyahara K, Mizunuma M, Hirata D, Tsuchiya E, Miyakawa T: **The involvement of the Saccharomyces cerevisiae multidrug resistance transporters Pdr5p and Snq2p in cation resistance**. *Febs Letters* 1996, **399**(3):317-320.

32. Dreyer I, Horeau C, Lemaillet G, Zimmermann S, Bush DR, Rodriguez-Navarro A, Schachtman DP, Spalding EP, Sentenac H, Gaber RF: **Identification and characterization of plant transporters using heterologous expression systems**. *Journal of Experimental Botany* 1999, **50**:1073-1087.

33. Apse MP, Aharon GS, Snedden WA, Blumwald E: **Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis**. *Science* 1999, **285**(5431):1256-1258.

34. Fukuda A, Nakamura A, Tanaka Y: **Molecular cloning and expression of the Na+/H+ exchanger gene in Oryza sativa**. *Biochimica Et Biophysica Acta-Gene Structure and Expression* 1999, **1446**(1-2):149-155.

35. Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR: **The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can**

function in cation detoxification in yeast. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(4):1480-1485.

36. Cramer GR, Lynch J, Lauchli A, Epstein E: **Influx of Na+, K+, and Ca-2+ into roots of salt-stressed cotton seedlings effects of supplemental Ca2+**. *Plant Physiology* 1987, **83**(3):510-516.

37. Knight H, Trewavas AJ, Knight MR: **Calcium signalling in Arabidopsis thaliana responding to drought and salinity**. *Plant Journal* 1997, **12**(5):1067-1078.

38. Lynch J, Polito VS, Lauchli A: **Salinity stress increases cytoplasmic Ca activity in maize root protoplasts**. *Plant Physiology* 1989, **90**(4):1271-1274.

39. Mendoza I, Rubio F, Rodrigueznavarro A, Pardo JM: **The protien phosphatase calcieurin is essential for NaCl tolerance of Saccharomyces cerevisiae**. *Journal of Biological Chemistry* 1994, **269**(12):8792-8796.

40. Brown AD, Simpson JR: **Water relations of sugar tolerant yeasts - role of intracellular polyols**. *Journal of General Microbiology* 1972, **72**(OCT):589-&.

41. Ford CW: **Accumulation of low-molecular weight solutes in water-stressed tropical legumes**. *Phytochemistry* 1984, **23**(5):1007-1015.

42. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN: **Living with**

water stress - evolution of osmolyte systems. *Science* 1982, **217**(4566):1214-1222.

43. Delauney AJ, Verma DPS: **Proline biosynthesis and osmoregulation in plants**. *Plant Journal* 1993, **4**(2):215-223.

44. Louis P, Galinski EA: **Characterization of genes for the biosynthesis of the compatible solute ectoine from Marinococcus halophilus and osmoregulated expression in Escherichia coli**. *Microbiology-Uk* 1997, **143**:1141-1149.

45. Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P: **Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation**. *Plant Cell* 1998, **10**(3):451-459.

46. Yoshiba Y, Kiyosue T, Nakashima K, YamaguchiShinozaki K, Shinozaki K: **Regulation of levels of proline as an osmolyte in plants under water stress**. *Plant and Cell Physiology* 1997, **38**(10):1095-1102.

47. Zhang CS, Lu Q, Verma DPS: **Removal of feedback inhibitionof delta(1)-pyrroline-5carboxylate synthetase, a bifunctional enzyme catalyzing the first 2 steps of proline biosynthesis in plants**. *Journal of Biological Chemistry* 1995, **270**(35):20491-20496.

48. Ding L, Zhu JK: **Reduced Na+ uptake in the NaCl-hypersensitive sos1**

mutant of Arabidopsis thaliana. *Plant Physiology* 1997, **113**(3):795-799.

49. Bohnert HJ, Shen B: **Transformation and compatible solutes**. *Scientia Horticulturae* 1999, **78**(1-4):237-260.

50. Hare PD, Cress WA: **Metabolic implications of stress-induced proline accumulation in plants**. *Plant Growth Regulation* 1997, **21**(2):79-102.

51. Jain RK, Selvaraj G: **Molecular genetic improvement of salt tolerance in plants**. *Biotechnology Annual Review, Vol 3* 1997, **3**:245-267.

52. Asada K: **The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons**. *Annu Rev Plant Physiol Plant Molec Biol* 1999, **50**:601-639.

53. Niyogi KK: **Photoprotection revisited: Genetic and molecular approaches**. *Annu Rev Plant Physiol Plant Molec Biol* 1999, **50**:333-359.

54. Noctor G, Foyer CH: **Ascorbate and glutathione: Keeping active oxygen under control**. *Annu Rev Plant Physiol Plant Molec Biol* 1998, **49**:249-279.

55. Halliwell B: **Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life**. *Plant Physiology* 2006, **141**(2):312-322.

56. Navrot N, Rouhier N, Gelhaye E, Jacquot J-P: **Reactive oxygen species generation and antioxidant systems in plant mitochondria**. *Physiologia Plantarum* 2007, **129**(1):185-195.

57. del Rio LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB: **Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling**. *Plant Physiology* 2006, **141**(2):330-335.

58. Foyer CH, Noctor G: **Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses**. *Plant Cell* 2005, **17**(7):1866-1875.

59. Barkla BJ, Vera-Estrella R, Pantoja O, Kirch HH, Bohnert HJ: **Aquaporin localization - how valid are the TIP and PIP labels?** *Trends Plant Sci* 1999, **4**(3):86-88.

60. Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O: **Salt stress in Mesembryanthemum crystallinum L cell suspensions activates adaptive mechanisms similar to those observed in the whole plant**. *Planta* 1999, **207**(3):426-435.

61. Azaizeh H, Gunse B, Steudle E: **Effects of NaCl and CaCl2 on water transport across root cells of maize (Zea mays L) seedlings**. *Plant Physiology* 1992, **99**(3):886-894.

62. Johansson I, Larsson C, Ek B, Kjellbom P: **The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are**

phosphorylated in response to Ca2+ and apoplastic water potential. *Plant Cell* 1996, **8**(7):1181-1191.

63. Leung J, Giraudat J: **Abscisic acid signal transduction**. *Annu Rev Plant Physiol Plant Molec Biol* 1998, **49**:199-222.

64. Tardieu F, Zhang J, Davies WJ: **What information is conveyed by an ABA signal from maize roots in drying field soil**. *Plant Cell Environ* 1992, **15**(2):185-191.

65. Ecker JR: **The ethylene signal transduction pathway in plants**. *Science* 1995, **268**(5211):667-675.

66. Hare PD, Cress WA, van Staden J: **The involvement of cytokinins in plant responses to environmental stress**. *Plant Growth Regulation* 1997, **23**(1-2):79-103.

67. Normanly J, Bartel B: **Redundancy as a way of life - IAA metabolism**. *Curr Opin Plant Biol* 1999, **2**(3):207-213.

68. Theologis A: **Ethylene signalling: Redundant receptors all have their say**. *Current Biology* 1998, **8**(24):R875-R878.

69. Kende H, Zeevaart JAD: **The five "classical" plant hormones**. *Plant Cell* 1997, **9**(7):1197-1210.

70. Morgan PW, Drew MC: **Ethylene and plant responses to stress**.

Physiologia Plantarum 1997, **100**(3):620-630.

71. Roitsch T: **Source-sink regulation by sugar and stress**. *Curr Opin Plant Biol* 1999, **2**(3):198-206.

72. Estelle M: **Polar auxin transport: New support for an old model**. *Plant Cell* 1998, **10**(11):1775-1778.

73. Jones AM: **Botany: Auxin transport: Down and out and up again**. *Science* 1998, **282**(5397):2201-2202.

74. Strizhov N, Abraham E, Okresz L, Blickling S, Zilberstein A, Schell J, Koncz C, Szabados L: **Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis**. *Plant Journal* 1997, **12**(3):557-569.

75. Nelson DE, Rammesmayer G, Bohnert HJ: **Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance**. *Plant Cell* 1998, **10**(5):753-764.

76. Berna A, Bernier F: **Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H2O2-producing enzyme**. *Plant Molecular Biology* 1999, **39**(3):539-549.

77. Droog F: **Plant glutathione S-transferases, a tale of theta and tau**. *Journal*

of Plant Growth Regulation 1997, **16**(2):95-107.

78. Foyer CH, LopezDelgado H, Dat JF, Scott IM: **Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling**. *Physiologia Plantarum* 1997, **100**(2):241-254.

79. Chen WQJ, Zhu T: **Networks of transcription factors with roles in environmental stress response**. *Trends Plant Sci* 2004, **9**(12):591-596.

80. Rushton PJ, Somssich IE, Ringler P, Shen QXJ: **WRKY transcription factors**. *Trends Plant Sci* 2010, **15**(5):247-258.

81. Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L: **MYB transcription factors in Arabidopsis**. *Trends Plant Sci* 2010, **15**(10):573-581.

82. Kim SY: **The role of ABF family bZIP class transcription factors in stress response**. *Physiologia Plantarum* 2006, **126**(4):519-527.

83. Olsen AN, Ernst HA, Lo Leggio L, Skriver K: **NAC transcription factors: structurally distinct, functionally diverse**. *Trends Plant Sci* 2005, **10**(2):79-87.

84. Fujita Y, Yoshida T, Yamaguchi-Shinozaki K: **Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants**. *Physiologia Plantarum* 2013,

147(1):15-27.

85. Kielbowicz-Matuk A: **Involvement of plant C2H2-type zinc finger transcription factors in stress responses**. *Plant Sci* 2012, **185**:78-85.

86. Lata C, Prasad M: **Role of DREBs in regulation of abiotic stress responses in plants**. *Journal of Experimental Botany* 2011, **62**(14):4731-4748.

87. Kreps JA, Wu YJ, Chang HS, Zhu T, Wang X, Harper JF: **Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress**. *Plant Physiology* 2002, **130**(4):2129-2141.

88. Chinnusamy V, Schumaker K, Zhu JK: **Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants**. *Journal of Experimental Botany* 2004, **55**(395):225-236.

89. Rizhsky L, Liang HJ, Shuman J, Shulaev V, Davletova S, Mittler R: **When Defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress**. *Plant Physiology* 2004, **134**(4):1683-1696.

90. Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T *et al*: **Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray**. *Plant Journal* 2002, **31**(3):279-292.

91. Wu X, Shiroto Y, Kishitani S, Ito Y, Toriyama K: **Enhanced heat and**

drought tolerance in transgenic rice seedlings overexpressing OsWRKY11 under the control of HSP101 promoter. *Plant Cell Reports* 2009, **28**(1):21-30.

92. Qiu Y, Yu D: **Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in Arabidopsis**. *Environ Exp Bot* 2009, **65**(1):35-47.

93. Li S, Fu Q, Huang W, Yu D: **Functional analysis of an Arabidopsis transcription factor WRKY25 in heat stress**. *Plant Cell Reports* 2009, **28**(4):683-693.

94. Jiang Y, Deyholos MK: **Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses**. *Plant Molecular Biology* 2009, **69**(1-2):91-105.

95. Zhou Q-Y, Tian A-G, Zou H-F, Xie Z-M, Lei G, Huang J, Wang C-M, Wang H-W, Zhang J-S, Chen S-Y: **Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants**. *Plant Biotechnology Journal* 2008, **6**(5):486-503.

96. Harmer SL, Hogenesch LB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA: **Orchestrated transcription of key pathways in**

Arabidopsis by the circadian clock. *Science* 2000, **290**(5499):2110-2113.

97. Mari M, Morales A, Colell A, Garcia-Ruiz C, Fernandez-Checa JC: **Mitochondrial glutathione, a key survival antioxidant**. *Antioxid Redox Signal* 2009, **11**(11):2685-2700.

98. Gill SS, Tuteja N: **Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants**. *Plant Physiol Biochem* 2010, **48**(12):909-930.

99. Dixon DP, Skipsey M, Edwards R: **Roles for glutathione transferases in plant secondary metabolism**. *Phytochemistry* 2010, **71**(4):338-350.

100. Lim PO, Kim HJ, Nam HG: **Leaf senescence**. In: *Annual Review of Plant Biology.* vol. 58. Palo Alto: Annual Reviews; 2007: 115-136.

101. Hayes JD, Flanagan JU, Jowsey IR: **Glutathione transferases**. In: *Annual Review of Pharmacology and Toxicology.* vol. 45. Palo Alto: Annual Reviews; 2005: 51-88.

102. Gichner T, Patkova Z, Szakova J, Demnerova K: **Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves**. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* 2004, **559**(1-2):49-57.

103. Badawi GH, Yamauchi Y, Shimada E, Sasaki R, Kawano N, Tanaka K:

Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (Nicotiana tabacum) chloroplasts. *Plant Sci* 2004, **166**(4):919-928.

104. Zhao F, Zhang H: **Salt and paraquat stress tolerance results from co-expression of the Suaeda salsa glutathione S-transferase and catalase in transgenic rice**. *Plant Cell Tissue and Organ Culture* 2006, **86**(3):349-358.

105. Matsumura T, Tabayashi N, Kamagata Y, Souma C, Saruyama H: **Wheat catalase expressed in transgenic rice can improve tolerance against low temperature stress**. *Physiologia Plantarum* 2002, **116**(3):317-327.

106. Prashanth SR, Sadhasivam V, Parida A: **Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant Avicennia mayina in indica Rice var Pusa Basmati-1 confers abiotic stress tolerance**. *Transgenic Research* 2008, **17**(2):281-291.

107. Wang YH, Ying Y, Chen J, Wang XC: **Transgenic Arabidopsis overexpressing Mn-SOD enhanced salt-tolerance**. *Plant Sci* 2004, **167**(4):671-677.

108. Xu W-F, Shi W-M, Ueda A, Takabe T: **Mechanisms of salt tolerance in transgenic Arabidopsis thaliana carrying a peroxisomal ascorbate**

peroxidase gene from barley. *Pedosphere* 2008, **18**(4):486-495.

109. Katsuhara M, Otsuka T, Ezaki B: **Salt stress-induced lipid peroxidation is reduced by glutathione S-transferase, but this reduction of lipid peroxides is not enough for a recovery of root growth in Arabidopsis**. *Plant Sci* 2005, **169**(2):369-373.

110. Gruszka Vendruscolo EC, Schuster I, Pileggi M, Scapim CA, Correa Molinari HB, Marur CJ, Esteves Vieira LG: **Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat**. *Journal of Plant Physiology* 2007, **164**(10):1367-1376.

111. Melchiorre M, Robert G, Trippi V, Racca R, Lascano HR: **Superoxide dismutase and glutathione reductase overexpression in wheat protoplast: photooxidative stress tolerance and changes in cellular redox state**. *Plant Growth Regulation* 2009, **57**(1):57-68.

112. Shao H-B, Chu L-y, Shao M-a, Jaleel CA, Mi H-m: **Higher plant antioxidants and redox signaling under environmental stresses**. *Comptes Rendus Biologies* 2008, **331**(6):433-441.

113. Foyer CH, Noctor G: **Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria**. *Physiologia Plantarum* 2003, **119**(3):355-364.

114. Shao HB, Liang ZS, Shao MA, Sun SM, Hu ZM: **Investigation on dynamic changes of photosynthetic characteristics of 10 wheat (Triticum aestivum L.) genotypes during two vegetative-growth stages at water deficits**. *Colloids and Surfaces B-Biointerfaces* 2005, **43**(3-4):221-227.

115. Li J, Jin H: **Regulation of brassinosteroid signaling**. *Trends Plant Sci* 2007, **12**(1):37-41.

116. Sgherri CLM, Navariizzo F: **Suflower seedlings subjected to increasing watre deficit stress oxidative stress and defense mechanisms**. *Physiologia Plantarum* 1995, **93**(1):25-30.

117. Shalata A, Neumann PM: **Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation**. *Journal of Experimental Botany* 2001, **52**(364):2207-2211.

118. Kocsy G, Szalai G, Galiba G: **Induction of glutathione synthesis and glutathione reductase activity by abiotic stresses in maize and wheat**. *TheScientificWorldJOURNAL* 2002, **2**(Cited July 15, 2002):1726-1732.

119. Jain M, Choudhary D, Kale RK, Bhalla-Sarin N: **Salt- and glyphosate-induced increase in glyoxalase I activity in cell lines of groundnut (Arachis hypogaea)**. *Physiologia Plantarum* 2002, **114**(4):499-505.

120. Atkinson HJ, Babbitt PC: **Glutathione transferases are structural and functional outliers in the thioredoxin fold**. *Biochemistry* 2009, **48**(46):11108-11116.

121. Edwards R, Dixon DP, Walbot V: **Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health**. *Trends Plant Sci* 2000, **5**(5):193-198.

122. Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B: **Identification of human prostaglandin E synthase: A microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(13):7220-7225.

123. Matsushita N, Aritake K, Takada A, Hizue M, Hayashi K, Mitsui K, Hayashi M, Hirotsu I, Kimura Y, Tani T *et al*: **Pharmacological studies on the novel antiallergic drug HQL-79: II. Elucidation of mechanisms for antiallergic and antiasthmatic effects**. *Japanese Journal of Pharmacology* 1998, **78**(1):11-22.

124. Evans JF, Leveille C, Mancini JA, Prasit P, Therien M, Zamboni R, Gauthier JY, Fortin R, Charleson P, Macintyre DE *et al*: **5-Lipoxygenase-activating protein is the target of a quinoline class of leukotriene synthesis**

inhibitors. *Molecular Pharmacology* 1991, **40**(1):22-27.

125. Leiers BR, Kampkotter A, Grevelding CG, Link CD, Johnson TE, Henkle-Duhrsen K: **A stress-responsive glutathione S-transferase confers resistance to oxidative stress in Caenorhabditis elegans**. *Free Radical Biology and Medicine* 2003, **34**(11):1405-1415.

126. Veal EA, Toone WM, Jones N, Morgan BA: **Distinct roles for glutathione S-transferases in the oxidative stress response in Schizosaccharomyces pombe**. *Journal of Biological Chemistry* 2002, **277**(38):35523-35531.

127. Desikan R, Mackerness SAH, Hancock JT, Neill SJ: **Regulation of the Arabidopsis transcriptome by oxidative stress**. *Plant Physiology* 2001, **127**(1):159-172.

128. Marrs KA: **The functions and regulation of glutathione S-transferases in plants**. *Annu Rev Plant Physiol Plant Molec Biol* 1996, **47**:127-158.

129. Dixon DP, Lapthorn A, Edwards R: **Plant glutathione transferases**. *Genome Biol* 2002, **3**(3).

130. Kitamura S, Shikazono N, Tanaka A: **TRANSPARENT TESTA 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in Arabidopsis**. *Plant Journal* 2004, **37**(1):104-114.

131. Dixon DP, Hawkins T, Hussey PJ, Edwards R: **Enzyme activities and**

subcellular localization of members of the Arabidopsis glutathione transferase superfamily. *Journal of Experimental Botany* 2009, **60**(4):1207-1218.

132. Dixon DP, Edwards R: **Enzymes of tyrosine catabolism in Arabidopsis thaliana**. *Plant Sci* 2006, **171**(3):360-366.

133. Diao GP, Wang YC, Yang CP: **Functional characterization of a gluthathione S-transferase gene from Limonium bicolor in response to several abiotic stresses**. *Afr J Biotechnol* 2010, **9**(32):5060-5065.

134. Wang ZY, Song FB, Cai H, Zhu YM, Bai X, Ji W, Li Y, Hua Y: **Over-expressing GsGST14 from Glycine soja enhances alkaline tolerance of transgenic Medicago sativa**. *Biol Plant* 2012, **56**(3):516-520.

135. Zhao F, Zhang H: **Expression of Suaeda salsa glutathione S-transferase in transgenic rice resulted in a different level of abiotic stress resistance**. *J Agric Sci* 2006, **144**:547-554.

136. Lee GJ, Boerma HR, Villagarcia MR, Zhou X, Carter TE, Li Z, Gibbs MO: **A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars**. *Theoretical and Applied Genetics* 2004, **109**(8):1610-1619.

137. Li WYF, Shao G, Lam HM: **Ectopic expression of GmPAP3 alleviates oxidative damage caused by salinity and osmotic stresses**. *New Phytol*

2008, **178**(1):80-91.

138. Wang HM, Zhou L, Fu YP, Cheung MY, Wong FL, Phang TH, Sun ZX, Lam HM: **Expression of an apoplast-localized BURP-domain protein from soybean (GmRD22) enhances tolerance towards abiotic stress**. *Plant Cell Environ* 2012, **35**(11):1932-1947.

139. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method**. *Methods* 2001, **25**(4):402-408.

140. Brears T, Liu C, Knight TJ, Coruzzi GM: **Ectopic overexpression of asparagine synthetase in transgenic tobacco**. *Plant Physiology* 1993, **103**(4):1285-1290.

141. Gerber IB, Dubery I, A.: **Fluorescence microplate assay for the detection of oxidative burst products in tobacco cell suspensions using 2',7'-dichlorofluorescein**. *Methods Cell Sci* 2003, **25**:115-122.

142. Moran R: **Formulas for determination of chlorophyllous pigments extracted with N,N-dimethylformamide**. *Plant Physiology* 1982, **69**(6):1376-1381.

143. Dixon DP, Edwards R: **Roles for Stress-inducible Lambda Glutathione Transferases in Flavonoid Metabolism in Plants as Identified by Ligand**

Fishing. *Journal of Biological Chemistry* 2010, **285**(47):36322-36329.

144. Chang Q, Wong YS: **Identification of flavonoids in hakmeitau beans (*Vigna sinensis*) by high-performance liquid chromatography-electrospray mass spectrometry (LC-ESI/MS)**. *J Agric Food Chem* 2004, **52**(22):6694-6699.

145. McGonigle B, Keeler SJ, Lau S-MC, Koeppe MK, O'Keefe DP: **A Genomics Approach to the Comprehensive Analysis of the Glutathione S-Transferase Gene Family in Soybean and Maize**. *Plant Physiology* 2000, **124**:1105-1120.

146. Heim KE, Tagliaferro AR, Bobilya DJ: **Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships**. *Journal of Nutritional Biochemistry* 2002, **13**(10):572-584.

147. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F: **Reactive oxygen gene network of plants**. *Trends Plant Sci* 2004, **9**(10):490-498.

148. Anderson JV, Davis DG: **Abiotic stress alters transcript profiles and activity of glutathione S-transferase, glutathione peroxidase, and glutathione reductase in Euphorbia esula**. *Physiologia Plantarum* 2004, **120**(3):421-433.

149. Dixon DP, Sellars JD, Edwards R: **The Arabidopsis phi class glutathione**

transferase AtGSTF2: binding and regulation by biologically active heterocyclic ligands. *Biochemical Journal* 2011, **438**:63-70.

150. Smith AP, Nourizadeh SD, Peer WA, Xu JH, Bandyopadhyay A, Murphy AS, Goldsbrough PB: **Arabidopsis AtGSTF2 is regulated by ethylene and auxin, and encodes a glutathione S-transferase that interacts with flavonoids**. *Plant Journal* 2003, **36**(4):433-442.

151. Brewer MS: **Natural antioxidants: sources, compounds, mechanisms of action, and potential applications**. *Compr Rev Food Sci Food Saf* 2011, **10**(4):221-247.

152. **Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods** [http://www.ars.usda.gov/services/docs.htm?docid=15866]

153. Maheshwari DT, Kumar MSY, Verma SK, Singh VK, Singh SN: **Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (Hippophae rhamnoides L.) leaves**. In: *Food and Chemical Toxicology.* vol. 49; 2011: 2422-2428.

154. Nabavi SF, Nabavi SM, Latifi AM, Mirzaei M, Habtemariam S, Moghaddam AH: **Mitigating role of quercetin against sodium fluoride-induced oxidative stress in the rat brain**. *Pharm Biol* 2012, **50**(11):1380-1383.

155. Lee SYH, Munerol B, Pollard S, Youdim KA, Pannala AS, Kuhnle GGC,

Debnam ES, Rice-Evans C, Spencer JPE: **The reaction of flavanols with nitrous acid protects against N-nitrosamine formation and leads to the formation of nitroso derivatives which inhibit cancer cell growth**. *Free Radical Biology and Medicine* 2006, **40**(2):323-334.

156. Lozano C, Torres JL, Julia L, Jimenez A, Centelles JJ, Cascante M: **Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells**. *FEBS Letters* 2005, **579**(20):4219-4225.

157. Salucci M, Stivala LA, Maiani G, Bugianesi R, Vannini V: **Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2)**. *Br J Cancer* 2002, **86**(10):1645-1651.

158. Cheynier V: **Phenolic compounds: from plants to foods**. *Phytochem Rev* 2012, **11**(2-3):153-177.

159. Winkel-Shirley B: **Biosynthesis of flavonoids and effects of stress**. *Curr Opin Plant Biol* 2002, **5**(3):218-223.

160. Haslam E: **Vegetable tannins - Lessons of a phytochemical lifetime**. *Phytochemistry* 2007, **68**(22-24):2713-2721.

161. Jambunathan N: **Determination and detectionof reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants.** *Methods in Molecular Biology* 2010, **639**: 292-298.

Appendix – Buffer and medium formulation

10x SSC	1.5 M NaCl, 150 mM sodium citrate, pH 7.0
B5 vitamin 1000x	1g myo-inositol; 100mg thiamine; 10 mg nicotine acid; 10 mg pyridoxine
BY-2 vitamin 1000x	0.02g 2,4-dichloropheoucetic acid; 0.05g thiamine; 5.0g myo-inositol
Detection buffer	100 mM Tris-HCl pH 9.5, 100 mM NaCl
Dulbecco's PBS	2.7 mM KCl, 1.5 mM KH2PO4, 150 mM NaCl, 8.9 mM Na2HPO4, pH 7.4
High stringency washing buffer	0.5x SSC, 0.1% SDS
Homemade buffer QG	6M Guanidine thiocyanate, 50mM Tris-HCl, 20mM EDTA pH 7.5
Homemade buffer QTB	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton X-100 (v/v)
LB	25 g/L LB broth
Low stringency washing buffer	2x SSC, 0.1% SDS

MS medium (*A. thaliana*) per liter	4.3g MS salt; 0.5g MES; 5g sucrose; 1x B5 vitamin; pH 5.7
MS medium (BY-2) per liter	4.3g MS salt; 30g sucrose; 0.255g KH2PO4; pH 5.0 Supplemented with 1x BY-2 vitamin
Pre-hybridization buffer	50% formamide, 5x SSC, 2% blocking solution, 50 mM sodium phosphate pH 7.0, 0.1% N-lauroylsarcosine, 7% SDS
RNA extraction buffer	200 mM Tris base, 400 mM KCl, 200 mM sucrose, 35 mM MgCl2, 25 mM EGTA, pH 9.0
YEP	10 g Bacto peptone, 10 g yeast extrat, 5 g NaCl