# The role of cytosolic glutamine synthetases in abiotic stress and development in *Arabidopsis thaliana*

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# ABSTRACT

Glutamine (Gln), a major nitrogen source in plants, is considered a central intermediate that coordinates carbon-nitrogen assembly for plant growth and development. To maintain a sufficient Gln supply, plant cells employ glutamine synthetases (GS), including cytosolic GS1 and plastidic GS2 for Gln production. Previous work has shown that the *GS1* is responsive to various environmental stresses. This study demonstrated the involvement of *GS1*s in Gln homeostasis and the role of GS1 in abiotic stress tolerance in *Arabidopsis*. The *GS1* family is comprised of five isoforms in *Arabidopsis thaliana*. Gene expression profiling showed that *GLN1;1*, *GLN1;3* and *GLN1;4* had similar expression patterns and were upregulated by abiotic (salinity and cold) stresses, whereas *GLN1;2* exhibited constitutive expression and no *GLN1;5* transcript was detected under any of the conditions tested. Null T-DNA insertion mutants for the five *GS1* genes were obtained. Only the *gln1;1* mutant displayed enhanced sensitivity to a GS inhibitor, phosphinothricin, and to cold and salinity treatments, suggesting a nonredundant role for GLN1;1. Increased stress sensitivity in *gln1;1* was associated with accelerated accumulation of reactive oxygen species (ROS), particularly in chloroplasts. To better understand the role of cytosolic GS isoforms, we generated two different triple mutant combinations. Triple mutant *gln1;1*/*gln1;2*/*gln1;3* showed reduced growth at an early stage. The *gln1;1*/*gln1;3*/*gln1;4* mutant is pollen lethal, indicating an essential role of Gln in plant gametophyte development. Collectively, our results establish a link between cytosolic Gln production, ROS accumulation, plant stress tolerance and development.

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# LIST OF ABBREVIATIONS

2,4-D: 2,4-dichlorophenoxyacetic acid

2D: two dimension

2-OG: 2-oxoglutarate

3-PGA: 3-phosphoglycerate

AAP: amino acid permease

AMT: ammonium transporter

ANT1: aromatic and neutral amino acid transport 1

AOX: alternative oxidase

APC: amino acid-polyamine-choline

APX: ascorbate peroxidase

ATP: adenosine triphosphate

ATF: amino acid transport family

AUX1: auxin-resistance

C: carbon

CAT: catalase

CATs: cationic amino acid transporters

CCA1: circadian clock associated 1

CDPK: calcium-dependent protein kinase

cHATS: constitutive high affinity transport system

DCFDA: dichlorodihydrofluorescein diacetate

DHA: dehydroascorbate

DHAR: DHA reductase

ETC: electron transport chain

Fd: ferredoxin

Fd-GOGAT: ferredoxin-glutamate synthase

FNR: ferredoxin-$NADP^+$ reductase

FTR: Fd:thioredoxin reductase

GABA: γ-aminobutyric acid

GDH: glutamate dehydrogenase

Gln: glutamine

Glu: glutamate

GOGAT: glutamate synthase

GPX: glutathione oxidase

GR: glutathione reductase

GS1/GLN1: cytosolic glutamine synthetase

GS2: plastidic glutamine synthetase

HATS: high affinity transport system

•HO: hydroxyl radical

$HO_2^-$: hydroperoxide

$H_2O_{2:}$ hydrogen peroxide

HR: hypersensitive response

HSTFs: heat shock transcription factors

IAA: indole-3-acetic acid

iHATS: inducible high affinity transport system

KCN: potassium cyanide

LATS: low affinity transport system

LATs: L-type amino acid transporters

LHT: lysine/histidine transporter

LSU: large subunit of rubisco

MEP: methylammonium permease

MAPK: mitogen-activated protein kinase

MD: menadione

MSX: L-methionie-S-sulfoximine

MV: methyl viologen

N: nitrogen

$NAD^+$: nicotinamide adenine dinucleotide

$NADP^+$: nicotinamide adenine dinucleotide phosphate

$NH_4^+$: ammonium

NiR: nitrite reductase

$NO_3^-$: nitrate

NR: nitrate reductase

$O_2^-$: superoxide radical

$^1O_2$: singlet oxygen

P5CS1: pyrroline-5-carboxylate synthase 1

PETC: photosynthetic electron transport chain

PSI: photosystem I

PSII: photosystem II

PKA: protein kinase

PPT: phosphinothricin

ProTs: proline transporter

PUFAs: polyunsaturated fatty acids

RB: rose bengal

Rh: rhesus

ROS: reactive oxygen species

RuBP: ribulose 1,5-bisphosphate

SA: salicylic acid

SHAM: salicylhydroxamic acid

SOD: superoxide dismutase

Trx: thioredoxin

UQ: ubiquinone

UV: Ultraviolet

# 1. General Introduction

## 1.1 Nitrogen Assimilation and Transport in Plants

Nitrogen (N) is one of the most important elements for all organisms. Many biochemical compounds required for cell development and reproduction contain N. For example, N is found in the nucleoside phosphates and amino acids which comprise nucleic acids and proteins. In plants, the availability and uptake of N is considered a major factor affecting plant productivity, biomass, and crop yield (Coruzzi, 2003). In order to synthesize more protein for growth and development, plants are able to assimilate N by taking up nitrate ($NO_3^-$), ammonium ($NH_4^+$) and amino acids from soil directly or through the symbiotic action of N-fixing bacteria. In this introduction, I will focus on the three major N sources ($NO_3^-$, $NH_4^+$ and amino acids) and introduce how they are assimilated and transported in the plant.

### 1.1.1 Nitrate Uptake and Transport

Plants obtain $NO_3^-$ from the soil by transporting it across the plasma membrane of epidermal and cortical cells of the root using $NO_3^-$ transporters (Forde, 2002). $NO_3^-$ concentration varies by different types of soil depending on many environmental factors, such as rainfall, pH value, temperature. There are large changes when soil is sampled within a small area (Miller *et al*., 2007). Physiological investigations of $NO_3^-$ uptake by roots have revealed that several $NO_3^-$ transport systems are employed to cope with the variations in $NO_3^-$ concentration in cultivated soils (Crawford, 1995). These transport systems include the low affinity transport system (LATS) (above 1 mM) and the high affinity transport system (HATS) (1μM to 1mM), which is comprised of the constitutive (cHATS) and inducible systems (iHATS). The cHATS is present regardless of

$NO_3^-$ supply, while iHATS is only stimulated by external application of $NO_3^-$ (Crawford, 1995). In *Arabidopsis thaliana*, two genetically linked genes, *AtNRT2.1* (*NITRATE TRANSPORTER*) and *AtNRT2.2*, were cloned (Zhuo *et al*., 1999). A number of studies have reported that mutants with impaired expression of *AtNRT2.1* and *AtNRT2.2* are defective in HATS activity (Filleur *et al*., 2001; Orsel *et al*., 2004; Zhuo *et al*., 1999). Dramatic up-regulation of *AtNRT2.1* and a transient increase in *AtNRT2.2* at the mRNA level are observed following reapplication of $NO_3^-$ to plants in $NO_3^-$-deprived conditions, indicating these two nitrate transporters might belong to iHATS (Okamoto *et al*., 2003). However, a recent study by Li *et al*., (2007) identified a new allele of *Arabidopsis* mutant uniquely disrupted in *AtNRT2.1*, and this mutant *Atnrt2.1* and *Atnrt2.2* were used to investigate the relative contribution of iHATS and cHATS. Their results defined that *AtNRT2.1* is the major contributor to iHATS and *AtNRT2.2* makes a minor contribution to iHATS, but *AtNRT2.2* can partially compensate for a loss of *AtNRT2.1*. Unlike HATS, there are more than 50 candidate genes involved in LATS in *Arabidopsis*. Six genes in the *AtNRT1* family were characterized in different tissues and found to be responsible for various functions. For example, AtNRT1.2 and AtNRT1.4, two low-affinity transporters, are involved in $NO_3^-$ uptake and petiole $NO_3^-$ storage respectively. Transcripts of *AtNRT1.2* were primarily detected in roots (Huang *et al*., 1999), whereas *AtNRT1.4* is expressed in leaf petioles (Chiu *et al*., 2004). Recently, *AtNRT1.6*, a new member of LATS, was characterized by Almagro *et al*., (2008). The expression of *AtNRT1.6* is only detectable in reproductive tissues. The *Atnrt1.6* mutant showed reduced $NO_3^-$ accumulation in mature seeds and an increased seed abortion rate suggesting that AtNRT1.6 is involved in delivering $NO_3^-$ to the developing embryo (Almagro *et al*., 2008). AtNRT1.5 and AtNRT1.8 were identified in the same tissue of transport system playing a role in long-distance transport from root to shoot (Li *et al*., 2010). Interestingly,

AtNRT1.1 was originally found to be a member of LATS, but it appears to possess dual affinity, with a phosphorylation switch between high and low affinity ranges of $NO_3^-$ uptake by cAMP-dependent protein kinase (PKA) (Liu and Tsay, 2003).

### 1.1.2 Ammonium Uptake and Transport

Plant roots are usually exposed to more $NO_3^-$ than $NH_4^+$ (Miller *et al*., 2007). High levels of $NH_4^+$ are toxic to plants and result in growth inhibition (Britto and Kronzucker, 2001; Walch-Liu *et al*., 2001). Thus, the uptake of $NH_4^+$ needs to be precisely regulated in order to adjust the cellular $NH_4^+$ concentration. In most plant species, $NH_4^+$ transporters belong to AMT1- and AMT2/MEP-type (AMMONIUM TRANSPORTER/METHYLAMMONIUM PERMEASE) classes of proteins and are encoded by gene families (Ludewig *et al*., 2001). Accumulating evidence indicates that AMT-type $NH_4^+$ transporters represent the major entry pathways for $NH_4^+$ assimilation in roots (Loque and von Wiren, 2004). In *Arabidopsis*, the AMT family is comprised of five isoforms (AMT1;1~AMT1;5). A few studies have provided evidence that the tightly-regulated high-affinity $NH_4^+$ uptake in *Arabidopsis* roots relies on the spatial arrangement of AMT1;1, AMT1;2 and AMT1;3. AMT1;1 and AMT1;3 are epidermis-located proteins, that can take up $NH_4^+$ directly from the soil. GFP fusion protein driven by the *AMT1;2* promoter showed that AMT1;2 is expressed in endodermal and cortical cells where the substrate ($NH_4^+$) must be delivered by other transporters from the soil (See Figure 1.1) (Wang *et al*., 2007, Yuan *et al*., 2007). Studies in single insertion mutants of *amt1;1, amt1;2* and *amt1;3* indicated that AMT1;1 and AMT1;3 contribute approximately two thirds of total $NH_4^+$ influx in N-deficient *Arabidopsis* roots, while AMT1;2 is responsible for 18 to 26% of the overall $NH_4^+$ uptake capacity (Loqué and von Wiren, 2004; Wang *et al*., 2007). In *Arabidopsis*, AMT2;1 is the only isoform in the

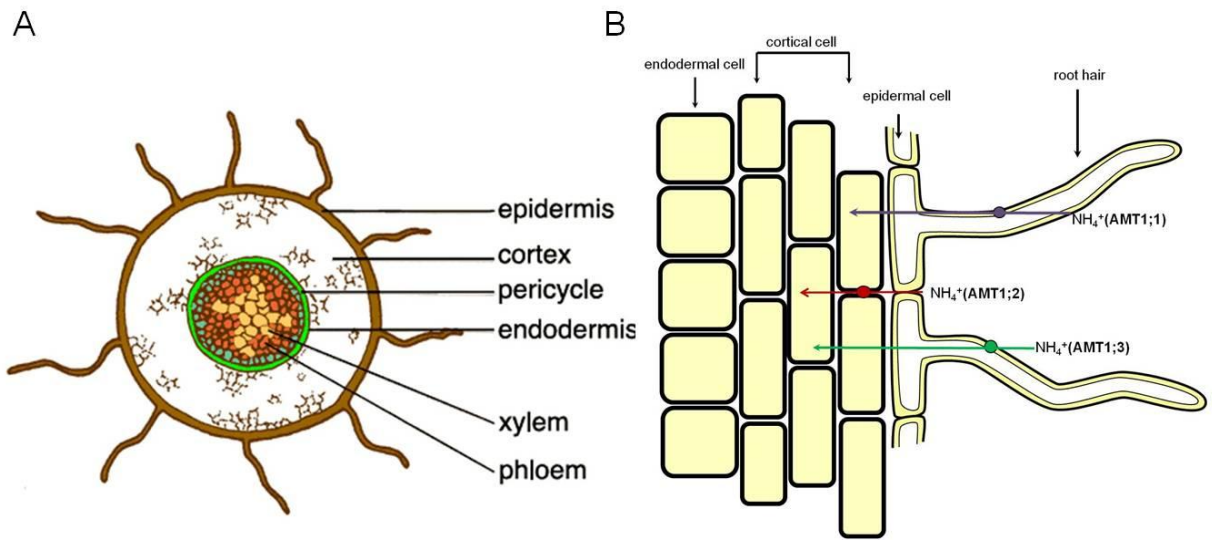


**Figure 1.1 Uptake of $NH_4^+$ is completed by the spatial arrangement of AMTs in *Arabidopsis* roots.**
(A). Cross section of plant root. Xylem and phloem form the core of vascular tissue and surrounded by a cortex tissue. Cortex tissue is represented by several layers of loosely arranged parenchyma cells. These cells are mainly for storage of water. The endodermis separates the cortex from the vascular tissue. The outermost covering of root is formed by epidermal cells which produce long unicellular projections called root hairs to enhance the root surface area for water absorption.
(B). Schematic representation of $NH_4^+$ uptake by AMTs and their spatial expression in root tissues. AMT1;1 and AMT1;3 are localized in root hair and epidermal cells. AMT1;2 proteins expressed at the plasma membrane of endodermal and cortical cells (Yuan *et al*., 2007).

MEP subfamily. Although *AMT2;1* transcripts are upregulated under N deficiency, RNA interference (RNAi) –mediated repression of *AMT2;1* revealed that *AMT2;1* was unable to contribute to overall $NH_4^+$ influx indicating an additional role of *AMT2;1* (Sohlenkamp *et al*., 2002). While much of the research on molecular aspects of $NH_4^+$ transport has been performed using *Arabidopsis*. Analysis for AMT-homologous sequences from tomato and rice showed three *LeAMT* genes and ten putative *OsAMT* genes, respectively (Loqué and von Wiren, 2004).

### 1.1.3 Amino Acid Uptake and Transport

In all organisms, amino acids play fundamental roles in various processes, such as protein synthesis, hormone metabolism, cell growth, nucleotide synthesis and N metabolism and serve as principal, long distance transport forms of organic N (Wipf *et al*., 2002). So far, according to different substrate specificities and affinities, as well as distinct subcellular localizations, amino acid transporters have been divided into at least two major superfamilies: (1) The ATF (amino acid transporter) family and (2) the APC (amino acid-polyamine-choline) family. In addition, some amino acid transporters localized at the organelle membrane were also identified; for example, DiT2.1, a chloroplast envelope membrane protein, functions in glutamate/malate exchange in the photorespiratory pathway (Renne *et al*., 2003).

The ATF family was the first subfamily described in plants and constitute five sub-classes: (i) AAP (amino acid permease), with eight members in *Arabidopsis*, mediate uptake of a broad spectrum of amino acids. All AAP members in *Arabidopsis* except AAP7 have been characterized. *AAP1* was the first gene encoding an amino acid transporter to be cloned from plants (Frommer *et al*., 1993; Hsu *et al*., 1993). With the capability to mediate transport of neutral and acidic amino acids into root cells, AAP1 is localized to the plasma membrane (Lee *et*

*al*., 2007). AAP3, a member of AAPs with high expression in roots, was detected at the nuclear membrane, ER (Endoplasmic Reticulum), Golgi bodies, endosomal vesicles and plasma membrane by histochemical analysis of epitope-tagged AAP3 in *Arabidopsis* which indicates that AAP3 might be involved in the trafficking pathway (Okumoto *et al*., 2004). Although AAPs mediate uptake of a broad spectrum of amino acids, analyses from Fischer *et al*. (2002) indicated that AAP1 and AAP5 were less efficient in delivering amino acids with aromatic side chains (tryptophan, phenylalanine), β-methyl groups (threonine, valine and isoleucine) and cyclic amino acids, whereas AAP3 and AAP6 mediated high levels of tryptophan transport. With the exception of AAP6, AAPs fail to recognize aspartate (Fischer *et al*., 2002).

(ii) LHTs ('lysine/histidine' transporters) are also high affinity transporters (μM level). In *Arabidopsis*, there are 10 putative members AtLHT1-AtLHT10. Tissue specificity expression revealed that AtLHT1, AtLHT2, and AtLHT3 are the predominant isoforms in *Arabidopsis*. *AtLHT2* shows a high expression in flower buds, *AtLHT3* localizes specifically in stem and the transcript of *AtLHT1* is present in a broad range of tissues with a relative high level of expression in root and leaf (Liu *et al*., 2010, Lee *et al*., 2004). Analysis of amino acid transport by the expressed protein in yeast revealed that *At*LHT1 (the first characterized member of LHT in *Arabidopsis*) was a high-affinity transporter for both lysine and histidine (Chen and Bush, 1997). More recently, AtLHT1 was shown to mediate the transport of a variety of amino acids with a much higher affinity than AAPs (Hirner *et al*., 2006). Biochemical analysis of AtLHT2 in yeast indicated that AtLHT2 plays an important role in the transport of uncharged and negatively charged amino acids (Lee *et al*., 2004).

(iii) ProTs (proline transporters) serve to transport the amino acid proline and glycine betaine as well as γ-aminobutyric acid (GABA). Unlike other amino acids, glycine betaine and GABA

are not able to incorporate into proteins. Originally, ProTs were described as specific transporters for Pro (Fischer *et al*., 1995; Rentsch *et al*., 1996). However, competition studies using *LeProT1* expressed in yeast indicated that other amino acids besides Pro might be potential substrates (Schwacke *et al*., 1999). Indeed, direct transport assays demonstrated that LePro1 is able to mediate the transport of glycine betaine, GABA and Pro (Schwacke *et al*., 1999). Moreover, expression of AtProTs in a transport-deficient yeast mutant strain (22574d) which carries mutations in the general amino acid (*gap1*), Pro (*put4*), and the GABA (*uga4*) permeases demonstrated that, besides Pro, all three AtProTs have the highee affinity for glycine betaine and a lower affinity for GABA (Grallath *et al*., 2005).

(iv) ANT1 (aromatic and neutral amino acid transporter 1) was the first amino acid transporter identified in this subfamily and only characterized in *Arabidopsis* so far (Chen *et al*., 2001). Sequence comparisons among the known amino acid transporters in plants suggest that *ANT1* is not a member of previously described amino acid transporter subfamilies due to the low similarity with them (Chen *et al*., 2001). Analysis of ANT1 transport activity showed that ANT1 transports aromatic and neutral amino acids, as well as arginine, indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (Chen *et al*., 2001).

(v) AUX1 (auxin-resistance) was named after the *aux1* mutant, which displays an auxin-resistant root growth phenotype and lacks root gravitropic curvature. The similarity of protein sequence and structure between AUX1 and AAP1 suggests that AUX1 mediates the transport of an amino acid-like signalling molecule (Bennett *et al*., 1996). Using *Xenopus* oocytes, Yang *et al*. (2006) showed that IAA, which is structurally similar to tryptophan, is a substrate of AUX1 (Yang *et al*., 2006).

The APC transporter family is functionally, but not structurally, related to ATF transporters

and can be also classified into two subgroups including cationic amino acid transporters (CATs) and L-type amino acid transporters (L-AATs) (Rentsch *et al*., 2007; Su *et al*., 2004). So far, L-AATs have only been identified in animals, but nine putative CATs have been characterized in *Arabidopsis* (Su *et al*., 2004). AtCAT5 is a high-affinity basic amino acid transporter and may function in reassimilation of leaking amino acids at the leaf margin (Su *et al*., 2004). AtCAT6 mediates the transport of large, neutral and cationic amino acids with moderate affinity in preference to other amino acids. Sink tissues, including developing seeds, lateral roots and nematode-induced feeding structures, are specific localizations for AtCAT6 (Hammes *et al*., 2006).

### 1.2 Nitrogen Assimilatory Pathway

$NO_3^-$ is the common form of inorganic N in soil, but cannot directly be used as substrate for protein synthesis in plants. To be used in plants, $NO_3^-$ is first reduced to $NO_2^-$ by nitrate reductase (NR) and $NO_2^-$ is further catalyzed by nitrite reductase (NiR) to $NH_4^+$. Because it is toxic to plant cells, $NH_4^+$ needs to be efficiently assimilated into amino acids. $NH_4^+$ is incorporated into glutamine (Gln) as the primary assimilation pathway through the sequential action of glutamine synthetase (GS) and glutamine:2-oxoglutarate amidotransferase (glutamate synthase, GOGAT), called the GS/GOGAT cycle. Glutamate dehydrogenase (GDH) incorporates 2-oxoglutarate (2-OG) and $NH_4^+$ to produce glutamate (Glu). Gln and Glu are the two entry points to further amino-acid metabolism. Plants are able to employ both Gln and Glu as N donors for the biosynthesis of other essential amino acids (Coruzzi, 2003). Since GS, GOGAT and GDH constitute the checkpoints, partitioning organic and inorganic N metabolism, I provide an overview of these key enzymes below (see Figure 1.2).

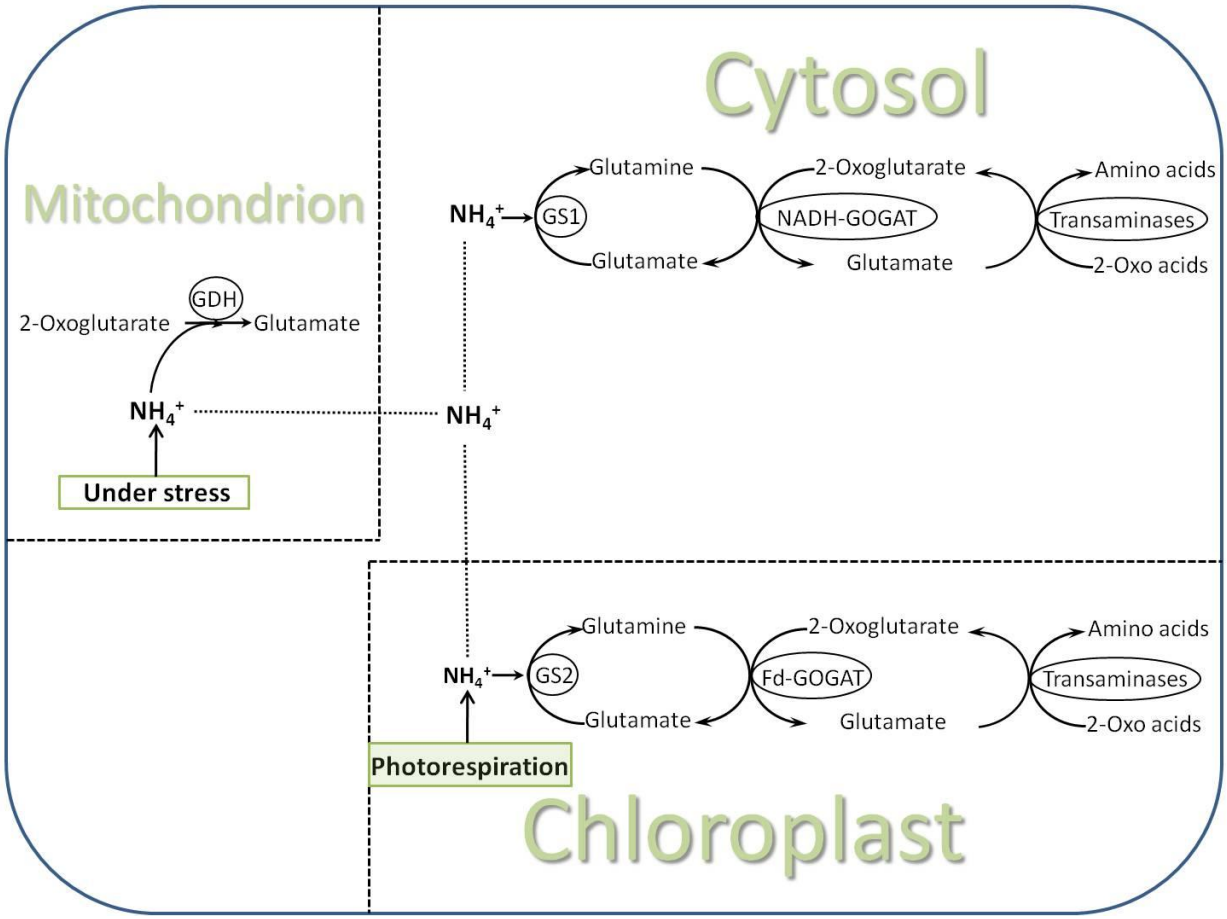


**Figure 1.2 Nitrogen assimilatory pathway**
A schematic figure to show the main N metabolic fluxes occurring in various organelles in leaf cell. $NO_3^-$ and $NH_4^+$ are two major inorganic N sources for plants. GS/GOGAT pathway is thought to be the principal mechanism of primary and secondary $NH_4^+$ assimilation in cytosol and chloroplast. In mitochondrion, GDH has a function in $NH_4^+$ assimilation under stress (from Forde and Lea, 2007). GS: glutamine synthetase; Fd: ferredoxin; NADH: nicotinamide adenine dinucleotide (reduced); GOGAT: glutamine oxoglutarate aminotransferase; GDH: glutamate dehydrogenase;

### 1.2.1 Glutamine Synthetase (GS)

GS takes charge of primary $NH_4^+$ assimilation in plants by catalyzing the ATP-dependent conversion of Glu into Gln with scavenging $NH_4^+$. Two forms of GS are present in higher plants, and are categorized according to their different cellular localization. GS1, the major cytosolic form of GS in plant roots, plays a dominant role in $NH_4^+$ assimilation from the environment. GS2 is resident as a soluble protein in chloroplasts and mitochondria for $NH_4^+$ scavenging during photorespiration (Taira *et al*., 2004).

Molecular biological studies identified multiple isoenzymes for GS1 in higher plants. Different plant species have unequal numbers of cytosolic GS. For example, there are five isoenzymes of GS1 (*GLN1-1*, *GLN1-2*, *GLN1-3*, *GLN1-4* and *GLN1-5*) in maize (Martin *et al*., 2006); rice plants possess three *GS1* genes; *OsGS1;1*, *OsGS1;2* and *OsGS1;3* (Tabuchi *et al*., 2005); and in *Arabidopsis*, five putative genes for GS1 have been identified, *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5* (Ishiyama *et al*., 2004a). The presence of multiple *GS1* genes has made the analysis of contributions by each individual difficult. The rice *OsGS1;1* knockout mutant shows severely retarded growth throughout its lifespan and has reduced grain filling (Tabuchi *et al*., 2005). The maize mutants *gln1-3* and *gln1-4* display reduced kernel number and size, respectively, and the double mutant *gln1-3*/*gln1-4* shows both phenotypes (Martin *et al*., 2006). Although the functional importance of some individual GS1 isoforms has been demonstrated, accumulating preliminary experiments revealed that the cytosolic GS1 genes were differentially expressed in distinct tissues suggesting nonredundant roles of each isoform (Ishiyama *et al*., 2004a; Peterman and Goodman, 1991; Sakakibara *et al*., 1996). Furthermore, GS1 isoforms were identified that are responsive to abiotic stress. For instance, the activity of GS1 in tomato increased after exposure to different NaCl concentrations (Debouba *et al*., 2006).

Two dimensional (2D) gel electrophoresis analysis of proteins revealed GS1 accumulation in response to cold treatment (Kwon *et al*., 2007). Thus, characterization of the roles of individual GS1 genes and their combined functions will greatly enhance our understanding of the importance of the GS1 family in N assmiliation and abiotic stress response.

Most plants possess only one nuclear gene for GS2. GS2 is predominantly expressed in leaves, where photorespiration occurs. Mutants defective in GS2 were originally identified in barley by screening under photorespiratory conditions. These mutants were unable to reassimilate photorepiratory $NH_4^+$ even in the presence of cytosolic GS (Wallsgrove *et al*., 1987). A more recent study using transgenic GLN2:GFP plants of *Arabidopsis* found that the fusion proteins was present not only in leaf chloroplasts, but also in mitochondria indicating a dual trafficking property of GS2 (Taira *et al*., 2004).

### 1.2.2 Glutamine Oxoglutarate Aminotransferase (GOGAT)

Since the GS/GOGAT pathway ultimately controls the N flux in plant cells, GOGAT has been studied intensively for decades and advances have been made in recent years (Forde and Lea, 2007). Plants possess two distinct GOGATs: the ferredoxin (Fd)- and NADH-dependent forms. Fd-GOGAT is the major isoform in higher plants that contributes 95% to total GOGAT activity, and is normally present in photosynthetic tissues, where it mediates primary N-assimilation or N-reassimilation in photorespiration (Somerville and Ogren, 1980). Genes encoding Fd-GOGAT have been characterized in many plants including maize, tobacco, barley and *Arabidopsis* (Avila *et al*., 1993; Coschigano *et al*., 1998; Sakakibara *et al*., 1991; Zehnacker *et al*., 1992). *GLU1* (formerly called *GLS*) was the first gene encoding Fd-GOGAT identified in *Arabidopsis*. Mutant *glu1* has less than 5% of wild-type GOGAT activity in leaves and shows a chlorotic and

conditional lethal phenotype (photorespiratory condition), suggesting an essential role for *GLU1* in photorespiration. However, this conditional lethal phenotype could be rescued by suppressing photorespiration (1% $CO_2$) (Somerville and Ogren, 1980). *GLU2* is expressed at low levels and cannot compensate for the absence of GLU1 activity when photorespiration occurs (Coschigano *et al*., 1998).

The other form of GOGAT relies on NADH as an electron donor. NADH-GOGAT has a different expression pattern from Fd-GOGAT, and is predominantly in developing tissues. For example, rice *OsNADH-GOGAT1* is mainly expressed in root-tips and in the premature leaf blade and spikelet at an early stage of ripening (Tabuchi *et al*., 2007). It has been proposed that NADH-GOGAT and cytosolic GS play a major role in initial N assimilation and also N transport in root because of the coincident expression of NADH-GOGAT and cytosolic GS (Tobin and Yamaya, 2001). The *glt1*-T mutant, which lacks NADH-GOGAT mRNA and enzyme activity, only exhibited growth inhibition under repressed photorespiration, whereas the Fd-GOGAT mutant *glu1* exhibited severe growth retardation, indicating nonredundant roles of NADH-GOGAT and Fd-GOGAT in $NH_4^+$ assimilation (Lancien *et al*., 2002).

### 1.2.3 Glutamate Dehydrogenase (GDH)

GDH mediates a reversible reaction of either synthesis of Glu by amination or catabolism of Glu by deamination. At an early stage, in order to fully develop the basal part of the shoot, plants require a large amount of protein; in this case the GS/GOGAT cycle is much more active than GDH. Later, during senescence, GS and GOGAT activities decrease, accompanied by GDH protein accumulation and increased aminating activity (Loulakakis *et al*., 2002; Masclaux *et al*., 2000). Due to the dual enzyme activity of GDH giving either 2-OG or Glu as products, GDH is

expected to function in the regulation of carbon/nitrogen (C/N) balance during the whole life cycle of the plant (Miyashita *et al*., 2008). To date, there are at least two types of *GDH* genes identified in plant species, each of them encoding distinct polypeptides α or β subunits of GDH. Seven isoforms of GDH can be produced by a random assembling of the α and β subunits (Reviewed by Purnell *et al*., 2005). Since the significance of the GS/GOGAT cycle in primary N assimilation has been accepted, it is generally thought that GDH plays only a small or negligible role in $NH_4^+$ assimilation instead of facilitating Glu catabolism (Magalhães *et al*., 1990). This is evidenced by studies on the *Arabidopsis* mutant *gdh1-1* defective in the α subunit of the enzyme which revealed that GDH functions in the direction of Glu catabolism under C-limiting conditions (Melo-Oliverira *et al*., 1996). However, the potential role of GDH in $NH_4^+$ assimilation under certain physiological conditions cannot be ignored. A recent study in tobacco and grapevine showed that salinity-induced reactive oxygen species (ROS) and methionine sulfoximine (GS inhibitor) inhibition result in an induction of α-GDH subunit expression, thus initiating the $NH_4^+$ assimilatory direction of GDH (Skopelitis *et al*., 2006). These lines of evidences demonstrate that GDH acts as a C-skeleton supplier when C is limiting or a stress-responsive protein that offers an alternative route for ammonium assimilation in case of the malfunction of the GS/GOGAT pathway.

### 1.3 Carbon and Nitrogen Partitioning

C and N are two major nutrient resources in plants. In all known lifeforms, C is the second most abundant element by mass after oxygen. Particularly in plants, the biomass of crops is highly affected by the composition of C, in terms of C-containing organic compounds such as sugar, starch, and fat. N is found in various amino acids and proteins that are critically important for

plant growth. It should not be surprising that N assimilation is highly interconnected to C metabolism in higher plants because the assimilation of N requires not only energy (eg: reduced Fd, NADPH, ATP), but also C skeletons.

### 1.3.1 Interconnection between C Metabolism and N Metabolism during Photosynthesis

Photosynthesis produces organic compounds from inorganic C by coupling the energy of absorbed light with endothermic metabolic processes. In eukaryotic plants, both light and dark reactions of photosynthesis are carried out in chloroplasts. Light reactions are made up of multiple reactions to convert light energy to chemical energy in the form of ATP and NADPH while producing $O_2$. The Calvin cycle, also called dark reaction because it does not require light, incorporates C from inorganic $CO_2$ into carbohydrates using the energy and reducing power (ATP and NADPH) generated in the light reactions. These two phases take place in distinct regions of the chloroplast; the thylakoid membrane for light reactions and the stroma for the dark reactions. In most plants, a three-C compound, 3-phosphoglycerate (3-PGA) is found as the first product in the Calvin cylce. In this case, plants that fix C into 3-PGA are termed $C_3$ plants. In addition, several plant species such as maize, sugarcane and some dicotyledonous plants initially fix C into four-C oxaloacetate and are therefore called $C_4$ plants. Both $C_3$ and $C_4$ plants employ a key enzyme, ribulose bisphosphate carboxylase/oxygenase (rubisco), to catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP) to form C intermediate in photosynthesis. In fact, rubisco is the main N-containing component accounting for close to 50% of the total protein in photosynthetic plant cells (Krapp *et al*., 2005). Studies have shown that N deprivation can reduce photosynthetic C fixation (Miller *et al*., 2010).

On the other side, in order to transfer N and build up reserves for subsequent use in growth,

plants assimilate inorganic N into N-transport amino acids which depends not only on energy but also C skeletons. Since the assimilation and transport of N is reviewed in part 1.1 and 1.2, the next paragraph will focus on the regulation of N assimilation by C metabolism.

As early as 20 years ago, it was showed that the uptake of $NO_3^-$ by roots depends on the efficiency of photosynthesis and carbohydrate supply (Pace *et al*., 1990). Considering nitrate transport requires energy, it is logical to think that $NO_3^-$ uptake may need photosynthesis as power supply. In recent years, more studies have indicated that not only does N transport demand energy, amino acid biosynthesis does as well. There are a number of C skeleton backbones that are required for amino acid biosynthesis, such as 2-OG, oxaloacetate, 3-PGA and pyruvate. In the GS/GOGAT cycle, 2-OG is the receptor for the amide group of Gln and the product for the reaction is Glu. Since the Gln and Glu produced in GS/GOGAT cycle are donors for the biosynthesis of all amino acids, 2-OG is of crucial importance for N metabolism. Mutants lacking *NAD-DEPENDENT ISOCITRATE DEHYDROGENASE* (*IDH*), the gene which produces 2-OG, have reduced amounts of certain free amino acids in comparison to the wild type (Lemaitre *et al*., 2007). Decreased rates of $NO_3^-$ reduction was also observed in *Arabidopsis* plants with antisense repression of *DIT1,* which encodes a translocator of 2-OG on the chloroplast (Schneidereit *et al*., 2006). In this case, the supply of C skeletons is one of the limiting factors for N assimilation. Therefore, it appears that C and N metabolisms are interdependent on each other for substantial growth.

### 1.3.2 Nitrogen Metabolism and Cellular Redox

In oxygenic photosynthetic eukaryotes, many important biological processes involve oxidation/reduction reactions, for instance, the process of photorespiration is highly dependent

on the oxidation/reduction between $NAD^+$ and NADH in order to maintain the supply of energy. The pathway of N assimilation in oxygenic photosynthetic plants also involves a series of redox reactions. These are initiated with a two-electron reduction of $NO_3^-$ to $NO_2^-$ by NR. This is followed by a six-electron reduction of $NO_2^-$ to $NH_4^+$ by NiR. Finally, in the GS/GOGAT cycle, a two-electron reductive reaction is catalyzed by GOGAT producing Glu from Gln and 2-OG (Suzuki and Knaff, 2005). All three enzymes (NR, NiR and GOGAT) in cyanobacteria catalyzing redox reactions involved in N metabolism are Fd dependent, while the higher plants NiR and GOGAT utilize reduced Fd as the electron donor.

Plant Fds are small acidic proteins containing a single 2Fe-2S cluster acting as ubiquitous redox carriers. Fds can interact with associated proteins, forming electrostatic complexes with Fds providing negative charges and their partners contributing complementary positive charges (Hase *et al*., 2006). Due to the very nature of its function, Fd interacts with many different proteins, such as NADP reductase, sulfite reductase, thioredoxin (Trx) reductase, as well as NiR and GOGAT (Schürmann *et al*., 2008). However, Fd does not function alone, a redox regulatory system called Fdx/Trx system comprised of reduced Fd, a Trx, and the enzyme, Fd-thioredoxin reductase (FTR) is more likely proposed playing an important role in N metabolism (Buchanan *et al*., 2005). This regulatory system is light-dependent and only present in oxygenic photosynthetic organisms. Light signal is perceived to initiate the redox cascades and reduced Fd forms. The interaction between reduced Fd and enzyme FTR transmits the electron to Trxs thus producing the reduced form of Trx. Once reduced, Trx interacts with specific disulfide sites on target proteins thereby influencing enzyme activities (Schürmann and Buchanan, 2008). Although this mechanism is not fully demonstrated in plants, the characterization of a mutant lacking FTR, in *Arabidopsis*, exhibiting phenotypic perturbations and more sensitive to oxidative

stress indicates an important role of the Fdx/Trx system (Keryer *et al*., 2004). In addition, results from spinach revealed that Fd-GOGAT is significantly activated by reduced Trx, further implying that the N flow appears to be regulated by cellular redox via the Fdx/Trx system (Lichter *et al*., 1998).

### 1.4 Stress Responses and Reactive Oxygen Species (ROS)

#### 1.4.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen. They form as a natural byproduct of aerobic metabolism. There are four types of ROS, hydrogen peroxide ($H_2O_2$), superoxide radical ($O_2^-$), hydroxyl radical (•HO), and singlet oxygen ($^1O_2$). Different ROS have very different properties: $HO^-$ reacts rapidly with all types of cellular components, while the main target for $O_2^-$ is Fe-S centers of proteins and $^1O_2$ is particularly reactive with conjugated double bonds such as those found in polyunsaturated fatty acids (PUFAs) (Moller *et al*., 2007). Among all the ROS, $H_2O_2$ is the most stable and can exist in plant tissue at low millimolar concentrations (Moller *et al*., 2007). Moreover, some of the ROS can transform into different types of ROS in the presence of redox-active metals; •HO can be formed from $H_2O_2$ either through the fenton reaction or the Haber-Weiss reaction (Jaspers *et al*., 2010). Since ROS are so active, influencing many cellular components, the mechanism of the generation and removal of ROS and their roles have been studied extensively.

#### 1.4.2 Generation of ROS during Stress Responses in Plants

Although the production of ROS is normally maintained at a low level, the equilibrium between

ROS production and scavenging systems can be disrupted by stresses such as high light, salt, chilling, heat shock, drought, and pathogen attack. Most cellular compartments overproduce ROS under such stress conditions. For example, in green parts of plants, chloroplasts, and peroxisomes are major ROS producers, while in non-green parts and in darkness mitochondria plays the major role in ROS production (Foyer and Noctor, 2003; Moller, 2001).

In photosynthetic cells, the photosynthetic electron transport chain (PETC) is the major place of ROS production. When plants are exposed to environmental stresses, the Calvin cycle is restricted and cannot consume NADPH rapidly enough, thus resulting in the accumulation of excess NADPH in the thylakoid membrane. Meanwhile, an over reduction of the reaction centres of photosystem I (PSI) and photosystem II (PSII) in PETC takes place under such environments, with high levels of NADPH resulting from the accumulation of excess electrons. As a terminal electron acceptor, $O_2$ is reduced leading to the formation of $O_2^-$ or hydroperoxide ($HO_2^-$) (Apel and Hirt, 2004, Miller *et al*., 2010). Although excessive production of ROS is detrimental to the plant cell, the reduction of $O_2$ is able to prevent overreduction of the electron transport chain (Dat *et al*., 2000).

In addition to chloroplasts, peroxisomes that depend on the photorespiratory glycolate oxidase reaction are the other major site of ROS production during photorespiration in green parts of plants. Under conditions of restricted $CO_2$ fixation, the oxygenase activity of rubisco increases and produces more glycolate. The excess glycolate is translocated from chloroplasts to peroxisomes and glycolate oxidase generates $H_2O_2$ as a by-product of glycolate oxidation (Tsanko *et al*., 2006). $H_2O_2$ is also produced during fatty acid β-oxidation. In plants, β-oxidation is the major pathway for degradation of fatty acids and some branched chain amino acids, and only takes place in peroxisomes where $H_2O_2$, NADH, and acetyl-CoA are generated as major end

products (Baker *et al*., 2006; Nyathi and Baker, 2006).

Mitochondria are a major source of ROS in mammalian cells; however, in green plant tissues, mitochondria do not make a large contribution to ROS production, but influence a fraction of total ROS production in nonphotosynthetic cells (Foyer and Noctor, 2003). Although in $C_3$ plants, chloroplastic and peroxisomal $H_2O_2$ production may be up to 30-100 times faster than $H_2O_2$ formation in the mitochondria, mitochondrial ROS are essentially associated with a number of cellular processes like stress adaptation and programmed cell death (Foyer and Noctor, 2003). So far, the ROS production in plant mitochondria is still not well known because of the few studies performed, but it is generally accepted that the rate of ROS production relies on the reduction level of the electron transport chain (ETC). Complex I and Complex III in the ETC are principal sites for ROS production. In addition, alternative oxidase (AOX) plays a role of maintaining the reduction state of the ubiquinone (UQ) pool and reducing the production of ROS. Inhibition of AOX by antimycin A or loss of the *AOX1* gene in *Arabidopsis* causes an increase in $O_2^-$ (Giraud *et al*., 2008 and Rhoads *et al*., 2006).

### 1.4.3 ROS Toxicity and Signalling in Abiotic Stresses

In plants, ROS plays a multiplicity of roles such as suicide agents, antimicrobial compounds as well as signaling molecules (Mehdy, 1994). The role of ROS can be viewed as a double-edged molecular sword in plant physiology and pathology. Over-accumulation of ROS modifies cellular components and causes damage to many signaling molecules (Moller *et al*., 2007). For instance, the rapid increase in ROS concentration, termed the “oxidative burst”, stimulated by environmental stress or pathogen invasion, leads to the death of plant cells due to the negative modifications of proteins, PUFAs, DNA and carbohydrates (Moller *et al*., 2007).

Although ROS can result in cellular damage, ROS can also act as a signal to initiate a series of responses under various abiotic stresses. Emerging evidence suggests that the signaling role of ROS is very complex and subtle. Microarray analysis conducted on mutants defective in important ROS-scavenging enzymes such as ascorbate peroxidase (APX1) and catalase (CAT) enabled more extensive understanding of the regulatory pathways related to abiotic stress-induced ROS (Davletova *et al*., 2005; Vandenabeele *et al*., 2004). Although a large number of genes responsive to stress are still a functional mystery, at least one signal transduction pathway was proposed to be affected by ROS. In this pathway ROS modify mitogen-activated protein kinases (MAPK) cascade and ultimately alter gene expression by modulating transcription factors (Jaspers *et al*., 2010).

MAPKs are serine/threonine kinases able to phosphorylate a wide range of substrates. Phosphorylation of proteins is one of the most common post-translational modifications in all organisms (Rodriguez *et al*., 2010). Molecular and genetic studies show that MAPKs are closely involved in signaling of various biotic and abiotic responses (Colcombet and Hirt, 2008). Recent studies have identified several MAPKs (MPK3, MPK4 and MPK6) activated in response to $H_2O_2$ (Desikan *et al*., 2001; Kovtun *et al*., 2000; Moon *et al*., 2003). However, the linkage between MAPKs and ROS from upstream receptors to downstream targets is still unclear. $H_2O_2$-dependent MPK3/MPK6 activation implies a signal sensor role for MAPK (Moon *et al*., 2003). On the other hand, an *Arabidopsis* MAPKKK, MEKK1, functions in integrating ROS homeostasis via MPK4, suggesting an additional role of MAPK as upstream regulator (Nakagami *et al*., 2006).

In addition to MAPK pathways, ROS accumulation can also be sensed by redox-response transcription factors. Heat shock transcription factors (Hstfs) are one class of stress-responsive

proteins. Hstfs are not only induced under heat conditions but also responsive to various stressful environments such as strong light, drought, and salinity (Miller and Mittler, 2006). Hstfs are involved in modulating ROS signalling networks during abiotic stress. For example, *Arabidopsis* HstfA4a and HstfA8 showed a dramatic induction in the *cytapx1* mutant (lacking cytosolic APX) during light stress (Davletova *et al*., 2005). Suppression of LpHstfA1 in tomato plants resulted in enhanced sensitivity to heat stress while overexpression of LpHstfA1 showed the opposite phenotype (Baniwal *et al*., 2004; Mishra *et al*., 2002). The promoter of *cytAPX1* contains an Hstf-binding motif and this binding site is functional suggesting a close link between Hstf and redox status (Panchuk *et al*., 2002; Rizhsky *et al*., 2004; Storozhenko *et al*., 1998).

## 2. Research Objectives

The main objective of my M.Sc. research is to determine the role of *Arabidopsis* cytosolic glutamine synthetase 1 (GS1/GLN1) in the regulation of Gln homeostasis response to biotic and abiotic stresses. Specific goals are:

a) To investigate the expression of *Arabidopsis GS1* with respect to tissue specificity and in response to various abiotic stresses and the pathogen infection.

b) To isolate and characterize T-DNA insertion knockout mutants for each GS1 isoform.

c) To determine the role of individual GS1 isoforms in abiotic stresses, and GS-inhibitor (phosphinothricin) tolerance.

d) To create double and triple mutants of major GS1 isoforms for investigation of their possible synergetic or additive function in plant growth, development and stress responses.

# 3. Materials and Methods

## 3.1 Plant Material and Growth Conditions

*Arabidopsis* Col-0 and their corresponding mutants, including *gln1;1* (Salk_000459), *gln1;2-1* (Salk_145235), *gln1;2-2* (Salk_102291), *gln1;3-1* (Salk_172283), *gln1;3-2* (Salk_038156), *gln1;4-1* (Salk_042546), *gln1;4-2* (Salk_147053), *gln1;5-1* (Salk_086579), and *gln1;5-2* (Salk_117504) single T-DNA insertion lines were obtained from the ABRC (Arabidopsis Biological Resource Center).

Growth on soil: Seeds (Col-0 and mutants) were sown in soil and kept in darkness for 2 d at 4$^{o}$C before being transferred to the growth chamber. The growth chamber (22 $^{o}$C) was managed under controlled conditions (16 hrs light/8 hrs dark for seed production, 12 hrs light/12 hrs dark for experiments).

Growth on agar plates: Seeds (Col-0 and mutants) were surface sterilized by 33% commercial bleach for 10 minutes and washed three times in sterile water. Sterilized seeds were placed on (-N)-Murashige and Skoog (MS) media with different N sources including 5 mM Gln or 20 mM $NH_4NO_3$, supplemented with 1% agar and 1% sucrose. Plates with seeds were then kept in darkness at 4$^{o}$C for 2 d and moved in incubator (22 $^{o}$C) for plant growth (11 hrs light/13 hrs dark).

## 3.2 Characterization of Homozygous T-DNA Insertional Mutants

Confirmation of null mutant was carried out by RT-PCR. The gene-specific primers and T-DNA-specific left-border primer LB are shown in Table 1. PCR conditions were 3 min at 94$^{o}$C and 40 cycles of 50 sec at 94$^{o}$C, 30 sec at 60$^{o}$C and 70 sec at 72$^{o}$C. After 40 cycles, a final extension at

72$^{o}$C for 5 min was performed.

**Table 1 Primer sequences for PCR amplification of *GLN1* genes**

| Name | Primer sequence |
|---|---|
| GLN1;1F | 5'-CAAAATTGTAACCTGGTGTGTCT-3' |
| GLN1;1R | 5'-TCATGTCCATTCCAGAACCA-3' |
| GLN1;2-1F | 5'-TGGTGGTTCTGGTATGGACA-3' |
| GLN1;2-1R | 5'-CCAAGAACGGAGAATCGAAA-3' |
| GLN1;2-2F | 5'-GGACACCATGAAACTGCTGA-3' |
| GLN1;2-2R | 5'-CCCCATTGAGATCCACTTTG-3' |
| GLN1;3-1F | 5'-TGTTTCGTTGTCCTGCTTTG-3' |
| GLN1;3-1R | 5'-ACCAATTGGCCAGTTCACAT-3' |
| GLN1;3-2F | 5'-AGGCACAACGCTGCTAAGAT-3' |
| GLN1;3-2R | 5'-CAGCTGAAGCTTCCCTATCG-3' |
| GLN1;4-1F | 5'-CGTTGAAAGATTTTGGGTTGA-3' |
| GLN1;4-1R | 5'-CGTGCAAGTAGGGGAAACAT-3' |
| GLN1;4-2F | 5'-ATATACACCGGCAGGAGAGC-3' |
| GLN1;4-2R | 5'-CCCACACAAGCTCAGTTTCA-3' |
| GLN1;5-1F | 5'-TCTTTCGGAACAAAAATGACG-3' |
| GLN1;5-1R | 5'-AAGGCTCTTCAGCCTTCACA-3' |
| GLN1;5-2F | 5'-CACTAGGTTGGCCTCTTGGT-3' |
| GLN1;5-2R | 5'-TCTCGTTGCCTTCACCATAA-3' |
| LB | 5'-GGCAATCAGCTGTTGCCCGTCTCACTGGT-3' |

### 3.3 Stress Treatment

Seeds of wild-type Col-0 and mutants were placed on MS medium following the procedure described in section 3.1. After seed germination and seedling establishment (7 d after stratification), seedlings were exposed to different stress treatments. In the salt stress treatment, *Arabidopsis* seedlings were transferred from MS medium to filter papers saturated with 100 mM NaCl (stressed) or $H_2O$ (control) in covered Petri dishes. The seedlings were collected at 0.5, 2, and 4 hrs time points after treatment in both conditions. In the cold stress treatment, seedlings in MS medium were exposed to 4 $^{o}$C, materials were harvested at 0, 2, and 5 hrs time points after treatment. All materials collected were flash frozen in liquid $N_2$ and stored in -80$^{o}$C freezer until

required.

### 3.4 Pathogen Inoculation

Four-week-old *Arabidopsis* plants in soil were sprayed with a conidial suspension of *Colletotrichum higginsianum* ($1 \times 10^3$ spores $\mu L^{-1}$ in distilled water). After inoculation, each tray was covered with a clear plastic lid. The plants inoculated with *C. higginsianum* were maintained in 100% relative humidity by regular spraying with distilled water in a growth chamber (Narusaka *et al*., 2004). Materials were collected at different intervals from 0 to 168 hrs post inoculation (hpi), flash frozen in liquid $N_2$ and stored at -80$^o$C until required.

### 3.5 RNA Extraction, cDNA Generation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 100 mg of materials using the TRIzol reagent (Invitrogen). cDNA was generated using the SuperScript$^{TM}$ II Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. cDNA derived from 2 μg total RNA was used as a template for each PCR. Semi-quantitative RT-PCR analysis of transcripts of selected genes was performed by using gene-specific primers shown in Table 2. PCR conditions were 3 min at 94$^o$C and 29 cycles of 50 sec at 94$^o$C, 30 sec at 60$^o$C and 1 min at 72$^o$C. After 29 cycles, a final extension at 72$^o$C for 5 min was performed.

### 3.6 Abiotic and Oxidative Stress Assays

Sterilized seeds were plated on MS medium supplemented with one of the following chemicals: 0.5 mg/L Phosphinothricin (PPT), 100 mM NaCl, 0.1 μM methyl viologen, 100 μM menadione,

500 μM salicylhydroxamic acid, and 9 μM rose bengal. Every treatment included an MS plate without any supplement grown under the same condition as the control. In the case of the cold stress assay, sterilized seeds were plated on MS medium and kept in 22 $^{o}$C in an incubator with controlled condition (see section 3.1). Seven-day-old seedlings were then transferred to -18$^{o}$C for 4hrs and recovered in 5 $^{o}$C for 16 d. All the Petri plates were vertically positioned.

**Table 2 Primer sequences for RT-PCR amplification of cDNA**

| Name | Primer sequence |
|---|---|
| cGLN1;1F | 5'-CCGAAACCGATTCCCGGTGA-3' |
| cGLN1;1R | 5'-AAAAGCAGAATAAGCAGAGCAAA-3' |
| cGLN1;2F | 5'-CAATGGGAGTTCCAAGTCGGC-3' |
| cGLN1;2R | 5'-GATCATCCTTTCAAGGGTTCC-3' |
| cGLN1;3F | 5'-CTGAGATCTCTGGTGTAATTG-3' |
| cGLN1;3R | 5'-ACAACAATACGCCCATAATAA-3' |
| cGLN1;4F | 5'-CGAACATGGATCCTTACACT-3' |
| cGLN1;4R | 5'-AATCAAACATAATAAACAAA-3' |
| cGLN1;5F | 5'-CCAATCCAGGGTGATTGGAA-3' |
| cGLN1;5R | 5'-ACCGTAACCGGATCAAACAA-3' |
| EF1α-F | 5'-CGTTGCCTCTAACTCCAAGG-3' |
| EF1α-R | 5'-TCCTTCTTGTCCACGCTCTT-3' |
| 18S-F | 5'-ATGGCCGTTCTTAGTTGGTG-3' |
| 18S-R | 5'-GTACAAAGGGCAGGGACGTA-3' |

### 3.7 Western Blot Analysis

*Arabidopsis* seedlings were ground in liquid N and proteins were extracted with a solution (50mM Tris, pH8.0, 2mM EDTA, 10% (v/v) glycerol and 0.01% 2-merceptoethonal). The amount of total protein was determined by Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard. About 8 μg of protein were loaded on a 12% SDS polyacrylamide gel and separated by electrophoresis. Protein profiles were analyzed according to the procedure of Laemmli (1970) followed by Coomassie blue staining. Protein were also electro-blotted onto PVDF membrane (Bio-Rad) and blocked by 4% skim milk TTBS (20mM

Tris, pH7.6, 137mM NaCl, 0.1% (v/v) Tween 20). The blocked PVDF membrane was then incubated with 18 μg of lyophilised anti-GS serum (Bennett and Cullimore, 1989) in 10 ml of TTBS at 37$^{o}$C for 2 hrs following with 4 washes with TTBS (15 min each). The anti-GS blotted membrane was incubated with horseradish peroxidase-conjugated anti-Rabbit IgG (1:15000) (Amersham) at 37$^{o}$C for 2 hrs followed by 3 washes with TTBS (15 min each), and detected by enhanced chemiluminescence reagents.

### 3.8 ROS Stimulation and Detection by Confocal Microscopy

$H_2O_2$ production was triggered by spraying 10 μM methyl viologen on 3-week-old *Arabidopsis* plants. Leaves were collected 4 hrs after treatment. Microscopic observations of ROS was performed using a Zeiss META 510 confocal laser scanning microscope. *Arabidopsis* leaves were stained with 50 μM 2'7'-dichlorohydrofluorescin diacetate ($H_2$DCFDA) and washed twice in K-phosphate buffer (20 mM, pH6.0). $H_2$DCFDA signals were visualized with excitation at 488 nm (emission: 498 to 532nm). Chloroplast autofluorescence was visualized with excitation/emission: 488/738-793 nm.

# 4. Result

## 4.1 Tissue Specific Expression of GLN1;1 and GLN1;4

In *Arabidopsis, GS2* is expressed specifically in the shoot and GS2 protein is restricted to chloroplasts and mitochondria (Taira *et al*., 2004). The function of GS2 is for the assimilation of ammonium released from photorespiration. Unlike GS2, GS1 has a number of isoforms expressed in both shoots and roots (Ishiyama *et al*., 2004). Studies have shown that cytosolic GS localizes mainly in the cytoplasm of mesophyll and vascular cells (Avila *et al*., 2001). Thus, it is generally believed that GS1 is responsible for primary ammonium assimilation and transport in plants. However, it is also important to note that although all the GS1 are expressed in the cytosol of plant cells, they may have different roles due to distinct expression patterns in tissues.

Since previous work showed a tissue-specific pattern of GS accumulation and GS activity in soybean (Fei *et al*., 2006), it may thus of interest to localize each of the isoforms with respect to tissue-specificity in *Arabidopsis* in order to gain further insight into their individual physiological roles. To this end, reverse transcriptase (RT)-PCR was used to examine the expression of all five *GS1* genes at the transcript level in specific tissues of *Arabdiopsis*. Because the cDNA sequences of all 5 genes are highly similar, it is important to know the specificity of each pair of primers. This issue is dealt more specifically in section 4.4, the gene specific primers were able to amplify an expected band from wild-type plant, but not from the corresponding mutants indicating the specificity of the primers (See Figure 4.5). These specific primers as shown in Table 2 were used in RT-PCR analysis. As shown in Figure 4.1A, except *GLN1;5*, transcript of all the other 4 isoforms can be detected. Interestingly, no expression of *GS1* genes was detected in siliques. GLN1;3 was one of the major isoforms among all of the GS1 proteins in *Arabidopsis* seedlings (Figure 4.5B) and its transcripts appeared have the highest level of

expression among the *GS1* genes (Figure 4.1A). Like *GLN1;3*, *GLN1;2* transcripts could be detected in different tissues. However, *GLN1;1* and *GLN1;4* showed more restricted pattern of expression. A low level of *GLN1;1* transcripts was detected in flowers, but high levels detected in roots and seedlings. The only organ in which *GLN1;4* transcripts were detected was the stem (Figure 4.1A). Proteins were also extracted from different tissues including root, leaf, stem and flower of 3 independent lines: wild-type, *gln1;2-2*/*gln1;3-1*, *gln1;1-1*/*gln1;2-2*/*gln1;3-1* (the mutants are described in section 4.7) and western blot was performed using GS antibody. Figure 4.1B shows the gel stained by coomassie blue indicating that similar amounts of total protein were loaded. It is worthy to note that I have determined total protein content in each sample by using the Bradford protein assay and loaded similar amounts of total protein in each well, even though amounts of the large subunit of Rubisco (LSU) did not appear equivalent among different tissue samples. Therefore, LSU is only good for protein normalization in the same tissues. The expression level of GS2 protein varied in different tissues of *Arabidopsis* with a higher level of expression in shoots than in roots. Because the transcript of *GLN1;5* was never detected under any condition, I assume that there is no expression of the GLN1;5 polypeptide in *Arabidopsis* under the experimental conditions I examined. In leaves and flowers, western blot showed a band in the *gln1;2-2*/*gln1;3-1* double mutant but not in the *gln1;1-1*/*gln1;2-2*/*gln1;3-1* triple mutant indicating the band was GLN1;1. In roots and stems, the band in the *gln1;2-2*/*gln1;3-1* double mutant was stronger than in the triple mutant, which means GLN1;1 also expressed in roots and stems. The broad expression of GLN1;1 in all tissues analyzed by western blot is consistent with the RT-PCR results. The weak band in roots and stems of the triple mutant suggested that GLN1;4 was present in these two types of tissue. When comparing GLN1 proteins in Col-0 and *gln1;2-2*/*gln1;3-1* double mutant among all the tissues, a dramatic decreased expression level of

GLN1 was observed in the *gln1;2-2*/*gln1;3-1* double mutant which suggested the expression of GLN1;2 and GLN1;3 were not restricted in any specific tissue. Interestingly, comparing the expression of GLN1 protein in *gln1;2-2*/*gln1;3-1* mutant to the wild-type plant, it appears that GLN1;1 and GLN1;4 contribute major GLN1 expression in roots and stems suggesting a possible role for them in primary N assimilation and transport.

### 4.2 Expression of *GS1* Genes in Response to Abiotic Stresses

A number of studies have indicated an enhanced expression of GS1 protein is responsive to various abiotic stresses, such as cold (Kwon *et al*., 2007), salt (Debouba *et al*., 2006), and $H_2O_2$ (Scarpeci *et al*., 2008). To determine which genes are specifically induced under stress conditions, I treated *Arabidopsis* seedlings with salt and cold stresses and detected transcripts of the 5 *GS1* genes by RT-PCR. *RD29A* gene which is known to be induced by cold and salt stresses was used to validate the treatments. As shown in Figure 4.2, no *GLN1;5* expression was observed in seedlings growing in normal conditions, neither was observed under the abiotic stresses. This observation indicates that *GLN1;5* is not expressed under normal and stress conditions. *GLN1;1*, *GLN1;3* and *GLN1;4* were all induced by the 100 mM NaCl treatment at different times. Transcript levels of *GLN1;2* were maintained at a relatively steady level under salt stress, which is higher than that in control plants treated with $H_2O$. *GLN1;1*, *GLN1;3* and *GLN1;4* showed similar expression patterns following the transfer of plants to 4 $^{o}$C, while *GLN1;2* exhibited a constitutive expression with low temperature exposure.

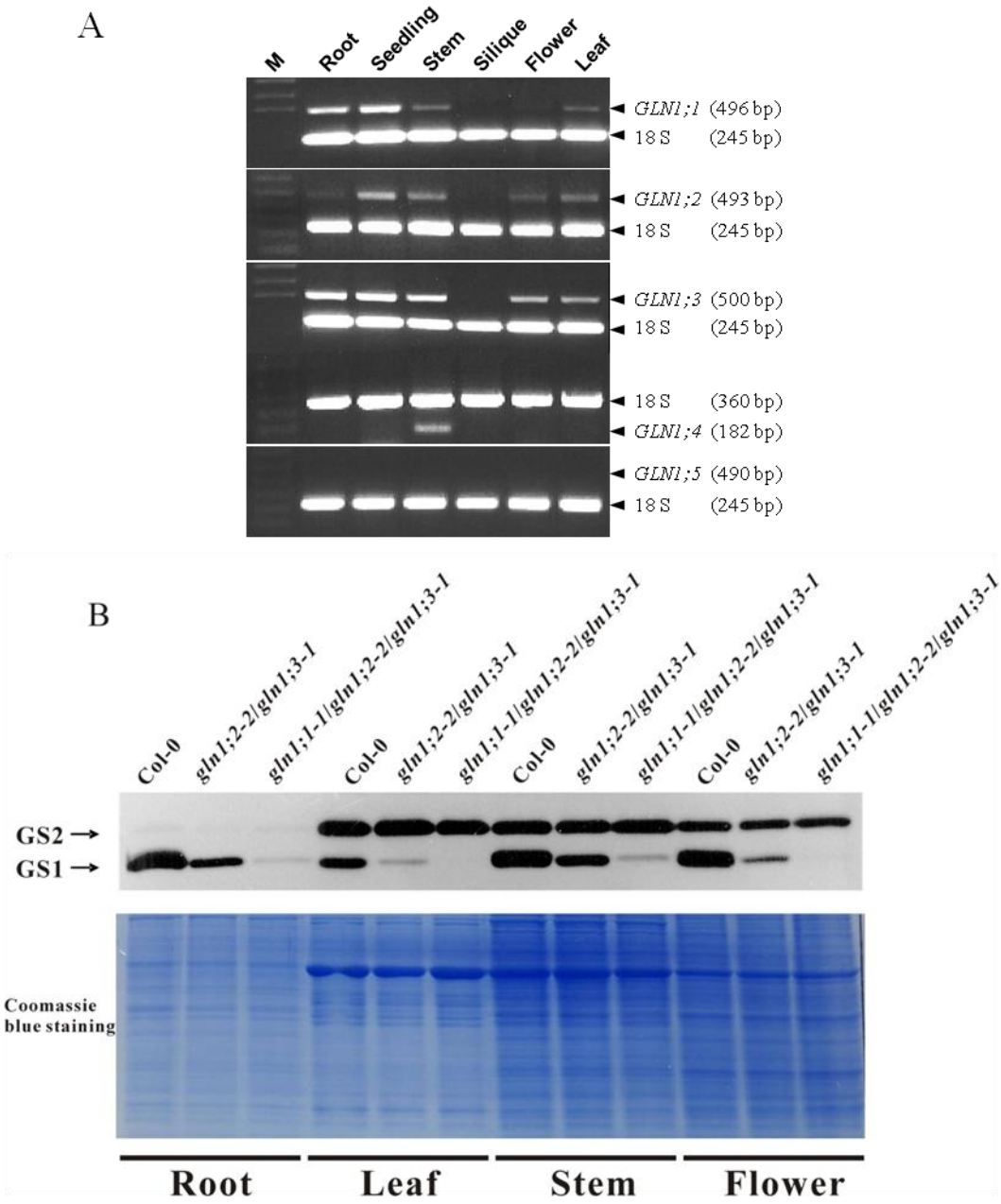


**Figure 4.1 Tissue specific expression of GLN1;1 and GLN1;4**
A. RT-PCR analysis of tissue-specific expression of *GLN1;1*-*GLN1;5*. cDNAs were normalized by using the *18S* gene. Total RNAs were extracted from roots of 2-week-old seedlings, fully expanded leaves, stems, flowers, and developing siliques of Col-0 *Arabidopsis* (M indicates marker, 27 cycles). B. Determination of GLN1;1 and GLN1;4 protein levels in different tissues. GS2 (44 kDa) and GS1 (~ 40 kDa) proteins were shown in the western blot. Proteins were extracted from roots, leaves, stems and flowers of Col-0, *gln1;2-2*/*gln1;3-1* and *gln1;1-1*/*gln1;2-2*/*gln1;3-1* plants and separated by using SDS-PAGE. Proteins were transferred to membrane and immunoblotted with GS antibodies. Coomassie blue staining of the gel showed the amount of protein loaded in each lane.

### 4.3 Expression of *GS1s* in Response to Pathogen Infection

In addition to responsiveness to abiotic stress, *GS1* expression has been identified to be associated with fungal and viral infection (Tavernier *et al*., 2007 and Pageau *et al*., 2006). Moreover, Gln is a crucial nutrient for both plants and pathogens. Competition in acquisition of Gln between them has a significant impact on disease development. Thus, investigation of the response of *GS1* genes is important for further characterizing the role of each *GS1* gene involved in defense against pathogens.

*C. higginsianum* is a hemibiotrophic fungus possessing a biotrophic phase and a necrotrophic phase, and the invasion of fungus is a sophisticated process including spores germination, appressorium formation, and primary and secondary hyphae generation. After plant death, *C. higginsianum* employs a necrotrophic behavior for long-lasting survival (Liu *et al*., 2007). Therefore the expression of each *GS1* following *C. higginsianum* infection was analyzed in a detailed time course investigation. The temporal expression pattern of the five *GS1* genes was examined over a 168 h post inoculation (hpi) period by RT-PCR. *PR1* which is induced by pathogen attacks was used as marker gene to show that the plants were responding to pathogen infection. As shown in Figure 4.3, the transcripts of *GLN1;1*, *GLN1;3* and *GLN1;4* accumulate in response to infection from 24 to 72 hpi. Whereas levels of *GLN1;1* started to decrease after 72 hpi, *GLN1;3* and *GLN1;4* transcripts remained at a relatively high level until the plants died (144 hpi). These results suggest that *GLN1;1*, *GLN1;3* and *GLN1;4* might have roles in disease development during *C. higginsianum* infection.

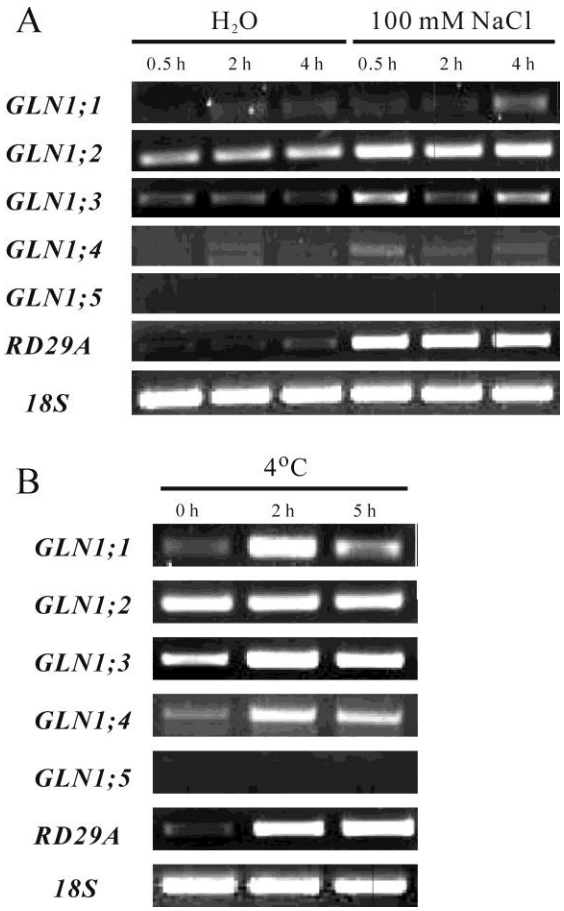


**Figure 4.2 RT-PCR analysis of expression patterns of *GLN1* genes in response to different abiotic stresses in *Arabidopsis* seedlings.**
(A). Time course of *GLN1;1-GLN1;5* gene transcript accumulation in *Arabidopsis* seedlings following salt treatment. RNA extracted from seedlings with $H_2O$ treatment at corresponding time points were used as controls. cDNA were synthesized and RT-PCR performed. The amounts of cDNA used were normalized based on the expression level of *18S*. The expression level of *RD29A* was shown to indicate that the seedlings were exposed in stress conditions (27 cycles). (B). Time course of *GLN1;1-GLN1;5* genes expression following cold treatment (27 cycles).

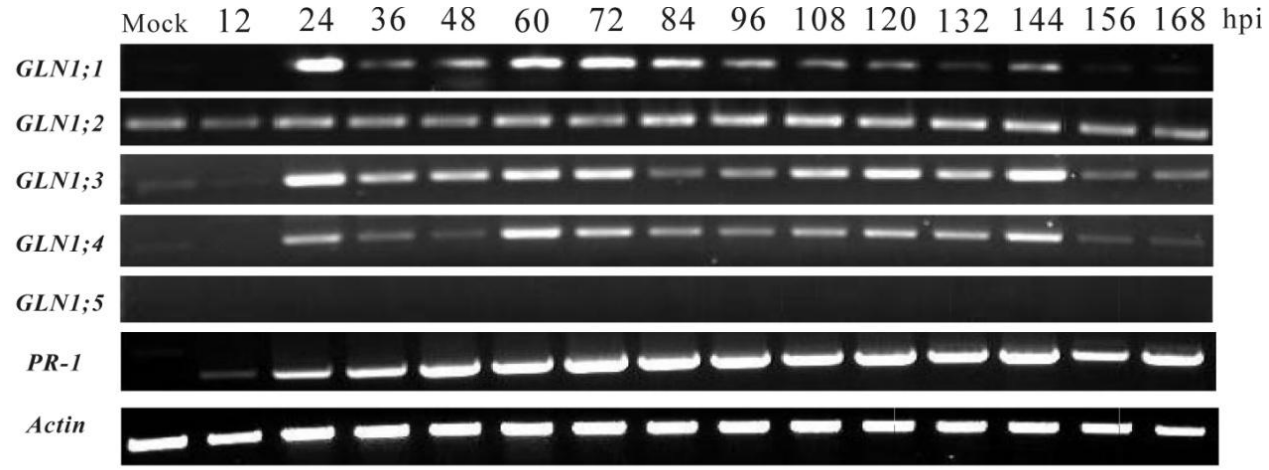


**Figure 4.3 RT-PCR analysis of *GLN1* expression in response to *Colletotrichum higginsianum* infection.**
Total RNA was isolated in a time course (0-168 hpi) from 4-week-old *Arabidopsis* leaves inoculated with *C. higginsianum* by spraying ($1 \times 10^3$ spores $\mu L^{-1}$ in distilled water). RNAs from uninfected leaves at 0 hpi were used as controls (Mock). cDNAs were normalized by using *Actin 2* specific primers. The transcript levels of the *PR-1* gene indicated a successful *C. higginsianum* inoculation of *Arabidopsis* plants (27 cycles).

### 4.4 Generation of *gln1;1~gln1;5* Mutants

Nine independent T-DNA insertion lines were ordered from the SALK collection and named *gln1;1* (Salk_000459), *gln1;2-1* (Salk_145235), *gln1;2-2* (Salk_102291), *gln1;3-1* (Salk_072283), *gln1;3-2* (Salk_038156), *gln1;4-1* (Salk_042546), *gln1;4-2* (Salk_147053), *gln1;5-1* (Salk_086579), *gln1;5-2* (Salk_117504). The putative T-DNA insertion sites associated with individual GS1 genes are shown in Figure 4.4. In order to obtain homozygous mutants, T-DNA insertions were identified at first by using combinations of gene- and T-DNA-specific primer pairs (see materials and methods). In order to confirm the null mutation of each gene, RT-PCR and western blotting were performed: Results show the absence of the full length transcript of each gene and protein of each isoform in the perspective mutants, with the exception of GLN1;5, for which neither the transcript nor the protein could be detected in Col-0 (Figure 4.5). Moreover, the data demonstrate that in wild-type plants the total GS1 proteins could be separated into 2 different bands by SDS-PAGE/western blot analysis by using the anti-GS antibody, including GLN1;3 (top) and a mixture of GLN1;1, GLN1;2, and GLN1;4 (bottom). In the *gln1;2* mutant, there was still a weak band at the bottom representing GLN1;1 and GLN1;4 which means GLN1;1 and GLN1;4 could not be separated by the SDS-PAGE and overlapped with GLN1;2. The results also showed that GS2 was equally expressed in all of the single mutants and was not affected by the absence of any of the GS1 isoforms (Figure 4.5B). In addition, because I had only one mutant line (Salk_000459) for isoform GLN1;1, confirmation of single T-DNA insertion was conducted by using a cross between the *gln1;1* homozygous and wild-type (Col-0) plant. The F1 offsprings (*GLN1;1*/*gln1;1* heterozygote) were then grown in soil to obtain the F2 generation. The seeds of the F2 generation were placed on the MS medium supplemented with 50 μg/mL kanamycin. After 2-week growth, the ratio between resistant and sensitive seedlings

was approximately 3:1 (93 resistant seedlings, 32 sensitive seedlings) indicating that it is highly possible for *gln1;1* mutant to have a single T-DNA insertion in the genome.

### 4.5 *GLN1;1* Is Required for Phosphinothricin (PPT) Tolerance

PPT is a common herbicide used in agriculture. The main target of PPT is GS. For the purpose of inhibiting the activity of GS, PPT binds to GS and occupies the position where glutamate is supposed to be (Lea *et al*., 1984). In order to overcome the inactivation of GS, enhanced GS expression is employed for improving N acquisition in plants. Studies on plants subjected to PPT treatment have revealed that overexpression of GS enhances tolerance to PPT (Pascual *et al*., 2008). Thus, in order to determine the tolerance of each mutant and the role of each gene, six independent lines including wild type (Col-0) were grown on MS medium with 0.5 mg/L PPT. After seeds were plated on the medium, a one-day delay of germination was observed among some of the mutants. This also happened in the control plate and was probably due to seed quality. After germination, as shown in Figure 4.6A, growth and development of leaf tissue and root of *gln1;1* seedlings were inhibited significantly. In contrast, the other 4 mutants (*gln1;2~gln1;5*) had a similar growth rate and phenotype as wild-type plants. Because GS1 proteins are dominantly expressing in the root, the involvement of each isoenzyme in primary root growth in response to PPT treatment was investigated by measuring the root length. Roots of seedlings growing on plates with PPT were compared to control plates (without PPT). This analysis showed that the degree of inhibition was much greater in *gln1;1* and other mutants including *gln1;2*, *gln1;3*,and *gln1;4* also exhibited statistically shorter root length compared to wild-type plants (Figure 4.6B). In conclusion, among all 5 homologs of *GS1* family, *GLN1;1* is the most important member for PPT tolerance in both leaves and roots.

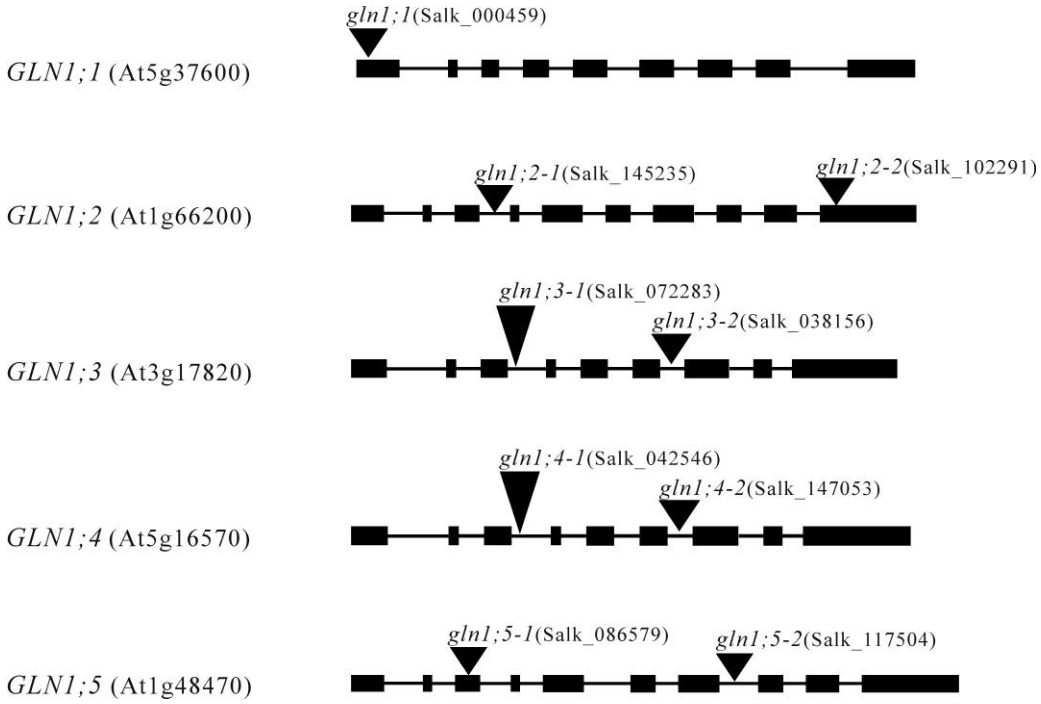


**Figure 4.4 Schematic diagrams of *GLN1* genes.**
T-DNA insertion sites in *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5* and their corresponding AGI codes are illustrated. T-DNA insertions are indicated with triangles, and exons and introns are indicated as black boxes and lines, respectively.

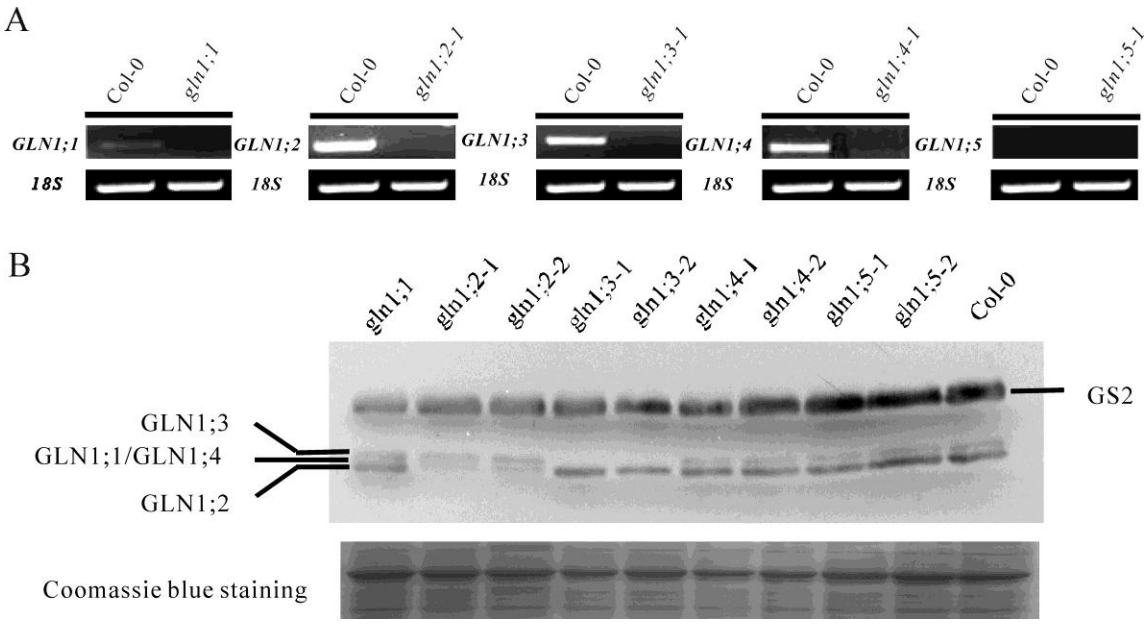


**Figure 4.5 Characterization of null mutations for *GLN1* isoforms.**
(A). RT-PCR analysis of indicated genes from wild-type (WT), *gln1;1*, *gln1;2-1 gln1;3-1*, *gln1;4-1* and *gln1;5-1* seedling plants (27 cycles).
(B). Western blot analysis of GS proteins extracted from *gln* mutants. Proteins were extracted from seedlings of wild-type (WT), *gln1;1*, *gln1;2-1*, *gln1;2-2*, *gln1;3-1*, *gln1;3-2*, *gln1;4-1*, *gln1;4-2*, *gln1;5-1* and *gln1;5-2* and separated using SDS-PAGE. Proteins were transferred to membrane and immunoblotted with an GS antibody. Coomassie blue staining indicated equal amount of protein was loaded in each well.

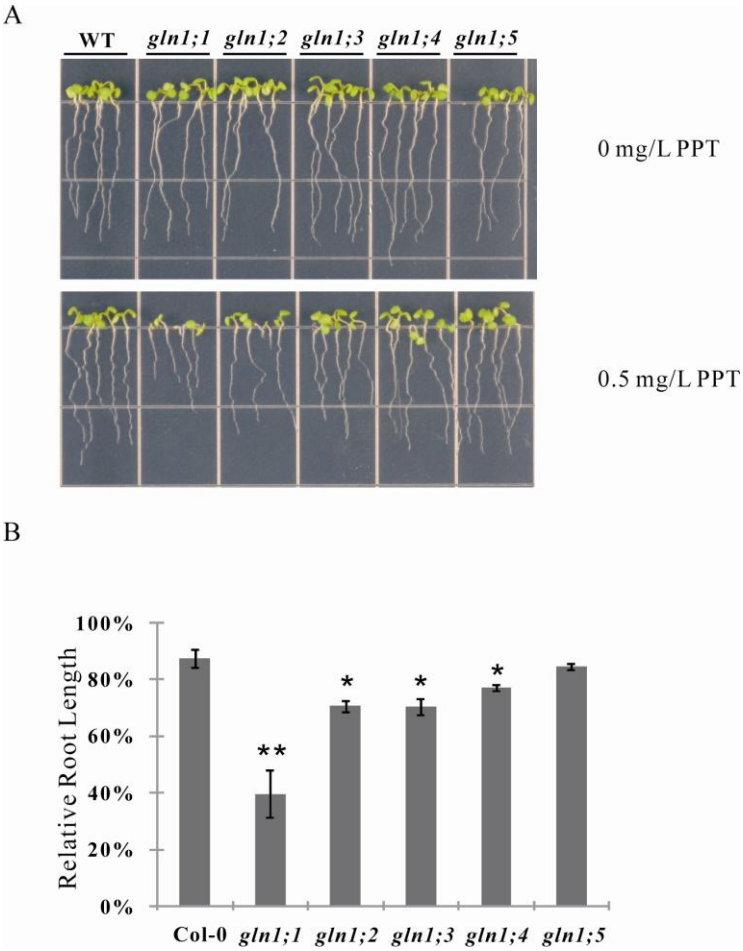


**Figure 4.6 *gln1;1* mutants are more sensitive to PPT.**
(A). 9-day-old seedlings of the wild-type, *gln1;1, gln1;2, gln1;3, gln1;4* and *gln1;5* mutant lines grown on MS medium with (bottom) or without (top) 0.5 mg/L PPT.
(B). Quantification of root growth of WT and *gln1* mutants on PPT. Asterisk indicates a P value (T-test). *, P value < 0.05; **, P value < 0.01. Error bars represent standard error for three independent experiments and twenty roots were measured respectively.

### 4.6 *gln1;1* Mutant is Susceptible to Salt, Cold and Oxidative Stresses

The lack of *GLN1;1* resulted in PPT sensitivity, suggesting that *GLN1;1* might be important for cell protection. Based on previous studies, GS1 is induced by salt (Debouba *et al*., 2005) and cold (Kwon *et al*., 2007) stresses. Furthermore, our results also showed that the transcripts of *GLN1;1*, *GLN1;3* and *GLN1;4* are up-regulated by abiotic stresses. Disruption in the expression of *GLN1;1*, *GLN1;3* or *GLN1;4* may therefore affect salt or cold tolerance. Thus, it is of interest to study the role of the *GS1* genes in *Arabidopsis* seedlings against these abiotic stresses.

As shown in Figure 4.7A, the presence of 100 mM NaCl somewhat hindered the greening of leaves and elongation of roots for all the genotypes tested. Compared with wild type, the effect in the *gln1;1* mutant was more severe, with growth and development of leaves being significantly affected. The responses to low temperature and salinity stress in plant cells share similar regulation pathway in which *GS1* might be involved. With the expectation of similar consequences of cold treatment as salt stress, seedlings were subjected to a cold shock stress after 7 days of growth at room temperature on MS medium plate. Not surprisingly, the *gln1;1* mutant developed more extensive necrosis and root growth inhibition resulting from cold stress than wild-type plants and the other mutants (Figure 4.7B). These results indicate that the enhanced expression of *GS1* triggered by stresses plays an essential role in both salt and cold stresses.

The cellular and molecular responses of plants to abiotic stress (including low temperature, drought and salt) have been studied intensively (Xiong *et al*., 2002). ROS are considered as a common signal involved in all of these stresses. Based on our results, the salt-sensitive phenotype of *gln1;1* suggested a possibility of enhanced ROS levels and prompted us to further characterize the role of *GLN1;1* in the ROS scavenging systems. Because external

application of different ROS generators is able to trigger a rapid accumulation of $H_2O_2$ or $O_2^-$ in different cellular locations (Apel and Hirt, 2004, Schwarzländer *et al*., 2009), I therefore analyzed the effect of different ROS generators on the all the mutants. First, the response of *gln1;1* to methyl viologen (MV), an inducer of ROS that specifically produces $O_2^-$ in chloroplasts in a light-dependent fashion was tested. As Figure 4.8 shows, only the growth of the *gln1;1* mutant was significantly inhibited when compared to wild type in plates supplemented with 0.1 μM MV. This inhibition was also reflected in root length. All seedlings, including wild type and *gln1;1*, exhibited similar growth on MS control plates.

Since differences in root length between wild type and *gln1;1* and dramatic variation of leaf development by MV treatment was observed, there is a question of whether *GLN1;1* is specific for chloroplast protection. In order to answer this question, another two ROS inducers, menadione (MD) and salicylhydroxamic acid (SHAM) (Schwarzländer *et al*., 2009), both producing ROS in mitochondria were selected to apply to the mutants. I attempted a series of concentrations of these two compounds, but did not observe any of them showing altered inhibition of *gln1* mutants compared to wild type (Figure 4.8). These results indicate that the *gln1;1* mutation enhances sensitivity specifically to methyl viologen, and that GLN1;1 might be a key factor for protection of chloroplast under oxidative stress.

To confirm that GLN1;1 is only functional for defense against oxidative stress in chloroplast, another ROS generator, rose bengal (RB), also targeting ROS in chloroplasts was employed to treat all the lines for further investigation. Based on our prediction, I expected similar growth behaviour as MV treatment; however, none of the mutants showed any more serious inhibition than wild type in all of our plates supplemented with RB (Figure 4.8). It is worth noting that even though MV and RB both contribute to ROS production in chloroplast, the

exact locations of ROS generation by these two inducer are different (see Figure 4.9). MV was suggested to release the flavoprotein from the thylakoid membrane into the stroma, particularly for flavoenzyme ferredoxin-$NADP^+$ reductase (FNR), and results in $O_2^-$ over-accumulation (Palatnik *et al*., 1997). In contrast, application of RB leads to high $^1O_2$ formation in the stroma (Fischer *et al*., 2007). The different results caused by these two ROS producers may due to the different localizations of ROS production in the chloroplast.

The results above shows that the *gln1;1* mutant has a prominent phenotype from MV treatment. In order to confirm whether ROS are involved in growth inhibition of the *gln1;1* mutant and where they are localized, I further examined the $H_2O_2$ generation in chloroplasts. Leaves of wild type plant and the *gln1;1* mutant were treated with 10 μM MV by spraying and collected after 4 hrs of treatment. The non-fluorescence compound 2′, 7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) producing a highly fluorescent compound dichlorofluorescein (DCF) was used as a dye for intracellular $H_2O_2$ staining (Jakubowski and Bartosz, 2000). As shown in Figure 4.10, $H_2O_2$ was not detected by confocal microscopy in the control treatments for the wild type and *gln1;1* mutant. However, 4 hrs treatment with 10 μM MV, $H_2O_2$ signals were dramatically enhanced in the *gln1;1* mutant leaves compared with wild type, as shown by the bright green fluorescence resulting from staining with $H_2$DCFDA. To investigate the possible localization of $H_2O_2$, DCF signals were compared to chloroplast autofluorescence derived from chlorophyll excitation by using a second channel of laser scanning confocal microscopy. The fluorescence for $H_2O_2$ and chlorophyll showed a co-localization pattern, suggesting that the $H_2O_2$ generation in *gln1;1* mutants induced by MV took place in the chloroplast.

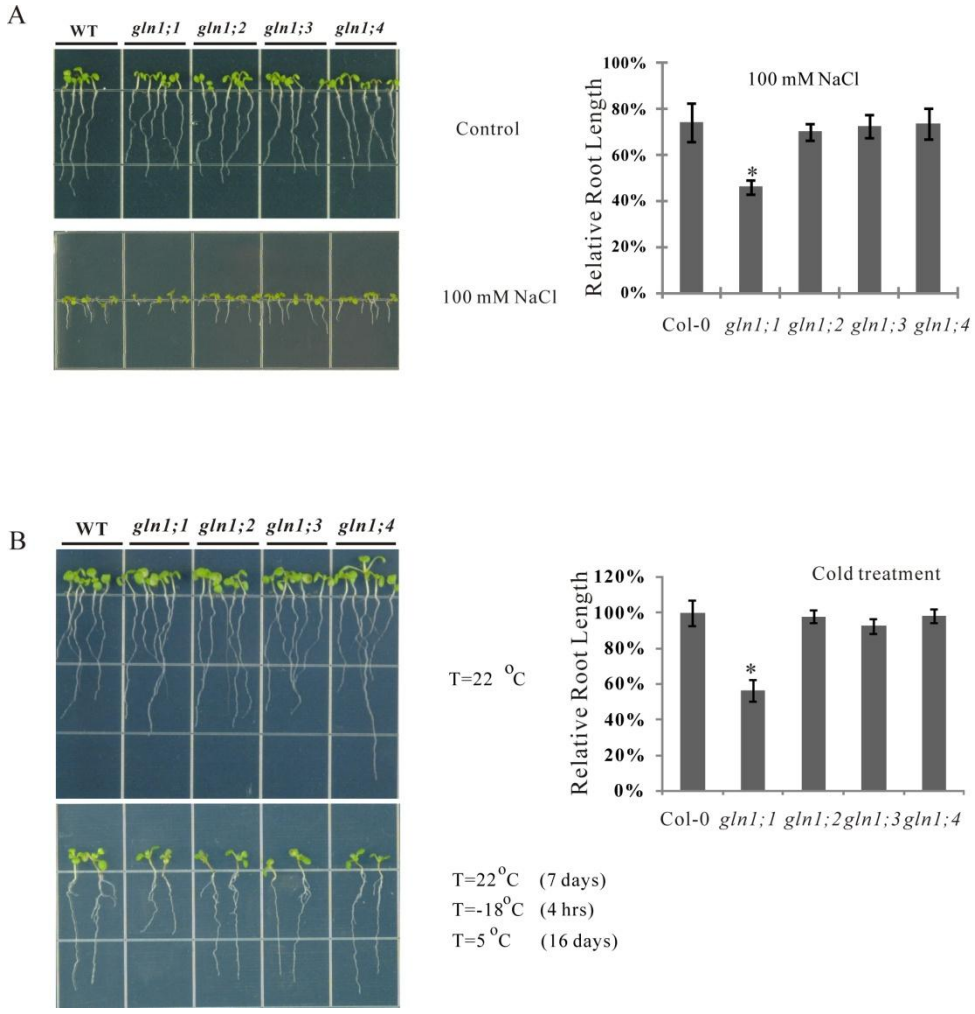


**Figure 4.7 Response of *gln1* mutants to abiotic stresses.**
(A). Left: 7-day-old seedlings of Col-0, *gln1;1, gln1;2, gln1;3* and *gln1;4* mutant lines grown in MS medium with (bottom) or without (top) 100 mM NaCl. Right: Effect of NaCl on root length of the Col-0 and *gln1* mutants grown on MS medium plates.
(B). Left: 9-day-old seedlings of Col-0, *gln1;1, gln1;2, gln1;3* and *gln1;4* mutant lines grown in MS medium at 22 ℃ (top). 7-day-old seedlings were treated by cold shock at -18 ℃ for 4 hrs and recovered at 5 ℃ for 16 d. Right: Effects of low temperature on root length of the Col-0 and *gln1* mutants grown on MS medium plates.
Error bars represent SE for three independent experiments and twenty roots were measured respectively. *, P value < 0.001 (T-test). Error bars represent SE for three independent experiments.

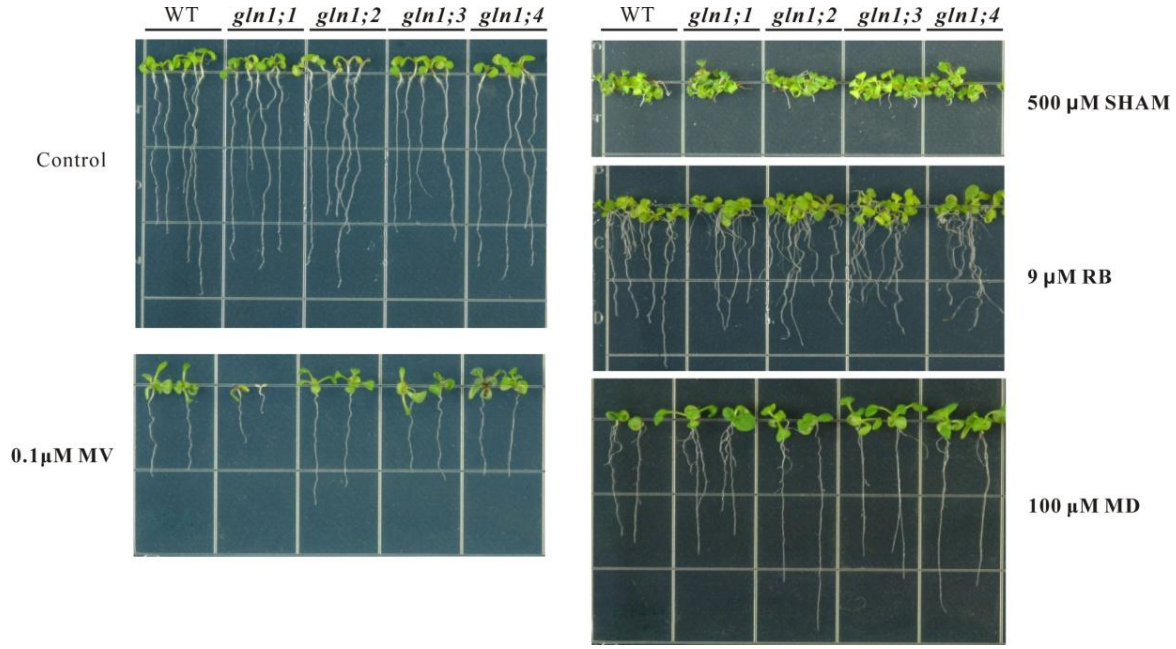


**Figure 4.8 Response of *gln1* mutants to different ROS generators.**
Seedlings of the wild-type, *gln1;1, gln1;2, gln1;3* and *gln1;4* mutant lines grown in MS medium with or without ROS generators (MV: methyl viologen; SHAM: salicylhydroxamic acid; RB: rose bengal; MD: menadione). Plates with treatment were photographed at 21 days after incubation. Control plates were photographed at 12 days after incubation.

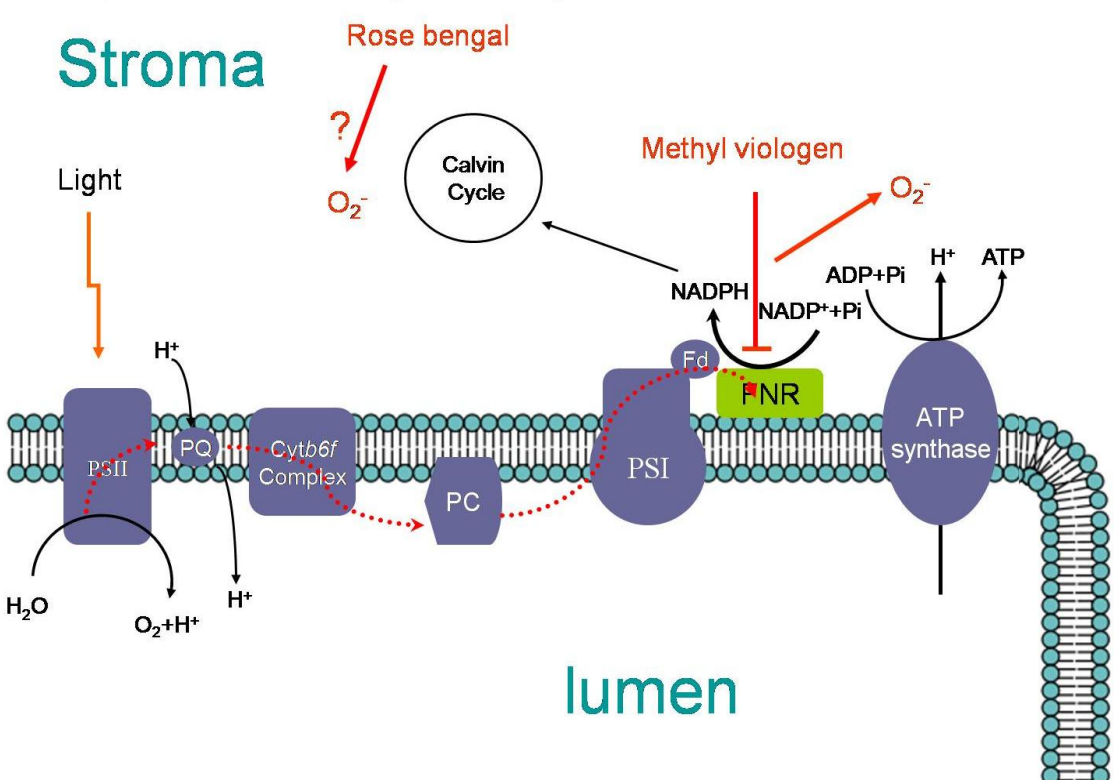


**Figure 4.9 Organization of the electron transport chain and ATP synthesis in the thylakoid membrane of plant chloroplast**

The components of the chloroplast electron transport chain and the ATP-synthesizing apparatus are shown. Electrons are transferred from $H_2O$ to $NADP^+$. Accompanying the electron transfer, a proton gradient is established across the membrane finally used for ATP synthesis. MV was suggested to release the FNR from the thylakoid membrane into the soluble fraction resulting in an induction of $O_2^-$ (Palatnik *et al*., 1997). RB leads to high $O_2^-$ formation in the stroma (Fischer *et al*., 2007). The diagram is based on Plant Physiology, Third edition by Lincoln Taiz and Eduardo Zeiger.

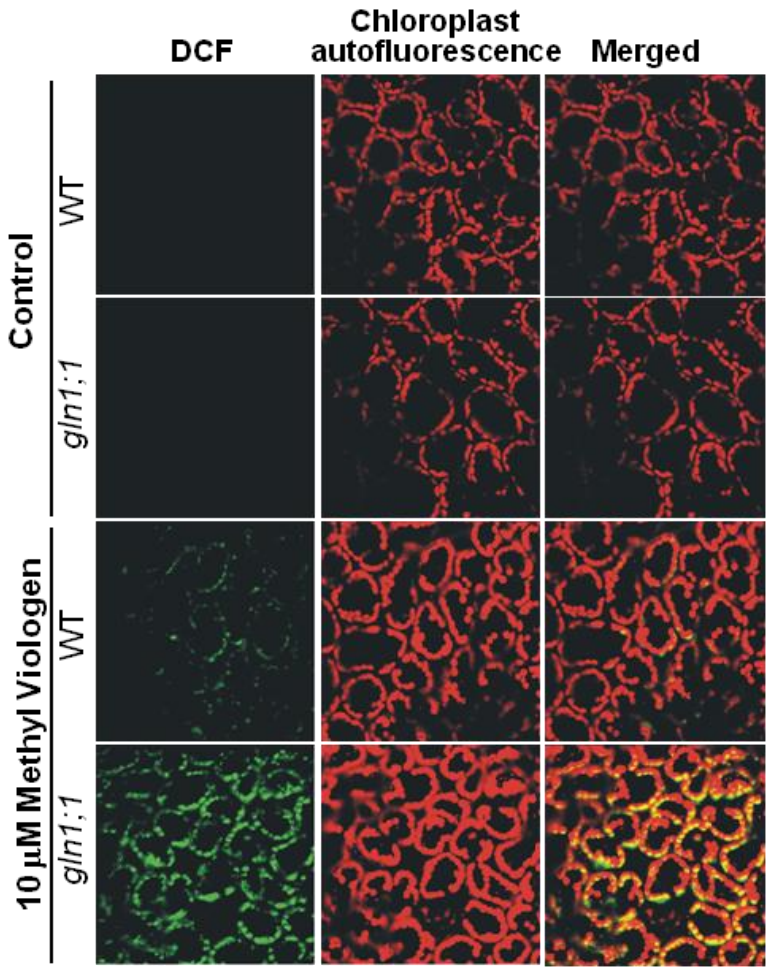


**Figure 4.10 ROS production in Col-0 and *gln1;1* mutant plants.**
Signals were detected by laser scanning confocal microscopy. Leaves from 3-week-old wild type and *gln1;1* mutant plants were treated with or without 10 µM MV and stained with $H_2$DCFDA (green). DCF signals were visualized with excitation/emission at 488/505-530 nm. Chloroplast autofluorescence (red) was visualized with excitation/emission: 488/>650 nm.

### 4.7 Isoforms GLN1;1, GLN1;2, and GLN1;3 Coordinate Primary Nitrogen Assimilation

Based on structural and biochemical studies, GS1 has been well-characterized as a key enzyme for ammonium assimilation. For example, shoots from GS-inhibited plants which were treated with MSX (L-methionine-S-sulfoximine) contained a higher $NH_4^+$ content than the control plants (Skopelitis *et al*., 2006). Similar induction of $NH_4^+$ concentration was observed in PPT treated tobacco plants (Purnell and Botella, 2007). In addition, biochemical assays also demonstrated that GS had high Gln synthetic activity by quantifying L-Gln synthesized from $NH_4^+$ and L-Glu (Ishiyama *et al*., 2004b). This evidence strongly suggests an essential role of GS in incorporating $NH_4^+$ into the Gln organic form of N.

In order to further characterize the function of cytosolic GS, I used the genetic material (*gln1* mutants) to illustrate the role of this enzyme in incorporating $NH_4^+$ into organic N. Since none of *gln1* mutants showed any difference in $NH_4^+$ assimilation under both normal condition (MS) and altered conditions with different forms of N (data not shown), the growth of double and triple mutants was assessed on media containing inorganic N ($NH_4NO_3$) or organic N (Gln). Because of functional redundancy of the five GS1 isoforms, the scarcity of mutants that are defective in several genes of *GS1* homologs somewhat impeded the characterization of GS1 functions. In order to comprehensively understand the principles underlying cooperative Gln synthesis via numerous isoforms, the double mutants *gln1;1-1*/*gln1;2-2*, *gln1;1-1*/*gln1;3-1*, *gln1;1-1*/*gln1;4-1*, *gln1;2-2*/*gln1;3-1*, *gln1;3-1*/*gln1;4-1* and triple mutant *gln1;1*/*gln1;2*/*gln1;3* were used in N assimilation assays. As I expected, all the single mutants (from *gln1;1* to *gln1;4*) exhibited similar growth as Col-0 wild type plants on MS medium with either $NH_4NO_3$ or Gln; while a reduced growth of seedlings was observed clearly in the double mutant *gln1;1*/*gln1;3* and more serious in the triple mutant

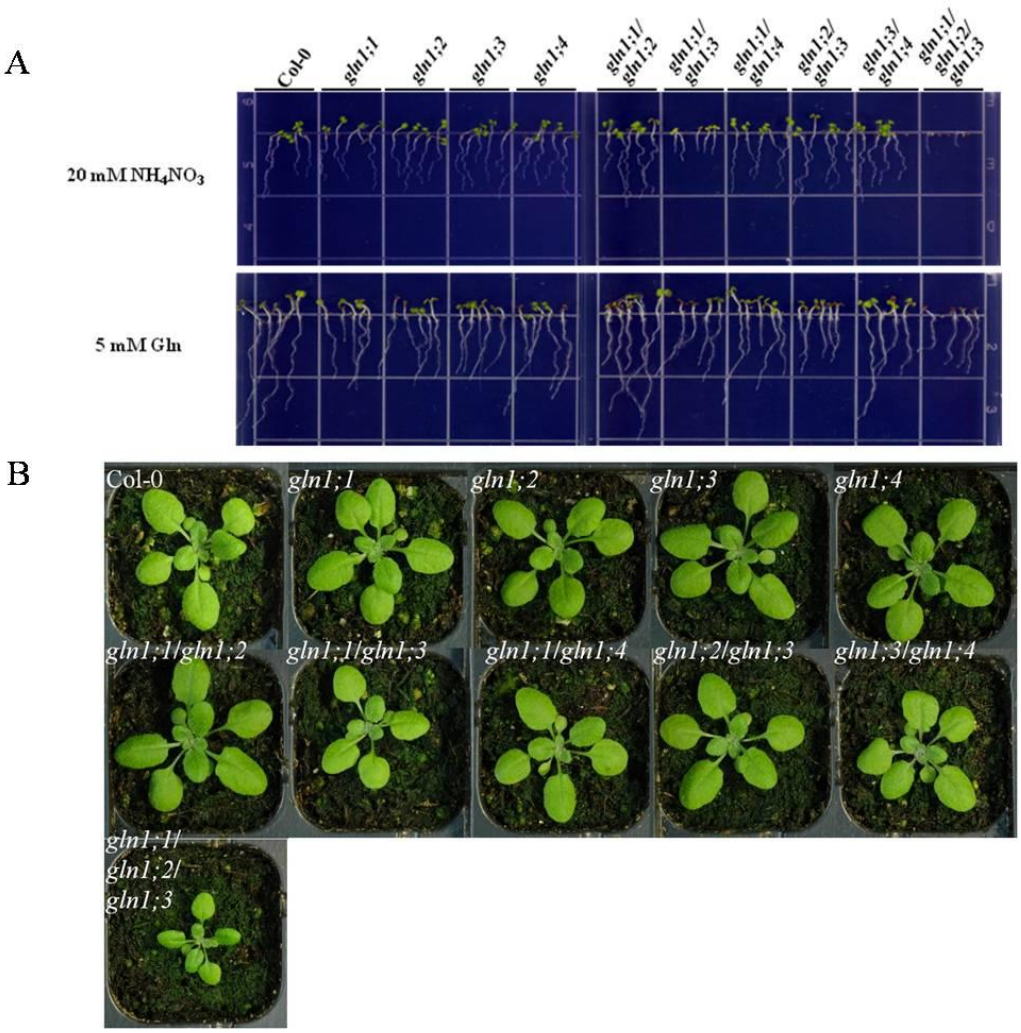


**Figure 4.11 GLN1 is involved in primary nitrogen assimilation.**
(A). *Arabidopsis* seeds were germinated and grown on MS medium (top lane) containing 20 mM $NH_4NO_3$ and MS medium supplemented with 5 mM Gln (bottom lane). The plates were incubated in a growth chamber with 11/13 hrs light/dark period at 22 ℃ and photographed at 5 days post incubation.
(B). Phenotype of 3-week-old *Arabidopsis* plant.

*gln1;1*/*gln1;2*/*gln1;3* compared to other lines in MS with inorganic N which contains 20 mM $NH_4NO_3$ (Figure 4.11A). In contrast, 5 mM Gln promoted the growth of *gln1;1*/*gln1;3* and *gln1;1*/*gln1;2*/*gln1;3*, partially restoring the phenotype of these two mutants (Figure 4.11A). These results strongly suggest that *Arabidopsis* cytosolic GSs are crucial for the conversion of N from inorganic ($NH_4^+$) to the organic (Gln) form in seedlings.

Cytosolic GS deficiency resulting in reduced growth and less grain were observed in wheat and maize *gln1* mutants (Martin *et al*., 2006; Tabuchi *et al*., 2005). Therefore, I proposed that cytosolic GS may also regulate the growth behaviour of *Arabidopsis* at later stages of development. In order to examine the effects of reduced *GLN1s* expression on plant growth, I observed all these mutants throughout the vegetative stage. Interestingly, *gln1;1*/*gln1;3* showed a little bit smaller size than Col-0 until 3 weeks old. The *gln1;1*/*gln1;2*/*gln1;3* mutant displayed reduced growth until the 4$^{th}$ week of growth (Figure 4.11B). These results further support the hypothesis that GS1 positively regulates plant growth, especially GLN1;1, GLN1;2 and GLN1;3.

### 4.8 GLN1;1, GLN1;3 and GLN1;4 Are Functionally Redundant for Pollen Development

Based on previous studies that *GS1* overexpressing tobacco plants exhibited enhanced growth phenotype (Oliveira *et al*., 2002) and a GS1 deficient mutant showed decreased biomass in maize (Martin *et al*., 2006) and male sterility in tobacco (Ribarits *et al*., 2007), I examined the phenotype of each *Arabidopsis* mutant line including single mutants, double mutants and triple mutants. Since reduced growth was observed in *gln1;1*/*gln1;3* and *gln1;1*/*gln1;2*/*gln1;3* mutants before flowering (Figure 4.11B), I continued to generate triple mutants by crossing *gln1;1-1*/*gln1;3-1* with *gln1;3-1*/*gln1;4-1* to obtain the *gln1;1*/*gln1;3*/*gln1;4* (knockout of 3 stress-induced isoforms) triple mutant. Interestingly, I was only able to only obtain *gln1;1*$^{+/-}$/*gln1;3*$^{+/-}$/*gln1;4*$^{-/+}$

heterozygous mutants, but not the *gln1;1*/*gln1;3*/*gln1;4* homozygous plants. According to Mendel's law, the segregation ratio of self-pollination by *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ heterozygous mutant was supposed to be 1 *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$ /*gln1;4*$^{+/+}$ : 2 *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$ /*gln1;4*$^{-/+}$ : 1 *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;*4$^{-/-}$. Theoretically, I had 25% possibility to isolation *gln1;1*/*gln1;3*/*gln1;*4 homozygous line. However, after several times of screening from the offspring of *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ , I was still unable to obtain the homozygous plants (numbers of plants screened each time was more than 100). Failure in obtaining a *gln1;1*/*gln1;3*/*gln1;4* homozygous line from these attempts suggests a possible situation that this triple mutant might be lethal before the embryo stage, because the F2 generation of *gln1;1*$^{-/-}$ /*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant plant showed 98~100% germination rate and normal growth (data not shown). In order to determine if the *gln1;1*/*gln1;3*/*gln1;4* homozygote is lethal at embryo stage, I examined the siliques from wild-type Col-0 and the *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant. As Figure 4.12A shows, small siliques and undeveloped ovules were observed in plants of the *gln1;1*$^{-/-}$ /*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant while wild type showed long and full siliques without any unfertilized ovules. This result strongly suggests that fertilization in flowers is disrupted in *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$ /*gln1;4*$^{-/+}$ mutant and results in unexpected segregation of genotypes.

Reduced fertility could be due to defects in either male or female gametes. Previous studies in tobacco have shown that inactivation of cytosolic GS causing aborted pollen results in male sterility (Ribarits *et al*., 2007). To test whether the male tissue is affected in the *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$ /*gln1;4*$^{-/+}$ mutant, I examined anthers in the opened flowers by Alexander's stain. As Figure 4.12B shows, compared to wild type, irregular size and shape of microspores in anthers from

*gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant were observed and most of the microspores were undeveloped, with some even collapsed. When I released the pollen grains out of the anther, normal pollen grains were stained with red color but the abnormal ones stained light blue (Figure 4.12B bottom). Moreover, fertilization requires guidance of the pollen tube to deliver the sperm cells to the ovules (Johnson and Preuss, 2002). Since significant aberrations of microspores in mature anthers were observed in the mutant, I further analysed the germination of pollen tubes in wild-type and mutant plants by using aniline blue staining. As Figure 4.12C shows, there were much fewer pollen tubes visualized in the *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant than in the wild type. Collectively, these results indicate that full fertility requires at least one of GLN1;1, GLN1;3 and GLN1;4 isoforms, otherwise the *gln1;1*/*gln1;3*/*gln1;4* null mutant will be lethal due to the impaired pollen development in *gln1;1*$^{-/-}$/*gln1;3*$^{-/-}$/*gln1;4*$^{-/+}$ mutant.

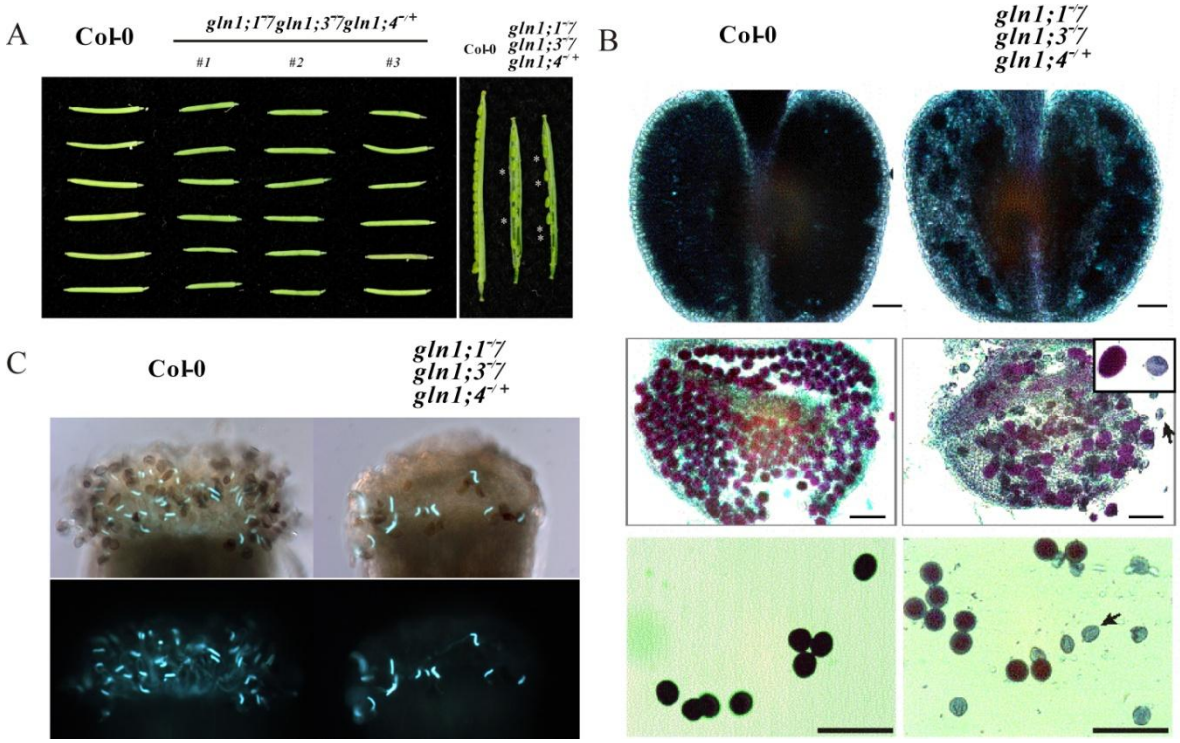


**Figure 4.12 Impaired pollen development in *gln1-1*$^{-/-}$*/gln1-3*$^{-/-}$*/gln1-4*$^{-/+}$ mutants.**
(A) Six siliques detached from the bottom of *Arabidopsis* stems of the Col-0 wild type and three randomly selected *gln1-1*$^{-/-}$*/gln1-3*$^{-/-}$*/gln1-4*$^{-/+}$ mutants were photographed (Left). Siliques from Col-0 and *gln1-1*$^{-/-}$*/gln1-3*$^{-/-}$*/gln1-4*$^{-/+}$ mutant plants. Small, presumably unfertilized, ovules were visible in the siliques of *gln1-1*$^{-/-}$*/gln1-3*$^{-/-}$*/gln1-4*$^{-/+}$ mutant (Right). Asterisks designate unfertilized ovules in the silique.
(B) Closed and opened anthers and pollen grains released from open anthers were stained with Alexander's stain and photographed under a microscope. Normal grains were stained red while abnormal ones were stained bright blue.
(C). Germinating tubes of pollens on stigma surface of open flowers of Col-0 and *gln1-1*$^{-/-}$*/gln1-3*$^{-/-}$*/gln1-4*$^{-/+}$ stained by aniline blue were visualized under a light microscope (top) and florescence microscopy (bottom). Bars=50 μM.

# 5. Discussion

## 5.1 GLN1;1 as a Major Cytosolic GS1 Isoform Contributes to Plant Tolerance against Abiotic Stresses

An important aspect of current research in plant biology is using reverse genetic techniques to identify key regulators related to abiotic stress tolerance. Although a large number of genes respond to various abiotic stresses, only a fraction of them have been characterized to be involved in stress tolerance while the physiological role for others is still speculative. Although transcriptional regulation somehow varies between different stress stimuli, it is generally thought that the response to cold, drought, and salt stress share a generic signalling pathway including the following components: 1) Induction of $Ca^{2+}$ influx which may reflect perception of the stress signal, 2) Activation of phosphoprotein cascades, completed by protein kinases such as calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK), 3) Transcription factors which aim to trigger or repress downstream genes to activate adaptive responses (Sreenivasulu *et al*., 2007; Xiong *et al*., 2002). So far, many genes encoding CDPKs, MAPKs, and transcription factors have been identified such as *OsCDPK7*, *OsMAPK5*, and *AtCBFs* (CRT-binding factors) (Martin and Busconi, 2001; Saijo *et al*., 2000; Xiong and Yang, 2003). In the case of cytosolic GS, accumulating studies report an induction of GLN1 both at the RNA and protein levels under various stress conditions (Debouba *et al*., 2006; Kwon *et al*., 2007). It has also been observed that GS1 in leaves of *Brassica napus* can be post-translationally regulated by reversible phosphorylation and that both protein kinase and protein phosphatase are able to catalyze the reactions (Finnemann and Schjoerring, 2000). This type of post-translational modification was also observed for *Arabidopsis* GLN1;1 by CDPK-related protein kinase (Li *et al*., 2006). The phosphorylation status of GS1 is closely associated with its activity and stability,

while active GS1 always presents as the phosphorylated form (Finnemann and Schjoerring, 2000). All these results suggest that GS1 may be involved in the response to abiotic stress.

In my current work, I have employed molecular and genetic approaches to demonstrate the physiological roles of cytosolic GS in abiotic stress and its involvement in ROS signalling in *Arabidopsis*. With the co-operation of GOGAT, GS plays an important role in N assimilation in plants. The product of GS, Gln, contributes to the biosynthesis of various organic N compounds such as amino acids, nucleotides and chlorophyll. Under stress conditions, intracellular hyperammonium would be produced and be toxic to the plant (Skopelitis *et al*., 2006). In order to efficiently remove toxic ammonium and in defense to an extreme environment, plants require a rearrangement of critical enzymes involved in N assimilation. The results from current work show that *GLN1;1*, *GLN1;3* and *GLN1;4* of *Arabidopsis* are induced by both of salt and cold treatments, which confirm the previous studies demonstrating that GS1 was responsive to salt treatment and cold stress (Debouba *et al*., 2006; Kwon *et al*., 2007),.

Although the GLN1;1, GLN1;3 and GLN1;4 are likely to be involved in assimilation of hyperammonium under stressful conditions, there is no genetic evidence in the current literature to demonstrate the significance of each gene associated with stress responses. The project results show that *GLN1;1* is broadly expressed in different tissues, while western blot hybridization showed a relative high expression in vascular-rich tissues (Figure 4.1B). The physiological role of vascular-localized cytosolic GS was proposed to be related to N storage under stress conditions in a previous study (Brugiere *et al*., 1999). Quantitative investigation of gene expression of four *GS1* genes in *Arabidopsis* roots revealed that *GLN1;1* mRNA accumulated abundantly compared to other genes in roots after a 3-day deprivation of N (Ishiyama *et al*., 2004b). According to our results, unlike other mutants, *gln1;1* mutant showed a serious growth

inhibition upon PPT treatment, as well as under abiotic stresses. This observation not only confirms the functional importance of cytosolic GS in stress conditions, but emphasizes a major role of *GLN1;1* among the *Arabidopsis* GS1 members in tolerance of abiotic stresses.

It is important to note that there is a currently controversial conclusion made in rice. While overexpressing plastidic GS in transgenic rice conferred tolerance to salt stress which is consistent to our results (Hoshida *et al*., 2000), *GS1;2*-overexpressing lines of rice, produced by an *Agrobacterium tumefaciens*-mediated transformation, exhibited higher sensitivity to salt and cold stress conditions (Cai *et al*., 2009). These contrasting results might be explained by two factors. First, rice is highly adapted to ammonium-based N nutrition. The uptake and transport of $NH_4^+$ in rice primarily relies on GS/GOGAT cycle and OsAMTs. Previous studies found that OsAMT1;2, which likely functions in $NH_4^+$ uptake from $NH_4^+$-rich soils, was up-regulated by Gln rather than $NH_4^+$ (Sonoda *et al*., 2003). The induction of *OsGS1;2* expression therefore produced more Gln and triggered an accumulation of *OsAMT1;2*, leading to hyperammonium in plant cells. Second, the distinct expression pattern and tissue specificity of *OsGS1;1*, *OsGS1;2* and *OsGS1;3* indicates an different functional roles of the three genes in rice (Ishiyama *et al*., 2004b; Tabuchi *et al*., 2005). The question as to why *OsGS1;2* is important in sensitivity to abiotic stress in rice still needs to be elucidated.

### 5.2 The Roles of Cytosolic GS in Plant Growth and Development

The effects of cytosolic GS on plant growth and yield have been fully investigated by analyzing the phenotypes of GS1 deficiency mutants in rice and maize plants (Martin *et al*., 2006; Tabuchi *et al*., 2005). Knocking out one key gene in both of these two species results in obvious growth retardation or decreased biomass. In contrast, *Arabidopsis* mutants lacking any one GS1 gene

failed to show any significant growth inhibition under normal conditions and N-deficient environment suggesting functional redundancy of these four detectable *GS1* genes. Many key enzymes like GS1 are encoded by duplicated genes, which are thought to perform redundant roles in many aspects, preventing lethal auxotrophy caused by single gene mutation (Briggs *et al*., 2006). Accordingly, I generated double and triple mutants by genetic crossing. I observed growth retardation in the *gln1;1*/*gln1;3* and *gln1;1*/*gln1;2*/*gln1;3* mutants, and pollen lethality in *gln1;1*/*gln1;3*/*gln1;4*, shedding light on the individual role of each gene.

*GLN1;1*, encoding a high affinity cytosolic GS, plays a major role in N assimilation among the *Arabidopsis* GS1 members when plants are exposed to abiotic stress and the herbicide PPT. The predominant function of *GLN1;1* is likely interrelated with its unique enzymatic properties or localization. Biochemical studies on the kinetic properties of each *Arabidopsis* GS1 isoenzyme revealed that GLN1;1 possesses the highest affinity for ammonium among the four GS1 isoforms, suggesting a high efficiency of toxic ammonium attenuation (Ishiyama *et al*., 2004b). With the regard of its localization, transgenic *Arabidopsis* plants (*GLN1;1* promoter-GFP) showed abundant accumulation of GFP at the root surface where the initial regulatory steps of ammonium assimilation take place (Ishiyama *et al*., 2004b). Moreover, although our results showed a broad expression pattern for *GLN1;1* in the tissues I examined (including root, stem, leaf, and flower), the relative protein expression of GLN1;1 varied a lot among different tissues, with vascular-rich tissues such as root and stem possessing higher levels of expression (Figure 4.1). These results suggest that GLN1;1 might play a pivotal role in primary assimilation in roots and N transport from source to sink tissue. Besides an essential role in vascular-rich organs, GLN1;1 may also function in sink tissue. I have shown that, unlike other mutants, only *gln1;1* displayed enhanced sensitivity specifically to methyl viologen, indicating that GLN1;1 might be

involved in reassimilation of ammonium produced by the photorespiratory pathway.

Although *GLN1;3*, encoding a low affinity isoform, did not show any significant role in tolerance to abiotic stress in single mutants analysis, even though its expression is responsive to such stresses, a synergic effect between *GLN1;1* and *GLN1;3*, indicated by the obvious growth retardation of the *gln1;1*/*gln1;3* seedlings on MS medium, suggests an important role for *GLN1;3* as well as *GLN1;1* (Figure 4.11A ). Unlike GLN1;1, which is expressed predominantly in vascular-rich tissues, GLN1;3 mainly accumulated in sink tissues such as leaves and flowers. In addition, recent studies by systems approaches showed that *GLN1;3* is a light-responsive gene and regulated by a master circadian clock control gene *CCA1* (Gutierrez *et al*., 2008). This result implies a sink-tissue specific role of GLN1;3. Moreover, the synergic phenotype of *gln1;1*/*gln1;3* seedlings also suggests a partial function of GLN1;3 in seeds; because the growth inhibition of *gln1;1*/*gln1;3* was first observed in germinating seeds at a stage when there are no functional chloroplast and leaf tissue. Therefore, I propose that GLN1;1 and GLN1;3 are the two major cytosolic GS present in *Arabidopsis*.

GLN1;4, another isoform with high affinity for ammonium, appears not function in seedlings, because no *GLN1;4* transcripts could be detected at the seedling stage and the *gln1;1*/*gln1;4* double mutant grows as well as the wild-type plant (data not shown). Even in the mature *Arabidopsis* plant, the mRNA of *GLN1;4* is less abundant than other isoforms (Ishiyama *et al*., 2004b). In fact, the pollen lethality of the *gln1;1*/*gln1;3*/*gln1;4* triple mutant and the reversion of lethality by applying any one of these 3 genes suggests that these 3 isoforms are equally significant in the mature plant, particularly at the reproductive stage. However, GLN1;4 is not completely redundant to GLN1;1 and GLN1;3 due to its tissue specificity shown in our western blot results (Figure 4.1B). GLN1;4 was identified as the only vascular-specific isoform

with an abundant expression and restricted localization in the root and stem, which is more similar to GLN1;1. However, GLN1;4 was not detected in leaves and flowers, on the one hand, implying its limited role in reproductive tissues, on the other hand, indicating that GLN1;4 might be responsible for signal transferring, because in the *gln1;1*/*gln1;2*/*gln1;3* mutant, the presence of GLN1;4 in stem can restore the lethality even though there is no GS1 protein in leaves and flowers.

GLN1;2 is classified as the lowest affinity isoform for ammonium among GS1 protein with the $K_m$ values for GLN1;2 being almost twice as low as that of the other low affinity isoform GLN1;3 (Ishiyama *et al*., 2004b). As the major form of cytosolic GS in *Arabidopsis* at seedling stage (Figure 4.5B), GLN1;2 appears to contribute less to N assimilation than other isoforms. Although the *gln1;2* mutant did not show any difference in phenotype to abiotic stress and oxidative stress compared to the wild type, transcripts of *GLN1;2* were induced during leaf senescence, implying a potential role of *GLN1;2* in senescence (Diaz *et al*., 2008).

In addition, the function of GS for inorganic N assimilation in plants has been established mainly based on biochemical studies in vitro. GS1 isoforms have been shown previously to possess high affinity to ammonium in *Arabidopsis* by measuring enzyme activities (Ishiyama *et al*., 2004b). In our work, the *gln1;1*/*gln1;2*/*gln1;3* mutant showed a growth retardation in MS-N media with $NH_4NO_3$ which implies that the cytosolic GS-deficiency mutants was not able to utilize inorganic N to synthesize amino acids. However, this kind of growth inhibition could be reversed by application of Gln to the MS-N medium indicating an essential role of GS1 in N conversion from the inorganic to orgranic form. To the best of our knowledge, this is the first genetic evidence showing that GS1 facilitates this conversion from inorganic N to organic N.

### 5.3 Involvement of Gln Metabolism in Regulation of Cellular Redox

Low temperature and high salinity are two different stresses stimulating distinct responses in plant cells. For example, low temperature is known to modify membrane fluidity (Murata and Los, 1997), while salt is known to change ionic and osmotic components (Cheeseman, 1988; Munns and Tester, 2008; Tuteja, 2007). However, the generation of ROS including $O_2^-$, $H_2O_2$, and $HO^-$, is a general signal involved in most stresses due to the reduced rate of photosynthesis (Apel and Hirt, 2004). Since *GLN1;1*, *GLN1;3*, and *GLN1;4* showed similar responses to salt and cold stress and enhanced sensitivity was observed in NaCl-treated *gln1;1* mutants, we hypothesize that the product of *GLN1;1*, Gln might play an important role in ROS scavenging.

Methyl viologen and rose bengal are two ROS elicitors both triggering ROS formation in the chloroplast but in different locations (Fischer *et al*., 2007; Palatnik *et al*., 1997). Menadione and salicylhydroxamic acid are also ROS propagators but targeting mitochondria (Shen *et al*., 2006; Sweetlove *et al*., 2001). Our plate assays did not show any significant inhibition of the *gln1;1* mutant by elicitor challenge other than by methyl viologen. This finding uncovered the involvement of Gln in attenuating ROS levels possible resulting from solubilization of FNR in chloroplasts and confocal images further confirmed that Gln is specifically functional in the chloroplast.

What is the mechanism underlying such an efficient ROS scavenging pathway? It is well known that plants possess two systems for ROS detoxification in the chloroplast. One is an enzymatic system, with superoxide dismutase (SOD), APX, and CAT being the major enzymes involved in removing ROS. Most of these enzymes are located in many cellular compartments (Myouga *et al*. 2008). The responsibility of these enzymes is to efficiently catalyze the reactions for converting the toxic forms of ROS ($O_2^-$ or $H_2O_2$) to low-toxic status. The other ROS

detoxification system is non-enzymatic, employing carotenes, tocopherol, ascorbic acid and glutathione as antioxidants in most cellular compartments for defense against oxidative stress (Mittler, 2002). Gln, one of the major amino acids present in the vascular tissue of plants, has only been reported to influence the redox potential of the cell via enhancing the generation of glutathione in animals but not in plants (Curi *et al*., 2007; Roth, 2007; Roth, 2008). Previous studies in plants revealed that down-regulations were observed in both the protein and activity of GS2 under different stress conditions, whereas GS1 protein was induced (Pageau *et al*., 2006; Scarpeci *et al*., 2008; Simonovic and Anderson, 2007). The opposing behavior of GS1 and GS2 might be due to the fact that plants require Gln to reduce ROS production resulting from the inhibition of photosynthesis in chloroplasts. Since Gln is consumed in chloroplasts under stress conditions, unknown signals may somehow activate *GS1* genes to compensate for Gln deficiency. One possible purpose of Gln consumption is Pro accumulation; proline accumulation is a common physiological response when plants are exposed to a wide range of environmental stresses, such as salt, drought, high/low temperature, UV irradiation, and pathogen infection (Hare and Cress, 1997; Saradhi *et al*., 1995; Verbruggen and Hermans, 2008). Pro is believed to be an important osmolyte that functions as a molecular chaperone stabilizing the structure of proteins, and to regulate the cellular redox potential by controlling free radical levels (Hare *et al*., 1999). The biosynthesis of Pro primarily relies on the Glu pathway in plant cells (Ashton and Desh Pal, 1993). Gln is the main source of Glu production through the GS/GOGAT cycle. A previous study in tobacco has demonstrated that, instead of Glu, Gln is the limiting factor for Pro production because $^{15}NH_4^+$-labeling experiments showed that the accumulation rate of $^{15}$N-Pro was similar to the rate of $^{15}$N-Gln production while both of them were much lower in GS1-deficient transgenic plants than in control plants. By contrast, the use of $^{15}$N-Glu in GS1-

deficient transgenic plants was similar to that of the control plant (Brugiere *et al*., 1999). In addition, a recent study indicated the transcript of *P5CS1*, encoding a enzyme catalyzing the rate-limiting step from Glu to Pro, was strikingly enhanced in chloroplasts under high salinity condition (Szekely *et al*., 2008). Based on these lines of evidence, glutamine might be involved in ROS scavenging through the Pro pathway.

Moreover, FNR mediates the final step of photosynthetic electron transfer between ferredoxin (Fd) and $NADP^+$ and produces NADPH (Lintala *et al*., 2007). Balancing the ATP/NADPH ratio in order to prevent excess accumulation of electrons is important for ROS control, because excess electrons can be transferred to $O_2$ thus generating ROS (Foyer and Noctor, 2005; Mittler, 2002). ROS stimulation by methyl viologen is due to the release of FNR from the thylakoid to the stroma thus resulting in the block of electron transport (Palatnik *et al*., 1997). Indeed, malfunction of FNR and Fd both result in the enhanced accumulation of ROS in plants (Hajirezaei *et al*., 2002; Palatnik *et al*., 2003; Voss *et al*., 2008). In this regard, *Arabidopsis fnr1* and *fnr2* mutants with impaired function of FNR showed a transcriptional upregulation of *NR* and *GLN1;1*, as well as an enhancement of N metabolism, suggesting that Gln might be involved in alternative electron transfer pathways in the chloroplast (Lintala *et al*., 2009; Lintala *et al*., 2007). Moreover, in vitro experiments demonstrated that the efficient oxidation of NADPH relies on Gln and 2-oxoglutarate (Valadier *et al*., 2008). In this study, I showed that the *gln1;1* mutant was only sensitive to methyl viologen and displayed high levels of ROS formation in chloroplast, further implying a significant role of Gln in the chloroplastic redox system.

The function of GLN1;1 suggested to take place in chloroplast, is interesting to our understanding of ROS scavenging systems. In addition to chloroplasts, mitochondria are also

major compartments for ROS production. Like GS2, Fd-GOGAT was reported to localize to mitochondria and the *glu1* mutant, which is defective in Fd-GOGAT, exhibited photorespiratory chlorosis at ambient $CO_2$ (Jamai *et al*., 2009). Thus, the chloroplast and mitochondrion both necessitate the presence of the GS2/Fd-GOGAT cycle for reassimilation of photorespiratory ammonium as well as for primary N assimilation. In this case, it is possible that *GLN1;1* may be required for ROS detoxification not only in the chloroplasts but also in mitochondria. However, it is interesting that our observation is not in such prediction indicating the role of GS2/Fd-GOGAT cycle may be distinct in these two compartments.

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