SEDE AMMINISTRATIVA: UNIVERSITÀ DEGLI STUDI DI PADOVA
DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI RISORSE NATURALI E AMBIENTE

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE ANIMALI E AGROALIMENTARI
INDIRIZZO: PRODUZIONI AGROALIMENTARI
CICLO XXVII

γ-glutamyl cycle in plant's adaptation to environment

Direttore della Scuola: Ch.ma Prof.ssa Viviana Corich
Coordinatore di Indirizzo: Ch.ma Prof.ssa Viviana Corich
Supervisore: Ch.mo Prof. Antonio Masi

Dottoranda: Anna Rita Trentin

CONTENTS

LIST OF ABBREVIATIONS

2-DE, two-dimension polyacrylamide gel electrophoresis;

ANOVA Analysis of variance,

ASC ascorbate,

CAT catalase,

DEPMPO5-(diethoxyphosphoryl)-5-methyl-1-pyroline N-oxide,

DETAPAC or DTPA Diethylenetriaminepentaacetic acid,

DHA Dehydroascorbate,

DHAR dehydroascorbate reductase,

DNA Deoxyribonucleic acid,

DNPH, 2,4-dinitrophenylhydrazine;

DP dipeptidase,

DTT dithiothreitol,

ECWF, extracellular washing fluid;

EPR Electron Paramagnetic Resonance,

ES, enrichment score;

FDR False discovery rates,

FW, fresh weight;

GGCT γ-glutamylcyclo-transferase,

GGT, gamma-glutamyl transferase/transpeptidase;

GGT1 gamma-glutamyl transferase 1 isoform,

GHs Glycosyl hydrolases,

GLM General linear models,

GO, gene ontology;

GPX glutathione peroxidase,

GR glutathione reductase,

GRX, glutaredoxins;

GSH, reduced glutathione;

GSNO S-Nitrosoglutathione,

GSSG, oxidized glutathione;

GSTs Glutathione S-Transferases,

GUS β-glucuronidase fusion proteins,

HL High Light,

HPLC High pressure liquid chromatography,

HR hypersensitive response,

ICP-OES Inductively Coupled Plasma Optical Emission Spectroscopy,

IEF, isoelectric focusing;

iTRAQ Isobaric tags for relative and absolute quantification,

LC-MS-MS Liquid Chromatography Mass Spectrometry,

LMW low molecular weight;

MBDs membrane bound dipeptidases,

mRNA messenger Ribonucleic acid,

NAC, N-acetylcysteine;

NADH β-Nicotinamide adenine dinucleotide, reduced form,

NADPH Nicotinamide adenine dinucleotide phosphate reduced salt,

NO nitric oxide,

PCD programmed cell death,

PM, plasma membrane;

PNPs Plant natriuretic peptides,

POD peroxidase

PRPs Pathogenesis-related proteins,

PRX, peroxyredoxins;

PUFAs polyunsaturated fatty acid,

ROS, reactive oxygen species;

RT, room temperature;

SAG, senescence-associated gene;

SBD-F, ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate;

SCX, strong cation exchange;

SDS Sodium dodecyl sulfate,

SOD Superoxide dismutase,

TEAB Triethyl ammonium bicarbonate,

TEM, transmission electron microscopy;

TRX, thioredoxins;

UV-B Ultraviolet-B radiation,

UVR8 UV-B photoreceptor 8,

WT, wild-type.

ABSTRACT

ABSTRACT

This thesis work focusses on the gamma-glutamyl cycle in plants, with the aim to address the physiological significance of this cycle in plant adaptation to the environment. It is composed of three sections, where different approaches have been developed to understand different aspects of the cycle. In consideration that alternative and converging strategies may provide tools for deciphering plant metabolism,two main approaches were adopted: the application of stress conditions, and the use of mutants.

In the first work, integrated biochemical, immunocytochemical, and quantitative proteomics analyses were performed in leaves of *Arabidopsis thalianaggt1* knockout mutant (lacking apoplastic GGT1 isoform) and itscorresponding wild-type (WT). The *ggt1* knockout leaves exhibited an increased ascorbate and GSH content, increased apoplastic GSH content, and enhanced protein carbonylations in the low-molecular-weight range compared to WT. Proteome data showed that disruption of gamma-glutamyl cycle in *ggt*1 knockout-leaves was associated with the induction of genes encoding four GSTs, a GSH peroxidase (GPX1), and glyoxylase II, suggesting that GGT1 plays a role in redox signaling. The disruption of the gamma-glutamyl cycle in the *ggt*1 mutant results in pleiotropic effects related to biotic and abiotic stress response, antioxidant metabolism, senescence, carbohydrate metabolism and photosynthesis, with strong implications for plant's adaptation to environment.

The objective of the second contribution wastounderstand how the *ggt1* mutant line responds when it is exposed to an external oxidative stress by UV-B radiation. The response of *ggt1* knockout *Arabidopsis* leaves to UV-B radiation was assessed by investigating changes in extracellular glutathione and ascorbate content and their redox state, and in apoplastic protein composition.Results show that, upon UV-B exposure, soluble antioxidants are altered in both genotypes. Rearrangements occur in their apoplastic protein composition, both in the wildtype under UV-B and in the *ggt*1 mutant in physiological conditions. This suggeststhe involvement of H_2O_2, which may ultimately act as a signal. I argue that oxidative stress conditions imposed by UV-B and disruption of the gamma-glutamyl cycle result in similar stress-induced responses, to some degree at least.

Since the gamma-glutamyl transferase operates in the extracellular space, aim of the third contribution was to better investigate the reactions involvingLMW thiols (glutathione,cysteine and cysteinyl-glycine), metals and enzymes related to ROS metabolism in the cell wall. Resultsindicate that LMW thiolsexhibit quenching capacity for reactive oxygen species generated in the apoplastic

spaceandpoint to a role for LMW thiols, which are metabolically related to each other in the gamma-glutamyl cycle, in modulating redox reactions in plant cell walls.

RIASSUNTO

L'argomento della mia tesi di dottorato è stato il ciclo del gamma-glutammile nelle piante con lo scopo generale di investigare il significato fisiologico di questo ciclo nell'adattamento delle piante all'ambiente. Il lavoro è composto da tre contributi sperimentali, nei qualidifferentiapprocci sono stati adottati per capire diversi aspetti del ciclo. Quando si vogliono approfondire le conoscenze per decifrare il metabolismo si possono usare strategiealternative e convergenti, due sono i principali approcci che sono stati adottati: sono stati imposti degli stress ossidativi esterni per valutare la risposta della pianta e si è fatto uso di mutanti.

Nel primo lavoro, sono state eseguite analisi biochimiche, immunocitochimiche e proteomiche in foglie di *Arabidopsis thaliana* del mutante *ggt1* (mancante dell'isoforma apoplastica di GGT1) e nel corrispondente wild-type (WT). Comparando lefoglie wild-tipe con il mutante *ggt1*,quest'ultimo presentava un incremento del contenuto di ascorbato e glutatione, anche il GSH apoplastico risultava aumentato e un cambiamento nelle carbonilazioni delle proteinea basso peso molecolare. I dati proteomici evidenziavano che l'interruzione del ciclo del gamma-glutammile nelle foglie del mutante *ggt*1 era associato con l'induzione di geni codificanti per quattro glutatione-sulfo-transferasi (GSTs), una glutatione perossidasi (GPX1), e la gliossilasi II, suggerendo che la proteina GGT1 ha un ruolo nel redox signaling. Quindi l'interruzione del ciclo del gamma glutammile nel mutante *ggt*1 porta ad effetti pleiotropici legati alla risposta a stress biotici e abiotici, altera il metabolismo degli antiossidanti, la senescenza, il metabolismo dei carboidrati e la fotosintesi, con forti implicazioni nell'adattamento delle piante all'ambiente.

L'obiettivo del secondo contributo era capire come il mutante *ggt*1 risponde quando è esposto ad un stress ossidativo esterno, è stato scelto di usare la radiazione UV-B. La risposta del mutante *ggt1*alla radiazione UV-B è stata valutata investigando i cambiamenti nello spazio apoplastico della composizione proteica e del contenuto di glutatione e ascorbato e il loro stato redox. I risultati evidenziano che, l'esposizione all'UV-B, altera gli antiossidanti solubili in entrambi i genotipi. I riarrangiamentiche avvengono nella composizione proteica dell'apoplasto, nel wild-type sottoposto a UV-B e nel mutante *ggt*1 in condizioni fisiologiche, suggeriscono un coinvolgimento del perossido di idrogeno (H_2O_2), il quale potrebbe agire come segnale. Questo mi porta a dedurre che le condizioni di stress ossidativo (imposte con l'UV-B) a l'interruzione del ciclo del gamma glutammile,in una certa misura, portano a una simile risposta indotta da stress.Poichè la gamma-glutammil transferase

agisce nello spazio extracellulare, scopo del terzo contributo è stato investigare le reazioni che avvengono tra i tioli a basso peso molecolare (glutathione, cisteina e cisteinil-glicina), i metalli e gli enzimi legati al metabolismo dei ROS nella parete cellulare. I risultati indicano che i tioli LMW sono in grado di quenchare le specie attive dell'ossigeno generate nell' apoplasto e evidenziano un ruolo per i tioli, i quali sono metabolicamente correlati tra loro nel ciclo del gamma-glutammile, nel modulare le reazioni redox nella parete cellulare.

INTRODUCTION

GENERAL INTRODUCTION

Climate changes are a selective force able to produce a pressure on natural populations. Effects of global changes are multiple: e.g. shift in geographical distribution, increase of parasites and/or competitor species, alteration in life-cycle (growth, reproduction and senescence), loss of habitat (change of sea-level rise, increased fire frequency, altered weather patterns, glacial recession), extinction or extirpation of species (Mawdsley *et al.*, 2009). In recent years, climate changes are rapid and unpredictable and they are likely to override plants' capacity to adapt. Due to their sessile life-style, plants must endure a range of biotic and abiotic stress conditions and they develop a modified tolerance to survive. Subsequently, these unfavourable situations can cause a restricted plant growth and development. Most notably, if we consider crop plants, these changes were reflected in reduction of productivity, that cause worldwide economic costs (Nakabayashi and Saito 2015;Suzuki *et al.*, 2014). To avoid damage from abiotic and biotic factors, plants adapt to the changes in their environment by activating evolved self-defense mechanisms. Adverse conditions increase the formation of reactive oxygen species (ROS), and consequently, plants develop enzymatic and non-enzymatic antioxidant molecules (Pitzschke *et al.*, 2006). Understanding plant responses to environmental changes is a need which demands modern and novel strategies. This research could represent a further step to help and improve plant's adaptation to the environment, with important consequences on crop productivity and crop-derived food quality and nutritional value.

1. REACTIVE OXYGEN SPECIES

The formation of reactive oxygen species is a common consequence of an oxygen-containing atmosphere; therefore, every organism, to survive, has developed some mechanisms to limit the damages of ROS. This class of molecules includes hydrogen peroxide (H_2O_2), superoxide anion radicals ($\bullet O_2^-$), hydroperoxyl radical ($\bullet HO_2$), hydroxyl radical ($\bullet OH$), singlet oxygen ($\bullet O_2$) and other highly oxidizing molecules. These molecules are generated from O_2 by energy transfer or electron transfer reactions; the first step requires an energy input, but afterwards, it occurs spontaneously (Karuppanapandian *et al.*, 2011). ROS exert their action by reacting with organic molecules, which in turn can be damaged or undergo redox modifications (Masi *et al.*, 2015).

Compared to O_2, $\bullet O_2$ is a highly reactive molecule (Mittler, 2002; Halliwell, 2006) and can interact with target biomolecules; the preferred ones are double bond moieties, such as polyunsaturated fatty acids (PUFAs) or guanine bases of DNA. In biological systems, $\bullet O_2$ is produced either by UV-B radiation or, in chloroplasts, due to chlorophyll photosensitization (Bischof *et al.*, 2003).

•O_2^- is a short-lived ROS (half-life of approximately 1 μs), it can not cross membranes and it is usually quickly dismutated to H_2O_2. •O_2^- can also react with •NO and give rise to peroxynitrite ($OONO^-$).

•HO_2· molecules are formed from O_2^- by protonation; they can cross biomembranes and react with PUFAs thus initiating lipid auto-oxidation (Halliwell and Gutteridge, 2006). H_2O_2 is a relatively long-lived molecule (half-life, 1 ms), moderately reactive and can diffuse short distances. By travelling across membranes, H_2O_2 can act as signal messenger in the stress response (Halliwell, 2006; Moller *et al.*, 2007).

•OH is the most reactive oxidant in the ROS family, and it is not considered to have signalling function. •OH reacts with all biomolecules (lipids and DNA, pigments, proteins). Plant cells can not scavenge this highly reactive ROS and its production in excess induce programmed cell death (PCD), (Vranova *et al.*, 2002; Manoharan *et al.*, 2005; Karuppanapandian *et al.*, 2011).

When exposed to biotic and abiotic stress, an increase in ROS concentration is often reported in plant cells, but they are also result from normal metabolic activity. Several metabolic pathways induce ROS generation in different cellular compartments such as chloroplasts (during photosynthesis), mitochondria (by electron transport in aerobic respiration), peroxisomes, cytosol, vacuoles, endoplasmic reticulum and plasma membranes (by oxidoreductase enzymes and metal catalyzed oxidation) (Corpas *et al.*, 2001; del Rio *et al.*, 2002; Mittler, 2002; Asada, 2006; Navrot *et al.*, 2007).

1.1 ANTIOXIDANTS SYSTEM IN PLANT

Cells have evolved a complex array of different biological strategies to ameliorate the harmful effects of ROS: one system is the prevention or avoidance of ROS formation, another way is scavenging ROS by enzymatic and non-enzymatic processes (e.g. low molecular weight antioxidants) to maintain and control low concentrations inside the cell (Nakabayashi and Saito 2015). Various enzymes are involved in ROS-scavenging, most notable are: superoxide dismutase (SOD) , catalase (CAT), ascorbate oxidase, glutathione peroxidase (GPX) reductase (GR) and sulfo-transferase (GSTs). Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2). In the peroxisomes, the enzyme catalase converts H_2O_2 to water and oxygen, and thus completes the detoxification initiated by SOD. Glutathione peroxidases is a group of selenium-enzymes, which also catalyze the degradation of hydrogen peroxide. Glutathione reductases convert oxidised glutathione to reduce form.

There are also a number of small molecules that are involved in ROS detoxification: ascorbic acid, glutathione, tocopherols are the main non-enzymatic antioxidants. However, there are also many secondary compounds (e.g. flavonoids, phenolic acids and other phenols, alkaloids, nitrogen compounds, aminoacids and amines, carotenoids and chlorophyll derivatives). They quench free radicals and ROS directly or by activation of scavenging defense systems controlled by cascades system (Gill and Tuteja 2010).

Ascorbic acid is a water soluble molecule capable of reducing ROS, while vitamin E (α-tocopherol) is a lipid soluble molecule that has been suggested to play a similar role in membranes. Glutathione plays an important role in the intra- and extra-cellular defense against the negative effects of reactive oxygen species. Reactions with ROS molecules oxidize glutathione, but the reduced form is regenerated in a redox reaction by an NADPH-dependent reductase. The ratio of the oxidized form of glutathione (GSSG) and the reduced form (GSH) is a dynamic indicator of the oxidative stress of an organism.

1.2 OXIDATIVE STRESS IN PLANT

In plants, various environmental perturbations induce oxidative stress, such as salinity, drought, high light intensity (HL), wind, heat, UV-B radiation, chilling, wounding, ozone (O_3), herbicides, parasites, heavy metals and pathogens. The overproduction of ROS results in an imbalance between the accumulation and the removal of these molecules in tissues and by plants antioxidant systems. This condition leads to non-specific damages to macromolecules such as DNA, proteins and lipids (Apel and Hirt, 2004; Munne-Bosch and Alegre, 2004; Karuppanapandian *et al.*, 2006a,b,c, 2008, 2009, 2011). One of the most known indicators of the presence of free radicals is lipid peroxidation. Unsaturated fatty acids, in cellular membranes, are a common target for these molecules. Lipid peroxides are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde. These injures can potentially result in cell death, and in the worst case lead to the organism death.

To check free radical formation, and consequently the presence of oxidative stress, it is common to investigate on antioxidants systems: they provide information on cellular redox state, and influence gene expression of defense response associated with biotic and abiotic stimuli (Foyer and Noctor 2005). In the cytosol, redox homeostasis is maintained thanks to a pool of low molecular weight antioxidants, mainly glutathione, ascorbate and tocopherol. The ability of these molecules to act as redox buffers in plant cells is one of their important function: glutathione reacts through its thiol (–SH) group and interacts with a number of other molecules in a series of redox reactions to scavenge

damaged species, and one of its main partner is ascorbate (GSH-ASC cycle). Ascorbate (or vitamin C) is synthesized in high concentrations in plant cells, and in addition to GSH contributes to redox buffering of hydrophilic molecules. Tocopherols (or vitamin E), due to its nature, is an important liposoluble redox buffer (Foyer and Noctor 2005).

1.3 ROS AS SIGNALS

Redox regulation has been demonstrated to be involved in different processes, not only in stress-regulated gene expression and disease resistance, but also in stomatal closure, organ development and control of the plant architecture, hormone signaling, and signal transduction (Potters *et al.*, 2010). Recent works provide evidence that ROS have a role in cell signalling, and they are involved in the regulation of various developmental and physiological processes and in pathogen defense (i.e., the HR - hypersensitive response), including apoptosis, gene expression, and the activation of cell signalling cascades (Guan and Scandalios, 2000; Pei *et al*., 2000; Mittler *et al.*, 2004; Foyer and Noctor, 2005; Vellosillo *et al.*, 2010). In many plant metabolic processes, ROS act as diffusible signals and secondary messengers in signal transduction pathways (Foyer and Noctor 2005). It should be noted that ROS can serve as both intra- and intercellular messengers. This evidence highlights in plants a delicate balance between ROS production and scavenging, that allows coexisting with different functions (Karuppanapandian *et al.*, 2011). Their combined action as metabolism signals and stress factors constitutes plant redox homeostasis, in which ROS and antioxidants acts as a metabolic interface (Foyer and Noctor 2005). Of course, not all reactive oxygen species have the same potential to act as signaling molecules, only ROS that are able to cross biomembranes are likely to be implicated. Most interesting from this point of view is H_2O_2 for a number of reasons: it is produced by different enzyme systems; its half-life is relatively long; and it is present and tolerated in higher concentration compared to other ROS. Moreover, H_2O_2 can transmit redox signals through the vascular system (long distance) or in the apoplast (short distance) (Foyer and Noctor 2005).

In plants, as in animals, a common mechanism to get rid of ROS as a relatively stable product is by the oxidation of thiol-containing domains; thiol oxidation plays a key role also on protein phosphorylation controlled by kinase pathway (Kovtun *et al.*, 2000; Gupta and Luan, 2003; Rentel *et al.*, 2004; Waszczak *et al.*, 2015; Reczek and Chandel 2015).Glutathione redox adjustments are as important as enhanced ROS pools in signalling (Creissen *et al.*, 1999; Mou *et al.*, 2003; Ball *et al.*, 2004; Gomez *et al.*, 2004; Evans *et al.*, 2005). Glutathione pool is oxidized and increased in catalase-deficient plants (Noctor *et al.*, 2002a; Rizhsky *et al.*, 2002). Moreover, different works demonstrated a relationship between salicylic acid, catalase, ascorbate peroxidase, and glutathione

in oxidative and reductive steps during plant–pathogen interactions (Vanacker *et al.*, 2000; Mou *et al.*, 2003). GSH status influences also cytosolic calcium concentration (Gomez *et al.*, 2004; Evans *et al.*, 2005), which is known to increase together with ROS production in early events of pathogen responses (Dangl and Jones, 2001; Lecourieux *et al.*, 2002).

2. GLUTATHIONE FUNCTIONS IN PLANTS

Glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is a tripeptide, the main and most abundant low molecular weight thiol and one of the major non-protein antioxidant molecules in plant cells. It is involved in a plethora of metabolic pathways and biological functions: e.g. it regulates protein function, flowering and lateral root growth, cell division, mRNA translation, xenobiotic detoxification, sulphur nutrition and storage, cellular redox state and signalling; moreover it is a precursor of phytochelatins (Potters *et al.*, 2010) (see Fig.1). All these functions make glutathione a node point for regulation of plant development and responses to the environment (Noctor *et al.*, 2011). GSH can move through plasma membrane, and because of its thiol (–SH) moiety, it is involved both in short and long distance sulphur transport by xylem and phloem fluids. Indeed, cysteine amine group is linked to the carboxyl group of the glutamate to form a gamma peptide that can not be cleaved by proteases.

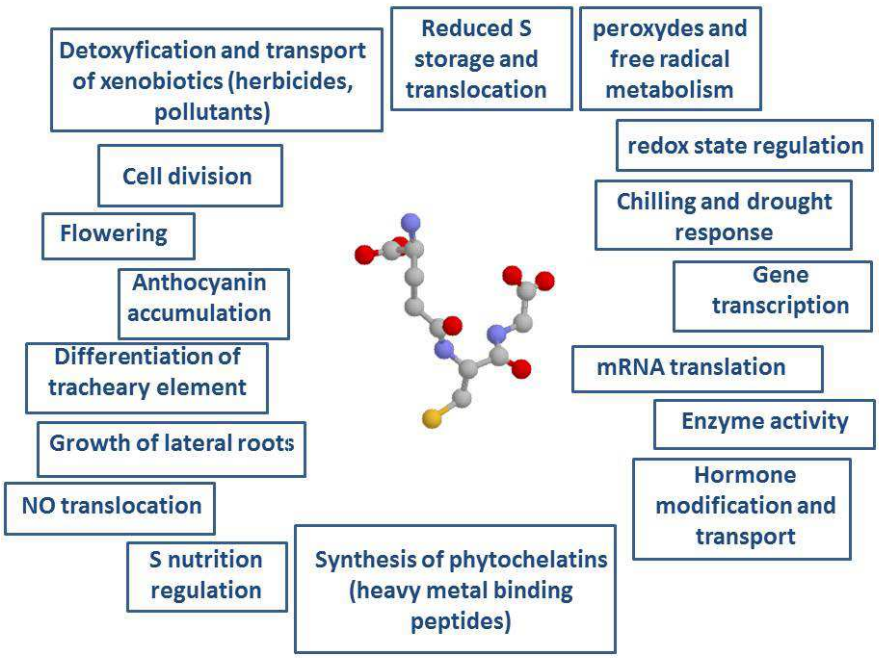


Fig. 1: An overview of glutathione functions.

2.1 GLUTATHIONE HOMEOSTASIS

The subcellular distribution of glutathione in plants is important for plant growth and development (Kocsy *et al.*, 2013). Its concentration, inside cells, occurs between 3 and 10 mM and varies due to different tissue and developmental stage of plants (Leustek and Saito 1999). Glutathione is involved in the regulation of sulphur metabolism in plants: it constitutes the main non-protein sink for reduced sulphur, being the major sulfur reservoir (Kopriva and Rennenberg2004).

The contribution of GSH to cellular homeostasis is demonstrated by the use of *A. thaliana* mutants in several functional genomic studies, that indicate a correlation between a decreased glutathione content and a series of damaging events. Among others, they include auxin transport and metabolism disruption, camalexin content decrease, root apical meristem developmental failure, increased sensitivity to cadmium, loss of apical dominance and reduced secondary root production, and enhanced sensitivity to pathogens (Bloem *et al.*, 2007; Pivato *et al.*, 2014). Environmental stress conditions provoke alterations in subcellular glutathione contents, therefore GSH is used also as stress marker. These information are helpful to understand the role of this metabolite during stress condition in plants(Zechmann, 2014).

The ratio of GSH-GSSG is the central indicator of cellular redox state: notably GSSG is accumulated in oxidative stress, whereas glutathione is in its reduced form (GSH) under physiological condition (Pivato *et al.*, 2014). Moreover, in plants glutathione cooperates with ascorbate in the so called ascorbate–glutathione cycle (Fig 2)to detoxify reactive oxygen species (ROS) by direct chemical interaction. Ascorbate is oxidized by ROS to monodehydroascorbate and dehydroascorbate (DHA), whose reduction is coupled to glutathione oxidation (then GSSG is enzymatically reduced by glutathione reductase GR). Since glutathione is deeply connected to ascorbate, knowing the amount of ascorbate and glutathione and their redox states (reduced vs. oxidized) is very important in the study of plant responses to stress (Zechmann, 2014).

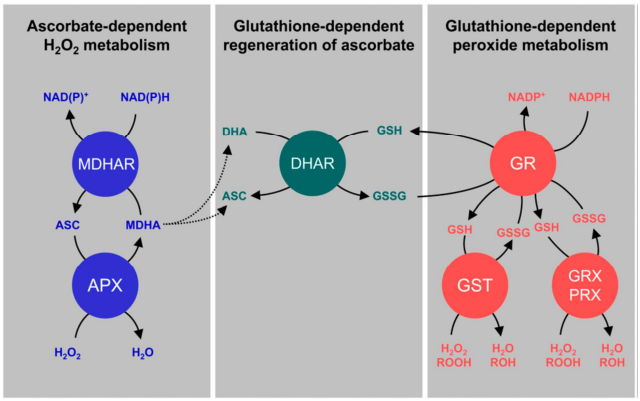


Fig. 2:Ascorbate–glutathione cycle (from Foyer and Noctor 2011).

2.2 GSH: PHYSICO-CHEMICAL PROPERTIES AND BIOCHEMICAL MECHANISMS OF REACTION

Glutathione is linked at the γ-carboxyl group of glutamate to the amino group of cysteine: this property confers stability to the molecule (proteases resistance) and allows programmed degradation by amino acid transferases. Inside cells GSH is predominantly maintained in its reduced form by glutathione reductases, that are present in cytosol, plastids, mitochondria, and peroxisomes (Halliwell and Foyer, 1978;Smith *et al.*, 1989; Edwards *et al.*, 1990; Jiménez *et al.*, 1997; Chew *et al.*, 2003; Kataya and Reumann, 2010).

Oxidized glutathione is produced by the formation of a disulfide bond between the cysteine thiol moieties of two glutathione molecules. The reversible redox reactions of cysteine ensure many GSH functions. In general, oxidized forms includes either disulfides with another glutathione molecule (GSSG) or with a different thiol to form mixed disulfides. ROS are the molecules mainly involved in GSH oxidation, that serves as scavenger or sacrificial nucleophile. Glutathione oxidation can also be catalysed by enzymes, that reduce H_2O_2 or peroxides to water or to the corresponding alcohol (Pivato *et al.*, 2014), or that oxidize glutathione for ascorbate regeneration (dehydroascorbate reductase, DHAR) (Foyer and Mullineaux, 1998).

Glutathione forms conjugates with a vast array of endogenous electrophilic species and with xenobiotics, acting as a detoxification agent (Wang and Ballatori, 1998; Dixon and Edwards, 2010). In particular, with nitric oxide (NO) it forms a conjugate (GSNO) which is receiving particular attention for its physiological significance as signalling molecule (Lindermayr *et al.*, 2005).

Therefore, in its oxidized/reduced forms, glutathione constitutes a redox buffer that on the one hand guarantees cellular redox homeostasis, and on the other hand it participates in signalling processes. A clear example of this double function is the interaction between glutathione and proteins: on the one hand oxidized protein thiols can be reverted to their reduced state thanks to glutathione (redox homeostasis), on the other hand the linkage between glutathione and proteins (glutathionylation) can act as signalling process (e.g. it can control activity of transcription factors) (Mejer and Hell 2005).

2.3 GSH BIOSYNTHESIS AND CATABOLISM

Glutathione biosynthesis is similar between plants and other organisms (Rennenberg and Filner 1982; Meister 1988; Noctor *et al.*, 2002b). The tripeptide is formed from Glu, Cys and Gly by two ATP-dependent enzymes (namely GSH1 and GSH2). The first step occurs in the plastid with the

synthesis of γ-glutamylcysteine (γ-Glu-Cys). This intermediate molecule is exported in the cytosol and/or cloroplast where the addition of glycine occurs (Pivato *et al.*, 2014). Notably, the γ-Glu-Cys synthetase (GSH1) is the rate limiting enzyme of GSH production: the increase of glutathione contents was shown both by artificial elevation of cysteine content by exogenous supplementation and by the overexpression of genes and enzymes involved in cysteine synthesis (Gullner *et al.*, 1999; Harms *et al.*, 2000; Bloem *et al.*, 2004 and 2007; Zechmann *et al.*, 2007 and2008; Noji and Saito, 2002; Wirtz and Hell, 2007).

In plant cells there are two alternative degradation pathways for GSH. In the cytosol a γ-glutamylcyclo-transferase (GGCT) pathway operates (Ohkama-Ohtsu *et al.*, 2008), meanwhile in apoplast and vacuole γ-glutamyltransferase/transpeptidases are active (GGT, EC 2.3.2.2) (Ohkama-Ohtsu *et al.*, 2007a,b; Tolin *et al.*, 2013;Masi *et al.*, 2015). The two degradation pathways coexist and operate independently of one another, and have therefore distinct physiological significance and regulation. Indeed, the gamma glutamyl cycle is functional to the recovery of extracellular glutathione, while the γ-glutamylcyclo-transferase/5 oxoproline pathway participates to the control of cytosolic glutathione homeostasis.

2.4 γ- GLUTAMYL CYCLE IN PLANTS

The existence of the extracellular enzyme gamma-glutamyl-transferase (GGT; E.C. 2.3.2.2) degrading GSH has been reported in plants as in animals (Martin *et al.*, 2007; Meister and Anderson 1983). This is a crucial enzyme in the gamma-glutamyl cycle, consisting of intracellular glutathione synthesis, extrusion to the extracellular space and recovery by gamma-glutamyltransferase (GGT) and cys-glydipeptidase (DP). The degradation into its constituent amino acids has now been demonstrated both in animals (Fig. 3) (Meister and Anderson 1983) and also in plants (Martin *et al.*,2007; Ferretti *et al.*, 2009). Amino acids are then taken up and glutathione is reassembled inside the cell. GSH/GGT-dependent processes have been described of pivotal importance in redox homeostasis in modulation of health and stress condition in evolutionarily distant organisms (Pennacchio *et al.*, 2014).

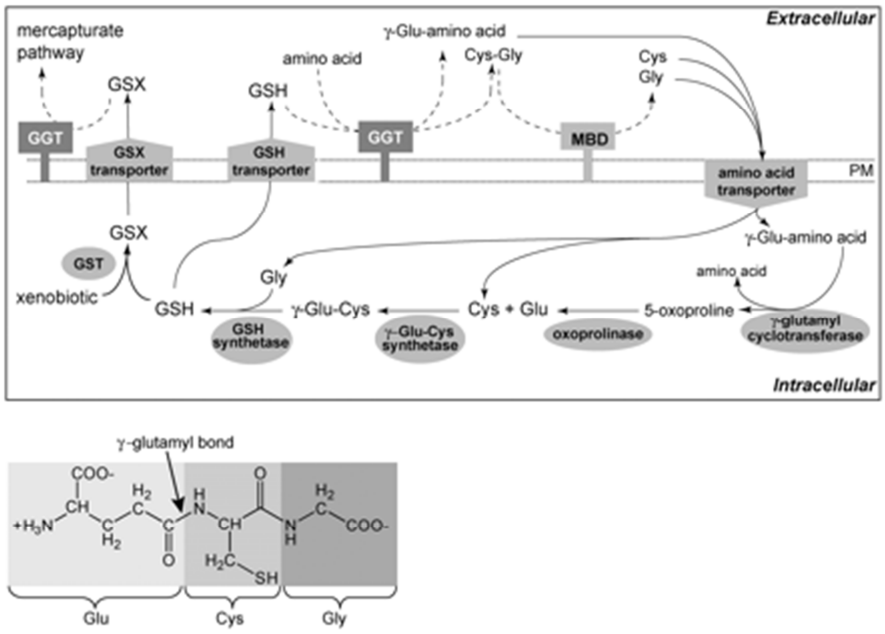


Fig. 3: The γ-glutamyl cycle as it operates in animals. Extracellular GSH is hydrolyzed by GGTs and dipeptidase to amino acids and dipeptides. The resulting amino acids and dipeptides are transported into the cell, thus making them available for a wide range of functions including intracellular synthesis of GSH and protein synthesis.Intracellularly, GSH also serves as substrate for glutathione S-transferase-catalyzed conjugation reactions. Glutathione S-transferase conjugates of many xenobiotics are exported and hydrolyzed by GGTs and MBDs prior to excretion (from Martin *et al.*, 2007).

3. GAMMA-GLUTAMYLTRANSFERASES IN ARABIDOPSIS

In *Arabidopsis thaliana*, four gamma-glutamyltransferase genes (At4g39640, At4g39650,At1g69820 and At4g29210) have been identified based on homology with animal GGTs (EC 2.3.2.2) (Martin *et al.*, 2007). GGT1 and GGT2 proteins are associated with the cell wall and the plasma membrane, respectively, whereas GGT3 does not seem to have a function, and GGT4 occurs only in vacuoles (Ohkama-Ohtsu *et al.*, 2007a,b; Ferretti *et al.*, 2009; Destro *et al.*, 2011; Tolin *et al.*, 2013). GGTs cleave the amide bond linking the *γ*-carboxylate of glutamate to cysteine, are involved in hydrolysis and turnover of glutathione and assist in degrading GSH conjugates. In particular, GGT1 and GGT2 control cellular glutathione uptake and long-distance transport. GGT4 is localized inside vacuole and cleaves glutathione-S-conjugates (Grzam *et al.*, 2007). Martin and colleagues (2007) used plants containing GGT::*β*-glucuronidase fusion proteins to investigate temporal and spatial enzyme localization (Fig.4). Results demonstrated that GGT1 and GGT4 are coexpressed in most organs/tissues. Their expression was highest at sites of rapid growth (such as the rosette apex, floral stem apex, and seeds) and they showed pinpoint locations where glutathione

is delivered to sink tissues as cysteine supplier. GGT2 displayed short and limited expression mainly in immature trichomes, developing seeds, and pollen.

Fig. 4: Histochemical localization of GGT1 activity in plants expressing a GGT1:GUS fusion.
A, GUS activity was highest at the shoot apex and in the vascular tissue of the hypocotyl and cotyledons. B, Enlargement of the shoot apex where GUS expression was strongest showed that activity was particularly high at the base of immature trichomes. C, 11-d-old seedling showed differential, age-dependent staining of leaves. Expression was high at the rosette apex but absent in the older leaves or was restricted to the major veins. D, GUS activity was high in the floral shoot of 27-d-old plants at bolting. Cross sections of an elongated floral stem at the base (E) and apex (F), respectively, showed staining was strongest at the apex. G, cross section through a leaf and the stem at the base of a mature rosette showed activity only in the vascular tissue. H, GUS activity was present throughout roots of mature plants. I, Floral buds and nearly all parts (J) of mature flowers showed GUS activity. J, The pistil stained prior to fertilization and the ovary (silique) wall (K) stained at all stages after fertilization. L, Removal of the seed coat showed GUS activity in the mature embryo (from Martin*et al.*, 2007).

4. THE APOPLASTIC SPACE

All plant tissues can be divided into two main compartments: symplastic (located in the inner side of the plasma membrane) and apoplastic space (located in the outer side, extracellular) (Fig. 5).

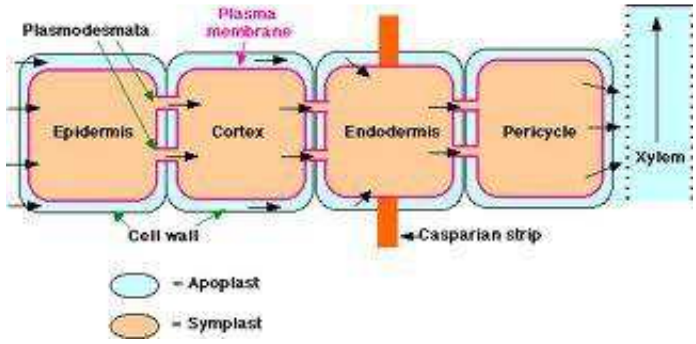


Fig. 5:Apoplast and symplast in plant cell.

Plasmodesmata are symplastic connection of cells that consist of microscopic channels used for the transport of small molecules (Giraldo and Valent 2013). The apoplastic route facilitates the transport of water and solutes across a tissue or organ: this process is known as apoplastic transport. It is also a site for cell-to-cell communication.

The apoplast includes the cell wall, with its interfibrillar and intermicellar space, and the xylem, with its gas and water-filled intercellular space (Sattelmacher 2000:). It contains proteins and other molecules involved in plant cell's sensing and signalling of biotic and abiotic stress (Dietz, 1997; Agrawal *et al.*, 2010).

It is at the interphase between the cell and the external environment, where fast fluctuations occur due to different stresses, such as salinity (Hernandez *et al.*, 2001), ozone (Jaspers *et al.*, 2005) drought (Hu *et al.*, 2005), UV-B radiation (Pristov *et al.*, 2013) and pathogens (Delaunois *et al.*, 2014). As a consequence, many molecules in the apoplastic space change their redox state and concentrations.

Any external environmental condition is sensed by the plant thanks to the generation of an extracellular signal, which is transmitted to the inner compartments and, following specific transduction pathways, induces plant response to readjust cell metabolism to the new condition. Many players are required for this system: specific genes expression, post-transcriptional and post-translational regulation, hormones and cell regulators (Masi *et al.*, 2015).

During local oxidative stress, hydrogen peroxide and superoxide anion can diffuse through apoplast and transmit a warning signal to neighbouring cells. In addition, a local alkalinization of the

apoplast due to such stress can travel within minutes to the rest of the plant via the xylem and trigger systemic acquired resistance.

REFERENCES

Agrawal G.K., Jwa N.S., Lebrun M.H., Job D., Rakwal R. (2010). "Plant secretome: unlocking secrets of the secreted proteins". *Proteomics* 10 pp. 799-827.

Apel K., Hirt H. (2004). "Reactive oxygen species: metabolism, oxidative stress, and signal transduction". *Annu Rev Plant Biol* 55 pp. 373–399.

Asada K. (2006)."Production and scavenging of reactive oxygen species in chloroplasts and their functions". *Plant Physiol* 141 pp. 391–396.

Ball L., Accotto G., Bechtold U., Creissen G., Funck D., Jimenez A., Kular B., Leyland N., Mejia-Carranza J., Reynolds H., Karpinski S., and Mullineaux P.M. *(*2004*).* "Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis". *Plant Cell* 16 pp. 2448–2462.

Bischof K., Janknegt P.J., Buma A.G.J., Rijstenbil J.W., Peralta G., Breeman A.M. (2003). "Oxidative stress and enzymatic scavenging of superoxide radicals induced by solar UV-B radiation in Ulvacanopies from southern Spain". *Sci Mar* 67 (3) pp. 353-359.

Bloem E., Haneklaus S., Salac I., Wickenhäuser P., Schnug E. (2007). "Facts and fiction about sulfur metabolism in relation to plantpathogen interactions". *Plant Biol*9 pp. 596–607.

Bloem E., Riemenschneider A., Volker J., Papenbrock J., Schmidt A., Salac I., et al. (2004). "Sulphur supply and infection with *Pyrenopeziza brassicae* influence l-cysteine desulphydrase activity in *Brassica napus* L". *J Exp Bot* 55 pp. 2305–2312.

Chew O., Whelan J., Millar A.H. (2003). "Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants". *J Biol Chem* 278 pp. 46869–46877.

Corpas F.J., Barroso J.B., del Río L.A. (2001). "Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells". *Trends Plant Sci* 6 pp. 145–150.

Creissen G., Firmin J., Fryer M., Kular B., Leyland M., Reynolds H., Pastori G., Wellburn F., Baker N.R., Wellburn A., and Mullineaux P. *(*1999*).* "Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco paradoxically causes increased oxidative stress".*Plant Cell*11 pp. 1277–1291.

Dangl J.L., and Jones J.D.G. (2001). "Plant pathogens and integrated defence responses to infection". *Nature* 411 pp. 826–833.

del Rio L.A., Corpas F.J., Sandalio L.M., Palma J.M., Gomez M., Barroso J.B. (2002). "Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes". *J Exp Bot* 53 pp. 1255–1272.

Delaunois B., Jeandet P., Clément C., Baillieul F., Dorey S., Cordelier S. (2014). "Uncovering plant-pathogen crosstalk through apoplastic proteomic studies". *Front PlantSci*5, 249. doi:10.3389/fpls.2014.00249.

DestroT., Prasad D., Martignago D., Bernet I. L., et al., (2011)."Compensatory expression and substrate inducibility of gamma-glutamyl transferase GGT2 isoform in *Arabidopsis thaliana"*. *J Exp Bot*62 pp. 805-814.

Dietz, K.J. (1997). Functions and responses of the leaf apoplast under stress. *Progress in Botany* 58 pp. 221-254.

Dixon D.P., Edwards R. (2010). "Glutathione *S*-transferases".*The Arabidopsis Book*. doi: 10.1199:tab.0131

Edwards E.A., Rawsthorne S., Mullineaux P.M. (1990). "Subcellular distribution of multiple forms of glutathione reductase in pea (*Pisum sativum* L.)". *Planta* 180 pp. 278–284.

Evans N.H., McAinsh M.R., Hetherington A.M., and Knight M.R. (2005). "ROS perception in *Arabidopsis thaliana*: The ozone-induced calcium response". *Plant J* 41 pp. 615–626.

Ferretti M., Destro T., Tosatto S.C.E., La Rocca N., Rascio N., Masi A. (2009). "Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast". *New Phytol* 181 pp. 115–126.

Foyer C.H., Mullineaux P.M. (1998). "The presence of dehydroascorbate and dehydroascorbate reductase in plant tissues". FEBS Lett425 pp. 528–529.

Foyer C.H., Noctor G. (2005). "Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses". *Plant Cell* 17(7) pp. 1866-75.

Foyer C.H., Noctor G. (2011). “Ascorbate and Glutathione: the heart of the redox hub”. *Plant Physiol*155 pp. 2-18.

Gill S.S., Tuteja N. (2010). “Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants”. *Plant Physiol Biochem* 48(12) pp. 909-30. doi: 10.1016/j.plaphy.2010.08.016.

Giraldo M.C., Valent B. (2013). “Apoplastic and symplastic compartments in plants”. *Nature Rev Microbiol* 11 pp. 800–814. doi:10.1038/nrmicro3119.

Gomez L.D., Noctor G., Knight M., and Foyer C.H. (2004). “Regulation of calcium signaling and gene expression by glutathione”. *J Exp Bot* 55 pp. 1851–1859.

Grzam A., Martin M.N., Hell R., Meyer A.J. (2007). “Gamma-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione S-conjugates in Arabidopsis”. *FEBS Lett* 581(17) pp. 3131-8.

Guan L.M., Scandalios J.G. (2000). “Hydrogen peroxide-mediated catalase gene expression in response to wounding”. *Free Rad Biol Med* 28 pp.1182–1190.

Gullner G., Tóbiás I., Fodor J., Kömives T. (1999). “Elevation of glutathione level and activation of glutathione-related enzymes affect virus infection in tobacco”. *Free Radic Res* 31(Suppl.), S155–S161. doi: 10.1080/10715769900301451.

Gupta R., and Luan S. (2003). “Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants”. *Plant Physiol*132 pp. 1149–1152.

Halliwell B. (2006). “Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life”. *Plant Physiol* 141(2) pp. 312–322. doi: 10.1104/pp.106.077073.

Halliwell B., Foyer C.H. (1978). “Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography”. *Planta* 139 pp. 9–17.

Halliwell B., Gutteridge J.M.C.(2006). “Free radicals in biology and medicine”. Oxford: Oxford University Press. UK, Fourth Edition.

Harms K., von Ballmoos P., Brunold C., Hofgen R., Hesse H. (2000). “Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione”. *Plant J* 22 pp. 335–343.

Hernandez J.A., Ferrer M.A., Jimenez A., Barcelo A.R., Sevilla F. (2001). “Antioxidant systems and O_2^-/ H_2O_2 production in the apoplast of Pea leaves. Its relation with salt-induced necrotic lesions in minor veins”. *Plant Physiol* 127 pp. 817–831.

Hu J.F., Li G.F., Gao Z.H., Chen L., Ren H B., Jia W.S. (2005). “Regulation of water deficit-induced abscisic acid accumulation by apoplastic ascorbic acid in maize seedlings”. *J IntegrPlant Biol* 47 pp. 1335-1344.

Jaspers, P., Kollist, H., Langebartels, C., Kangasjarvi, J. (2005). “Plant responses to ozone,” in *Antioxidants and reactive oxygen species in plants,* ed. N. Smirnoff (Oxford: Blackwell Publishing), pp. 268-292.

Jiménez A., Hernández J.A., del Río L., Sevilla F. (1997). “Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves”. *Plant Physiol* 114 pp. 275–284.

Karuppanapandian T., Sinha P.B., Kamarul Haniya A., Manoharan K. (2006a).“Differential antioxidative responses of ascorbate-glutathione cycle enzymes and metabolites to chromium stress in green gram (Vigna radiata L. Wilczek) leaves”. *J Plant Biol* 49 pp. 440–447.

Karuppanapandian T., Sinha P.B., Kamarul Haniya A,. Premkumar G., Manoharan K. (2006b).“Aluminium-induced changes in antioxidative enzyme activities, hydrogen peroxide content and cell wall peroxidase activity in green gram (Vigna radiataL. cv. Wilczek) roots”. *J Plant Biol* 33 pp. 241–246.

Karuppanapandian T., Sinha P.B., Premkumar G., Manoharan K. (2006c). “Chromiumtoxicity: Correlated with increased in degradation of photosynthetic pigments and total soluble protein and increased peroxidase activity in green gram (*Vigna radiata*L.) seedlings”. *J Swamy Bot-Cl* 23 pp. 117–122.

Karuppanapandian T., Saranyadevi A.R., Jeyalakshmi K., Manoharan K. (2008).“Mechanism, control and regulation of leaf senescence in plants”. *J Plant Biol* 35 pp. 141–155.

Karuppanapandian T., Sinha P.B., Kamarul Haniya A., Manoharan K. (2009).“Chromium-induced accumulationof peroxide content, stimulation of antioxidative enzymes and lipid peroxidation in green gram (*Vigna radiata* L. cv. Wilczek) leaves”. *Afr J Biotechnol* 8 pp. 475–479.

Karuppanapandian T., Moon J.C., Kim C., Manoharan K., Kim W. (2011). “Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms”.*AJCS*5(6) pp. 709-725.

Kataya A.M.R., Reumann S. (2010). “Arabidopsis glutathione reductase 1 is dually targeted to peroxisomes and the cytosol”. *Plant Signal Behav* 5 pp. 171–175.

Kocsy G., Tari I., Vankova R., Zechmann B., Gulyas Z., Poor P., Galiba G. (2013). “Redox control of plant growth and development”. *Plant Sci*211 pp. 77–91. doi: 10.1016/j.plantsci.2013.07.004.

Kopriva S., Rennenberg H. (2004). “Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism”. *J Exp Bot*55 pp. 1831–1842.

Kovtun Y., Chi, W.-L., Ten, G., and Shee, J. (2000). “Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants”. *Proc Natl Acad SciUSA* 97 pp. 2940–2945.

Lecourieux D., Mazars C., Pauly N., Ranjeva R., and Pugin A. (2002). “Analysis and effects of cytosolic free calcium increases in response to elicitors in Nicotiana plumbaginifolia cells”. *Plant Cell*14 pp. 2627–2641.

Leustek T., and Saito K. (1999). “Sulfate Transport and Assimilation in Plants”. *Plant Physiol* 120(3) pp. 637-644.

Lindermayr C., Saalbach G., Dürner J. (2005). “Proteomic identification of *S*-nitrosylated proteins in Arabidopsis”. *Plant Physiol*137 pp. 921–930.

Manoharan K., Karuppanapandian T., Sinha P.B., Prasad R. (2005). “Membrane degradation, accumulation of phosphatidic acid, stimulation of catalase activity and nuclear DNA fragmentation during 2,4-D-induced leaf senescence in mustard”. *J Plant Biol* 48 pp. 394–403.

Martin M.N., Saladores P.H., Lambert E., Hudson A.O., Leustek T. (2007). “Localization of members of the γ-glutamyl transpeptidase family identifies sites of glutathione and glutathione S-conjugate hydrolysis”. *Plant Physiol* 144 pp. 1715-1732.

Masi A., Trentin A.R., Agrawal, G.K., RakwalR. (2015). “Gamma-glutamyl cycle in plants: a bridge connecting the environment to the plant cell?” *Front Plant Sci* 16 April 2015 | http://dx.doi.org/10.3389/fpls.2015.00252.

Mawdsley J.R., O'Maley R., Ojima D.S. (2009)."A Review of Climate-Change Adaptation Strategies for Wildlife Management and Biodiversity Conservation". *Conservation Biology* 23(5) pp. 1080–1089.

Meister A., (1988). "Glutathione Metabolism and Its Selective Modification". *J Biol Chem* 263(33) pp. 17205-17208.

Meister A., and Anderson M.E. (1983). "Glutathione". *Annu Rev Biochem* 52 pp. 711-760.

Mejer A.J., Hell R. (2005). "Glutathione homeostasis and redox-regulation by sulfhydryl groups". *Photosynth Res* 86(3) pp. 435–457.

Mittler R. (2002). "Oxidative stress, antioxidants and stress tolerance". *Trends Plant Sci* 7(9) pp. 405-10.

Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. (2004)."Reactive oxygen gene network of plants". *Trends Plant Sci* 9 pp. 490–498.

Moller I.M., Jensen P.E., Hansson A. (2007). "Oxidative modifications to cellular components in plants". *Annu Rev Plant Biol* 58 pp. 459–481.

Mou Z., Fan W., and Dong X. (2003). "Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes". *Cell* 113 pp. 935–944.

Munne-Bosch S., Alegre L. (2004). "Die and let live: leaf senescence contributes to plant survival under drought stress". *Funct Plant Biol* 31 pp. 203–216 .

Nakabayashi R. and Saito K. (2015). "Integrated metabolomics for abiotic stress responses in plants".*Curr Opin Plant Biol* 24 pp. 10–16.

Navrot N., Roubier N., Gelbaye E., Jacquot J-P. (2007)."Reactive oxygen species generation and antioxidant systems in plant mitochondria". *Physiol Plant* 129 pp. 185–195.

Noctor G., Veljovic-Jovanovic S., Driscoll S., Novitskaya L., and Foyer C.H. (2002a). "Drought and oxidative load in wheat leaves: A predominant role for photorespiration?" *Ann Bot* 89 pp. 841–850.

NoctorG., GomezL., VanackerH., FoyerC.H. (2002b). "Interactions between biosynthesis, compartimentation and transport in the control of glutathione homeostasis and signalling".*J Exp Bot* 53(372)pp. 1283–1304.

Noctor G., Queval G., Mhamdi A., Chaouch S., Foyer C.H. (2011). “Glutathione”. *Arabidopsis Book* doi: 10.1199/tab.0142.

Noji M., Saito K. (2002). “Molecular and biochemical analysis of serine acetyltransferase and cysteine synthase towards sulfur metabolic engineering in plants”. *Amino Acids* 22 pp. 231–243.

Ohkama-Ohtsu N., Radwan S., Peterson A., Zhao P., et al. (2007a). “Characterization of the extracellular γ-glutamyl transpeptidases, GGT1 and GGT2, in Arabidopsis”. *Plant J* 49 pp. 865-877.

Ohkama-Ohtsu, N., Zhao, P., Xiang, C., Oliver, D. J. (2007b). “Glutathione-conjugates in the vacuole are degraded by γ-glutamyl transpeptidase GGT3 in Arabidopsis”. *Plant J* 49 pp. 878-888.

Ohkama-Ohtsu N., Oikawa A., Zhao P., Xiang C., Saito K., Oliver D. J. (2008). “A gamma-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in *Arabidopsis”*. *Plant Physiol* 148 pp. 1603–1613.

Pei Z-M., Murata Y., Benning G., Thomine S., Klusener B., Allen G.J., Grill E., Schroeder J.I. (2000). “Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells”. *Nature* 406 pp. 731–734.

Pennacchio F., Masi A., Pompella A. (2014). “Glutathione levels modulation as a strategy in host-parasite interactions—insights for biology of cancer”. *Front Pharmacol* 2014; 5: 180. doi: 10.3389/fphar.2014.00180.

Pitzschke, A.,Fornazi, C., Hirt, H. (2006). Reactive oxygen species signalling in plants. *AntioxidRedox Sign* 8 pp. 1757–1764.

Pivato M., Fabrega-Prats M., Masi A. (2014). “Low-molecular-weight thiols in plants: Functional and analytical implications”.*Arch Biochem Biophys* 560 pp. 83–99.

PottersG., Horemans N., JansenM. K. (2010). “The cellular redox state in plant stress biology a charging concept”. *Plant Physiol Biochem* 48 pp. 292–300. doi:10.1016/j.plaphy.2009.12.007.

Pristov J.B., Jovanović S.V., Mitrović A., Spasojević I. (2013). “UV-irradiation provokes generation of superoxide on cell wall polygalacturonic acid”. *Physiol Plant* 148 pp. 574–581.

Reczek CR, Chandel NS. (2015). “ROS-dependent signal transduction”. *Curr Opin Cell Biol* 33 pp. 8-13. doi: 10.1016/j.ceb.2014.09.010.

RennenbergH., FilnerP. (1982).“Stimulation of h(2)s emission from pumpkin leaves by inhibition of glutathione synthesis”.*Plant Physiol* 69(4) pp. 766–770.

Rentel M.C., Lecourieux D., Ouaked F., Usher S.L., Peterson L., Okamoto H., Knight H., Peck S.C., Grierson C.S., Hirt H., and Knight M.R. (2004). “OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis”. *Nature* 427 pp. 858–861.

Rizhsky L., Hallak-Herr E., Van Breusegem F., Rachmilevitch S., Barr J.E., Rodermel S., Inzé D., and Mittler R. (2002). “Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase”. *Plant J*32 pp. 329–342.

Sattelmacher B. (2000). “The apoplast and its significance for plant mineral nutrition”. *New Phytol*149(2)pp. 167–192.

Smith I.K., Vierheller T.L., Thorne C.A.(1989). “Properties and functions of glutathione reductase in plants”. *Physiol Plant* 77 pp. 449–456.

Suzuki N., Rivero R.M., Shulaev V., Blumwald E., Mittler R. (2014). “Abiotic and biotic stress combinations”. *New Phytol* 203(1) pp. 32–43.

Tolin S., Arrigoni G., Trentin A.R., Veljovic-Jovanovic S., Pivato M., Zechman B., Masi A. (2013). “Biochemical and quantitative proteomics investigations in *Arabidopsisggt1* mutant leaves reveal a role for the gamma-glutamyl cycle in plant’s adaptation to environment”. *Proteomics* 13 pp. 2031–2045.

Vanacker H., Carver T.L.W., and Foyer C.H. (2000). “Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction”. *Plant Physiol* 123 pp. 1289–1300.

Vellosillo T., Vicente J., Kulasekaran S., Hamberg M., Castresana C. (2010). “Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens”. *Plant Physiol* 154 pp. 444–448.

Vranova E., Inze D., Van Breusegem F. (2002). “Signal transduction during oxidative stress”. *J Exp Bot* 53 pp. 1227–1236.

Wang W., Ballatori N. (1998). “Endogenous glutathione conjugates: occurrence and biological functions”. PharmacolRev50 pp. 335–355.

Waszczak C., Akter S., Jacques S., Huang J., Messens J., and Van Breusegem F. (2015). “Oxidative post-translational modifications of cysteine residues in plant signal transduction”. *J Exp Bot* doi: 10.1093/jxb/erv084.

Wirtz M., Hell R. (2007). “Dominant-negative modification reveals the regulatory function of the multimeric cysteine synthase protein complex in transgenic tobacco”. *Plant Cell* 19 pp. 625–639.

Zechmann B., Zellnig G., Urbanek-Krajnc A., Müller M. (2007). “Artificial elevation of glutathione affects symptom development in ZYMV-infected *Cucurbita pepo* L. plants”. *Arch Virol* 152 pp. 747–762.

Zechmann B., Müller M., Zellnig G. (2008). “Modified levels of cysteine affect glutathione metabolism in plant cells”.In *Sulfur Assimilation and Abiotic Stress in Plants*, eds Khan N. A., Singh S., Umar S., editors. (Berlin: Springer;) pp. 193–206.

ZechmannB. (2014).”Compartment-specific importance of glutathione during abiotic and biotic stress”.*Front Plant Sci* 5: 566. doi: 10.3389/fpls.2014.00566.

OBJECTIVES

OBJECTIVES

Plants growth and composition result from a complex interplay of genotypic features and environmental and nutritional factors. With the dual aim to improve crop production and plant-derived food quality, many scientists worldwide are studying the physiological determinants of plant adaptation to environmental stress conditions.

The appearance of reactive oxygen species (ROS) is an unavoidable consequence of life in oxygenic atmosphere, but their production is enhanced under unfavourable environmental conditions. In living cells, antioxidants act to contrast free radicals and permit to control and limit damages caused by toxins, medications, stress, pollution, poor diet, trauma, infections and radiation (Halliwell and Gutteridge, 2006).Oxidative stress conditions negatively affect plant growth and development, and accelerate scenescence.

In recent years, a great number of nutritionists and consumers have also shown an increasing interest towards food therapeutic value, long shelf life and consequently on natural plants antioxidants (Sreeramulu *et al.*, 2013). Plant foods, due to their antioxidant activity, help to stay healthy and prevent disease (Kaur and Kapoor, 2001).

All these considerations point to the importance of studying antioxidants metabolism in plant cells.

A major soluble antioxidant in plant cells is ascorbate,and human diet relies on plant food intake for correct supply.Another main non-proteic antioxidant is GSH, mainly localised intracellularly; however, it is also found in the apoplast, where it is involved in the so called gamma-glutamyl cycle.

An interesting peculiarity of the cycle is that it occurs between inside and outside the cell: GSH is synthetized in the cytosol, carried out trough plasma membranesto the extracellular space and here it is cleaved by gamma-glutamyltransferase (GGT) to produce cysteinyl-glycine (cys-gly) and glutamate.

A major aim of my thesis was to better clarify if the gamma-glutamyl cycle is involved in mechanisms that regulate redox responses and antioxidant levels in plants.

To do so, I decided to investigate: i) what are the metabolic consequences of the *gg*t1 mutation at proteomic level; ii) how *Arabidopsis thalianaggt1* mutants respond to oxidative conditions; iii) what are the signals arising in the apoplast, involving LMW thiols and cell wall components, that might mediate the redox responses.

REFERENCES

Halliwell B., Gutteridge J.M.C. (2006). “Free Radicals in Biology and Medicine”. Ed 4. Clarendon Press, Oxford.

Sreeramulu D., Reddy C.V.K., Chauhan A., Balakrishna N., Raghunath M. (2013). “Natural Antioxidant Activity of Commonly Consumed Plant Foods in India: Effect of Domestic Processing”. *Oxid Med Cell Longev* 2013 12;2013:369479. http://dx.doi.org/10.1155/2013/369479.

Kaur C., Kapoor H.C. (2001). “Antioxidants in fruits and vegetables—the millennium's health”. *IntJ Food Sci Tech* 36(7) pp. 703–725

ORIGINAL PUBLICATIONS

CHAPTER 1: BIOCHEMICAL AND QUANTITATIVE PROTEOMICS INVESTIGATIONS IN ARABIDOPSIS *GGT1* MUTANT LEAVES REVEAL A ROLE FOR THE GAMMA-GLUTAMYL CYCLE IN PLANT'S ADAPTATION TO ENVIRONMENT

Serena Tolin, Giorgio Arrigoni, **Anna Rita Trentin**, Sonja Veljovic-Jovanovic, Micaela Pivato, B. Zechman and Antonio Masi

Proteomics 2013 Jun;13(12-13):2031-45. doi: 10.1002/pmic.201200479

Biochemical and quantitative proteomics investigations in Arabidopsis *ggt1* mutant leaves reveal a role for the gamma-glutamyl cycle in plant's adaptation to environment

Serena Tolin[1,2], Giorgio Arrigoni[2,3], Anna Rita Trentin[1], Sonja Veljovic-Jovanovic[4], Micaela Pivato[1], Bernd Zechman[5] and Antonio Masi[1]

[1]DAFNAE, University of Padova, Viale dell'Universita' 16, 35020 Legnaro (PD).

[2]Proteomics Center of Padova University, VIMM, and Padova University Hospital, Via G. Orus 2b, 35129 Padova, Italy.

[3]Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy

[4]Institute of Multidisciplinary Research, Kneza Viseslava 1, University of Belgrade, Serbia

[5]Karl-Franzens-University of Graz, Institute of Plant Sciences, Schubertstrasse 51, 8010 Graz, Austria

Abbreviations: 2-DE, two-dimension polyacrylamide gel electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; ECWF, extracellular washing fluid; ES, enrichment score; FDR, false discovery rate; FW, fresh weight; GGT, gamma-glutamyl transferase/transpeptidase; GO, gene ontology; GRX, glutaredoxins; GSH, reduced glutathione; GSSG, oxidized glutathione; IEF, isoelectric focusing; LMW low molecular weight; NAC, N-acetylcysteine; PM, plasma membrane; ROS, reactive oxygen species; SCX, strong cation exchange; PRX, peroxyredoxins; RT, room temperature; SAG, senescence-associated gene; SBD-F, ammonium 7-fluoro 2,1,3-benzooxadiazole-4-sulfonate; TRX, thioredoxins; TEM, transmission electron microscopy; WT, wild-type.

Key words: antioxidants; differential proteomics; gamma-glutamyl cycle; glutathione; oxidative stress.

ABSTRACT

The existence of a gamma-glutamyl cycle consisting of intracellular GSH synthesis, extrusion to the apoplastic space and recovery by gamma-glutamyl transferase (GGT)-assisted degradation into its constituent amino acids, has been demonstrated in plants. To address the significance of this cycle in plant cells, we performed integrated biochemical, immunocytochemical, and quantitative proteomics analyses in the *Arabidopsis thaliana ggt1* knockout mutant (lacking apoplastic GGT1 isoform) and its corresponding wild-type (WT). The *ggt1* knockout leaves exhibited an increased ascorbate and GSH content, increased apoplastic GSH content, and enhanced protein carbonylations in the low-molecular-weight range compared to WT. The combined iTRAQ and LC-MS/MS based quantitative proteomics approach identified 70 proteins (out of 1,013 identified proteins) whose abundance was significantly different in leaves of *ggt1* mutant compared to WT, with a fold change ≥1.5. Mining of the proteome data for GSH-associated genes showed that disruption of gamma-glutamyl cycle in *ggt1* knockout-leaves was associated with the induction of genes encoding four GSTs in the *phi* class (*GSTF2, GSTF6, GSTF9, and GSTF10*), a GSH peroxidase (*GPX1*), and glyoxylase II. Proteins with a lower abundance compared to the WT are involved in chloroplast functions, carbohydrate/maltose metabolism and vegetative storage protein synthesis. Present findings suggest that GGT1 plays a role in redox signaling. The disruption of the gamma-glutamyl cycle in the *ggt1* mutant results in pleiotropic effects related to biotic and abiotic stress response, antioxidant metabolism, senescence, carbohydrate metabolism and photosynthesis, with strong implications for plant's adaptation to environment.

1. INTRODUCTION

Climate changes are so rapid and unpredictable in recent years that they are likely to override plants' capacity to adapt, resulting in restricted plant growth and development, and consequently in reduced productivity. Developing crop plants with a modified tolerance to abiotic and biotic stresses is therefore a necessity, which demands modern, novel strategies to gain a thorough understanding of how plants respond to environmental changes. Intrinsically, plants adapt to the changes in their environment and avoid damage from abiotic and biotic factors, by activating evolved self-defense mechanisms.

There is now a wealth of evidence to indicate that many adverse environmental factors affecting plants at the cellular level take effect, at least in part, by promoting oxidative stress [1-3]. These effects are mediated by changes in the level of reactive oxygen species (ROS) in the apoplast - a complex structure surrounding plant cells. The role and properties of extracellular gamma-glutamyl transferase/transpeptidase (GGT, (5-L-glutamyl)-peptide:amino-acid 5-glutamyl transferase; EC 2.3.2.2.) in oxidative stress have been widely studied in animal cells [4], and more recently in plants [5-10]. The GGT is an ectoenzyme promoting cleavage of the gamma-glutamyl moiety of GSH and gamma-glutamyl-related compounds.

GGTs exist in all organisms and in multiple isoforms, sharing the characteristic of being extra-cytosolic. In animals, they are located on the plasma membrane (PM) with the catalytic site facing outwards [4]. In *Arabidopsis thaliana*, the two isoforms GGT1 and GGT2 are reportedly apoplastic, where GGT1 is cell-wall bound [10] and GGT2 is PM associated; GGT4 is vacuolar and assists in degradation of the GSH conjugates [6,9]. A fourth isoform, GGT3 is believed to be non-functional because it contains a truncated sequence [8].

While GGT is known to promote GSH degradation to cysteinylglycine (cys-gly) and glutamic acid, the significance of this reaction in plant metabolism is largely unclear. This step is part of the

gamma-glutamyl cycle consisting of the extrusion of GSH to the extracellular space, degradation into its constituent amino acids, followed by their reabsorption by means of amino acid transporters. In mammals, this cycle has been correlated with antioxidative responses involving GSH and has been implicated in intercellular and inter-organ cysteine delivery [11]. The GGT knockout mice exhibit abnormal growth and die prematurely, within 2 months of birth [12].

In plants, the involvement for GGT in antioxidant response remains poorly characterized. Under photo-oxidative stress induced by ultraviolet B exposure, cys-gly content in leaves was reported to increase throughout the period of exposure to radiation [13]. In another study aiming to isolate genes involved in protection against oxidative damage, a cDNA from *Arabidopsis* encoding a putative GGT was isolated and its expression in yeast conferred an enhanced tolerance to the thiol-oxidizing drug diamide [14]. While the vacuolar AtGGT4 has been demonstrated to drive the metabolism of GSH-conjugates, the functions of the apoplatic isoforms are less clear.

A functional genomics approach using knockout mutant lines has indicated no clear phenotype in *ggt1A. thaliana* lines, apart from a shorter life cycle represented by early flowering and premature senescence [15,8]. This phenotype was interpreted as the result of a difficulty in adapting to the environment, the shorter life cycle being an escape mechanism similar to the strategy adopted by plants that survive in harsh conditions, e.g. drought [16]. It should be noted that, in terms of their dependence on cysteine availability, animal cells behave very differently from plant cells: the former rely on external GGT for intracellular cysteine availability, whereas plant cells have an autonomous capacity for cysteine biosynthesis. The GGT mutations may therefore have less dramatic effects on plants than on animals.

With an aim to shed light on the metabolic readjustments due to the mutation in the *ggt1* knockout, we applied biochemical, immunocytochemical and quantitative proteomics approaches on leaf proteins from the *ggt1* and wild-type (WT) plants. For the quantitative proteomics, we utilized iTRAQ isobaric tags for relative and absolute quantitation in combination with liquid

chromatography tandem mass spectrometry (LC-MS/MS) on a high mass accuracy Orbitrap mass spectrometer. Our results indicate that the gamma-glutamyl cycle is part of the cell's coordinated response to the environment.

2. MATERIALS AND METHODS

2.1 Plant materials

After 4 days of stratification at 4°C in the dark, seeds from *A. thaliana* L. ecotype Columbia (Col-0) and a *ggt1* knockout mutant line were sown on soil and grown in a greenhouse. The *ggt1* knockout mutant (ecotype Columbia) was identified in the mutant collection [17] established at the Salk Institute, and is available from the Nottingham *A. thaliana* Stock Centre (http://nasc.nott.ac.uk; polymorphism SALK_080363). Leaf samples were harvested at the fully-expanded rosette stage, approximately one week before bolting, and stored at -80°C until use. For proteome analysis, samples were obtained after pooling leaves from ten independent plants per genotype.

2.2 Electron microscopy/immunogold labeling of ascorbate and GSH

Sample preparation for electron microscopy and immunogold labeling of ascorbate and GSH was performed as described previously [18,19]. Fixation of leaves was performed for 90 minutes in a mixture of 2.5% paraformaldehyde and 0.5% glutardialdehyde dissolved in 0.06 M phosphate buffer (pH 7.2). Samples were then washed for 60 minutes in buffer and dehydrated for 20 minutes at each step in increasing concentrations (50%, 70%, and 90%) of acetone. Infiltration was performed with LR-White resin (30%, 60% and 100%; London Resin Company Ltd., Berkshire, UK) and the samples were polymerized for 48 hours at 50 °C. Sections with a thickness of 80 nm were blocked for 20 minutes with 2% bovine serum albumine (BSA) dissolved in phosphate buffered saline (PBS, pH 7.2). Subsequently they were treated for 120 minutes with the primary antibodies (anti ascorbate rat polyclonal IgG, Abcam plc, Cambridge, UK and anti GSH rabbit polyclonal IgG, Millipore Corp., Billerica, MA, USA) diluted 1:300 (ascorbate antibody) and 1:50

(GSH antibody) in PBS containing 1% BSA (for ascorbate labeling) and 1% goat serum (for GSH labeling). After three short rinses in PBS sections were incubated for 90 minutes with 10 nm gold-conjugated secondary antibodies (goat anti rat IgG for ascorbate, goat anti rabbit IgG for GSH, British BioCell International, Cardiff, UK) diluted 1:100 (for ascorbate labeling) and 1:50 (for GSH labeling) in PBS. Sections were finally rinsed with distilled water. At least 20 (vacuoles and peroxisomes) to 60 (all other cell compartments) sectioned cell structures of a minimum of 15 different cells from at least four different samples per plant were analyzed for gold particle density. The data were statistically evaluated with the Mann-Whitney U test using Statistica (Stat-Soft, Tulsa, OK, USA, 2002) and presented as the number of gold particles per μm^{-2}.

2.3 Soluble antioxidant extraction

Frozen leaf samples (250 mg) from at least five biological replicates were ground with a mortar and pestle to extract soluble antioxidants with 0.1 N HCl and 1 mM EDTA. Following centrifugation at 10,000 g for 10 min, extracts were rapidly tested for ascorbate and low-molecular-weight (LMW) thiol levels.

2.4 Extracellular washing fluid extraction

A vacuum infiltration procedure was used for apoplast protein extraction [20]. Briefly, one gram of leaf tissue was washed in chilled H_2O and then submerged in 100 ml chilled vacuum infiltration buffer (50 mM KPi, pH 6.1) containing 5 µM N-acetylcysteine (NAC) in a vacuum desiccator. A vacuum was applied for 10 min at a pressure of 20 kPa using a vacuum pump to remove the gas from the apoplastic spaces. Excess buffer was removed from the WTand *ggt1* mutant leaves. Each leaf was then positioned vertically in a 20 ml syringe. The syringes were placed in centrifuge tubes and centrifuged at 200 g for 20 min at 4°C. Apoplastic extracellular washing fluid (ECWF) extracts were collected from the bottom of the tubes. NAC was used as a tracer and internal standard. It elutes in a chromatographic region devoid of other peaks, several minutes after the elution of GSH,

which is usually the last endogenous thiol compound to remain visible in chromatograms. ECWF extracts were then used to measure the LMW thiol and ascorbate content.

2.5 Ascorbate content

The ascorbate content was determined spectrophotometrically by measuring the absorbance at 265 nm, according to the Hewitt and Dickes method [21].

2.6 LMW thiol content

Prepared extract (50 µL) was derivatized with SBD-F fluorophore (Sigma-Aldrich, St. Louis, USA). LMW thiols were separated by isocratic HPLC using the method described in Masi et al. [13] with some adaptations. The mobile phase was 3% methanol in 75 mM NH_4^+-formiate, pH 2.9.

2.7 Carbonylated proteins analysis

Proteins were extracted from leaves of the *ggt1* mutant and WT plants using extraction buffer A (20 mM Tris pH 8.0, 3 M NaCl, 1 mM EDTA, and 1% SDS) in a ratio of 1:5 (w/v). Extracted proteins were subsequently precipitated with acetone. The pellet thus obtained was then resuspended in a solution (5 M urea, 2 M thiourea, 2% CHAPS, and 0.4% ampholites) compatible to two-dimension polyacrylamide gel electrophoresis (2-DE). Collected supernatant was subjected to protein quantification using the modified Lowry total protein kit (Sigma-Aldrich, St. Louis, USA), according to the manufacturer's instructions. Total protein (100 µg) from each sample was loaded onto IPG strips (7 cm, 3-10 pH range) (GE Healthcare Bio-Science AB, Uppsala, Sweden) for isoelectric focusing (IEF). After IEF, the strips were incubated with 10 mM DNPH solution (to derivatize the carbonylated proteins) in 10% TFA, followed by two washes with a washing solution (8 M urea, 20% glycerol, 1% SDS, and 0.5 M Tris HCl, pH 6.8). Treated IPG strips were then subjected to 2-DE using a 12% SDS-PAGE.

The 2D gels were then subjected to Western blot analysis, where the protein spots were transferred onto a nitrocellulose membrane (using Hoefer mini-VE transblot apparatus, Amersham Biosciences, Piscataway, USA) and saturated with the T-PBS blocking buffer (Triton X100 0.1% in PBS pH 7.4, plus 3% skimmed milk) for 1 h at room temperature (RT). The membranes were incubated with rabbit anti-DNP antibodies (Serologicals Corporation, Norcross, USA) (dilution 1:20,000) in T-PBS plus 3% milk for 1 h at RT, followed by washing with T-PBS. The membranes were incubated with secondary antibody (anti-goat anti-rabbit, dilution 1:50,000) for 1 h at RT. The peroxide-luminol reaction was used to detect cross-reacting proteins; the chemiluminescent reaction buffer was mixed with chemiluminescent reagent in a ratio of 2:1 (v/v) (Sigma-Aldrich, St. Louis, USA). Membranes were incubated in the prepared chemiluminescent solution for 5 min at RT and developed in a dark room.

2.8 Statistical analysis

Data from at least five replicates were submitted to an analysis of variance (ANOVA). The Tukey honest significant difference multiple comparisons procedure was used to discriminate between means. A $p < 0.05$ was considered significant for all comparisons.

2.9 Total leaf protein extraction for iTRAQ labeling and MS analyses

Leaves (0.5 g) were homogenized in liquid nitrogen to fine powder. Leaf powder was suspended in an extraction buffer (50 mM HEPES pH 8, 1% Triton X100, 1M NaCl, 1mM phenylmethanesulfonylfluoride, 1 mM benzamidine) with thorough mixing, followed by centrifugation at 10,000 rpm for 15 min at 4°C. To the collected clear supernatant, cold acetone was added to precipitate proteins at -20° C overnight, and centrifuged at 12,000 rpm for 15 min at 4°C. The protein pellet thus obtained was used for protein quantification using the modified Lowry method, as described above, for iTRAQ labeling.

2.10 iTRAQ labeling

The principle behind the method is described in Ross et al. [22]. Labeling was done with an iTRAQ® Reagents Multiplex Kit (AB Sciex, MA, USA). The iTRAQ experiment was performed on protein samples derived from pooled leaves (collected from 10 independent plants of each *ggt1* mutant and WT) as described above. Two technical replicates were carried out with tag swapping for each pooled sample. Protein content from each sample (WT and *ggt1* mutant leaves) was carefully quantified using the bicinchoninic acid (BCA) method (Sigma-Aldrich kit, St. Louis, USA). 500 μg of proteins from each sample were precipitated with cold acetone and kept overnight at -20 °C. The protein pellets were resuspended in an iTRAQ-compatible buffer (TEAB 0.5 M, SDS 0.1%) to a final concentration of 2-5 μg/μL. To avoid any variability in the precipitation and resuspension processes, protein content was quantified again using the bicinchoninic acid method. 100 μg of each sample was reduced, alkylated and digested with trypsin, according to the iTRAQ manufacturer's protocol. To check the digestion efficiency, 1 μg of each sample was analyzed by LC-MS/MS (details of the instruments and instrumental methods are given in the following section).Control and mutant samples were split into two identical 50 μg aliquots and labeled with different iTRAQ tags: the WT sample was labeled with tags 114 and 116, whereas the *ggt1* mutant sample with tags 115 and 117. Before labeling, to each sample, trypsin-digested BSA (2.5 pmol) was spiked as an internal standard for monitoring labeling efficiency. The labeling procedure was completed as specified in the iTRAQ manufacture's protocol. Prior to mixing the samples in a 1:1:1:1 ratio, 1 μg of each labeled sample was analyzed separately by LC-MS/MS to test labeling efficiency. The resulting data were checked against the database as explained below, setting the iTRAQ labeling as a variable modification. All the peptides were correctly identified as being iTRAQ-modified at the *N*-terminus and at each lysine residue. The samples were then pooled and dried under vacuum.

2.11 Strong cation exchange fractionation

Strong cation exchange (SCX) chromatography was performed on a SCX cartridge (AB Sciex, MA, USA). The labeled samples were dissolved in 500 μL of buffer A (10 mM KH_2PO_4, 25% acetonitrile, pH 3) and loaded onto the cartridge using a syringe pump with a 50 μL/min flow rate. The cartridge was washed 3 times with 500 μL of buffer A. Peptides were eluted in a stepwise manner with increasing concentrations of KCl in buffer A. The labeled peptides were eluted in 8 fractions (500 μL per fraction) with the following concentrations of KCl in the buffer A: 40, 60, 80, 100, 130, 170, 200, and 350 mM. The volume of each fraction was reduced under vacuum to remove acetonitrile. Samples were desalted using C18 cartridges (Sep-Pack, C18, Waters, Milford, MA, USA) according to the manufacturer's instructions. Samples were finally dried under vacuum and kept at -20°C until MS analysis.

2.12 LC-MS/MS analyses

Samples were resuspended in H_2O/0.1% formic acid and 1 μg of each fraction underwent LC-MS/MS analysis. The MS analyses were conducted with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Pittsburgh, CA, USA) coupled online with a nano-HPLC Ultimate 3000 (Dionex-Thermo Fisher Scientific). Samples were loaded onto a homemade 10 cm chromatographic column packed into a pico-frit (75 mm I.D., 10 mm tip, New Objectives) with C18 material (ReproSil, 300 Å, 3 μm). Peptides were eluted with a linear gradient of acetonitrile/0.1% formic acid from 3% to 50% in 90 min at a flow rate of 250 nL/min. According to the method described by Köcher et al. [23], the instrument performed a full scan at high resolution (60000) on the Orbitrap, followed by MS/MS scans on the three most intense ions with CID fragmentation on the linear trap. MS/MS scans were performed on the same ions with HCD fragmentation on the Orbitrap (with a resolution of 7500) to obtain low mass range data suitable for protein quantification. The peptides reliably identified in each sample were inserted in an exclusion list that was used to perform (under

the same chromatographic and instrumental conditions) a second LC-MS/MS run for each sample fraction.

2.13 Data analysis

The raw LC-MS/MS files were analyzed using Proteome Discoverer 1.2 (Thermo Fisher Scientific, Pittsburgh, USA). The software was connected to a Mascot Search Engine server, version 2.2.4 (Matrix Science, London, UK). The spectra were searched against an *A. thaliana* database (downloaded from TAIR version dated February 2012 with 33,596 entries and 13,743,691 residues) concatenated with a database of contaminant proteins commonly found in proteomic experiments. Enzyme specificity was set to trypsin with 2 missed cleavages. Peptide and fragment tolerance was set to 10 ppm and 0.6 Da, respectively. Methylthiocysteine, 4-plex iTRAQ at the *N*-terminus and Lys were set as fixed modifications, while methionine oxidation was selected as a variable modification. Based on the search against the corresponding randomized database, false discovery rates (FDR) of 5% and 1% were calculated by the Proteome Discoverer. The data were pre-filtered to exclude MS/MS spectra containing less than 5 peaks or with a total ion count below 50. Were considered as positive hits all proteins identified and quantified with at least two independent peptides with a high degree of confidence (FDR 1%). The quantification was performed normalizing the results on the median value of all measured iTRAQ reporter ratios. The list of quantified proteins was exported to Excel for further filtering and statistical analyses, which were conducted after removing from the final list the proteins that showed a discordant trend in the replicates. A ratio of mutant to WT $\geq$ 1.5 (fold change $\geq$ +50%) was set as the threshold for increased content, while a ratio of WT to mutant $\geq$ 1.5 was taken to indicate decreased protein content.

2.14 Bioinformatics analysis

The QuickGO web-based browser, provided by the UniProt-GOA group, was used to analyze Gene Ontology (GO) terms and annotations. The GO-term functional annotation was done using DAVID [24], which relies on a modified Fisher's exact *p*-value to cluster GO-terms in functional annotation groups, establishing whether the term is over-represented in a given proteomic data set. DAVID generates an enrichment score (ES) for each cluster, defined as the geometric mean of all the enrichment *p*-values for each annotation term associated with the gene members in the background group [25]. An ES higher than 1.3 is considered statistically significant (1.3 is equivalent to *p*-value < 0.05).

3. RESULTS

Consistent with previous findings [15], the *ggt1* mutants exhibited no obvious phenotype, rather only a shorter life cycle, presumably due to a general difficulty in adapting to the environment. On average, the *ggt1* knockout plants flowered three to four days earlier than the WT, followed by a premature senescence.

3.1 Soluble antioxidant content in leaves and in the leaf apoplastic space

Total ascorbate content, on a total leaf content basis, was approximately 35% higher in *ggt1* mutants (2.71±0.28 μmol per g FW^{-1}) than in WT (1.99±0.22 μmol per g FW^{-1}). The *ggt1* knockout leaves also carried higher total GSH, but not cys-gly, content compared to WT (Table 1).

	Leaf		**ECWF**	
	GSH	**Cys-Gly**	**GSH**	**Cys-Gly**
Col- 0	855.72 (± 44.61)	5.03 (± 0.14)	8.59 (± 1.19)	0.87 (± 0.23)
ggt1-	1183.25 (± 37.69) *	4.76 (± 0.21)	13.74 (± 3.64)*	0.11(± 0.05)**

Table 1.GSH and cysteinylglycine content measured by chromatographic separation and quantitation of their SBD-derivatives. Analyses were carried out on total leaf extracts and on leaf extracellular washing fluids (ECWF) obtained from *ggt1* mutant and WT plants. Values are calculated as the mean of at least 5 different experiments and are expressed in nmol·(g-1) FW. * and **, indicate significance at the 0.05, 0.01 level of confidence, respectively.

The LMW thiols were measured in the ECWF. Contamination level of the extracts identified by the infiltration-centrifugation technique was assessed by means of malic dehydrogenase activity measurements, and was consistently below 2% (data not shown). The quality of the extracts was also indirectly confirmed by the absence of γEC, a precursor in GSH biosynthesis and metabolically restricted in the cytoplasm, which was not detected in the ECWF. Our results point to a higher GSH content, and a drop in cys-gly content (approximately to one tenth) in *ggt1* mutant compared to WT extracts, which is due to the loss of apoplastic GGT activity.

NAC was used as a tracer and internal standard in this study (Supporting Figure S1). When leaves were infiltrated with a 5 μM NAC solution, the resulting concentration of NAC in the extract after centrifugation was approximately 4 μM, as a result of dilution with the fluid already existing in the apoplast. This result suggests that the actual concentration of the metabolites measured in the apoplastic space could be approximately 5 times higher. Under normal physiological conditions, in fact, the leaf apoplast is filled with air, which is replaced by the buffer solution during the infiltration step. This evidence may enable a better definition of the apoplastic redox potential for the GSH/GSSG couple, since redox potential depends on GSH concentration and the GSH/GSSG ratio.

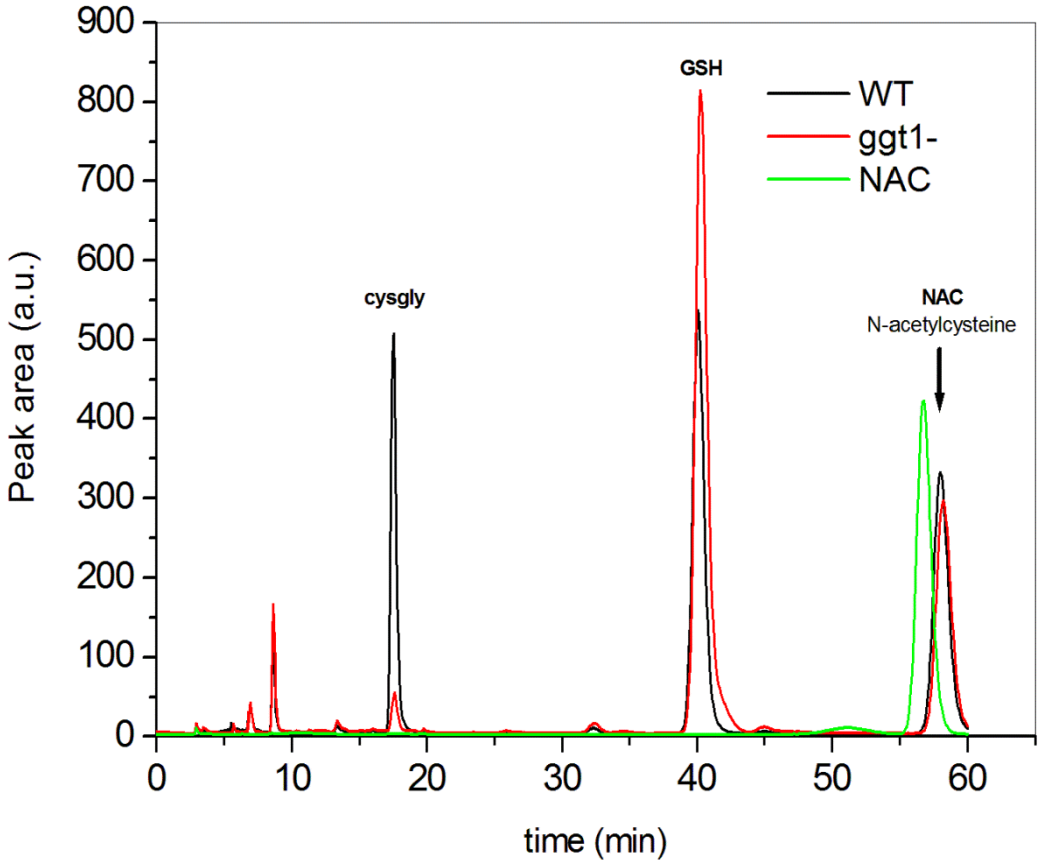


Figure S1. Representative chromatograms showing separation of low-molecular weight thiols from WT and *ggt1* mutant ECWF extracts. Leaves were infiltrated with infiltration buffer (50 mM KCl KPi, pH 6.1) containing 5 µM NAC. Following infiltration and centrifugation, 50 µL of ECWF were derivatized with SBD-F for chromatographic separation. Green line: NAC containing buffer solution; red line: ECWF from *ggt1* mutant leaves; black line: ECWF from WT mutant leaves.

3.2 Ascorbate and GSH labeling

Subcellular changes in ascorbate and GSH labeling were investigated by transmission electron microscopy (TEM) in leaves of the *ggt1* knockout and WT plants (Fig. 1). The distribution of ascorbate and GSH specific gold labeling in the WT leaves (Fig. 1a, c) was much the same as observed in previous studies [18,19], but compartment-specific changes were observable in

*ggt1*mutant (Fig. 1b, d). The ascorbate content was significantly higher in plastids (118%), peroxisomes (60%) and cytosol (44%), and remained at the same levels as in WT in the other cell compartments. The GSH content in *ggt1* mutant was significantly higher in mitochondria (23%), while remaining unchanged in nuclei and peroxisomes, but significantly lower in plastids (-62%) and cytosol (-28%). Most strikingly, GSH-specific labeling was common in the *ggt1* mutant apoplast (Fig. 1d), but not seen in control (Fig. 1c).

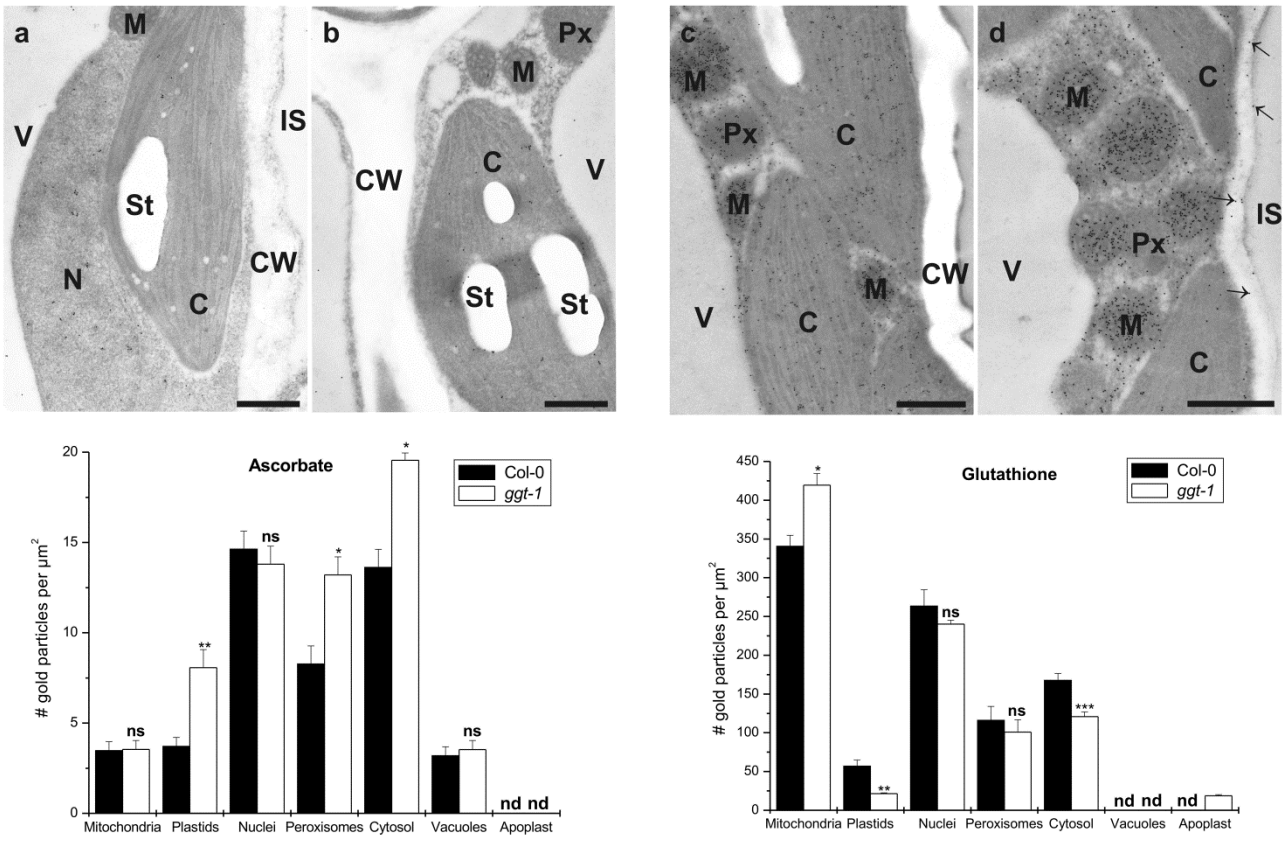


Figure 1. Subcellular localization of ascorbate and GSH in leaves of the *ggt1* knockout and WT plants. Transmission electron micrographs (upper panels) and quantitative analysis (lower graphs) show the overall distribution of gold particles bound to ascorbate (left panel) and GSH (right panel) in leaf cells of WT (a, c) and *ggt1* mutants (b, d). Values are represented as means with standard errors and document the amounts of gold particles bound to GSH per square micrometer in different cell compartments of mesophyll cells. Significant differences were calculated using the Mann–Whitney *U* test; *, ** and *** indicate significance at the 0.05, 0.01 and 0.001 level of confidence,

respectively. $n > 20$ for peroxisomes and vacuoles and $n > 60$ for all other cell structures. Abbreviations: Ns, non significance; nd, not detected; C, chloroplasts with or without starch (St); CW, cell walls; IS, intercellular spaces; M, mitochondria; N, nuclei; Px, peroxisomes; V, vacuoles, scale bar= 0.5µm.

3.3 2-DE analysis of carbonylated proteins

Proteins may undergo cleavage by hydroxyl radicals, and side chain modifications such as tyrosine hydroxylations and nitrations, and methionine or cysteine oxidation (by hydroxyl radicals or hydrogen peroxide). However, one of the most common modifications involves carbonyl formation, which has been widely used as an indicator of oxidative damage in several organisms and has been shown to increase in older tissues [26, 27]. The presence of oxidative damage on proteins under conditions of oxidative stress is revealed by detecting carbonyls through the immunodetection of coupled dinitrophenylhydrazine (DNPH). The great sensitivity of immunodetection enables the detection of even minute amounts of modified proteins.

We characterized the oxidized proteome in leaves (Fig. 2). The 2-DE combined with Western blotting allowed us to detect carbonylated protein spots as 2,4-dinitrophenylhydrazone (DNP)-derivatives. There were several spots of greater intensity in the LMW range, suggesting that protein carbonylations increased in the *ggt1* knockout leaf.

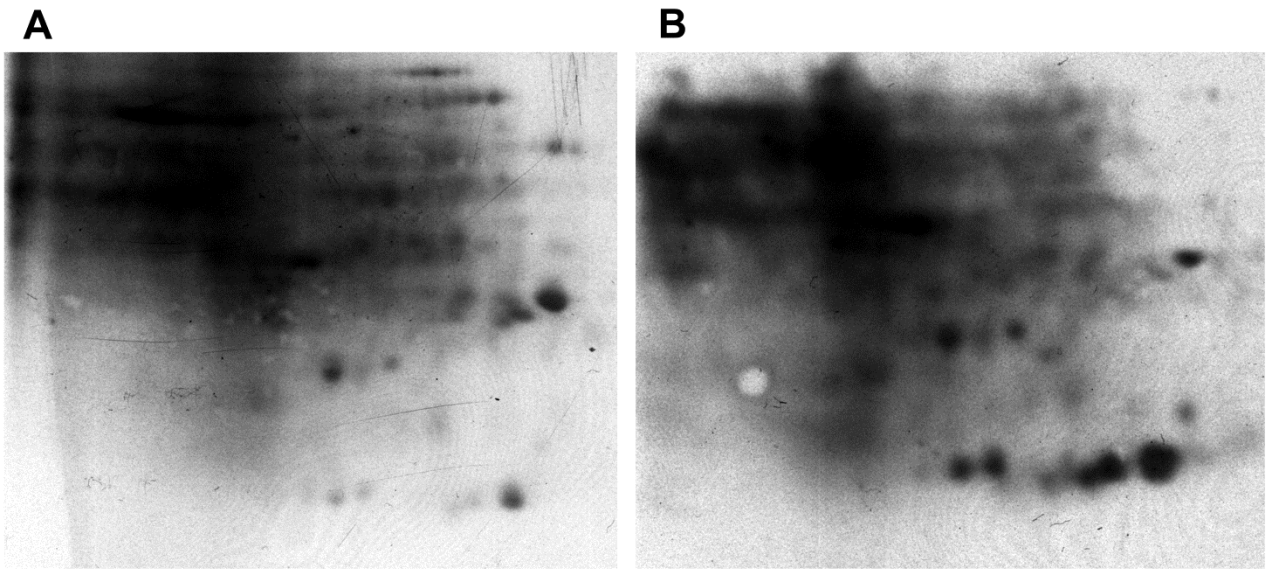


Figure 2. Detection of carbonylated proteins. 2-DE coupled withWestern blot analysis detects carbonylated proteins in leaves of the WT (A) and *ggt1* knockout (B) plants.

3.4 The iTRAQ and MS-based identification of differentially-accumulated proteins

The LC-MS/MS analysis of iTRAQ-labeled peptides resulted in identification of 1,013 proteins. Of which, 70 proteins were found to accumulate differentially with fold changes of ≥ 1.5 between *ggt1* mutant and WT (Tables 2 and 3) and 1% FDR. 46 proteins showed a higher abundance (fold change ≥ 1.5, measured as *ggt1*/Col-0 ratio), while 24 showed a lower abundance (fold change ≥ 1.5, measured as Col-0/*ggt1* ratio) (Tables 2 and 3, respectively). The full list of identified proteins and peptides is provided as a supporting material. Graphs showing the quality of the quantitative results obtained in the experiment are reported in Supplementary Figure S2.

UniProt ID/AC	Locus name	*ggt1*-/ Col-0 ratio	Description	% Cov.	# Pep.	Localization[a]
P46422	At4g02520	3.4	GSH S-transferase F2	47.64	9	A, C, c, ER/G, PM, V
P28493	At1g75040	2.8	Pathogenesis-related protein 5	38.08	7	A, CW, Ex, V
P42760	At1g02930	2.5	GSH S-transferase F6	28.37	6	A, CW, c, M, V

Q9LJR2	At3g15356	2.3	Lectin-like protein	22.51	7	A, CW
Q9STT3	At3g47800	2.2	AT3g47800/T23J7_130, Aldose 1-epimerase-like protein	5.03	2	ER/G
Q9XI36-2	At1g15340	2.1	Isoform 2 of Methyl-CpG-binding domain-containing protein 10	9.64	3	c, N
P31168	At1g20440	2.0	Dehydrin COR47 OS	14.34	3	c, ER/G
Q9LK72	At3g16530	2.0	AT3g16530/MDC8_16, Lectin-like protein	25.72	8	A, CW, N
O23138	At1g22840	2.0	Probable cytochrome c	18.42	3	c, M, V
Q96262	At4g20260	1.9	AT4G20260 protein, Endomembrane-associated protein	55.11	13	c, PM
Q9S7E4	At5g14780	1.8	Formate dehydrogenase	19.27	10	C, M
Q9SMU8	At3g49120	1.8	Peroxidase 34	25.78	9	A, CW, Ex, V
Q9FKK7	At5g57655	1.8	Xylose isomerase	18.24	8	ER/G, V
P43082	At3g04720	1.8	Hevein-like protein	14.62	3	not known
Q9C8L4	At1g53580	1.7	HydroxyacylGSH hydrolase 3	8.16	2	M
O49292	At1g77090	1.7	PsbP domain-containing protein 4	5.77	2	C
O80858	At2g30930	1.7	Expressed protein	37.8	5	C, PM
P42763	At1g76180	1.7	Dehydrin ERD14	25.41	4	C, c, PM
Q9LVI9	At3g17810	1.6	Putative dehydrogenase	12.91	5	C, c
O22160	At2g44920	1.6	Thylakoid lumenal 15 kDa protein 1	18.75	4	C
P31169	At5g15970	1.6	Stress-induced protein KIN2	39.39	2	C, N, PM
O81826	At4g27230	1.6	Probable histone H2A.3	19.85	3	N
Q93W28	At4g15545	1.6	AT4g15540/dl3810w, expressed protein	10.98	3	not known
B9DG18	At1g20620	1.6	AT1G20620 protein, Catalase 3	38.97	17	not known
P52032	At2g25080	1.6	Phospholipid hydroperoxide GSH peroxidase 1	28.39	6	C
Q9LEV3	At5g10860	1.5	CBS domain-containing protein CBSX3	12.14	3	M
Q9ZQ80	At2g03440	1.5	Nodulin-related protein 1	25.67	4	not known
Q9XI93	At1g13930	1.5	At1g13930/F16A14.27, uncharacterized	36.77	5	C
Q8L7R2	At2g17265	1.5	Homoserine kinase 1	4.86	2	C, c
Q9LZP9	At3g62410	1.5	CP12 domain-containing protein 2	26.72	4	C
P42799	At5g63570	1.5	Glutamate-1-semialdehyde 2,1-aminomutase 1	20.25	7	A, C

O49304	At1g23130	1.5	At1g23130/T26J12_10, Polyketide cyclase/dehydrase and lipid transport-like protein	15	2	not known
Q9SW21	At4g25050	1.5	Acyl carrier protein	20.44	3	C
F4I0N7	At1g75750	1.5	Gibberellin-regulated protein 1	34.02	4	not known
P42761	At2g30870	1.5	GSH S-transferase F10	30.23	6	CW, C, c, ER/G
Q56WK6	At1g72150	1.5	Patellin-1	39.97	25	A, C, c, ER/G, PM, V
Q9LIN0	At3g26450	1.5	Major latex protein, putative	33.55	5	not known
Q8VXZ7	At3g56310	1.5	Alpha-galactosidase	9.15	3	CW, c, V
Q9M2D8	At3g61260	1.5	Uncharacterized protein At3g61260	41.98	7	c, PM, V
O65660	At4g39730	1.5	AT4g39730/T19P19_120, Dehydration stress-induced protein	17.13	3	C, c, PM, V
Q9SVG4-2	At4g20830	1.5	Isoform 2 of Reticuline oxidase-like protein	8.33	5	A, c, CW, Ex, M, PM, V
Q9LW57	At3g23400	1.5	Probable plastid-lipid-associated protein 6	31.69	9	C, N, PM
O80852	At2g30860	1.5	GSH S-transferase F9	40.00	8	A, C, c, PM, V
Q9XFT3-2	At4g21280	1.5	Isoform 2 of Oxygen-evolving enhancer protein 3-1	43.50	10	C
Q42589	At2g38540	1.5	Non-specific lipid-transfer protein	44.07	6	A, C, CW, Ex,
O82291	At2g35490	1.5	Probable plastid-lipid-associated protein 3	9.57	3	C

Table 2. List of proteins whose content is increased in *ggt1* mutants. Proteins are listed in decreasing fold-change order, as defined by the *ggt1*/Col-0 ratio. The UniProtKB and TAIR accession ID are provided. Abbreviations: Cov., coverage; Pep., peptide; A, apoplast; CW, cell wall; C, chloroplast; c, cytosol and/or plasmodesma; ER/G, endoplasmatic reticulum/Golgi apparatus; Ex, extracellular region; M, mitochondrion; N, nucleus; PM, plasma membrane; and V, vacuole.

UniProt ID/AC	Locus name	Col-0/ ggt1 ratio	Description	% Cov.	# Pep.	Localization[a]

P21218	At4g27440	2.7	Protochlorophyllide reductase B	14.21	7	C
O49195	At5g24780	2.6	Vegetative storage protein 1	18.15	7	V
F4KII6	At5g24770	2.2	Vegetative storage protein 2	15.38	4	not known
F4K410	At5g13650	2.1	Elongation factor family protein	4.89	2	not known
Q8L7S8	At5g26742	1.9	Isoform 2 of DEAD-box ATP-dependent RNA helicase 3	5.35	4	C, N
P25853	At4g15210	1.8	Beta-amylase 5	28.51	11	c
F4HRB4	At1g45201	1.7	Triacylglycerol lipase-like 1 protein	4.91	2	not known
Q9SJL8	At2g36880	1.7	S-adenosylmethionine synthase 3	23.59	8	c, PM
Q9LUT2	At3g17390	1.7	S-adenosylmethionine synthase 4	21.12	8	CW, c, PM, V
P19456	At4g30190	1.6	ATPase 2, plasma membrane-type	25.11	22	c, ER/G, PM, V
Q8RY94	At4g18440	1.6	Adenylosuccinate lyase	33.4	18	C
Q39099	At2g06850	1.6	Xyloglucan endotransglucosylase/hydrolase protein 4	7.09	2	A, CW, C, Ex
Q9XFS9	At5g62790	1.6	1-deoxy-D-xylulose 5-phosphate reductoisomerase	11.74	6	C
P56804	AtCg00330	1.5	30S ribosomal protein S14	29	3	C, c
O50008	At5g17920	1.5	5methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	35.95	29	C, c, ER/G, PM, V
Q9LK36	At3g23810	1.5	Adenosylhomocysteinase 2	18.97	11	c, ER/G, PM, V
Q6NQA8	At1g14250	1.5	AT1G14250 protein	10.45	4	V
F4KFA3	At5g03360	1.5	DC1 domain-containing protein	1.12	3	not known
P24636	At5g44340	1.5	Tubulin beta-4 chain	17.57	8	CW, C, c, ER/G
Q94K48	At3g62530	1.5	Armadillo/beta-catenin-like repeat-containing protein	7.24	2	C, ER/G, M, N
P59259	At1g07660	1.5	Histone H4	50.49	5	N
Q8LPR9	At1g06950	1.5	Protein TIC110	5.31	5	C
Q9CA67	At1g74470	1.5	Geranylgeranyl diphosphate reductase	17.56	8	C
P16127	At4g18480	1.5	Magnesium-chelatase subunit chlI	20.05	8	C, CW

Table 3. List of proteins whose content is decreased in *ggt1* mutants. Proteins are listed in decreasing fold-change order, as defined by the Col-0/*ggt1* ratio. Rest is the same as mention in Table 2.

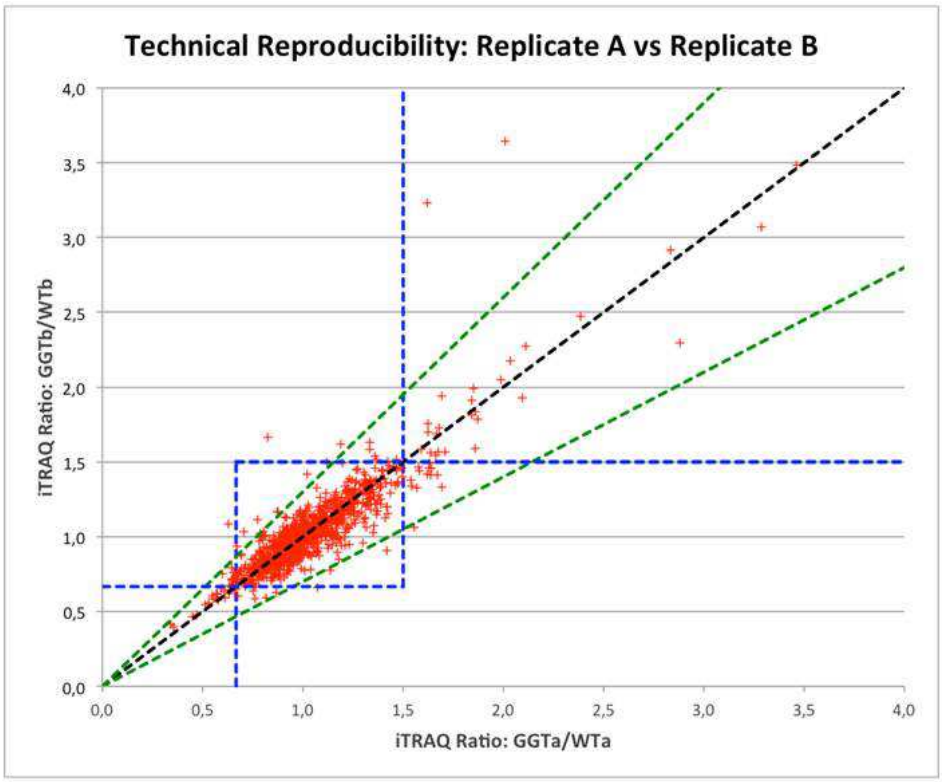


Figure S2. The graphs show the quality of the quantitative data and the reproducibility obtained for the iTRAQ experiment.

Proteins showing significantly different abundance in mutant *vs* WT leaves were subjected to GO annotations. Their localization is shown in Tables 2 and 3, and graphically presented in Fig. 3a and b. The biological processes in which the proteins are involved are listed in Tables S2 and S3 (Supporting Material), and also presented as pie charts in Fig. 3c and d. Notably, a considerable number of the proteins whose abundance was increased, including those with the highest fold change, localize in apoplast. This observation is consistent with the DAVID analysis. The DAVID functional annotation tool enables a given set of proteins to be classified in clusters of protein groups that are significantly enriched by comparison with the background data set (i.e., all

identified proteins). An algorithm calculates the ES for each cluster (see Materials and Methods). DAVID analysis identified four clusters with a significant ES for proteins with increased level and two clusters for proteins with decreased level.

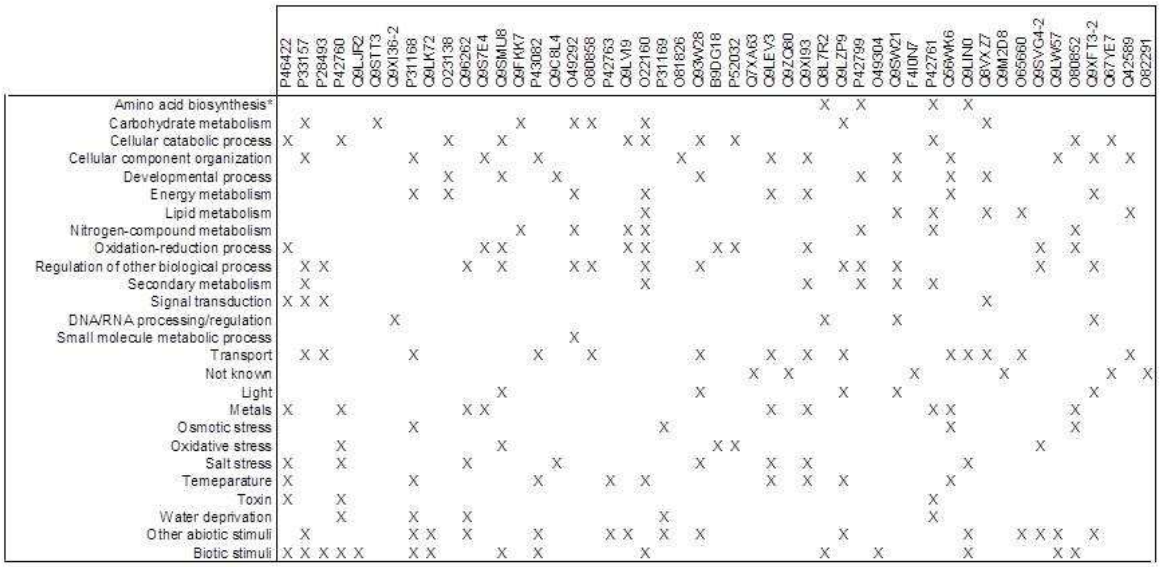
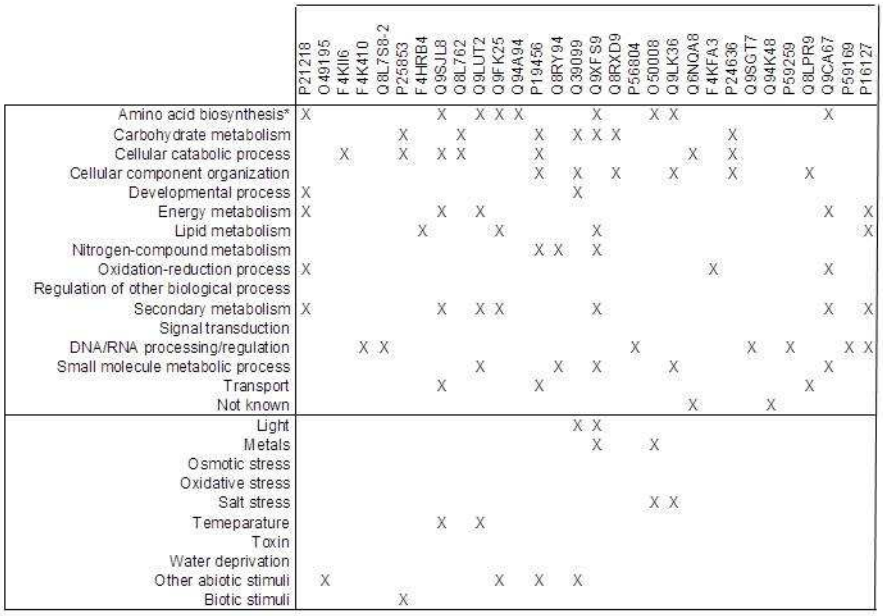


Table S2. Biological processes of proteins whose content is increased. Table lists the biological process in which the proteins are involved, according to GO annotations. The lower box indicates the response to several abiotic and biotic stimuli.

* Amino acid biosynthesis category included also proteins involved in amino acid modification processes.



	P21218	O49195	F4KII6	F4K410	Q8L7S8-2	P25853	F4HRB4	Q9SJL8	Q8L762	Q9LUT2	Q9FK25	Q94A94	P19456	Q8RY94	Q39099	Q9XFS9	Q8RXD9	P56804	O50008	Q9LK36	Q6NQA8	F4KFA3	P24636	Q9SGT7	Q94K48	P59259	Q8LPR9	Q9CA67	P59169	P16127
Amino acid biosynthesis*	X							X		X	X	X				X			X	X								X		
Carbohydrate metabolism						X			X				X		X	X	X						X							
Cellular catabolic process			X			X		X	X				X								X		X							
Cellular component organization													X		X		X			X			X				X			
Developmental process	X														X															
Energy metabolism	X							X		X																		X		X
Lipid metabolism							X				X					X														X
Nitrogen-compound metabolism													X	X		X														
Oxidation-reduction process	X																					X						X		
Regulation of other biological process																														
Secondary metabolism	X							X		X	X					X												X		X
Signal transduction																														
DNA/RNA processing/regulation				X	X													X						X		X			X	X
Small molecule metabolic process										X				X		X				X								X		
Transport								X					X														X			
Not known																					X				X					
Light															X	X														
Metals																X			X											
Osmotic stress																														
Oxidative stress																														
Salt stress																			X	X										
Temeparature								X		X																				
Toxin																														
Water deprivation																														
Other abiotic stimuli		X									X		X		X															
Biotic stimuli						X																								

Table S3. Biological processes of proteins whose content is decreased. Table lists the biological process in which the proteins are involved, according to GO annotations. The lower box indicates the response to several abiotic stimuli and the biotic ones.

* Amino acid biosynthesis category included also proteins involved in amino acid modification processes.

Concerning the proteins showing higher abundance, one cluster refers to localization, and shows that proteins belonging to the extracellular compartment (apoplast, cell wall and extracellular region) are present in significant amounts (ES 1.57). This result indicates that the main perturbation due to the *ggt1* mutation occurs in the same compartment where the protein is localized (see Table 2 and Fig. 3a). Other major clusters of protein groups relate to defense and response to pathogens (ES 1.71), and abiotic stress (ES 1.52). The remaining cluster of induced proteins includes enzymes metabolically related to GSH and/or involved in detoxification processes (ES 1.43). Proteins with lower content in mutant leaves are mostly related to one-carbon and cysteine metabolism (ES 1.42), and carbohydrate metabolic processes (ES 1.36). This analysis is further supported by the visualization shown in Fig. 3 c and d.

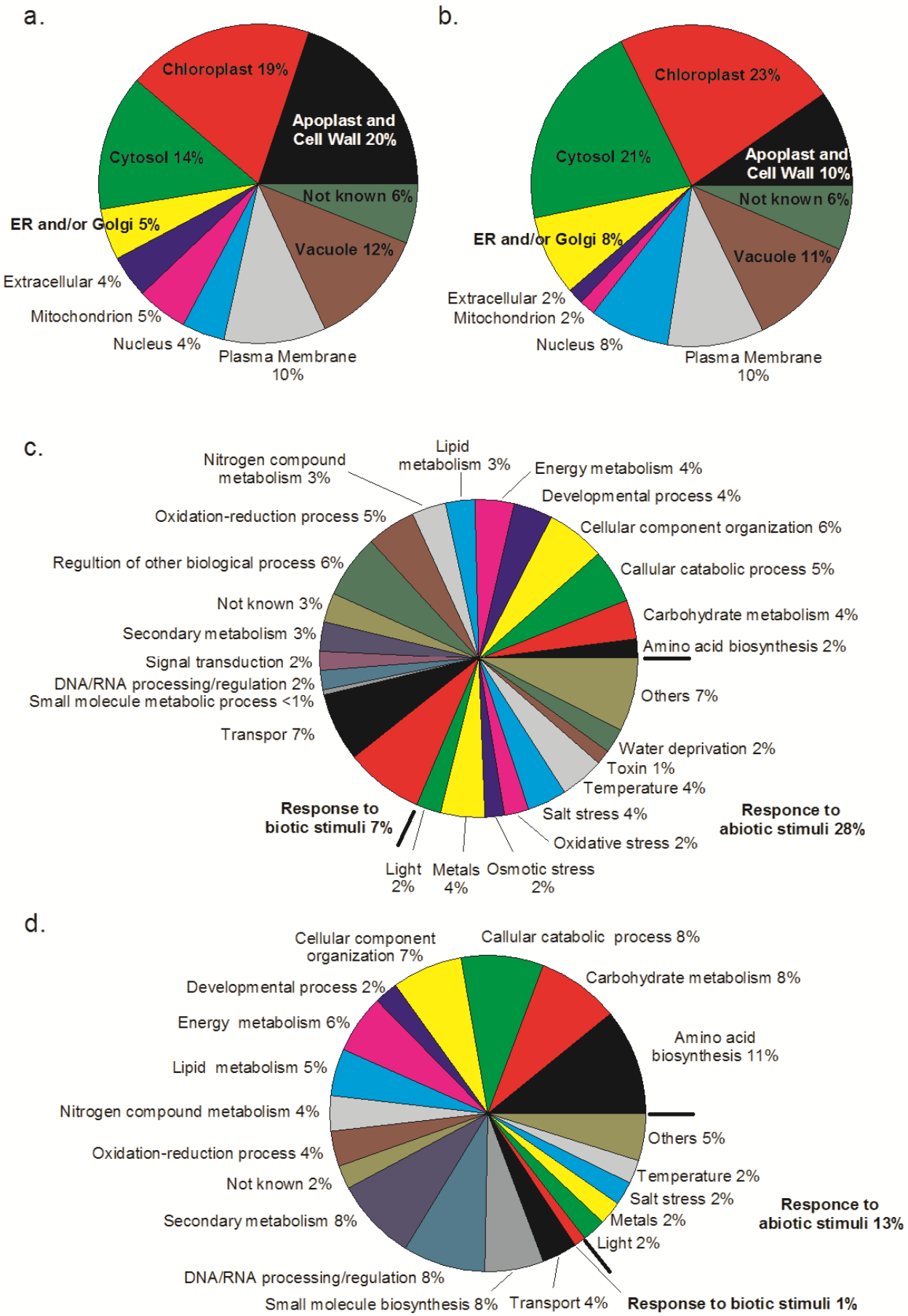

a.
Chloroplast 19%
Apoplast and Cell Wall 20%
Cytosol 14%
Not known 6%
ER and/or Golgi 5%
Vacuole 12%
Extracellular 4%
Mitochondrion 5%
Nucleus 4%
Plasma Membrane 10%
b.
Chloroplast 23%
Cytosol 21%
Apoplast and Cell Wall 10%
Not known 6%
ER and/or Golgi 8%
Vacuole 11%
Extracellular 2%
Mitochondrion 2%
Nucleus 8%
Plasma Membrane 10%
c.
Nitrogen compound metabolism 3%
Lipid metabolism 3%
Energy metabolism 4%
Developmental process 4%
Oxidation-reduction process 5%
Cellular component organization 6%
Regultion of other biological process 6%
Callular catabolic process 5%
Not known 3%
Carbohydrate metabolism 4%
Secondary metabolism 3%
Amino acid biosynthesis 2%
Signal transduction 2%
DNA/RNA processing/regulation 2%
Small molecule metabolic process <1%
Others 7%
Transpor 7%
Water deprivation 2%
Toxin 1%
Temperature 4%
Response to biotic stimuli 7%
Salt stress 4%
Responce to abiotic stimuli 28%
Oxidative stress 2%
Light 2%
Metals 4%
Osmotic stress 2%
d.
Cellular component organization 7%
Callular catabolic process 8%
Carbohydrate metabolism 8%
Developmental process 2%
Energy metabolism 6%
Amino acid biosynthesis 11%
Lipid metabolism 5%
Nitrogen compound metabolism 4%
Others 5%
Oxidation-reduction process 4%
Temperature 2%
Not known 2%
Salt stress 2%
Secondary metabolism 8%
Metals 2%
Responce to abiotic stimuli 13%
Light 2%
DNA/RNA processing/regulation 8%
Small molecule biosynthesis 8%
Transport 4%
Response to biotic stimuli 1%

Figure 3. GO annotation of identified differential proteins. Cellular component (a, b) and functional (c, d) classification of identified proteins with higher expression (a, c) and lower expression (b, d) proteins in *ggt1* mutant *vs* WT. Classification was carried out using GO database at UniProtKB (http://www.uniprot.org/). Percentage values indicate the proportion of total number of proteins within that category with respect to the total hits. The biological processes in which each protein is involved are listed in Table S2 and S3 (Supplemental Material).

4. DISCUSSION

As reported in the literature and confirmed here, the *ggt1*knockout plants exhibit no obvious phenotype except for early flowering and accelerated senescence. It should be noted that plants used in this study were at the stage of fully-expanded leaf, before bolting, and had no visible signs of senescence in the rosette. Therefore, the observed variations cannot be attributed to an age-dependent or developmental senescent state, but rather to unknown redox signals arising from the apoplast which trigger plant's adaptation to environment. The implication of a redox control by the gamma-glutamyl cycle has been highlighted in a previous work [10], in which exposure to the thiol oxidizing drug diamide with a concomitant GGT inhibition by serine-borate resulted in net GSH extrusion from barley roots.

In accordance with that, we show that GSH degradation in apoplast is required for intracellular redox balance, since disrupting GGT function also results in oxidative damage to some protein components. Consequently, there is an up-regulation of a number of enzymes involved in antioxidative and stress response. Proteomics analysis revealed metabolic adjustments in the *ggt1* mutants, which in turn affected different compartments and functions.

4.1 The *ggt1*-induced metabolic alterations

4.1.1 Chloroplast functions

A number of chloroplastic proteins were found at increased level in the *ggt1* mutant samples, such as PsbP-like protein (At1g77090), 15 kDa thylakoid lumen protein 1 (At2g44920), chloroplastic lipase/lipoxygenase (At4g39730), and a chloroplastic aminotransferase (At5g63570) involved in chlorophyll biosynthesis. Among others, it is worth noticing the up-regulation of a chloroplastic (stromal) dehydrogenase (At3g17810), which is a senescence-related protein; and of CP12-2 (At3g62410), a small protein acting as a linker in the assembly of a core complex of PRK/GAPDH. CP12-2 has been shown to undergo conformational changes depending on redox conditions, thus causing the reversible inactivation of GAPDH and PRK, and which is also reportedly involved in cellular response to heat, cold, and anoxia [28]. The level of a protein involved in defensive response to bacteria and ozone, namely chloroplastic plastid- and lipid-associated protein 6 (At3g23400) was also increased. Although the chloroplastic peptide methionine sulfoxide reductase B2 (At4g21860) was only slightly up-regulated with a fold change of 1.4, this protein has a protective role against oxidative stress by reactivating proteins that have been inactivated by methionine oxidation [29].

Some chloroplast protein components were also repressed, and the most strongly down-regulated protein was protochlorophyllide reductase B (At4g27440). Two more proteins directly involved in the chlorophyll biosynthetic process are less abundant in *ggt1*. One is a redox-sensitive magnesium-chelatase subunit (At4g18480) that is active under reducing conditions but inactive under oxidizing conditions. The other one is At1g74470 protein, which catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate, providing phytol for both tocopherol and chlorophyll synthesis. Other chloroplastic proteins with lowered content were TIC110 (At1g06950), involved in protein precursor importing, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (At5g62790), required for

chloroplast development, and RNA helicase 3 (At5g26742), which contains a motif that controls ATP binding and hydrolysis.

4.1.2 Stress response

Several altered proteins were found to be involved in the stress response, such as a dehydrin (At1g76180) that acts as a chaperone, preventing the aggregation and/or inactivation of various substrates. Protein KIN2 (At5g15970) is almost identical to KIN1, a cold-regulated *Arabidopsis* protein. KIN1 has been suggested to have amino acid sequence similarities with type I fish antifreeze proteins [30]; it is induced by ABA, drought, cold and salinity stresses [31,32]. The polyketide cyclase/dehydrase and lipid transport-like protein (At1g23130) are also involved in response to stress and biotic stimuli. The gene product of At1g13930 is an uncharacterized chloroplastic protein, whose expression reportedly correlates with the plant's exposure to cadmium [33].

We also identified catalase 3 (AT1G20620) and CBSX3, a protein involved in cell redox homeostasis (that is known to participate in the mitochondrial NADP-thioredoxin system) [34]. The isoform 2 of reticulin oxidase-like protein (At4g20830) is believed to be secreted in response to oxidative stress.

4.1.3 Plant-pathogen response

Several proteins accumulating differentially are related to plant-pathogen interactions. For example, glucan endo-1,3-beta-glucosidase (PR2, At3g57260) and PR5 protein (AT1G75040) are implicated in defending plants against pathogens and establishing a systemic acquired resistance (SAR). The lectin-like proteins (At3g15356 and AT3g16530) and the hevein-like protein (At3g04720) are involved in recognizing pathogens, and defending against fungi and incompatible reactions. ACP4 (acyl carrier protein, At4g25050) plays a major part in the biosynthesis of fatty acids in leaves,

being essential not only in the biosynthesis of the cuticular wax and cutin polymers in leaves, but also in establishing a SAR.

4.1.4 GSH metabolism

Analyzing the list of proteins with a higher abundance in *ggt1* mutant samples revealed the presence of several phi-class GSH S-transferases. In particular, GSTF2 (At4g02520) and GSTF6 (At1g02930) showed the greatest variation. The rapid induction of these two GSH S-transferases was demonstrated in *Arabidopsis* following infection by an avirulent strain of *Pseudomonas syringae*, and also as a result of combined salicylic acid and ethylene signaling [35]. GSH S-transferase GSTF9 (At2g30860) and GSTF10 (At2g30870) are implicated in detoxification and stress response. GSTF10 is reportedly involved in stress tolerance. Its overexpression conferred a greater tolerance to salt and a disturbed redox status in transgenic plants, while its down-regulation by RNA interference reduced the plants' tolerance to abiotic stress and accelerated their senescence [36]. Besides having a major role in xenobiotic conjugation and detoxification, GSTs might behave like a peroxidase and catalyze other GSH-dependent reactions, such as dehydroascorbate reduction. They may therefore help to maintain the cells' redox state. In particular, GSTF9 has a GSH peroxidase activity [37].

In addition to the four phi-class GSTs, two additional GSH-metabolizing enzymes were found to be altered in the *ggt1* mutant. One is the chloroplastic AtGPX1 (glutathione peroxidase 1, At2g25080), which prevents oxidative damage by catalyzing hydrogen peroxide, lipid peroxides and organic hydroperoxide reduction by GSH. Another is hydroxyacylglutathione hydrolase 3, also annotated to encode glyoxalase 2, a thiolesterase (At1g53580) that catalyzes the hydrolysis of S-D-lactoyl-glutathione to release GSH and lactic acid. This latter reaction is relevant in the removal of toxic methylglyoxal, an oxygenated short aldehyde produced by glycolysis or lipid peroxidation

that accumulates in plants under environmental stresses [38]. This enzyme is located in mitochondria, where immunocytochemical analysis reveals an increased GSH content (Fig 1).

The level of several proteins involved in cys and met biosynthesis was lower in *ggt1* mutant samples (Fig. 3d, Table S3), suggesting a slower rate of sulfur amino acid biosynthesis. This evidence may correlate with an increase in the extracellular GSH caused by GGT mutation, acting as a negative signal for the sulfur assimilation pathway [39,40].

4.1.5 Thiol redox homeostasis

Other proteins metabolically related to GSH and also involved in redox homeostasis are peroxiredoxins (PRX), glutaredoxins (GRX) and thioredoxins (TRX). While several of the proteins were identified in the MS/MS analysis, none of these components are included in Table 2 because the relative change in their expression fell below the established threshold fold change. However, a better look at the full list (Supplemental Table S4) shows some variations that may be relevant. While adopting as a threshold a fold change of 1.5 represents a trade-off enabling a better screening of statistically significant variations, from a biological perspective it may underestimate the effect of variations in proteins with a higher coefficient of flux control over metabolism, such as regulatory enzymes.

The fold change variation in the GRX, PRX and TRX is shown in Fig. 4. All six PRX were up-regulated (1.2-1.4 folds) as were the eight TRX (see Supplemental Table S4), but only one of the three GRX showed a comparable up-regulation. Together with GSH, these proteins participate in redox homeostasis; GSH has been shown to be the preferred electron donor for PRX [41]. The redox potentials determine whether enzymes are more reducing or more oxidizing, and differ significantly among the various enzymes. In general, however, TRX are better disulfide bond reducers than GRX [42].

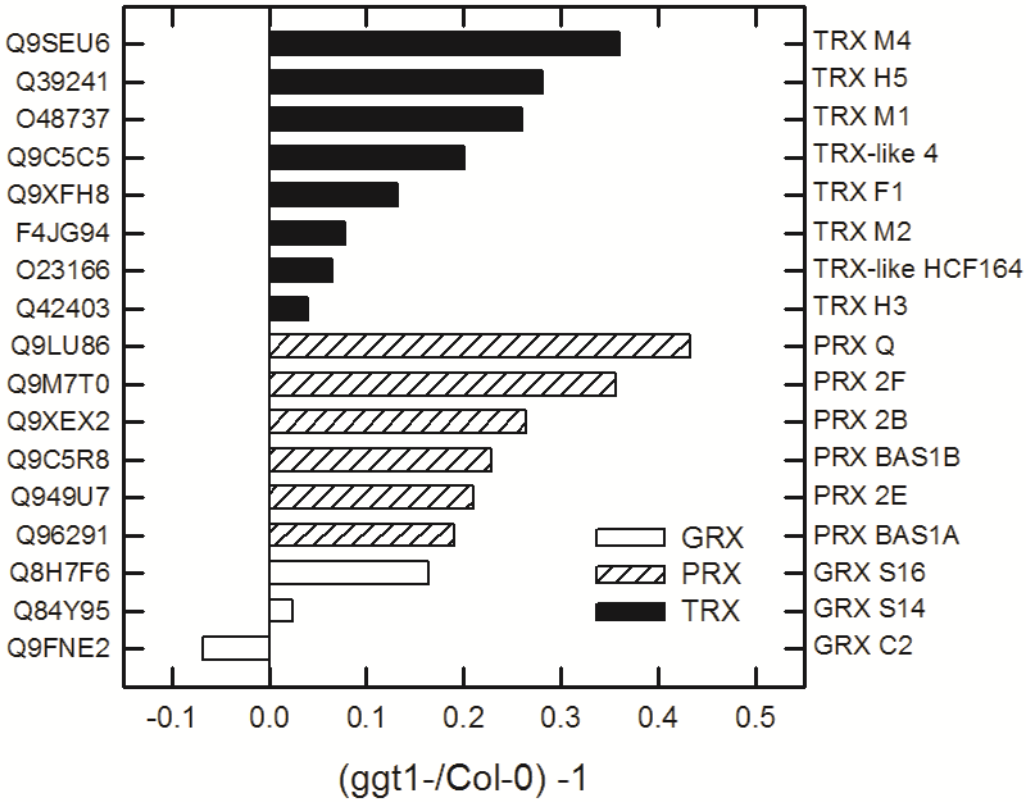


Figure 4. Thioredoxins (TRX), peroxiredoxins (PRX) and glutaredoxins (GRX) enzymes are indicated both by UniProt entry name/accession number (on the left) and by protein name abbreviation (on the right). The histogram shows the fold change-variation for these enzymes in *ggt1* mutant *vs* WT plants calculated as (*ggt1*/Col.0)-1 (data from Table S1, Supplementary Material).

4.1.6 Carbohydrate metabolism

Carbohydrate metabolism, and maltose metabolism in particular, was found to be suppressed. For example, a protein BAM5 (At4g15210) promotes hydrolysis of alpha-D-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains. Consistent with this function, another protein with lower expression in maltose metabolism is amylomaltase (At2g40840).

4.1.7 Others

Among the proteins whose level was increased in *ggt1* mutants, we identified a nuclear MBD10 (At1g15340). This protein is probably a transcription regulator with a potential role in controlling chromatin structure mediated by CpG methylation. Hormonal disturbances might explain the alteration of GASA1 (At1g75750), a gibberellin-regulated protein. The accumulation of cytosolic aldose 1-epimerase (At3g47800), a component of exose metabolism, cytochrome c (At1g22840) in the mitochondrial respiratory chain, and mitochondrial formate dehydrogenase, suggests that energy metabolism and primary processes are also affected. Formate dehydrogenase, which may also use formyl-GSH as a substrate [43], was reportedly induced by stress [44] and ROS [45]. Formate derived from the detoxification of formaldehyde generated by glycine decarboxylase under light can be oxidized to generate NADH in the mitochondrial matrix.

4.2 Alteration of redox homeostasis in the *ggt1* mutant: similarities with senescence, stress and antioxidant response

Our results point to a metabolic scenario having some similarities with senescence. For example, the strong suppression of chlorophyll biosynthesis can be gauged by the down-regulation of protochlorophyllide reductase B. On the other hand RuBisCO expression is typically depressed in senescence, but it was unaffected in this study. This view is further confirmed by other observations. Comparing our protein expression list with a previous large-scale study on senescence-associated genes [46] it was found that only some of the proteins whose content is increased in our study (xylose isomerase, glyoxalase II, catalase 3, and the CBSX3 of unknown function) are perhaps associated with senescence. The level of vegetative storage proteins VSP1 (At5g24780) and VSP2 (At5g24770), two sequentially expressed genes associated with senescence, and thought to serve as a storage buffer between nitrogen losses from senescing leaves and during grain filling [47] - strongly decreased in leaves of the *ggt1* mutant. We argue that energy is needed

to cope with the stressful conditions induced by the *ggt1* mutation, and this is diverted from storage processes.

One more difference relates to the senescence-associated genes (SAGs). SAG2 and SAG12 have been found to be associated with senescence [48]. In our study, SAG2 (thiol protease aleurain, At5g60360) expression was higher, but only with a fold change of 1.3 (see supplemental list S1 and S4), which meant that it was not included in Table 2. Surprisingly, we did not observe a change of SAG12 (At5g45890), which is considered to be a senescence-induced marker [49]. In another study, SAG12 was not induced by oxidative stress conditions, which is capable of triggering the expression of other senescence-associated genes [50]. These findings point to the onset of a complex array of metabolic adjustments in leaves of the *ggt1* mutant, as seen during senescence, and in response to biotic and abiotic stimuli, as well as other stress factors.

4.2.1 Hydrogen peroxide homeostasis in apoplast

The deployment of defense systems in the *ggt1* mutants is a consequence of altered GSH degradative metabolism in the apoplast. While GSH content in the apoplast is low (compared with ascorbate) [51,52], our study evidenced an higher content of GSH in *ggt1* mutant apoplast (see Table 1).

The accumulation of apoplastic hydrogen peroxide as a result of ROS generation by plasma membrane NADPH oxidases or cell wall peroxidases is a known response to various stress stimuli and is assumed to be involved in the signaling networks leading to transcriptional reprogramming [53]. It has been shown that ROS-generating peroxidases PRX33 and PRX34 are responsible for the oxidative burst [50] and play an important role in *Arabidopsis* resistance to pathogens [54]. In the *ggt1* mutant PRX34 (At3g49120) is more abundant (1.8 fold compared with the WT), suggesting that apoplastic glutathione, or some molecules associated with the GGT reaction, may regulate cell wall peroxidase expression and H_2O_2 formation; as such, they may therefore be considered as actors

in apoplastic redox signalling. Thiols are candidate molecules for signal transduction because they engage several redox reactions with target molecules [55]. It is tempting to speculate that this signal molecule might be GSH itself, accumulating in the apoplast when GGT is disrupted. Alternatively, it could be cys or cys-gly, downstream from the degradation process. In this regard, cys-gly is highly reactive and highly liable to oxidation; it seems a good candidate in redox signaling, which could interact with plasma membrane redox sensitive thiol cysteines [56,57].

4.3 Concluding remarks

A feature common to the above mentioned responses in the *ggt1* mutant could be redox alterations. Cellular redox equilibrium is maintained by a network of redox proteins and redox couples, such as NAD and NADP, along with the antioxidant molecules ascorbate and GSH, acting synergistically with other signaling molecules, such as salicylic acid [58,59]. The ascorbate-glutathione cycle is a key part of the network of reactions involving enzymes and metabolites with redox properties for the detoxification of ROS. The loss of function due to the *Arabidopsis* GGT1 knockout mutation triggers highly specific changes in compartmental interplay of ascorbate and GSH in cells. These changes accompanied remarkable rearrangements in proteome, which resemble those induced under specific abiotic and biotic stresses. To put it simply, a sort of “alert response” is activated in *ggt1* mutant leaves even in the absence of a real environmental threat, suggesting a failure in correct redox sensing. In line with this observation, further experiments will be carried out to describe the *ggt1* mutant adaptive response under oxidative conditions, which might result in a more severe phenotype.

Redox reactions in the extracellular space can accomplish several functions. They can participate in building the extracellular matrix or control redox sensitive enzymes. The latter function helps cells to adapt cell metabolism (in order to produce energy, build molecules, and defend themselves against oxidative damage) to events occurring in the outside environment, where unfavorable conditions may give rise to ROS with an altered redox balance and potentially detrimental effects

on plant functions and growth. Signals arising from the apoplast thus have a key role in plant's adaptation to environment [53].

Investigating the signal transduction pathway linking extracellular GSH degradation to the intracellular redox balance is an important goal of future research with a view to finding the key genes/proteins involved in the cross talk between the different stress responses, and to identifying candidates for future manipulation to improve plants' stress resistance.

ACKNOWLEDGMENTS

The authors wish to thank Prof. Massimo Ferretti (Pousada Horizonte Azul, Boipeba, Brazil) for his valuable comments and advice, and also the Cassa di Risparmio di Padova e Rovigo (Cariparo) for financing the purchase of the LTQ-Orbitrap XL mass spectrometer. This work was funded by the MURST ex-60%. B.Z. was supported by the Austrian Science Fund (FWF P22988).); G.A. was supported by a grant from University of Padova (CPDA082784/08). The authors have no conflict of interest to declare.

5. REFERENCES

[1] Scandalios, J. G., *Oxidative stress and the molecular biology of antioxidant defenses*, Cold Spring Harbor Laboratory Press, Plainview, NY, USA 1997.

[2] Noctor, G., Foyer, C. H., Simultaneous measurement of foliar glutathione, gamma-glutamylcysteine, and amino acids by high-performance liquid chromatography: comparison with two other assay methods for glutathione. *Anal. Biochem.* 1998, *1*, 98-110.

[3] Asada, K.,The water–water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. 1999, *50*, 601-639.

[4] Tate, S. S., Meister A., Serine-borate complex as a transition state inhibitor of □-glutamyl transpeptidase. *Proc. Natl. Acad. Sci.USA* 1978, *75*, 4806-4809.

[5] Storozhenko, S., Belles-Boix, E., Babiychuk, E., Hérouart, D., et al., □-Glutamyl transpeptidase in transgenic tobacco plants. Cellular localization, processing, and biochemical properties. *Plant Physiol.* 2002, *128*, 1109-1119.

[6] Grzam, A., Martin, M. N., Hell, R., Meyer, A. J., γ-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione S-conjugates in Arabidopsis. *FEBS Lett.* 2007, *581*, 3131-3138.

[7] Martin, M. N., Saladores, P. H., Lambert, E., Hudson, A. O., Leustek, T., Localization of members of the □-glutamyl transpeptidase family identifies sites of glutathione and glutathione S-conjugate hydrolysis. *Plant Physiol.* 2007, *144*, 1715-1732.

[8] Ohkama-Ohtsu, N., Radwan, S., Peterson, A., Zhao, P., et al., Characterization of the extracellular □-glutamyl transpeptidases, GGT1 and GGT2, in Arabidopsis. *Plant J.* 2007a, *49*, 865-877.

[9] Ohkama-Ohtsu, N., Zhao, P., Xiang, C., Oliver, D. J., Glutathione-conjugates in the vacuole are degraded by γ-glutamyl transpeptidase GGT3 in Arabidopsis. *Plant J.* 2007b, *49*, 878-888.

[10] Ferretti, M., Destro, T., Tosatto, S. C., La Rocca, N., et al., Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast. *New Phytol.* 2009, *181*, 115-126.

[11] Meister, A., Tate, S. S., Griffith, O. W., Gamma-glutamyl transpeptidase. *Methods Enzymol.* 1981, *77*, 237-253.

[12] Will, Y., Fischer, K. A., Horton, R. A., Kaetzel, R. S., Brown, M. K., et al., Gamma-glutamyltranspeptidase-deficient knockout mice as a model to study the relationship between glutathione status, mitochondrial function, and cellular function. *Hepatology* 2000, *32*, 740-749.

[13] Masi, A., Ghisi, R., Ferretti, M., Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. *Plant Physiol.* 2002, *159*, 499-507.

[14] Kushnir, S., Babiychuk, E., Kampfenkel, K., Belles-Boix, E., et al., Characterization of *Arabidopsis thaliana* cDNAs that render yeasts tolerant toward the thiol-oxidizing drug diamide. *Proc. Natl. Acad. Sci.USA* 1995, *92*, 10580-10584.

[15] Destro, T., Prasad, D., Martignago, D., Bernet, I. L., et al., Compensatory expression and substrate inducibility of gamma-glutamyl transferase GGT2 isoform in *Arabidopsis thaliana*. *J. Exp. Bot.* 2011, *62*, 805-814.

[16] Levitt, J., *Responses of plants to environmental stre*sses, New York: Academic Press, 1972.

[17] Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J. et al., Genomewide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 2003, *301*, 653-657.

[18] Zechmann, B., Müller, M., Subcellular compartmentation of glutathione in dicotyledonous plants. *Protoplasma.* 2010, *246*, 15-24.

[19] Zechmann, B., Stumpe, M., Mauch, F., Immunocytochemical determination of the subcellular distribution of ascorbate in plants. *Planta.* 2011, *233*, 1-12.

[20] Polle, A., Chakrabarti, K., Schuermann, W., Rennenberg, H., Composition and properties of hydrogen peroxide decomposing systems in extracellular and total extracts from needles of Norway spruce (*Picea abies* L., Karst). *Plant Physiol.* 1990, *94*, 312-319.

[21] Hewitt, E. J., Dickes, G. J., Spectrophotometric measurements on ascorbic acid and their use for the estimation of ascorbic acid and dehydroascorbic acid in plant tissues. *Biochem. J.* 1961, *78*, 384.

[22] Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., et al., Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 2004, *3*, 1154-1169.

[23] Köcher, T., Pichler, P., Schutzbier, M., Stingl, C. et al., High precision quantitative proteomics using iTRAQ on an LTQ Orbitrap: a new mass spectrometric method combining the benefits of all. *J. Proteome Res.* 2009, *8*, 4743-4752.

[24] Dennis, G., Sherman, B. T., Hosack, D. A., Yang, et al., DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003, *4*, P3.

[25] Huang, W., Sherman, B. T., Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 2009, *4*, 44-57.

[26] Nystrom, T., Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.* 2005, *24*, 1311-1317.

[27] Moller, I. M., Jensen, P. E., Hansson, A., Oxidative modifications to cellular components in plants. *Annu. Rev. Plant. Biol.* 2007, *58*, 459-481.

[28] Singh P., Kaloudas D., Raines, C. A., Expression analysis of the Arabidopsis CP12 gene family suggest novel roles for these proteins in roots and floral tissues. *J. Exp. Bot.* 2008, *59*, 3975-3985.

[29] Li C. W., Lee S.-H., Chieh P.-S., Lin C.-S., et al., Arabidopsis Root-Abundant Cytosolic Methionine Sulfoxide Reductase B Genes MsrB7 and MsrB8 are Involved in Tolerance to Oxidative Stress. *Plant Cell Physiol.* 2012, *53*, 1707-1719.

[30] Kurkela, S., Franck, M., Cloning and characterization of a cold- and ABA- inducible Arabidopsis gene. *Plant. Mol. Biol.* 1990, *15*, 137-144.

[31] Gilmour, S. J., Artus, N. N., Thomashow, M. F., cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*.*Plant Mol Biol.* 1992, *18*, 13-21.

[32] Kurkela, S., Borg-Frank, M., Structure and expression of kin2, one of two cold- and ABA-induced genes of *Arabidopsis thaliana*. *Plant Mol. Biol.* 1992, *19*, 689-692.

[33] Sarry, J. E., Kuhn, L., Ducruix, C., Lafaye, A., et al., The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics* 2006, *6*, 2180-98.

[34] Yoo, K. S., Ok, S. H., Jeong, B. C., Jung, K. W., et al., Single cystathionine β-synthase domain-containing proteins modulate development by regulating the thioredoxin system in Arabidopsis. *Plant Cell* 2011, *23*, 3577-94.

[35] Lieberherr, D., Wagner, U., Dubuis, P. H., Métraux, J. P., Mauch, F., The Rapid Induction of Glutathione S-Transferases AtGSTF2 and AtGSTF6 by Avirulent Pseudomonas syringae is the Result of Combined Salicylic Acid and Ethylene Signaling. *Plant Cell. Physiol.* 2003, *44*, 750-757.

[36] Ryu, H. Y., Kim, S. Y., Park, H. M. You, J. Y. et al., Modulations of AtGSTF10 expression induce stress tolerance and BAK1-mediated cell death. *Biochem. Biophys. Res. Commun.* 2009, *379*, 417-422.

[37] Nutricati, E., Miceli, A., Blando, F., De Bellis, L., Characterization of two *Arabidopsis thaliana* glutathione S-transferase. *Plant. Cell. Rep.* 2006, 997-1005.

[38] Hoque, T. S., Uraji, M., Tuya, A., Nakamura, Y., Murata, Y., Methylglyoxal inhibits seed germination and root elongation and up-regulates transcription of stress-responsive genes in ABA-dependent pathway in *Arabidopsis*. *Plant Biol.* 2012, *5*, 854-858.

[39] Lappartinent, A.G., Touraine, B., Demand-driven control of root ATP-sulfurylase activity and sulfate uptake in intact canola. *Plant Physiol.* 1996, *177*, 147-157.

[40] Lappartient, A. G., Vidmar, J. J., Leustek, T., Glass, A. D., Touraine, B., Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound.*Plant J.* 1999, *18*, 89-95.

[41] Finkemeier, I., Goodman, M., Lamkemeyer, P., Kandlbinder, A., et al., The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana* under stress. *J. Biol. Chem.* 2005, *280*, 12168-12180.

[42] Carmel-Harel O, Storz G., Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress. *Annu. Rev. Microbiol.* 2000, *54*, 439-61.

[43] Uotila, L., Koivusalo, M., Purification of formaldehyde and formate dehydrogenases from pea seeds by affinity chromatography and S-formylglutathione as the intermediate of formaldehyde metabolism. *Arch. Biochem. Biophys.* 1979, *196*, 33-45.

[44] Hourton-Cabassa C., Ambard-Bretteville F., Moreau F., Davy de Virville J., et al., Stress induction of mitochondrial formate dehydrogenase in potato leaves. *Plant Physiol.* 1998, *116*, 627-635.

[45] Rhoads, D. M., Umbach, A. L., Subbaiah, C. C., Siedow, J. N., Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiol.* 2006, *141*, 357-366.

[46] Gepstein, S., Sabehi, S., Carp, M. J., Hajouj, T., et al., Large-scale identification of leaf senescence-associated genes. *Plant J.* 2003, *36*, 629-642.

[47] Rossato L, MacDuff JH, Laine P, Le Deunff E, Ourry A., Nitrogen storage and remobilization in Brassica napus L. during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation.*J. Exp. Bot.* 2002, *53*, 1131-41.

[48] Grbic, V., SAG2 and SAG12 protein expression in senescing Arabidopsis plants. *Phys. Plant.* 2003, *119*, 263-269.

[49] Navabpour S., Morris K., Allen R., Harrison E., et al., Expression of senescence-enhanced genes in response to oxidative stress. *J. Exp. Bot.* 2003, *54*, 2285–2292.

[50] Bindschedler L.V., Dewdney J., Blee K.A., Stone J.M., et al., Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *Plant. J.* 2006, *47*, 851–863.

[51] Luwe, M., Antioxidants in the apoplast and synplast of beech (*Fagus sylvatica* L.) leaves: seasonal variations and responses to changing ozone concentrations in air. *Plant Cell Environ.* 1996, 19, 321-328.

[52] Pignocchi, C., Foyer, C. H., Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr. Opin. Plant Biol.* 2003, *6*, 379-389.

[53] Shapiguzov A., Vainonen J.P., Wrzaczek M., Kangasjärvi J., ROS-talk - how the apoplast, the chloroplast, and the nucleus get the message through. *Front. Plant Sci.* 2012, *3*, 292.

[54] Daudi A, Cheng Z, O'Brien JA, Mammarella N, et al., The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. *Plant Cell* 2012, *24*, 275-87.

[55] Noctor G., Mhamdi A., Chaouch S., Han Y., Neukermans J., Marquez-Garcia B., et al., Glutathione in plants: an integrated overview.*Plant Cell Environ*. 2012,*35*, 454-484.

[56] Shiu S. H., Bleecker A. B., Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. *Plant Physiol.* 2003, *132*, 530-543.

[57] Wrzaczek M., Brosché M., Salojärvi J., Kangasjärvi S., et al., Transcriptional regulation of the CRK/DUF26 group of Receptor-like protein kinases by ozone and plant hormones in Arabidopsis. *BMC Plant Biol*. 2010, *10*, 95.

[58] Han Y., Chaouch S., Mhamdi A., Queval G., et al., Functional Analysis of Arabidopsis Mutants Points to Novel Roles for Glutathione in Coupling H(2)O(2) to Activation of Salicylic Acid Accumulation and Signaling. Antioxid Redox Signal. 2013, (in press). doi:10.1089/ars.2012.5052.

[59] Foyer C.H., Noctor G., Redox Signaling in Plants. *Antioxidants & Redox Signaling*, 2013. (in press) doi:10.1089/ars.2013.5278.

CHAPTER 2: Trentin Anna Rita, Pivato Micaela, Mehdi Syed Muhammad Muntazir, Barnabas Leonard Ebinezer, Giaretta Sabrina, Fabrega-Prats Marta, Prasad Dinesh, Arrigoni Giorgio, Masi Antonio

PROTEOME READJUSTMENTS IN THE APOPLASTIC SPACE OF *ARABIDOPSISTHALIANAGGT1* MUTANT LEAVES EXPOSED TO UV-B RADIATION

Front Plant Sci. 2015 Mar 24;6:128. doi: 10.3389/fpls.2015.00128

Proteome readjustments in the apoplastic space of *Arabidopsisthalianaggt1* mutant leaves exposed to UV-B radiation

Trentin Anna Rita[1], Pivato Micaela[1,2], Mehdi Syed Muhammad Muntazir[1], Barnabas Leonard Ebinezer[4], Giaretta Sabrina[1], Fabrega-Prats Marta[1], Prasad Dinesh[1,3], Arrigoni Giorgio[2,5], Masi Antonio[1]*.

[1]Department of DAFNAE, University of Padova (Italy)
[2]Proteomics Center of Padova University (Italy)
[3]Department of Bio-Engineering, Birla Institute of Technology (India)
[4]Sugarcane Breeding Institute Coimbatore (India)
[5]Department of Biomedical Sciences, University of Padova (Italy)

Correspondence:
Dr. Antonio Masi
University of Padova
Dept. DAFNAE
Viale Universita' 16,
Legnaro (PD), 35020, Italy
antonio.masi@unipd.it

ABSTRACT

Ultraviolet-B radiation acts as an environmental stimulus, but in high doses it has detrimental effects on plant metabolism. Plasma membranes represent a major target for ROS generated by this harmful radiation. Oxidative reactions occurring in the apoplastic space are counteracted by antioxidative systems mainly involving ascorbate and, to some extent, glutathione. The occurrence of the latter and its exact role in the extracellular space are not well documented, however. In *Arabidopsis thaliana*, the gamma-glutamyl transferase isoform GGT1 bound to the cell wall takes part in the so-called gamma-glutamyl cycle for extracellular glutathione degradation and recovery, and may be implicated in redox sensing and balance.

In this work, oxidative conditions were imposed with UV-B and studied in redox altered *ggt1* mutants. The response of *ggt1* knockout *Arabidopsis* leaves to UV-B radiation was assessed by investigating changes in extracellular glutathione and ascorbate content and their redox state, and in apoplastic protein composition. Our results show that, on UV-B exposure, soluble antioxidants respond to the oxidative conditions in both genotypes. Rearrangements occur in their apoplastic protein composition, suggesting an involvement of H_2O_2, which may ultimately act as a signal. Other important changes relating to hormonal effects, cell wall remodeling, and redox activities are discussed. We argue that oxidative stress conditions imposed by UV-B and disruption of the gamma-glutamyl cycle result in similar stress-induced responses, to some degree at least.

Keywords: glutathione, gamma-glutamyl-transferase, oxidative stress, iTRAQ labelling, apoplast, ultraviolet-B radiation

Abbreviations: UV-B Ultraviolet-B radiation, ROS Reactive Oxygen Species, GGT1 gamma-glutamyl transferase 1 isoform, H_2O_2Hydrogen Peroxide, iTRAQ Isobaric tags for relative and absolute quantification, UVR8UV-B photoreceptor 8, GSHGlutathione, LC-MS-MS Liquid Chromatography Mass Spectrometry, ECWF Extracellular washing fluid,DHADehydroascorbate, SBD-F4-fluoro-7-sulfobenzofurazan ammonium salt fluorophore, LMWLow Molecular Weight, HPLCHighpressure liquid chromatography, ANOVAAnalysis of variance, GLMGeneral linear models, SDSSodium dodecyl sulfate, TEAB Triethyl ammonium bicarbonate, FDR False discovery rates, FW Formula Weight, GSSGGlutathione disulfide / Oxidized GSH, SOD Superoxide dismutase, PNPs Plant natriuretic peptides, GSTsGlutathione S-Transferases,GHsGlycosyl hydrolases, PRPsPathogenesis-related proteins.

INTRODUCTION

The apoplast - i.e. the extraprotoplastic matrix of plant cells, including the cell wall - contains a number of enzymatic and non-enzymatic components involved in many physiological processes and is therefore important in the plant cell's response to both abiotic and biotic stress (Dietz, 1997; Agrawal *et al.*, 2010). Being at the interface with the external environment, rapid fluctuations occur in this compartment as a consequence of unfavorable conditions, such as salinity (Hernandez *et al.*, 2001), ozone (Jaspers *et al.*, 2005) drought (Hu *et al.*, 2005), and UV-B radiation (Pristov *et al.*, 2013), with consequent changes in the concentrations and redox state of its components.

Ultraviolet-B radiation (UV-B, 280-315 nm) is a component of the solar electromagnetic spectrum reaching the Earth's surface, which has gained attention in recent years because it has increased as a consequence of ozone layer destruction by anthropogenic emissions.

As a component of the solar radiation reaching the leaf, UV-B also acts as an environmental stimulus for plant growth and development. Recent literature has demonstrated the existence of the UV-B photoreceptor 8 (UVR8), which controls the plant's photomorphogenic response to UV-B radiation. UVR8 promotes a signal cascade that mediates UV-B photomorphogenic responses in order to secure plant acclimation and survival in sunlight (Rizzini *et al.*, 2011).

While it is beneficial at low intensities (Hideg*et al.*, 2013), numerous studies have reported that excess UV-B radiation harms plants by causing oxidative damage to cellular targets (Brosche and Strid 2003), altering the structure and functions of the leaf epidermis, cell wall and membranes (Pristov *et al.*, 2013). A common consequence of many types of environmental stress in plants is a greater abundance of some reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Li and Van Staden, 1998). Increases in ROS are seen after UV-B exposure too (Noctor *et al.*, 2014), and result in lipid peroxidations and damage to plasma membranes. To prevent these detrimental effects, plant cells deploy an array of non-enzymatic and enzymatic antioxidant systems that act as biochemical barriers to counteract and deactivate ROS.

This complex interplay of several metabolites, enzymes, ROS, antioxidants and hormones gives rise to signals that are transferred inside the cell through the plasma membrane to activate adaptive and response mechanisms.

A major line of defense in the apoplast is represented by the antioxidant molecule ascorbate and, to a lesser extent, glutathione. While both are involved intracellularly in the Halliwell-Asada pathway for controlling ROS and thereby maintaining the cellular redox state and protecting the cellular components from oxidative threat (Smirnoff and Pallanca, 1996), (Schafer and Buettner, 2001) (Potters *et al.*, 2010) (Saruhan *et al.*, 2009), only ascorbate occurs in high micromolar, or even millimolar quantities in the apoplast (Potters *et al.*, 2010), where it can play a part in redox control.

The role of extracellular glutathione in the apoplastic space is controversial because it can only be found in traces under physiological conditions, but it can rise to 2% of the total leaf glutathione under pathogen attack (Vanacker *et al.*, 1998a).

There have been reports, however, of the extracellular enzyme gamma-glutamyl-transferase (GGT; E.C. 2.3.2.2) degrading GSH (Martin *et al.*,2007), which means that, like animals (Meister and Anderson, 1983), plants also have a gamma-glutamyl cycle involving intracellular glutathione biosynthesis, extrusion and extracellular degradation, with recovery of the constituent amino acids (Ferretti *et al.*, 2009).

These findings can explain the low levels of glutathione in the extracellular environment on the one hand, but also raise the question of the significance of a gamma-glutamyl cycle in plants. In barley roots, using GGT inhibitors in association with the thiol oxidizing molecule diamide resulted in a net glutathione extrusion and accumulation in the extracellular medium (Ferretti *et al.*, 2009). This leads us to wonder whether a gamma-glutamyl cycle could operate as a redox sensing or redox balancing system.

Another study (Tolin *et al.*, 2013) characterized the leaf proteome of *Arabidopsisthalianaggt1* mutant lines and showed that, even under physiological conditions, a number of antioxidant and defense enzymes were significantly upregulated as a result of impaired extracellular GGT activity. This also implies that GSH turnover involving apoplastic GSH degradation is needed for proper redox sensing and/or a coordinated response to the environment. We speculated that a feedback signal might be missing when the GGT cycle is disrupted, and this would trigger the altered response.

To shed light on these unknown GGT functions in the plant's adaptation to the environment, in this work we investigated the effects of UV-B radiation as an oxidizing stress condition affecting the apoplastic environment in wild type*Arabidopsis* and a previously-characterized *ggt1* knockout mutant line (Destro *et al.*, 2011).

To improve our understanding of protein regulation, it can be helpful to use fractionation (sub-cellular proteomics) to reduce the complexity of the total protein extract and enable the visualization of proteins occurring in low quantities (Brunet *et al.*, 2003).

Since apoplastic proteome analysis can afford a better understanding of the complex network of extracellular proteins involved in plant defense (Agrawal *et al.*, 2010), we investigated the changes occurring in the extracellular proteome as a consequence of the null mutation and/or UV-B treatment by means of iTRAQ labelling for relative peptide quantification and LC-MS-MS analysis. This strategy enables an accurate and sensitive protein quantification, which is essential for

theidentification of apoplastic proteins in small quantities or small variations in their level of expression.

Following extraction with the extracellular washing fluid (ECWF) technique, we also explored ascorbate and glutathione content and their redox state in the leaf apoplastic fluids.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *A. thaliana* and a *ggt1* knockout mutant line, both Columbia ecotype (Col-0), were sterilized and incubated at 4°C in the dark for four days to synchronize germination and ensure a uniform growth. The *ggt1* knockout mutant was established in the mutant collection identified by the Salk Institute (Alonso *et al.*, 2003), and was obtained from the Nottingham *A. thaliana* Stock Centre (http://nasc.nott.ac.uk; polymorphism SALK_080363). Seeds were sown in soil pots and grown in a greenhouse.

For the UV-B radiation experiments, plants in the phase of maximum expansion of the rosette (before bolting) were transferred to a climatic cell 2 days before the treatment to enable their acclimation. The growth chamber settings were: 12/12 h light/dark cycle, 21/21°C temperature, 300 µmol m^{-2} s^{-1} photosynthetically active radiation, and 60% relative humidity. The UV-B treatment was applied for 8 hours at the beginning of the light period. The radiation was provided by two Philips TL40W/12 lamps with an intensity, measured on a level with the plants, of 8.3 kJ m^{-2} d^{-1} (UVB_{BE}, biologically effective UV-B). After the 8h UV-B treatment, leaves were immediately harvested for ECWF andtotal leaf extraction. Following, both the infiltrate and the leaf extracts were analysed for ascorbate content by spectrophotometric method, as described, the same day. Aliquots of the extracts were stored in -80° for thiol measurements.

Apoplastic fluid extraction

Extracellular washing fluids (ECWF) were extracted by vacuum infiltration according to (Lohaus *et al.*, 2001). About 1g of fresh leaves were cut, rinsed, immersed in infiltration buffer and vacuum-infiltrated for 10 min at 20 kPa. After infiltration, the leaves were blot-dried, weighed and placed vertically in a 5 ml syringe. The syringes were placed in tubes and centrifuged at 200 g, 4°C for 20 min. Apoplastic fluids were collected from the bottom of the tubes. For ascorbate and thiol extraction, 10 µl 0.1N HCl were placed at the bottom of the tubes before centrifugation to prevent oxidation. The composition of the infiltration buffer used for the ascorbate and thiol measurements

was: KH_2PO_4 50 mM, KCl 50 mM and EDTA 2.5 mM, pH 4.5. For the GGT activity and proteomic analyses, the infiltration buffer contained: KH_2PO_4 50 mM, KCl 0.2 M and PMSF 1 mM, pH 6.2.
The contamination level of the extracts obtained with the infiltration/centrifugation technique was assessed by means of malic dehydrogenase activity measurements, and ranged between 1.6 and 2.5% among the replicate extractions (data not shown).

Total leaf extraction

Total leaf extraction for the thiol, ascorbate and DHA measurements was done using metaphosphoric acid 1.5% and EDTA 1 mM buffer: 1 g of fresh leaves were powdered in a mortar with liquid nitrogen and extracted in a leaves to buffer ratio of 1:4, then centrifuged at 10'000 rpm for 10 min at 4°C. The same extraction procedure was used for total GGT activity, but using the infiltration buffer.

Asc and DHA determination

Ascorbate and dehydroascorbate were measured by spectrophotometric analysis following the decrease in absorbance at 265 nm according to Hewitt and Dickes 1961.

Chromatographic low-molecular-weight thiol assay

To measure total thiol concentration extracts, 50 µL of total leaf extract and ECWF were derivatized with 4-fluoro-7-sulfobenzofurazan ammonium salt fluorophore (SBD-F) (Dojindo, Japan). LMW thiols were separated by isocratic HPLC using the method described elsewhere(Masi *et al.*, 2002) with some modifications. The mobile phase was 75 mM ammonium-formiate, pH 2.9 and 3% methanol (97:3, vol/vol). For oxidized thiol quantification, samples were pre-treated with 2-vinylpyridine according to Griffith 1980, thenbuffered to basic pH and treated with 2-vinylpyridine for 1 hour to protect the free thiol moieties. Afterwards, the samples were washed to remove the resulting complexes, and the remaining unreactedsamples (containing the oxidized thiols) were derivatized and analyzed by HPLC.

GGT activity measurements

GGT activity was determined spectrophotometrically according to (Huseby and Stromme 1974). Leaf extracts were reacted in a mix of solution A (5 mM g-glutamyl-p-nitroanilide 100 mM NaH_2PO_4, pH 8.0) and solution B (575 mM gly-gly in 100 mM NaH_2PO_4, pH 8.0) in a ratio of 10:1. Absorbance was recorded for 1 hour at 407 nm to measure p-nitroaniline release into the assay medium.

Statistical analysis

After checking for a normal distribution, data were tested with one-wayANOVA using the GLM procedure in SAS (SAS 9.2, 2008). Data with a non-normal distribution were submitted to a nonparametric test (Kruskal-Wallis) using XLSTAT (2014 version). In both cases, Bonferroni's test was used to ascertain differences between means. Significance was established at $P \leq 0.05$.

Proteomic analysis

Protein *in situ* digestion

Proteins obtained from infiltration were quantified by bicinchoninic acid spectrophotometric assay; 50 µg of proteins were loaded in a homemade 11% SDS gel and the electrophoretic run was stopped as soon as the protein extracts entered the runninggel. Bands were excised and washed several times with 50 mM TEAB (triethylammonium bicarbonate) and dried under vacuum after a short acetonitrile wash. Cysteines were reduced with 10 mM dithiothreitol (in 50 mM TEAB) for 1 hour at 56°C, and alkylated with 55 mM iodoacetamide (in 50 mM TEAB) for 45 min at room temperature in the dark. Gel pieces were then washed with alternate steps of TEAB and acetonitrile, and dried. Proteins were digested *in situ* with sequencing grade modified trypsin (Promega, Madison, WI, USA) at 37°C overnight (12.5 $ng \cdot \mu l^{-1}$ trypsin in 50mM TEAB). Peptides were extracted with three steps of 50% acetonitrile in water. One µg of each sample was withdrawn to check digestion efficiency using LC-MS/MS analysis, and the remaining peptide solution was dried under vacuum.

iTRAQ labeling and peptide fractionation

Peptides were labeled with iTRAQ reagents (ABSciex) according to the manufacturer's instructions. They were labeled with the four iTRAQ tags using a Latin panel strategy: wt UV-B, *ggt1* UV-B, wt ctrl and *ggt1* ctrl were labeled respectively with 114, 115, 116 and 117 tags in the first replicate; 115, 116, 117, 114 tags in the second and 116, 117, 114, 115 tags in the third. Prior to mixing the samples in a 1:1:1:1 ratio, 1 µg of each sample was analyzed separately to check label efficiency by LC-MS/MS analysis, setting the iTRAQ labeling as a variable modification in the database search. All the peptides were correctly identified as being iTRAQ-modified at the N-terminus and at each lysine residue. The samples were then pooled and dried under vacuum. The mixture of labeled samples (one per replicate) was suspended in 500 µl of buffer A (10 mM KH_2PO_4, 25% acetonitrile, pH 2.9) and loaded onto a strong cation exchange cartridge (AB Sciex)

for fractionation according to Tolin *et al.*, 2013. After a washing step with buffer A, the peptides were eluted stepwise with increasing concentrations of KCl in buffer A (25, 50, 100, 200, and 350 mM). The volume of each fraction (500 µl) was reduced under vacuum, and the samples were desalted using C18 cartridges (Sep-Pack, C18, Waters) according to the manufacturer's instructions. The samples were ultimately dried under vacuum and kept at -20 °C until MS analysis.

LC-MS/MS analysis, database search, and protein quantification

Samples were suspended in H_2O/0.1% formic acid and analyzed by LC-MS/MS. The MS analyses were conducted with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Pittsburgh, CA, USA) coupled online with a nano-HPLC Ultimate 3000 (Dionex- Thermo Fisher Scientific). Samples were loaded in a homemade 10 cm chromatographic column packed into a pico-frit (75 mm id, 10 mm tip, New Objectives) with C18 material (ReproSil, 300 Å, 3 µm). The LC separation and mass spectrometer settings used for the analyses were the same as those described in Tolin *et al.*, 2013, and the method was as described by Köcher *et al.*, 2009.

The raw LC-MS/MS files were analyzed using Proteome Discoverer 1.4 (Thermo Fisher Scientific), connected to a Mascot Search Engine server (version 2.2.4, Matrix Science, London, UK). The spectra were searched against a ARATH Uniprot protein database. Enzyme specificity was set to trypsin with two missed cleavages, and peptide and fragment tolerance was set to 10 ppm and 0.6 Da, respectively. Methylthiocysteine, 4-plex iTRAQ at the N-terminus and Lys were set as fixed modifications, while Met oxidation was selected as a variable modification. False discovery rates (FDR) were calculated by the software, based on the search against the corresponding randomized database. Only proteins identified and quantified with at least 2 unique peptides with 99% confidence (FDR 1%) were considered as positive identifications. A 5% FDR was adopted in only two cases (as shown in Table 2), in which the MS/MS spectra were manually inspected for confirmation. Data were pre-filtered to exclude MS/MS spectra containing less than 5 peaks or with a total ion count below 50. Quantification was done by normalizing the results on the median value of all measured iTRAQ reporter ratios.

Protein expression ratios are given as: wt (UV-B/ctrl), *ggt1* (UV-B/ctrl), ctrl (*ggt1*/wt) and UV-B (*ggt1*/wt) and they are the mean value of at least 2 biological replicates. To improve the statistical robustness of the data, all proteins were submitted to a two-tailed Z test with a confidence level of $p < 0.05$. The variations were further restricted to proteins exhibiting an at least ±50% fold change in their expression (1.5 for upregulated and 0.68 for downregulated proteins).

RESULTS

GGT activity

An increase in GGT enzymatic activity was found in wt plants after UV-B irradiation; this increase was greater in total leaf extracts (+35%, Table 1) than in ECWF (+10%, Fig 1A). Activity in the mutant was significantly lower in total leaf extracts and almost undetectable in the ECWF (as was to be expected because GGT1 is the only apoplastic isoform active in leaves), but no significant differences were observed after UV-B exposure (Fig 1A).

Antioxidant content (GSH and ascorbate)

Ascorbate was only found in its reduced form in total leaf extracts, and was increased by UV-B treatment (by approximately 20-30%) in both genotypes (Table 1). We found no reduction in the ascorbate in the apoplastic space, where we could only measure the oxidized form, dehydroascorbate (Fig. 1C).We found no significant differences between the genotypes or treatments in the total glutathione or cys-gly content in total leaf extract (Table 1). In ECWF total glutathione content was higher in the *ggt1* mutant than in the wild type; and supplementing UV-B radiation did not alter these values (Fig 1B). GSSG was lower in the ECWF from wt leaves under UV-B treatment, whereas oxidized cys-gly increased significantly under the same conditions (fig 1B, 1D).

It should be noted that apoplastic glutathione is only a small fraction of total leaf glutathione, so fluctuations in the apoplast are somewhat diluted during the extraction process. For the same reason, variations in the small amount of extracellular DHA may not have been reflected in total leaf extracts.

Table 1:

		ggt activity	**ascorbate**	**GSH**	**cys-gly**
		%	µmol/ g FW	nmol/g FW	nmol/g FW
wt	ctrl	100 ± 7.8	2.82 ± 0.03	261.9 ± 5.8	1.14 ± 0.04
ggt1	ctrl	5 ± 1.6	2.66 ± 0.03	275.7 ± 7.5	1.04 ± 0.04
wt	UV-B	136 ± 11	3.37 ± 0.05 *	296.2 ± 6.2	1.34 ± 0.05
ggt1	UV-B	7 ± 3	3.53 ± 0.06 *	293.7 ± 9.1	1.01 ± 0.03

Table 1: GGT activity, ascorbate, GSH and cys-gly content in total leaf extract. Values are the mean ± S.E. of 4 biological replicates from 3 technical replicates. For GGT activity, the reference value of the wild type control was 50.43 mU/g FW. Asterisks indicate P ≤ 0.05.

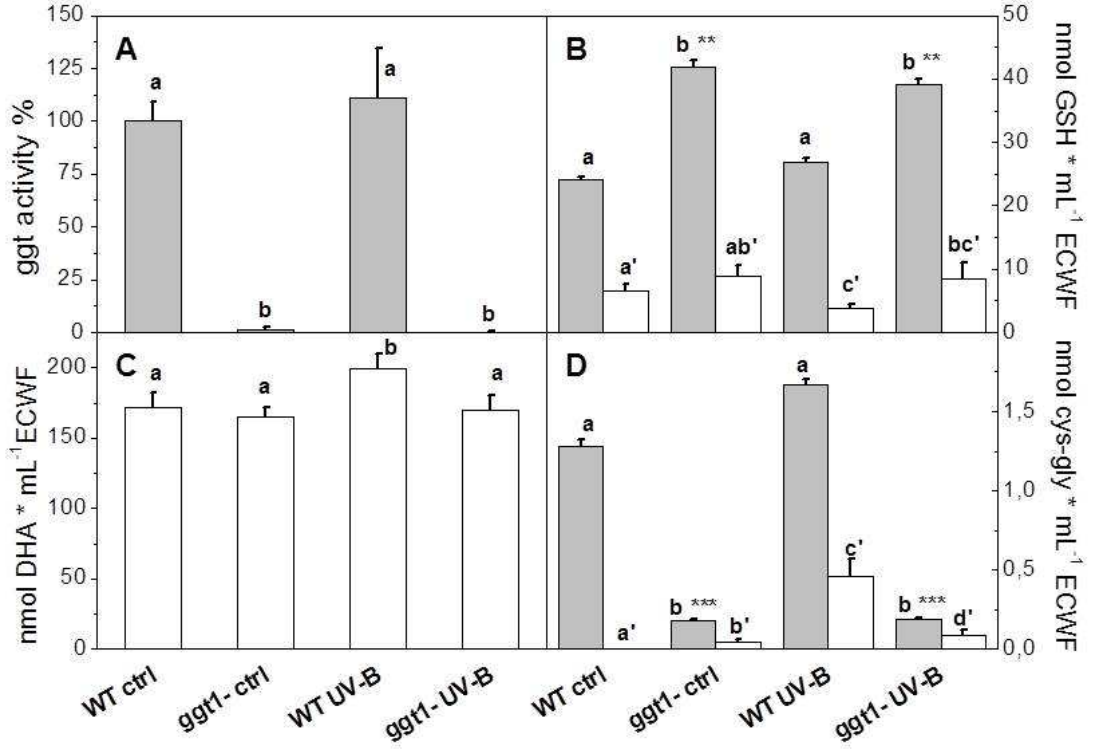


Fig 1: GGT activity (A), glutathione (B), ascorbate (C) and cys-gly (D) in ECWF.Grey bars show total content, white bars oxidized forms. Reported values are the mean ± S.E. of 3 technical replicates, each conducted with at least 4 biological replicates. Different letters indicate significant differences between conditions(P≤ 0.05 *; P≤ 0.01 **; P≤ 0.001 ***). For GGT activity, the reference value of the wild type control was 43.05 mU/mL ECWF.

Proteomic analysis

In total, 329 proteins were uniquely identified by the LC-MS/MS analyses; 208 were found in at least two biological replicates. Based on the Gene Ontology assignment for cellular compartmentalization (Uniprot 14, *www.uniprot.org*), we restricted our analysis to the 118 proteins that were either apoplastic or unlocalized, accounting for approximately 57% of the total.

Should be considered bearing in mind that several truly extracellular proteins have yet to be properly assigned to the apoplast. In fact, it has been reported (Ding *et al.*, 2012; Agrawal *et al.*, 2010) that about 50% of proteins secreted in the apoplast lack a leaderless secretory tag;. There are

consequently many unpredicted secretory proteins in plants, and their occurrence is often underestimated or they are even considered improperly as contaminants. Our decision to restrict our assignments according to the Uniprot database was therefore rigorous, but probably led to an underestimation of the truly apoplastic proteins.
The variations considered were further restricted to proteins exhibiting an at least ±50% fold change in expression.

Various information can be drawn from comparisons between the four experimental conditions: i) the effect of UV-B treatment on each genotype; ii) differential apoplastic protein composition in *ggt1* vs. wt; iii) possible differences in the behavior of the *ggt1* mutant and the wt under UV-B.Comparing the two genotypes, 23 proteins were downregulated and only 3 were upregulated in *ggt1*by comparison with the wt under physiological conditions (Table 2). UV-B treatment resulted in 8 proteins being downregulated in *ggt1*; and in 12 being downregulated and 11 being upregulated in the wt. When the *ggt1* and wt were compared after UV-B treatment, it emerged that 9 proteins were expressed less, and 10 were expressed more in the mutant than in the wild type. A condensed view of all these variations is given in Table 2.

Table 2:

Accession nr	Locus name	Description	FDR %	*WT* UVB/ctrl	*ggt1* UVB/ctrl	CTRL *ggt1/wt*	UV-B *ggt1/wt*
F4HR88	At1g33590	Leucine-rich repeat-containing protein	1	0.55		0.48	
O81862	At4g19810	At4g19810	1	0.55			
F4IAX0	At1g31690	Putative copper amine oxidase	1	0.57			
Q9M5J8	At5g06870	Polygalacturonase inhibitor 2	1	0.57			
Q9LMU2	At1g17860	At1g17860/F2H15_8	1	0.57		0.48	
B9DGL8	At5g08370	AT5G08370 protein	1	0.58			
F4HSQ4	At1g20160	Subtilisin-like serine endopeptidase-like protein	1	0.61			
F4IIQ3	At2g28470	Beta-galactosidase	1	0.62			
Q9ZVS4	At1g03220	Aspartyl protease-like protein	1	0.65		0.66	2.5
Q94F20	At5g25460	At5g25460	1	0.66		0.58	1.6
Q9FT97	At5g08380	Alpha-galactosidase 1	1	0.68			
Q940J8	At4g19410	Pectinacetylesterase family protein	1	0.68		0.68	1.9
O49006	At3g14310	Pectinesterase/pectinesterase inhibitor 3	1	1.5			0.55
O65469	At4g23170	Putative cysteine-rich receptor-like protein kinase 9	1	1.5			

P24806	At4g30270	Xyloglucan endotransglucosylase/hydrolase prot 24	1	1.6			
F4J270	At5g20950	Beta-1,3-glucanase 3	1	1.7			0.47
Q9ZV52	At2g18660	EG45-like domain containing protein 2	1	1.8			
P46422	At4g02520	Glutathione S-transferase F2	1	1.8			0.51
O22126	At2g45470	Fasciclin-like arabinogalactan protein 8	1	1.9			
F4JRV2	At4g25100	Superoxide dismutase	5	1.9		1.7	
P33157	At3g57260	Glucan endo-1,3-beta-glucosidase, acidic isoform	1	2.1	0.63		0.26
F4JBY2	At3g60750	Transketolase	1	2.7		2.2	
O80852-2	At2g30860	Isoform 2 of Glutathione S-transferase F9	1	2.9			
F4HUA0	At1g07930	Elongation factor 1-alpha	1	4.4			
Q9SG80	At3g10740	Alpha-L-arabinofuranosidase 1	1		0.35		
Q9FZ27	At1g02335	Germin-like protein subfamily 2 member 2	5		0.37		
F4K5B9	At5g07030	Aspartyl protease family protein	1		0.54		
O64757	At2g34930	Disease resistance-like protein/LRR domain-containing protein	1		0.31		
Q9S7Y7	At1g68560	Alpha-xylosidase 1	1		0.55		
Q9C5C2	At5g25980	Myrosinase 2	1		0.61		
Q9FKU8	At5g44400	Berberine bridge enzyme	1		0.50		0.68
Q9SMU8	At3g49120	Peroxidase 34	1			0.56	
Q9ZVA2	At1g78830	At1g78830/F9K20_12	1			0.57	2.3
P94072	At5g20630	Germin-like protein subfamily 3 member 3	1			0.52	
Q42589	At2g38540	Non-specific lipid-transfer protein 1	1			0.42	
Q9FW48	At1g33600	Leucine-rich repeat-containing protein	1			0.58	
Q9LXU5	At5g12940	Leucine-rich repeat-containing protein	1			0.51	
Q9LYE7	At5g11420	Putative uncharacterized protein At5g11420	1			0.55	
Q9M2U7	At3g54400	AT3g54400/T12E18_90	1			0.64	2.0
Q9LT39	At3g20820	Leucine-rich repeat-containing protein	1			0.68	
O24603	At2g43570	Chitinase class 4-like protein	1			0.34	0.17
P33154	At2g14610	Pathogenesis-related protein 1	1			0.34	
Q8W112	At5g20950	Beta-D-glucan exohydrolase-like protein	1			0.65	
P28493	At1g75040	Pathogenesis-related protein 5	1			0.30	
Q94K76	At5g18470	Curculin-like (Mannose-binding) lectin family protein	1			0.53	
Q9LEW3	At5g10760	Aspartyl protease family protein	1			0.44	
Q9LRJ9	At3g22060	Cysteine-rich repeat secretory protein 38	1			0.49	
Q9LV60	At5g48540	Cysteine-rich repeat secretory protein 55	1			0.5	
Q9C5M8	At4g24780	Probable pectate lyase 18	1			0.68	
O23255	At4g13940	Adenosylhomocysteinase 1	1			1.5	
O50008	At5g17920	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	1			2.4	
Q9SVG4-2	At4g20830	Isoform 2 of Reticuline oxidase-like protein	1				0.43
Q940G5	At4g25900	Aldose 1-epimerase family protein	1				0.61

Q9LFA6	At3g52840	Beta-galactosidase 2	1	0.59
Q9LU14	At3g16370	GDSL esterase/lipase APG	1	1.6
Q39099	At2g06850	Xyloglucan endotransglucosylase/hydrolase prot 4	1	1.8
Q9LFR3	At5g14920	Gibberellin-regulated protein 14	1	1.8
O04496	At1g09750	Aspartyl protease-like protein	1	1.9
Q9FH82	At5g45280	AT5g45280/K9E15_6	1	2.0

Table 2:Brief overview of expression changes in apoplastic and unlocalized proteins in the four conditions analyzed: wt (UV-B/ctrl), *ggt1* (UV-B/ctrl), ctrl (*ggt1*/wt) and UV-B (*ggt1*/wt).

To facilitate the interpretation of the results, we ran a bioinformatic analysis with Blast2GO, a tool for the functional annotation of sequences and data mining, based on the gene ontology (GO) vocabulary (Conesa and Götz, 2008).This made it easy to assess and visualize the relative abundance of functional terms (obtained from the pool of GO terms) in the category of biological processes, based on a score assigned by the Blast2GO algorithm. Within the category of biological processes, the GO terms involved under the four conditions, and either down- or upregulated, are shown in Figs. 2 and 3, respectively.

Based on the Blast2GO scores, UV-B in both the wild type and the *ggt1* mutant mainly seem to cause a lower expression of proteins in the "metabolic process" and "response to stimulus" categories (Fig. 2). Far fewer proteins were upregulated, but the analysis as a whole again showed that the "response to stimulus" and "metabolic process" categories scored highest, but only after the UV-B treatment in both genotypes (Fig. 3). Based on the results shown in Table 2, the variations observed were functionally grouped as explained below. For the sake of simplicity, the proteins listed in Table 2 were divided into 4 broad categories, but many of those described here could have been placed in more than one category (depending on whether we considered the protein's biological function or its catalytic activity, for instance).

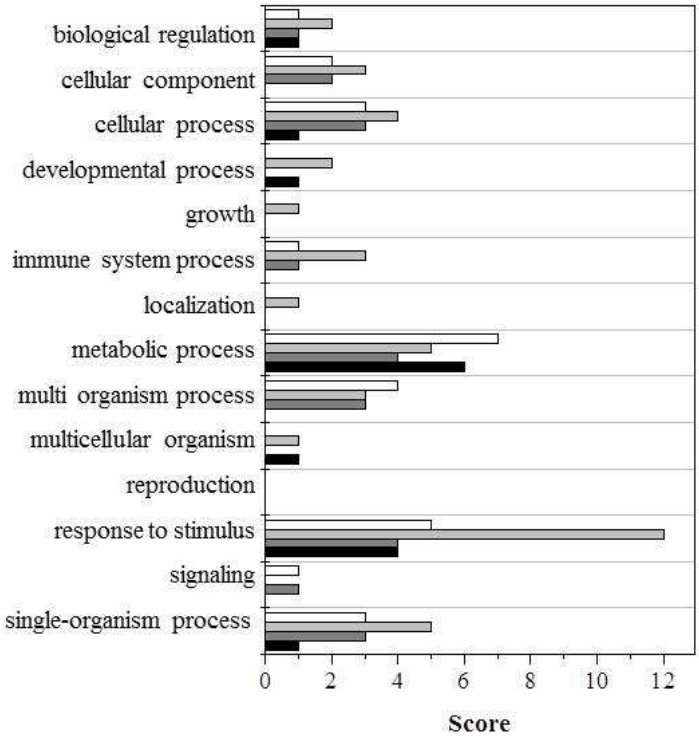


Fig 2: GO terms distribution in the biological process of downregulated proteins. Black bars shows wt (UV-B/ctrl), dark grey bars *ggt1* (UV-B/ ctrl), light grey is ctrl (*ggt1*/wt), and white bars is UV-B (*ggt1*/wt).

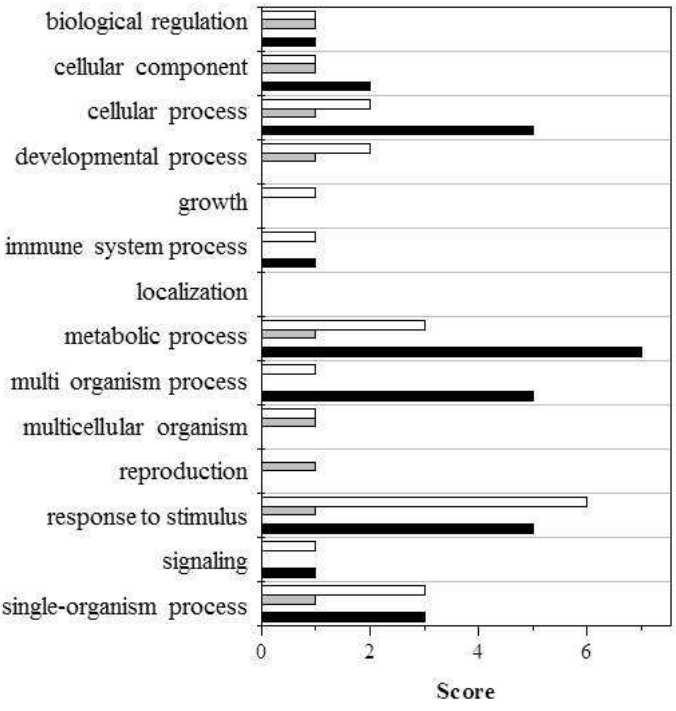


Fig 3: GO terms distribution in the biological process of upregulated proteins. Black bars shows wt (UV-B/ctrl), dark grey bars *ggt1* (UV-B/ ctrl), light grey is ctrl (*ggt1*/wt), and white bars is UV-B (*ggt1*/wt).

Pathogenesis and hormone-related proteins

Gibberellins are hormones that can be found in the apoplastic space too (Kramer 2006). Here, we found the gibberellin-regulated protein Q9LFR3 (At5g14920) upregulated by UV-B treatment in the mutant.

Among the proteins targeted by hormones there is a galactose-binding domain containing protein (At5g25460, Q94F20) with a putative function in response to karrikins, a novel group of plant growth regulators (Nelson *et al.*, 2011). This protein is downregulated in the wild type under UV-B treatment, andin the *ggt1* mutant in physiological conditions.

By comparison with the wild type, two pathogen-related proteins are less expressed in the *ggt1* mutant, i.e. PR-1 (At2g14610) and PR-5 (At1g75040), reportedly regulated by brassinosteroids (Sävenstrand et al., 2004). Another protein involved in lipid catabolism and response to pathogens is a GDSL esterase/lipase (At3g16370) that is expressedmore in the *ggt1* mutant than in the wild type under UV-B.

Proteolytic enzymes are directly or indirectly involved in several plant cellular processes, including resistance to pathogens and disease (Xia *et al.*, 2004). In our study, we identified four members of the aspartyl protease family, a class of enzymes acting as endopeptidases to remove aspartic residues from polypeptide chains. One of them (At1g03220) is downregulated in the wild type after UV-B treatment, andin the *ggt1* mutant in physiological conditions. This protein and At1g09750 are both upregulated in *ggt1* bycomparison with the wild type as an effect of UV-B treatment.At5g07030 is downregulated under UV-B in the mutant, and At5g10760 is downregulated in the mutant under control conditions. It could be hard to explain these opposite effects in the expression of members of the same aspartyl protease family, but it is worth noting that these enzymes are reportedly involved in plant defenses and development (Minic *et al..*, 2007).

Cell wall remodeling

Up to 90% of plant cell walls consist of three types of polysaccharide: cellulose, hemicelluloses, and pectins. Their composition and structure differ from one species to another, and change as plants develop and with environmental fluctuations (Cosgrove 1997; Popper and Fry 2003*et al.*, 2007). Six proteins belonging to the class of glycoside hydrolases are downregulated by UV-B

radiation in the wild type (At2g28470, At5g08380, At4g19410, At5g06870, At4g19810, and At5g08370) while another six proteins are upregulated (At3g14310, At2g45470, At3g57260, At4g30270, At5g20950 and At2g18660).

Comparing the *ggt1* mutant with the wild type clearly revealed a constitutive downregulation of proteins related to cell wall remodeling (At4g19410, At4g24780, At5g20950 and At2g43570). One of them is downregulated both in the mutant under physiological conditions and in the wild type after UV-B treatment. An opposite response to UV-B radiation emerged for beta-glucosidase At3g57260, which was higher in the wild type, and lower in *ggt1* after the treatment. UV-B radiation resulted in a lower expression in the *ggt1* mutant of other cell wall remodeling proteins, namely α-arabinofuranosidase At3g10740, α-xylosidase At1g68560, and the berberine bridge enzyme At5g44400. We also found a lower expression of chitinase At2g43570 in the *ggt1* mutant than in the wild type both under physiological conditions and after treatment with UV-B.

Signaling

In this study, we observed changes in four proteins containing leucine-rich repeats, and in two cysteine-rich secretory proteins belonging to a class acting as kinases. Leucine- and cysteine-rich proteins are transmembrane proteins that are reportedly induced by ROS and salicylic acid (Brandes *et al.*, 2009).

All leucine-rich proteins were downregulated in the mutant (At1g33600, At5g12940, At1g33590 and At3g20820) under physiological conditions. It seems particularly interesting that At1g33590 expression was also downregulated in the wild type under UV-B radiation.

The two cysteine-rich repeat secretory proteins, At3g22060 and At5g48540, were both downregulated in the *ggt1* mutant under physiological conditions. These proteins are also PM-associated receptor-like kinases, and At3g22060 interacts with one or more unknown PM-localized ABA receptor(s) (Xin *et al.*, 2005), whereas At5g48540 is involved in response to karrikins (Nelson *et al.*, 2011).

The EG45-like domain containing protein (At2g18660) is part of a class of small proteins that act as signaling molecules. In our study, At2g18660 was upregulated in the wild type under UV-B.

GSTs, redox regulation and ROS balance

One protein (At1g31690) involved in response to oxidative stress was downregulated under UV-B in the wild type. This protein is involved in H_2O_2 metabolism, acting as an oxidase. Also Peroxidase 34 (At3g49120) was downregulated in the *ggt1* mutant under control conditions.

Two GSTs proteins belonging to categories F2 and F9 (At4g02520 and At2g30860, respectively) were upregulated in the wild type plants after UV-B radiation. When the two genotypes were compared after the same treatment, the expression level was lower in the former.

Two proteins in the germin-like family (At5g20630 and At1g02335) were downregulated in *ggt1*, one in physiological conditions and the other after UV-B treatment. These proteins are involved in defending against biotic and environmental stress.

Superoxide dismutase proteins are reportedly involved in removing superoxide radicals from the apoplast following UV-B radiation (Alscher *et al.,* 2002). In our study, the expression of superoxide dismutase (At4g25100) was upregulated in the wild type after UV-B radiation and in *ggt1* under physiological conditions.

DISCUSSION

GGT activity and soluble antioxidants

Following excess UV-B exposure, plants deploy a wide array of morphological and biochemical defense mechanisms, including soluble antioxidants (Schiu 2005). The changes observed in this study are consistent with the view that, under UV-B radiation, oxidative conditions in the apoplastic space involve both ascorbate and glutathione, the two main soluble antioxidant molecules in plant cells. Ascorbate in ECWF was found fully oxidized, which is consistent with the view that oxidizing conditions prevail in the apoplastic space(Saruhan *et al.*, 2009; Vanacker *et al.*, 1998a; Vanacker *et al.*1998b).

UV-B radiation induced an increase in apoplastic ascorbate in both genotypes, suggesting that ascorbate is extruded as a means to counteract the artificially-imposed oxidative conditions. While glutathione content was substantially unchanged in total leaf extracts in all the conditions tested, it was altered in the ECWF from mutant leaves, where the effect of the *ggt1* null mutation results in a net increase in glutathione content, as a predictable effect of the reduced GGT degradation activity.

Under UV-B, the concurrent decrease in oxidized glutathione and increase in oxidized cys-gly can be interpreted as an enhanced gamma-glutamyl transferase activity; this is supported by the previous finding that GGT1 has a stronger preference for GSSG (Okhama-Otsu *et al.,* 2008).

GGT activity was barely detectable in the mutant, confirming that GGT1 is the main isoform contributing to over 90% of said activity in wild type leaves. Since the GGT2 isoform is not expressed in leaves (Destro *et al.,* 2011) and GGT3 is assumed to be non-functional (Martin *et al.,* 2007), this indicates that the activity found in the mutant represents the contribution of the remaining vacuolar isoform GGT4.

The increased GGT activity following UV-B treatment in the wild type therefore suggests that the rate of the gamma-glutamyl cycle is accelerated by this radiation. The involvement of the vacuolar GGT4 in the degradation of glutathione conjugates, e.g. with lipoperoxides and/or other damaged molecules, might be implicated too, but this seems unlikely since no significant increase in GGT activity was apparent in the mutant under the same conditions.

Collectively, these novel findings thus imply that the gamma-glutamyl cycle is accelerated under oxidative conditions imposed by ultraviolet-B radiation, and support the conviction that it is involved in oxidative stress sensing and/or response.

Apoplastic proteome readjustments

Proteome analysis has proved a powerful tool for deciphering cell metabolism under different perturbations and has been found useful in apoplastic studies too (Agrawal *et al.*, 2010). Apoplastic proteins establish a constitutive systemic defense network, with only a few of them changing under environmental and/or biotic stress (Delaunios *et al.*, 2014).

Two main approaches are currently adopted in plant physiology studies: the application of stress conditions, and the use of mutants. These alternative and converging strategies may provide tools for deciphering metabolism. In this work, oxidative conditions were imposed with UV-B and studied in redox-altered *ggt1* mutants. Subcellular fractionation and apoplastic proteome analysis were then used to arrive at a better understanding of the rearrangements in the extracellular compartment.

The experimental design adopted here could consequently help to describe and compare the effects of UV-B treatment on the two genotypes, and the differences in apoplastic proteome composition between the mutant and wild type leaves under control conditions.

In both genotypes, UV-B treatment caused a downregulation of different kinds of protein related to cell wall biosynthesis, response to stress and proteolysis. It promptedan upregulation, but only in the wild type, of other proteins involved in cell wall remodeling and two glutathione S-transferases, GST-F2 and GST-F9. No proteins were found upregulated in the *ggt1* mutant after UV-B (Table 2).

The hormonal changes occurring in the *ggt1* mutant, with or without exposure to UV-B radiation, were not considered in the experimental setup, and were beyond the scope of this work. Several proteins seen here to change in expression could be targets for hormones, however. For instance, one protein whose expression was stimulated by UV-B is reportedly a gibberellin-regulated protein (At5g14920), suggesting that gibberellins could be implicated in the response. The expression of a galactose-binding domain containing protein (At5g25460), which is stimulated by karrikins (a novel group of plant growth regulators (Nelson *et al.*, 2011), was also higher in the mutant than in the

wild type after UV-B. Interplay with hormones may also concern two PRPs (PR-1 and PR-5), whose expression was lower in the *ggt1* mutant under physiological conditions. A previous study (Sävenstrand *et al.*, 2004) had found their expression strongly reduced in brassinosteroid metabolism mutants. It would be interesting to see whether the brassinosteroid pathway is altered in *ggt1* mutants too.

Broadly speaking, cell wall modifying proteins such as glycosyl hydrolases (GHs), peroxidases, esterases, transglycosylases and lyases, are involved in the construction, remodeling or turnover of cell wall components (Cosgrove, 1997; Stolle-Smits *et al.*, 1999; Obel *et al.*, 2002; Reiter, 2002). Some of them may have other functions too, e.g. in the glycosylation state of target proteins (Kang *et al.*, 2008), which in turn could be involved in signaling processes (Minic *et al.*, 2007).

Taking a broader look at the changes found in this category suggests that UV-B affects the expression of some proteins in the wild type (e.g. pectine-acetylesterase and its inhibitor, xyloglucan endotransglucosylase, beta 1,3-glucanase, beta-galactosidase, alpha-galactosidase and a polygalacturonase inhibitor) and others in the *ggt1* mutant (alpha-xylosidase, myrosinase, alpha-arabinofuranosidase, and a berberine bridge enzyme), confirming the view that the cell walls are the target of this radiation. Notably, these remodeling processes are affected in the *ggt1* mutant not only by UV-B treatment, but also under physiological conditions. Since cell wall structure is reportedly altered during development and by exposure to stress (Potters *et al.*, 2009), our findings could be explained by the existence of a stress-like condition in the mutant, where some signals mimic the oxidative state induced by UV-B in the wild type.

Myrosinase, a protein in the class of glycoside hydrolases, was less expressed under UV-B in the *ggt1* mutant, and this could have ecophysiological consequences because in Brassicaceae myrosinases play a part in growth, development and defenses against microbes, as well as deterring insects and herbivores (Rodman, 1991). The two germin-like proteins that were downregulated in the *ggt1* mutant could also be consistent with alterations in the defense systems against biotic and environmental stress.

The expression of some other proteins was altered in opposite ways (up- or downregulated)after UV-B exposure, depending on the genotype considered: for instance, the stress-responsive glucanendo-1,3-beta-glucosidase was upregulated in the wild type, but downregulated in *ggt1*.

A group of proteins involved in response to stimuli, i.e. the leucine-rich and cysteine-rich proteins, wasdownregulated in *ggt1* in physiological conditions. Leucine-rich proteins contain a leucine-rich repeat (LRR) motif that has revealed a central role in recognizing different pathogen-associated molecules in the innate host defense of plants and animals (Gunawardena *et al.*, 2011).

In this study, we also identified 4 aspartyl proteases that were altered under our experimental conditions: this may mean that members of this category of enzymes related to plant defenses are sensitive to redox variations. Aspartyl proteases are important for plant development. They have been implicated in the ABA-dependent responsiveness to drought-induced stress (Yao *et al.*, 2012), and inArabidopsis agene encoding the aspartyl protease protein was found downregulated by cold and high-salinity stress (Seki *et al.*, 2002).

The EG45-like domain containing protein 2 (At2g18660) was upregulated in the wild type under UV-B. This protein belongs to the category of plant natriuretic peptides (PNPs), a novel class of small proteins showing homology with the N-terminus of expansins, though they are significantly shorter and lack the wall-binding domain (Ludidi *et al.*, 2002). Previous studies found PNPs upregulated under saline and osmotic conditions (Rafudeen *et al.*, 2003), but the effects of UV-B on this class of peptidic signaling molecules had not been reported before.

Among the variations in apoplastic enzyme expression found in the present study, some that particularly attracted our attention are closely related to ROS metabolism.

Superoxide anion formation is reportedly triggered by ultraviolet-B radiation (Alscher *et al.*, 2002). It seems noteworthy that no plant superoxide dismutase (SOD)identified to date contains a signal peptide, but extracellular SOD activity in stressed or pathogen-infected plants has been reported in many works (Hernández *et al.*, 2001; Karpinska *et al.*, 2001; Kaffarnik *et al.*, 2009; Pechanova *et al.*, 2010). SODsproduce H_2O_2, which is degraded to H_2O by ascorbate peroxidase. By removing superoxide anions, SODs may limit the duration of the oxidative burst to an early event in plant defense (Scheler *et al.*, 2013; Pristov *et al.*, 2013).

In this study, a superoxide dismutase (At4g25100) was found upregulated by UV-B radiation in wild type leaves: this can be interpreted as the need to improve scavenging activity to remove excess superoxide anions. Although its localization is not reported in the official databases, its occurrence in the apoplast was noted in other studies too (Ding *et al.*, 2012; Kwon *et al.*, 2005). Higher SOD levels combined with lower levels of the putative copper amine oxidase (At1g31690) may result in higher H_2O_2 levels. An increased GST expression could also result in its scavenging, however, so while it seems clear that ROS metabolism is affected by UV-B treatment in the wild type, it is hard to draw any conclusions on hydrogen peroxide levels, and further experimentation is needed to validate our hypothesis.

The ROS scavenging scenario in the *ggt1* mutant is undoubtedly more complex. The above-mentioned apoplastic SOD is upregulated under physiological conditions, and a peroxidase is downregulated. These effects may be interpreted as readjustments in the redox-altered

*ggt1*background. Such readjustments may be needed to sustain a higher H_2O_2 level, which could act as a signal.

Taken together, these effects may resultin higher H_2O_2 levels in the mutant under physiological conditions (schematically shown in Fig. 4), whereas the rise in H_2O_2 in the wild type is a direct consequence of oxidative stress conditions induced by UV-B radiation.

As a signaling molecule, H_2O_2 may cross membranes in a process facilitated by aquaporins (Bienert *et al.*, 2006), reaching internal cell compartments and the nucleus, where it can activate defense gene expression (Mullineaux *et al.*, 2006). If this assumption holds true, it might explain the "constitutive alert response" effect observed in a previous proteomic analysis of total leaf extracts from *ggt1* mutant leaves (Tolin *et al.*, 2013).

Future research is therefore needed to ascertain the level of ROS, and especially H_2O_2, in the apoplast of *ggt1* mutants, and the possible involvement of hormones (e.g. brassinosteroids and gibberellins) in the response. Both H_2O_2 and hormones are signals arising in the apoplast that can be transferred intracellularly and evoke the cell's responses. For this signal transduction function we could also consider four leucine-rich and two cysteine-rich proteins belonging to the superfamily of receptor-like kinases (RLKs), which are associated with the plasma membrane and contain redox-sensitive thiols,which were found at lower level in the ggt1 mutant.Disrupting of the gamma-glutamyl cycle could result in an altered signal perception pathway.

While hormonal and redox readjustments seem to be implicated in the modified metabolism of *ggt1* mutants, it remains to be seen how silencing the gamma-glutamyl transferase activity and consequently impairing the gamma-glutamyl cycle may lead to the effects reported here. Further experiments are needed to clarify the link between the gamma-glutamyl cycle and apoplastic redox events.

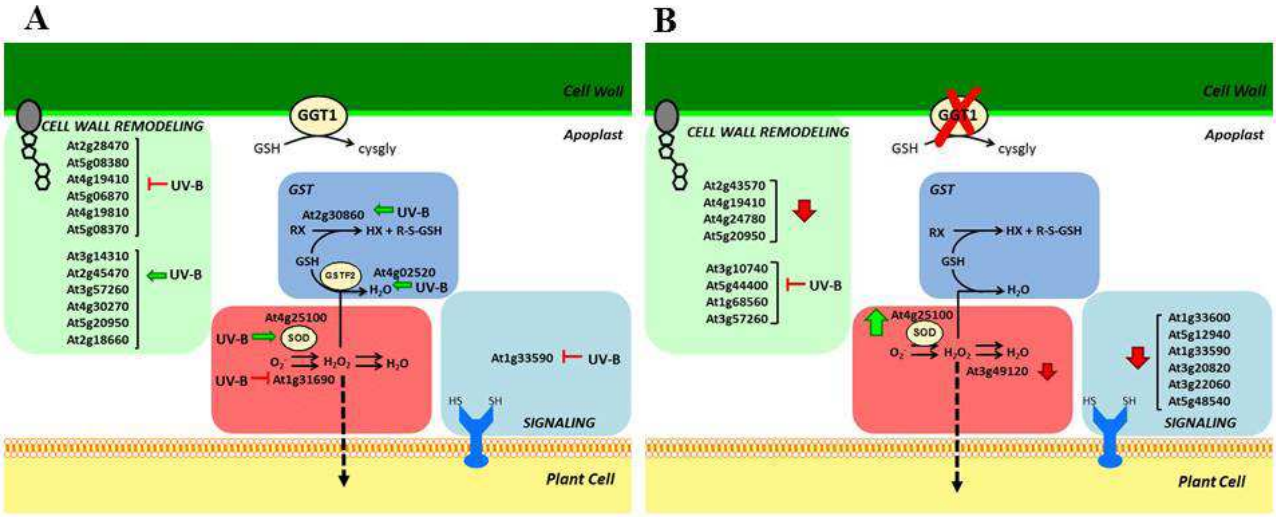


Fig. 4: Schematic overview of apoplastic proteome variations in: A) wild type, induced by UV-B; B) *ggt1* genotype due to the mutation and/or to UV-B treatment.

Vertical arrows refer to stimulation (↑) or repression (↓) caused by the mutation; horizontal arrows indicate repression (├) or stimulation (←) caused by UV-B treatment.

ACKNOWLEDGMENTS

The authors wish to thank Renato Millioni from the Proteomics Center of Padova University for his kind support, and Frances Coburn for text and style improvement. The authors also wish to thank the “Cassa di Risparmio di Padova e Rovigo” (CARIPARO) Foundation for funding the acquisition of the LTQ-Orbitrap XL mass spectrometer.

Dinesh Prasad was granted a University of Padova fellowship for Indian researchers; MS Mehdi was supported by an Erasmus Mundus - EMMA Fellowship. Leonard E. Barnabas was granted a “Fondazione A. Gini” fellowship. Micaela Pivato was funded by a University of Padova research fellowship. Marta Fàbrega-Prats was granted a fellowship for doctoral students from the CARIPARO Foundation. This work was funded by the University of Padova - MURST ex-60%.

REFERENCES

Agrawal, G. K., Jwa, N. S., Lebrun, M. H., Job, D., Rakwal, R. (2010). Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* 10, 799-827. doi: 10.1002/pmic.200900514

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., Ecker, J. R. (2003). Genome-wide insertional mutagenesis of *Arabidopsisthaliana*. *Science* 301 (5633), 653–657. doi:10.1126/science.1086391

Alscher, R. G., Erturk, N., Health, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* 53, 1331–1341. doi:10.1093/jexbot/53.372.1331

Bienert, G. P., Schjoerring, J. K., Jahn T. P. (2006). Membrane transport of hydrogen peroxide. *Biochimica et Biophysica Acta* 1758, 994-1004

Brandes, N., Schmitt, S., Jakob, U. (2009). Thiol-based redox switches in eukaryotic proteins. *Antiox. Redox Signal.* 11, 997–1014. doi:10.1089/ARS.2008.2285

Brosché, M., Strid, Å., (2003). Molecular events following perception of ultraviolet-B radiation by plants. *Physiol. Plant.* 117, 1-10. doi: 10.1034/j.1399-3054.2003.1170101.x

Brunet, S. Thibault, P., Gagnon, E., Kearney, P., Bergeron, J. J., Desjardins, M. (2003). Organelle proteomics: looking at less to see more. *Trends Cell Biol.* 13, 629–638. doi:10.1016/j.tcb.2003.10.006

Cheng, Z., Zheng, Z., Huang, J., Lai, Z., Fan, B. (2009). Biosynthesis of salicylic acid in plants. *Plant signal Behav.* 4:6, 493-496

Conesa, A., Götz, S. (2008). Blast2GO: a comprehensive suite for functional analysis in plant genomics. *Int. J. Plant Genomics* 2008:619832. doi:10.1155/2008/619832

Cosgrove, D. J. (1997). Assembly and enlargement of the primary cell wall in plants. *Ann. Rev. Cell Dev. Biol.* 13, 171-201

Delaunois, B., Jeandet, P., Clément, C., Baillieul, F., Dorey, S., Cordelier, S. (2014). Uncovering plant-pathogen crosstalk through apoplastic proteomic studies. *Front. Plant. Sci.* 5, 249. doi:10.3389/fpls.2014.00249

Destro, T., Prasad, D., Martignago, D., Bernet, I. L., Trentin, A. R., Renu I. K., Ferretti, M., Masi, A. (2011). Compensatory expression and substrate inducibility of γ-glutamyl transferase GGT2 isoform in *Arabidopsisthaliana*. *J. Exp. Bot*. 62, 805–814

Dietz, K.J. (1997). Functions and responses of the leaf apoplast under stress. *Progress in Botany* 58, 221-254

Ding, Y., Wang, J., Wang, J., Stierhof, Y. D., Robinson, D. G., Jiang, L. (2012). Unconventional protein secretion. *Trends in Plant Science* 17, 606-615. doi:10.1016/j.tplants.2012.06.004

Ferretti, M., Destro, T., Tosatto, S. C., La Rocca, N., Rascio, N., Masi, A. (2009). Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast. *New Phytol.* 181, 115–126. doi:10.1111/j.1469-8137.2008.02653.x

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207–212. doi:10.1016/0003-2697(80)90139-6

Gunawardena, H. P., Huang, Y.,Kenjale, R.,Wang, H., Xie, L., Chen, X. (2011). Unambiguous Characterization of Site-specific Phosphorylation of Leucine-rich Repeat Fli-I-interacting Protein 2 (LRRFIP2) in Toll-like Receptor 4 (TLR4)-mediated Signaling. *J Biol Chem*, 286, 10897-10910.

Henzler, T., Steudle, E. (2000). Transport and metabolic degradation of hydrogen peroxide in Chara corallina : model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. *J. Exp. Bot.* 51, 2053–2066

Hernandez, J. A., Ferrer, M. A., Jimenez, A., Barcelo, A. R., Sevilla, F. (2001). Antioxidant systems and O2.-/ H_2O_2 production in the apoplast of Pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiol.* 127, 817–831. doi:10.1104/pp.010188

Hewitt, E. J., Dickes, G. J. (1961). Spectrophotometric measurements on ascorbic acid and their use for the estimation of ascorbic acid and dehydroascorbic acid in plant tissues. *Biochem. J.* 78, 384–391

Hideg, E., Jansen, M. A. K., Strid, A. (2013). UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends Plant Sci.* 18, 107–115. doi:10.1016/j.tplants.2012.09.003

Hu, J.F., Li, G. F., Gao, Z. H., Chen, L., Ren, H. B., Jia, W. S. (2005). Regulation of water deficit-induced abscisic acid accumulation by apoplastic ascorbic acid in maize seedlings. *J. Integr. Plant Biol.* 47, 1335-1344. doi: 10.1111/j.1744-7909.2005.00165.x

Huseby, N. E., Stromme, J. H. (1974). Practical points regarding routine determination of γ - glutamyl transferase (γ-GT) in serum with a kinetic method at 37°C. *Scand. J. Clin. Lab. Invest.* 34, 357–363

Jaspers, P., Kollist, H., Langebartels, C., Kangasjarvi, J. (2005). "Plant responses to ozone," in *Antioxidants and reactive oxygen species in plants*, ed. N. Smirnoff (Oxford: Blackwell Publishing), 268-292

Kaffarnik, F. A., Jones, A. M., Rathjen, J. P.,. Peck, S. C. (2009). Effector proteins of the bacterial pathogen pseudomonas syringae alter the extracellular proteome of the host plant, *Arabidopsisthaliana*. *Mol. Cell. Proteomics* 8:145–56. doi:10.1074/mcp.M800043-MCP200

Kang, J. S., Frank, J., Kang, C. H., Kajiura, H., Vikram, M., Ueda, A., Kim, S., Bahk, J. D., Triplett, B., Fujiyama, K., Lee, S.Y., Von Schaewen, A., Koiwa, H. (2008). Salt tolerance of *Arabidopsisthaliana* requires maturation of N-glycosylated proteins in the Golgi apparatus. *Proc. Natl. Acad. Sci. U. S. A.* 105, 5933-5938. doi: 10.1073/pnas.0800237105

Karpinska, B., Karlsson, M., Schinkel, H., Streller, S., Su, K., Melzer, M., Wingsle, G. (2001). A novel superoxide dismutase with a high isoelectric point in higher plants. Expression, regulation, and protein localization. *Plant Physiol.* 126, 1668–1677

Köcher, T., Pichler, P., Schutzbier, M., Stingl, C., Kaul, A., Teucher, N., Hasenfuss, G., Penninger, J. M., Mechtler, K. (2009). High precision quantitative proteomics using iTRAQ on an LTQ orbitrap: a new mass spectrometric method combining the benefits of all. *J. Prot. Res.* 8, 4743–4752. doi:10.1021/pr900451u

Kramer, E. M. (2006). How far can a molecule of weak acid travel in the apoplast or xylem ? *Plant Physiol.* 141, 1233–1236. doi:10.1104/pp.106.083790.1

Kwon, H. K., Yokoyama, R., Nishitani, K. (2005).A proteomic approach to apoplastic proteins involved in cell wall regeneration in protoplasts of Arabidopsis suspension-cultured cells.*Plant Cell Physiol* 46, 843-857

Li, L., Van Staden, J. (1998). Effects of plant growth regulators on the antioxidant system in callus of two maize cultivars subjected to water stress. *Plant Growth Regulation* 24, 55–66

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., Muehling, K. H. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiol. Plant.* 111, 457–465. doi:10.1034/j.1399-3054.2001.1110405.x

Ludidi, N. N., Heazlewood, J. L., Seoighe, C., Irving, H. R., Gehring, C. A. J. (2002). Expansin-like molecules: novel functions derived from common domains. *Mol. Evol*. 54, 587-594

Martin, M. N., Saladores, P. H., Lambert, E., Hudson, A. O., Leustek, T. (2007). Localization of members of the gamma-glutamyl transpeptidase family identifies sites of glutathione and glutathione S-conjugate hydrolysis. *Plant Physiol.* 144, 1715–1732. doi:10.1104/pp.106.094409

Masi, A., Ghisi R., Ferretti M. (2002). Measuring low-molecular-weight thiols by detecting the fluorescence of fheir SBD-derivatives: application to studies of diurnal and UV-B induced changes in Zea Mays L. *J. Plant Physiol.*159, 499–507. doi:10.1078/0176-1617-00655

Meister, A., Anderson, M. E. (1983). Glutathione. *Annu. Rev. Biochem.* 52, 711–760. doi:10.1146/annurev.bi.52.070183.003431

Minic, Z., Jamet, E., Négroni, L., Arsene der Garabedian, P., Zivy, M., Jouanin, L. (2007). A sub-proteome of *Arabidopsisthaliana* mature stems trapped on Concanavalin A is enriched in cell wall glycoside hydrolases. *J. Exp. Bot.* 58, 2503–2512. doi:10.1093/jxb/erm082

Mullineaux, P. M., Karpinski, S., Baker, N. R. (2006). Spatial dependence for hydrogen peroxide-directed signalling in light-stressed plants. *Plant Physiol* 141, 346-350

Nelson, D. C., Scaffidi, A., Dun, E. A., Waters, M. T., Flematti, G. R., Dixon, K. W., Beveridge, C. A., Ghisalberti, E. L., Smith, S. M. (2011). F-Box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsisthaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8897–8902. doi:10.1073/pnas.1100987108

Noctor, G., Mhamdi, A., Foyer, C. H. (2014). The roles of reactive oxygen metabolism in drought: not so cut and dried. *Plant Physiol.* 164, 1636-1648. doi: 10.1104/pp.113.233478

Obel, N., Porchia, A. C., Scheller, H. V. (2002). Dynamic changes in cell wall polysaccharides during wheat seedling development. *Phytochemistry* 60, 603-610

Ohkama-Ohtsu, N., Oikawa, A., Zhao, P., Xiang, C., Saito, K., Oliver, D. J. (2008). A gamma-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in *Arabidopsis*. *Plant Physiol.* 148, 1603–1613. doi:10.1104/pp.108.125716

Pechanova, O., Hsu, C. Y., Adams, J. P., Pechan, T., Vandervelde, L., Drnevich, J., Jawdy, S., Adeli, A., Suttle, J. C., Lawrence, A. M., Tschaplinski, T. J., Séguin, A., Yuceer, C. (2010). Apoplast proteome reveals that extracellular matrix contributes to multistress response in Poplar. *BMC Genomics* 11: 674. doi:10.1186/1471-2164-11-674

Popper, Z. A., Fry, S.C. (2003). Primary cell wall composition of Bryophytes and Charophytes. *Ann. Bot.* 91, 1–12. doi:10.1093/aob/mcg013

Potters, G., Horemans, N., Jansen, M. K. (2010). The cellular redox state in plant stress biology a charging concept. *Plant Physiol. Biochem.* 48, 292–300. doi:10.1016/j.plaphy.2009.12.007

Potters, G., Pasternak, T. P, Guisez, Y, Jansen, M. A. (2009). Different stresses, similar morphogenic responses: integrating a plethora of pathways.*Plant Cell Environ* 32(2), 158-169. doi: 10.1111/j.1365-3040.2008.01908.x

Pristov, J. B., Jovanović, S. V., Mitrović, A., Spasojević, I. (2013). UV-irradiation provokes generation of superoxide on cell wall polygalacturonic acid. *Physiol. Plant.* 148, 574–581. doi:10.1111/ppl.12001

Rafudeen, S., Gxaba, G., Makgoke, G., Bradley, G., Pironcheva, G., Raitt, L., Irving H., Gehrin, C. (2003). A role for plant natriuretic peptide immuno-analogues in NaCl- and drought-stress responses. *Physiol. Plant.* 199, 554-562. doi: 10.1046/j.1399-3054.2003.00201.x

Reiter, W. D. (2002). Biosynthesis and properties of the plant cell wall. *Curr. Opin. Plant Biol.* 5, 536–542

Rizzini, L., Favory, J. J., Cloix, C., Faggionato, D., O'Hara, A., Kaiserli, E., Baumeister, R., Schäfer, E., Nagy, F., Jenkins, G. I., Ulm, R. (2011). Perception of UV-B by the *Arabidopsis* UVR8 protein. *Science* 332 (6025), 103–106. doi:10.1126/science.1200660

Rodman, J. E. (1991). A taxonomic analysis of glucosinolate-producing plants. Part 1: Phenetics, *Syst. Bot.* 16, 598-618

Saruhan, N., Terzi, R., Saglam, A., Kadioglu, A. (2009). The relationship between leaf rolling and ascorbate-glutathione cycle enzymes in apoplastic and symplastic areas of Ctenanthe Setosa subjected to drought stress. *Biol. Res.* 42, 315–326. doi:10.4067/S0716-97602009000300006

Sävenstrand, H., Brosché, M., Strid, A. (2004). Ultraviolet-B signalling: *Arabidopsis* brassinosteroid mutants are defective in UV-B regulated defence gene expression. *Plant Physiol. Biochem.* 42, 687–694. doi:10.1016/j.plaphy.2004.06.011

Schafer, F. Q., Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Rad. Biol. Med.* 30, 1191–1212. doi:10.1016/S0891-5849(01)00480-4

Scheler, C., Durner, J., Astier, J. (2013). Nitric oxide and reactive oxygen species in plant biotic interactions. *Curr. Opin. Plant Biol.* 16, 534–539. doi:10.1016/j.pbi.2013.06.020

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., Shinozaki K. (2002). Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high -salinity stresses using a full-length cDNA microarray. *Plant J* 31, 279-292

Shiu, C.-T. (2005). Ultraviolet-B-induced oxidative stress and responses of the ascorbate-glutathione cycle in a marine macroalga Ulva Fasciata. *J. Exp. Bot.* 56,2851–2865. doi:10.1093/jxb/eri277

Smirnoff, N., Pallanca, J. E. (1996). Ascorbate metabolism in relation to oxidative stress. *Biochem. Soc. Trans.* 24, 472–478

Stolle-Smits, T., Beekhuizen, J. G., Kok, M. T., Pijnenburg, M, Recourt, K., Derksen, J., Voragen, A. G. (1999). Changes in cell wall polysaccharides of green bean pods during development. *Plant Physiol.* 121, 363–372

Tolin, S., Arrigoni, G., Trentin, A. R., Veljovic-Jovanovic, S., Pivato, M., Zechman, B., Masi, A. (2013). Biochemical and quantitative proteomics investigations in *Arabidopsisggt1* mutant leaves reveal a role for the gamma-glutamyl cycle in plant's adaptation to environment. *Proteomics* 13, 2031–2045

Vanacker, H., Carver, T. L. W., Foyer, C. H. (1998a). Pathogen-Induced Changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiol.* 117, 1103–1114. doi:10.1104/pp.117.3.1103

Vanacker, H., Harbinson, J., Ruisch, J., Carver, T. L. W., Foyer, C. H. (1998b). Antioxidant defences of the apoplast. *Protoplasma* 205, 129-140

Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R. A., Lamb, C. (2004). An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *EMBO J.* 23, 980–988. doi:10.1038/sj.emboj.7600086

Xin, Z., Zhao, Y., Zheng, Z. L., (2005). Transcriptome analysis reveals specific modulation of abscisic acid signaling by ROP10 small GTPase in *Arabidopsis*. *Plant Physiol* 139, 1350–1365. doi:10.1104/pp.105.068064.dopsis

Yao, X., Xiong, W., Ye, T., Wu, Y. (2012). Overexpression of the aspartic protease *ASPG1* gene confers drought avoidance in *Arabidopsis. J Exp Bot* 1-15doi: 10.1093/jxb/err433

CHAPTER 2: Antonio Masi, **Anna Rita Trentin**,Ganesh Kumar Agrawal, and Randeep Rakwal

Gamma-glutamyl cycle in plants: a bridge connecting the environment to the plant cell?

[1]DAFNAE, University of Padova, Legnaro, Italy

[2]Research Laboratory for Biotechnology and Biochemistry (RLABB), Kathmandu, GPO Box 13265, Kathmandu, Nepal

[3]GRADE Academy Private Limited, Adarsh Nagar-13, Main Road, Birgunj, Nepal

[4]Organization for Educational Initiatives, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8577, Japan

[5]Department of Anatomy I, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa, Tokyo 142-8555, Japan

***Correspondence:** Dr. Antonio Masi, DAFNAE, University of Padova, Legnaro, Italy

E-mail: antonio.masi@unipd.it

Fax: +39-049-827-2929

APOPLAST AND REDOX COMPONENTS IN PLANTS ADAPTATION TO THE ENVIRONMENT

Being at the interphase between the plant cell and the environment, the apoplast represents a compartment where an extensive cross-talk occurs among different components, to generate signals that can pass through the plasmalemma and reach the symplast (Agrawal *et al.*, 2010). In this way, both abiotic and biotic stress conditions evoke defensive and adaptive responses. These responses are represented by the structural and metabolic readjustments that are driven by enzymes (proteins), whose coordinated expression is regulated by signals and signal transduction pathways(Foyer and Noctor 2005). For an environmental stimulus to be sensed and the response to occur, it follows that any environmental condition, which is external to the cell, must initiate an extracellular signal. Upon interaction of such signals with the receptors located on the plasma membrane, or their transfer to the inner compartment, a signal transduction pathway can be initiated to readjust cell metabolism to the new conditions. This task requires the concerted action of many players: specific genes expression, post-transcriptional and post-translational regulation, hormones and cell regulators.

When trying to explain the process of plant sensing and adaptation to the environment, key questions arise: what are the signals generated by the environment? How can they evoke the response? A widely accepted view is that many unfavourable conditions result in the appearance of reactive oxygen species (ROS) (Pitzschke*et al.*, 2006). ROS are the natural consequence of a life in an oxygen-containing atmosphere, and result from any imbalance in the electron flow in fundamental processes such as photosynthesis and respiration. They are represented by oxygen-containing radical species or hydrogen peroxide, H_2O_2, having an intrinsic reactivity with the organic molecules which can be consequently either damaged or undergo a redox modification. The increase of ROS in the apoplast, as a consequence of oxidative conditions, has been documented (Mittler *et al.*, 2004; Noctor, 2006; Potters *et al.*, 2010). Under physiological conditions, they are

involved in cell wall synthesis and remodeling, e.g., the ROS are also part of a cross-talk between host and pathogen during the infection process(Torres *et al.*, 2006).ROS and redox modifications thus seem to be good candidates in transferring the information related to the environment to the cell.

GAMMA-GLUTAMYL CYCLE AND GAMMA-GLUTAMYL-TRANSFERASES

In plants, the gamma-glutamyl cycle is a metabolic route of extra-cytosolic glutathione degradation by gamma-glutamyl-transferase (GGT) and cys-gly dipeptidase, followed by the re-uptake of constituent amino acids, intracellular re-synthesis and extrusion (Ferretti *et al.*, 2009). In Arabidopsis, a detailed description of the four GGT genes expression was obtained by GUS-staining of transformed lines (Martin *et al.*, 2007). GGT1 and GGT2 have high similarity and share large sequence identity, and they both are located in the apoplast; but while GGT1 is ionically cell-wall bound and expressed in most vascular tissues (Ferretti *et al.*, 2009), GGT2 seems to be preferentially associated to plasma membranes and expressed in specific tissues in seeds, flowers, and roots.GGT3 is believed to be a non-functional, truncated sequence, whereas GGT4 has been localised in the vacuole, where it assists in the degradation of GS-conjugates of toxic compounds and xenobiotics (Grzam *et al.*, 2007). The significance of GSH cycling between the extracellular and intracellular space was addressed in an *A. thaliana* knockout mutant line lacking the *ggt1* isoform, by comparative proteomic analyses of total leaf extracts (Tolin *et al.*, 2013). In that study, it was reported that the disrupture of the gamma-glutamyl cycle by *ggt1*silencing results in enhanced expression of an array of antioxidant and defense enzymes, which could be collectively described as a “constitutive alert response”.

The occurrence of glutathione in the apoplast has often been questioned in the past, but several evidences now indicate that it is indeed there, although at low level (Zechman, 2014); it seems puzzling, however, that a glutathione degradation activity, occurring outside the cell, can result in

redox alteration inside the cell. Due to its low extracellular concentration, it is unlikely that glutathione itself acts as an antioxidant outside the cell; that function might better be fulfilled by ascorbate, reportedly by far more abundant in the apoplast (Pignocchi and Foyer 2003), where in any case oxidizing conditions are prevalent.

GLUTATHIONE AND GLUTATHIONE DEGRADATION ACTIVITY

All this considered, what could be then the function of glutathione and glutathione degradation activity? Some key elements that are worth considering are: i) presence of a redox sensitive thiol group in the molecule; ii) apoplastic ROS production as a consequence of the unfavourable environmental conditions; iii) presence of the plasma-membrane bound receptors; iv) redox exchange reactions occurring between the low-molecular-weight thiols and cysteines of plasma-membrane bound proteins, acting as redox switches. In order for a molecule to act as a signal, its concentration should be low and un-buffered, such that perturbations may induce large variations in its pool size. The reversible conversion of reduced to oxidized form may also rapidly modify the GSH pool. The interaction and exchange reactions of low-molecular-weight thiols and cysteines of plasma-membrane receptors and components may secondarily amplify the signal. On the other hand, the possibility that gamma-glutamyl cycling be implicated in the response to oxidative stress might be inferred by some previous reports (Masi *et al.*, 2002; Ferretti *et al.*, 2009).

To better investigate the relationship between oxidizing stress conditions and GGT-driven glutathione degradation, apoplastic fluid proteins were extracted from *ggt1* mutant leaves following ultraviolet B (UV-B) treatment (Trentin *et al., submitted*). Comparative analysis with wild-type and control conditions, and data integration suggest that while expression of cell wall remodelling proteins is affected by both UV-B and *ggt1* silencing, the mutation itself resulted in reduced expression of a number of plasma-membrane associated genes (cys-rich, leucine-rich secretory proteins) which are involved in signalling and are assigned to the gene ontology category of

"response to stimulus". Alteration of expression of components related with ROS metabolism, such as superoxide dismutase (SOD), glutathione S-transferases (GSTs) or peroxidases, is also observed in one condition or another, but given the simultaneous presence of alternative pathways it is hard to predict, from that study, whether the level of apoplastic H_2O_2 is increased or not, and therefore if H_2O_2 is the molecule involved in transferring the signals arising from the apoplast.

PROTEOMICS AS A TOOL TO UNDERSTAND THE GAMMA-GLUTAMYL CYCLE

In the post-genomic era, understanding gene functions has been greatly facilitated by the availability of mutants and by other "omics" tools. Altering or silencing the expressionof selected genes, either constitutively or transiently, may result in a phenotype whose interpretation enables assignment of physiological roles and functions. In many cases, this is not an easy task; because of the absence of a clear phenotype or too complex ones, resulting from redundancy, compensatory and pleiotropic effects, etc. These effects are not so obvious or easily predictable. Thus, "omic" technologies prove to be highly valuable to decipher metabolism, in that they can describe re-adjustments in transcripts, protein or metabolite profiles resulting from gene manipulation or environmental constraints.

Proteomics has greatly assisted in elucidating the functions of the gamma-glutamyl cycle in GGT mutants. While it was clear that GGT4 mutants are unable to detoxify xenobiotics or toxic compounds, GGT2 mutants have no clear phenotype and GGT1 mutants exhibit a slightly shorter life cycle. In this case, it was proteomics to demonstrate, in *ggt1* mutants, alterations in the antioxidative and defense responses, and apoplastic components that may convey redox information from the extracellular milieu to internal compartments. In the future, proteomics may still contribute to pinpoint plasma membrane components that are clearly involved in this process. Proteomics may

also help in identifying one missing step in the gamma-glutamyl cycle, i.e., the cysteinyl-glycine dipeptidase, whose occurrence is inferred but not demonstrated yet (Kumada *et al.*, 2007).

CONCLUSIONS

The significance of the gamma-glutamyl cycle is not fully understood yet. Glutathione cycling between the symplast and apoplast may represent a way to transfer redox information. Functional genomics approaches indicate that disruption of the functional cell-wall bound GGT1 isoform results in a constitutive alert response where anti-oxidative enzymes are up-regulated, probably as an effect of the altered plasma membrane receptors level and the redox state. With the more general aim of understanding how environmental challenges are perceived by plant cells, it seems therefore important to conclusively assign a role for extracellular GGTs and the gamma-glutamyl cycle in controlling the redox signals generated in the apoplast, which are transferred to the symplast and activate antioxidant systems. To this end, further high-throughput and targeted proteomic approaches will be necessary to perform and compare under the diverse stresses as indicated in Fig.1.

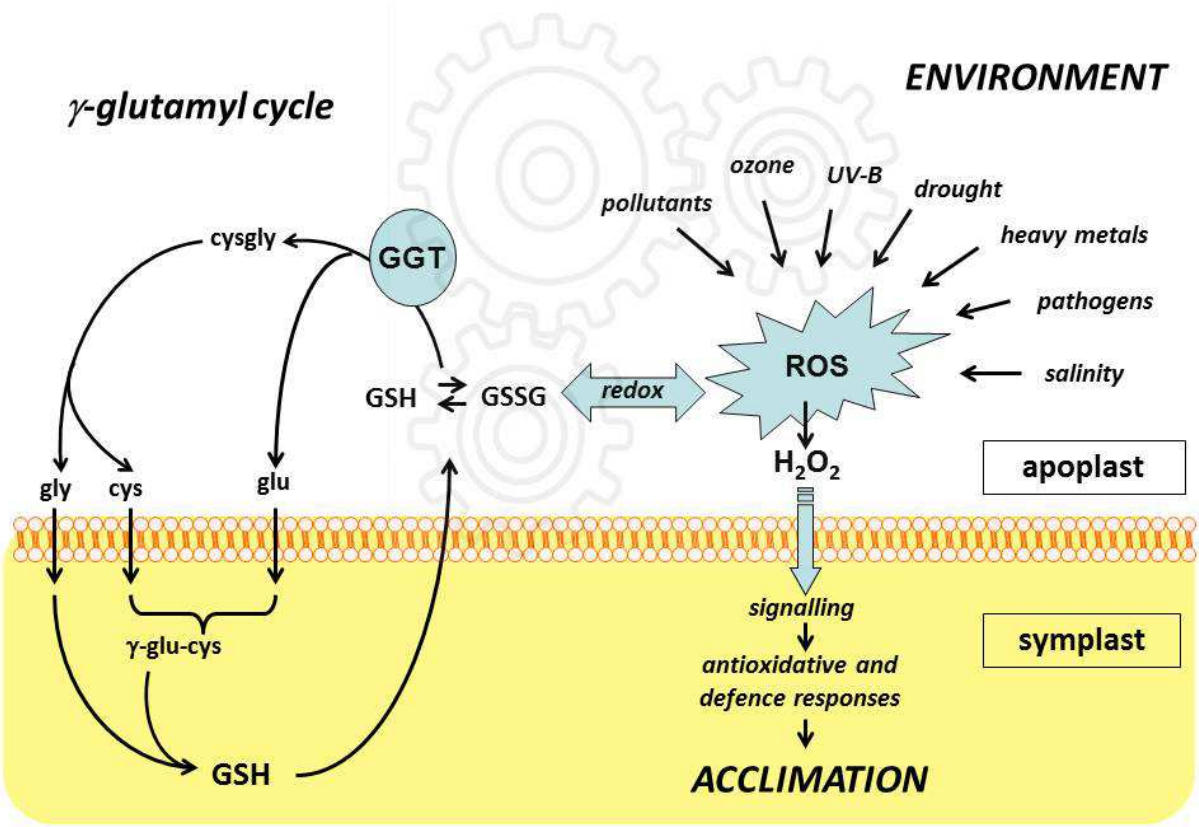


Figure 1. Integration of redox events in the apoplast with the γ-glutamyl cycle. Unfavourable environmental conditions result in formation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), which may intracellularly activate anti-oxidative and defense responses leading to plant adaptation.

ACKNOWLEDGMENTS

This work was supported by grants from University of Padova – "MURST ex-60%".

REFERENCES

1. Agrawal G. K., Jwa N. S., Lebrun M. H., Job D., Rakwal R. (2010). Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* 10, 1-29.
2. Ferretti M., Destro T., Tosatto S.C., La Rocca N., Rascio N., Masi A. (2009). Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast. *New Phytol.* 181, 115-126
3. Foyer C.H., Noctor G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17, 1866-1875
4. Grzam, A., Martin, M. N., Hell, R., Meyer, A. J. (2007). γ-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione S-conjugates in Arabidopsis. *FEBS Lett.* 581, 3131-3138.
5. Kumada H. O., Koizumi Y., Sekiya J. (2007). Purification and characterization of dipeptidase hydrolyzing L-cysteinylglycine from radish cotyledon. *Biosci. Biotechnol. Biochem.* 71, 3102-3104.
6. Martin M. N., Saladores P. H., Lambert E., Hudson A. O., Leustek T. (2007). Localization of members of the γ-glutamyl transpeptidase family identifies sites of glutathione and glutathione S-conjugate hydrolysis. *Plant Physiol.* 144, 1715-1732.
7. Masi A., Ghisi R., Ferretti M. (2002). Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. *J. Plant Physiol.* 159, 499-0507.
8. Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490-498.
9. Noctor G. (2006). Metabolic signaling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ.* 29, 409-425.

10. Pignocchi C., Foyer C. H. (2003). Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr. Opin. Plant Biol.* 6, 379-389.
11. Pitzschke A., Forzani C., Hirt H. (2006). Reactive oxygen species signaling in plants. *Antioxid. Redox Signal.* 8, 1757-1764.
12. Potters G., Horemans N., Jansen M. A. K. (2010). The cellular redox state in plant stress biology – a charging concept. *Plant Physiol. Biochem.* 48, 292-300
13. Tolin S., Arrigoni G., Trentin A. R., Veljovic-Jovanovic S., Pivato M., Zechman B., Masi A. (2013). Biochemical and quantitative proteomics investigations in Arabidopsis ggt1 mutant leaves reveal a role for the gamma-glutamyl cycle in plant's adaptation to environment. *Proteomics* 13, 2031-2045.
14. Torres M. A., Jones J. D. G., Dangl J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* 41, 373-378.
15. Trentin A. R. et al.(submitted to *Frontiers in Plant Science*, revision pending).
16. Zechmann B. (2014). Compartment-specific importance of glutathione during abiotic and biotic stress. *Front. Plant Sci.* 5, 566.

CHAPTER 3: REGULATION OF ROS HOMEOSTASIS IN APOPLASTIC SPACE BY THIOLS

ABSTRACT

Glutathione and low molecular weight thiols (cysteine and cysteinyl-glycine), with their –SH moiety, are strongly reactive nucleophilic molecules. They have a main role in plant metabolism, defense from biotic and abiotic stress and in redox homeostasis by contrasting excessive oxidation states.

In plant cell the apoplast is an interphase between the environment and symplast. Environmental oxidative stresses evoke in the apoplastic space production of reactive oxygen species (ROS) subsequently, in some way, a signal transduction pathways is activated. This allows to signal to pass through plasma membrane and reach the cytoplasm.

Aim of this work was to investigate the role of LMW thiols in root apoplast, by highlighting reactions involving low molecular weight thiols, metals and enzymes related to ROS metabolism.

Our results obtained with chromatography, ICP spectrometry, EPR spectroscopy and absorption spectroscopy analyses, and in presence of isolated cell walls, indicate that glutathione, cysteinyl-glycine and cysteine have quenching capacity for reactive oxygen species generated in the apoplastic space.

Our findings point to a role for LMW thiols, which are metabolically related to each other in the gamma-glutamyl cycle, in modulating redox reactions in plant cell walls.

INTRODUCTION

Reactive oxygen species (ROS) generation requires an activation energy but then it continues spontaneously (Karuppanapandian*et al.*, 2011); in plant tissues and cells they can be produced by photosynthesis and by various environmental stresses (Schofer *et al.*, 2001; Rodrigues-Serrano *et al.*, 2006). As a consequence, ROS concentration in the apoplast varies fastly and highly due to biotic and abiotic stresses (Hernandez *et al.*, 2001; Jaspers *et al.*, 2005; Hu *et al.*, 2005; Pristov *et al.*, 2013).

Notably, regulation of a plethora of important cell functions occurs by oxidation/reduction reactions in mammalian cells (Paolicchi *et al.*, 2002) and recent evidences have shown that ROS play a key role as a messenger in normal cell signal transduction and cell cycling (Rahantaniaina *et al.*, 2013). In cell wall and apoplast there are several enzymes, like oxidases and peroxidases, that produce ROS in response to development and environmental challenge. Moreover, it is known that superoxide has an important role in redox signalling and activates defense responses (Foyer and Noctor 2009). Plants present many and different strategies to improve defence responses by scavenging ROS with enzymatic and non-enzymatic processes (Sharma *et al.*, 2012).

One of the main non-protein antioxidants in cells is glutathione, a versatile molecule with a plethora of roles in plant metabolism and defense. It is known that GSH plays a crucial protective role in redox homeostasis (Foyer and Noctor 2011); it reacts with ROS thus protecting thiol groups to contrast excessive protein oxidation (Rahantaniaina *et al.*, 2013). Glutathione's functions depend on cysteine's thiol moiety, a strong nucleophilic group (Pivato *et al.*, 2014) that allows reacting with a wide spectrum of molecules to form not only GSSG but also GS-conjugates (Dixon and Edwards, 2010) and protein thiolation (Pivato *et al.*, 2014). Glutathione scavenging capacity is depending on pKa for the sulphydryl group (pKa = 8.83); when proton concentration decreases also glutathione redox potential is reduced. This property has relevant physiological implications, since pH differs significantly among cell compartments; consequently, also the GSH/GSSG ratio is expected to change (Rahantaniaina *et al.*, 2013). It is known that pH in the apoplastic space is around 4.5-5; therefore, in theory, glutathione will be completely reduced. However, the presence of oxidizing molecules are able to oxidize GSH to GSSG (e.g.: dehydroascorbate, ROS).

In plant tissues, GSH is mainly localised intracellularly; however, a limited amount is also found in the apoplastic space, where it is involved in the so called gamma-glutamyl cycle. In this cycle, it is synthetized in the cytosol, carried out to the extracellular space and cleaved by gamma-glutamyltransferase (GGT) to produce cysteinyl-glycine (cys-gly) and glutamate (Ferretti *et al.*, 2009). Subsequently, cysgly is cleaved to cysteine and glycine by an unknown yet dipeptidase

(Ohkama-Ohtsu *et al.*, 2008); single aminoacids can finally cross plasma membrane by means of aminoacid transporters, and be used in the cytosol to synthesize new GSH.

The presence of these GSH-related thiols in the apoplast may result in redox reactions and an altered redox balance in the extracellular space, due to their biochemical properties and reactivity.
Although GSH is commonly considered a major antioxidant in living cells, in the last few years it has been provided evidence for a prooxidant role of GSH and thiols deriving from its catabolism in animals, as a result of gamma-glutamyl transpeptidase (GGT) activity(Stark *et al.*, 1993; Pompella *et al.*, 2003; Dominici *et al.*, 2005). This novel evidence may have strong implications also in plantbiology and physiology.
In particular, GSH, cysteine and cysteinyl-glycine promote metal ions reduction and induce production of reactive species such as superoxide, hydrogen peroxide and other free radicals (Paolicchi et al., 2002). Metal ions are also present in the apoplast; their concentration depends on plant species, and they interact with cell wall components and thiols (Speisky *et al.*, 2008, 2011; Carrasco-Pozo *et al.*, 2008; Aliaga *et al.*, 2010, 2012). Notably, it is reported that redox active transition metals can react with H2O2 and convert it into hydroxyl radical via metal-catalyzed Fenton reaction (Karuppanapandian *et al.*, 2011).

Two categories of enzymes located on the cell wall are mainly involved in oxidative metabolism: peroxidases, which use H_2O_2 as a substrate, and superoxidase dismutases converting O_2^- to H_2O_2 and producing •OH(Higashi *et al.*, 2015; del Río 2015).However, it has been reported that PODs may also produce H_2O_2 by oxidation of different kinds of reductants as metal and phenolic compounds (Urbañski and Berêsewicz 2000; Šukalovic*et al.*, 2005; Kukavica *et al.*, 2009) and in presence of other reductants (such as NADH) PODs can produce •OH (Chen and Schoffer 1999; Schoffer *et al.*, 2002). Additionally, there is evidence for a superoxide radical involvement in hydroxyl radical production (Chen and Schoffer 1999; Liszkay *et al.*, 2004; Karkonen and Fry 2006). However, who are the naturally occurring reductants that in apoplast participate to ROS production? This question remains still unknown (Kukavica *et al.*, 2009).

Aim of this work is to investigate the role of LMW thiols in root apoplast, by highlighting reactions and interactions that occur in apoplastic space between low molecular weight thiols, metals and enzymes involved in response to oxidative metabolism.

In this study, various techniques have been used to determine structural variations occuring in the apoplastic space due to low molecular weight thiols: HPLCchromatography, ICP spectrometry, EPR spectroscopy and absorption spectroscopy.

MATERIAL AND METHODS

Plant material

Barley seeds were treated with bleach solution for 10 minutes, washed with water and then soaked to germinate in a dark room in controlled temperature and humidity for 4 days. Seedlings were transferred into a becker with a modified Hoagland solution (1/2 strength, as described in table 1) in a growth chamber set as follows 12/12 h light/dark cycle, 21/21 °C temperature, 300 µmol m-2 s-1 photosynthetically active radiation and 60% relative humidity.

Chromatographic low molecular weight thiols determination

10 days plants were used for the experiments. Roots where washed 2 times with 1 mM $CaCl_2$, dryed and weighed. About 1 g was used for the extraction of extracellular washing fluid (ECWF) obtained by centrifuging roots at 4°C, 200 g for 20 minutes. Total root extracts were obtained by powdering plant material in liquid N_2 with 0.1 M HCl in ratio 1:4 fresh weight : buffer(n=10 replicates). To determine low molecular weight (LMW) thiols concentration in the extracts, 50 µL of total leaf extract and ECWF were derivatized with 4-Fluoro-7-sulfobenzofurazan ammonium salt fluorophore (SBD-F) (Dojindo, Japan). Thiols were separated by isocratic HPLC using the method described by Masi *et al.*, 2002 with some modifications. The mobile phase was 75 mM NH_4-formiate, pH 2.9 and 3% methanol (97:3, vol/vol).

Metals determination

The elemental determination was conducted with ICP-OES, SPECTRO ARCOS EOP (Spectro A.I., Germany).

The ICP-OES was employed to determine 24 elements: Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Sr, Tl, V and Zn. All instrument operating parameters were optimized for nitric acid 3% solution.

Sample Preparation and Calibration Standards

The root samples were analysed after microwave closed vessel digestion (Ethos 1600 Milestone S.r.l. Sorisole, BG, Italy) . Roots (0,35 g)were added to hydrogen peroxide and nitric acid “suprapure” quality (Merck, Darmstadt, Germany). After microwave digestion, the so dissolved sample was diluted with ultrapure water to a final volume of 25 ml. Cell wall fractions were obtained by powdering roots in liquid $N_2$and extraction with buffer (50 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.05% Tween 80, 1 mM PMSF), and centrifugation at 4°C, 500 g for 20 minutes. The pellet phase contained cell wall fraction and was digested with microware as above.Calibration

standards were matched to 3% nitric acid “suprapure” quality. Priorto the final dilution, the elements to be determined were added from multielement solutions and stock solutions (Inorganic Ventures, Christiansburg, VA, USA). Concentrations of 0, 0.005, 0.02, 0.05, 0.2 and 0.5 mg/L of the analytes were prepared.

Validation

The accuracy and precision of both methods were investigated analyzing blank solution, low level control solution (recovery limits ± 30%), medium level control solution (recovery limits ± 10%) and the international standard reference material NIST SRM 1643e (Trace Elements in Natural Water) prepared like above described. The measured values and the certified values were in excellent agreement for all the elements.

EPR spectroscopy

EPR measurements were made for in vitro detection of •OH and superoxide radical ($\bullet O_2^-$) signals and to establish thiols quenching ability. EPR spectra were recorded at room temperature using a Elexsys II spektrometar operating at X-band (9.51 GHz) under the following settings: modulation amplitude, 0.2 mT; modulation frequency, 100 kHz; microwave power, 10 mW; centre of magnetic field, 341 mT; scan range, 20 mT; scan speed, 4 mT/min. Spectra were recorded and analyzed using Xepr (Bruker) software. Measurements at low temperature were performed at 125°K.

Superoxide Production

Superoxide radical was generated, in the presence of oxygen, by exposing riboflavin to light and detected using a spin-trapping reagent 5-(diethoxyphosphoryl)-5-methyl-1-pyroline N-oxide (DEPMPO).

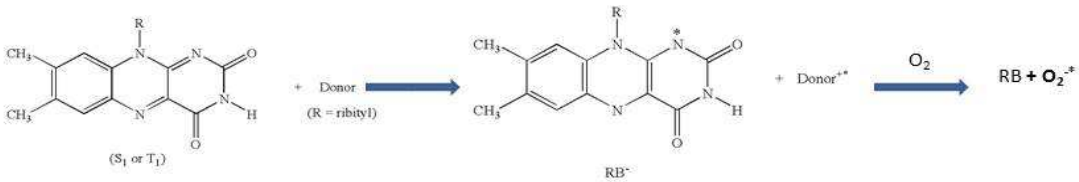


Fig. 1: Schematic mechanism of supeoxide radical production by riboflavin

Control reaction mixture had a final volume of 30 µL composed of DTPA 4 mM; DEPMPO 50 mM and Riboflavin 0,22 mM. To evaluated thiols quenching power, 0,3 mM of each tested molecule (cys, cys-gly and GSH) was added to the reaction mixture.

Hydroxyl radical

Hydroxyl radical was generated in Fenton reaction the presence of Cu^{+} and H_2O_2 was detected using a spin-trapping reagent 5-(diethoxyphosphoryl)-5-methyl-1-pyroline N-oxide (DEPMPO).

Hydroxyl radical formation was allowed by the reaction:

- $Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + OH^{-} + \bullet OH$
- DEPMPO + •OH $\longrightarrow$ DEPMPO•/OH

Reaction mixture was composed of 4 mM DTPA; 0.116 mM Cu^{+}; 50 mM DEPMPO and 4mM H_2O_2. To evaluated thiols quenching power, 0.03 mM of each tested molecule (cys, cys-gly and GSH) was added to the reaction.

Cell Wall Isolation

Barley roots were powdered in liquid N_2 and homogenized with buffer [50 mM Tris‑HCl (pH 7.2), 50 mM NaCl; 0.05% Tween-80; 1 mM phenylmethylsulfonyl fluoride (PMSF)] in ratio 1:2 according to Kukavica *et al.* (2009). The homogenate was filtered through two layers of cloth. The filtrate was centrifuged at 1,000× g for 20 min at 4°C. The cell wall pellet was washed four times in the same buffer without detergent and salt. In order to obtain ionically bound fraction, the cell wall pellet was suspended in 1 M NaCl, followed by incubation for 30 min at 4°C with continuous stirring. Then it was centrifuged at 1,000 ×g for 10 min. Both fractions were collected: in the supernatant there were ionically cell wall-bound proteins and in the pellet the covalently-bound fraction remained.

Determination of peroxidase activities

Oxidative activity of cell wall peroxidase (PODox) was determined as described by Sukalovic *et al.*, 2005 following the decrease in absorbance at 340 nm, due to oxidation of β-Nicotinamide adenine dinucleotide (NADH). Reaction mixture was composed of 0.25 mM $MnCl_2$, 0.2 mM p-coumaric acid, in 50 mM phosphate buffer pH 5.5 and 10 µL of cell wall isolate; reaction started by adding 0.2 mM NADH. Alteration of NADH oxidation by thiols was measured by adding them directly in cuvette to final concentrations of 1, 0.1 or 0.01 mM. Oxidative POD activity was also measured after incubation, for 30 minutes, of cell wall with 1 mM thiols, 1 mM Detapac, 1 mM DTT and 1 mM H_2O_2. Spectrophotometric measurements of NADH reduction/oxidation were performed as

described above except. All the assays were performed at 28°C using Shimadzu, UV-160 spectrophotometer (Japan).

RESULTS AND DISCUSSION

Thiols detection

Low molecular weight thiols were measured in a total roots extract and in the extracellular washing fluids. A representative chromatogram of thiols distribution is reported in Fig. 1 and contents are shown in table 1.

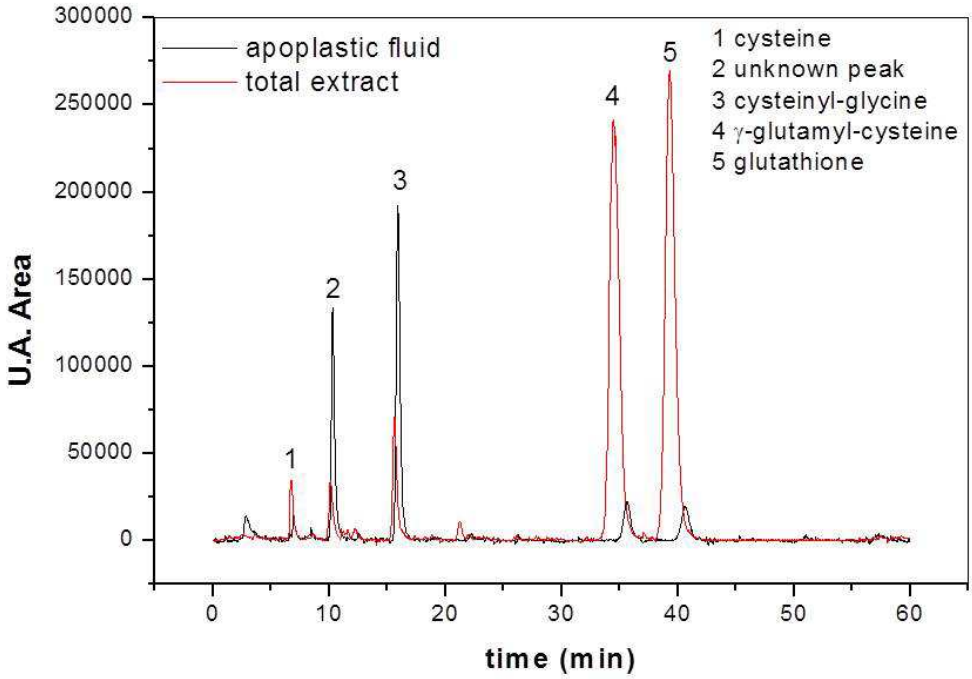


Fig. 1: A rapresentative chromatogram of thiols in barley roots in a total extract (red line) and in the apoplast (black line).

	Cys	Cys-Gly	γ-EC	GSH	
total extract	6,75 ± 0,94	1,79 ± 0,50	225,73 ± 88,03	89,60 ± 11,82	nmol/g FW
apoplastic fluid	2,10 ± 1,29	2,26 ± 1,18	1,94 ± 2,15	1,33 ± 0,75	nmol/mL
ratio apoplast/total	0,31 ± 0,22	1,26 ± 0,66	0,01	0,01	

Table 1: Thiols content in barley: in total roots, apoplastic space and relative distribution. Values are mean of 8 biological replicates + standard deviation.

Gamma-glutamylcysteine (γ-EC) is glutathione precursor, synthetized in the cytosol, and is usually low abundant and at the detection limits; here, the high γ-EC content highlights active glutathione synthesis in the root tissue. Glutathione extrusion is followed by degradation by gamma-glutamyl transferase in the gamma-glutamyl cycle, and its apoplastic content is consequently very low (comparing with cytosol). From Fig. 1 it is possible to argue that cysteine, cysteinyl-glycine and an unidentified peak are the thiols present in the extracellular compartment, and due to the nucleophilic properties of the –SH moiety they may react with a variety of molecules (e.g.: metals, enzymes, cell wall components).

Noticeably, the most abundant contents are measured in cysteinyl-glycine and the unknown peak; it seems interesting that these two thiols show a strict positive correlation (Fig. 2), suggesting that they may be metabolically related.

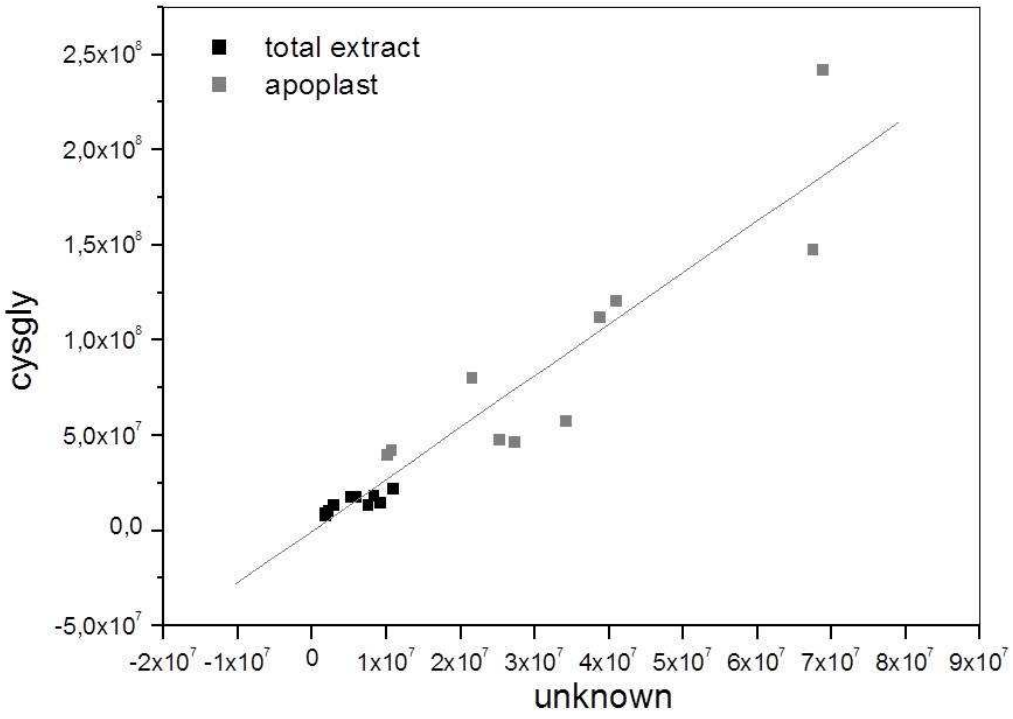


Fig. 2: Correlation between areas of cysteinyl-glycine and unknown peak.

Metals content

Metal ion contents were measured in root tissue and in cell wall compartment to evaluate ion distribution between inside and outside the cell. All metals, from nutrient solution, pass through cell walls and apoplastic space to reach the cytosol, where are implicated in a plethora of metabolic processes. However, ions are differently abundant in the apoplast: iron is the most abundant in

absolute values, whereas if we consider the ratio between the two compartments, copper and zinc concentrations are relatively higher in the apoplast (Table 2).

Iron is well known to be involved in redox balance and plant defense responses. Reactive Fe^{3+} ions are stored in cell walls, to contrast pathogen attack and mediate oxidative stress (Liu *et al.*, 2007). Copper and zinc are both required as cofactors of several apoplastic enzymes (e.g.: superoxide dismutase, ascorbate oxidase, laccases, dipeptidases), which may explain the high apoplastic ratio of these metals. However, free Cu^{2+} reacts with protein thiol bonds, causing disruption of secondary structure and moreover, catalyses reactive oxygen species production by activating Fenton reactions (Ducic and Polle 2005). This dual perspective makes copper interesting while evaluating cell wall interaction with low molecular weight thiols.

	Cu^{2+}	Fe^{3+}	Mn^{2+}	Mo^{6+}	Zn^{2+}	
roots content	1,63 ± 0,11	74,40 ± 10,76	4,35 ± 0,50	0,17 ± 0,03	7,36 ± 0,31	mg/Kg
cell wall content	1,21 ± 0,50	42,41 ± 16,67	1,20 ± 0,12	0,06 ± 0,01	5,77 ± 2,80	mg/Kg
ratio apoplast/total	0.74	0.57	0.28	0.35	0.78	

Table 2: Metals content in barley: in total roots, apoplastic space and relative distribution. Values are mean of 5 biological replicates + standard deviation.

EPR measurements

EPR spectroscopy technique was used for the in vitro detection of hydroxyl radical (•OH) and superoxide radical ($\bullet O_2^-$) signals to establish thiols quenching ability. Superoxide radical was generated, in the presence of oxygen, by exposing riboflavin to light, and detected using a spin-trapping reagent 5-(diethoxyphosphoryl)-5-methyl-1-pyroline N-oxide (DEPMPO). Cysgly, GSH and Cys were tested for the ability to quench the ROS species.

Results obtained in the study of superoxide radical (Fig. 3) evidence an effective ability of low molecular weight thiols to quench$\bullet O_2^-$ radical. Results show that it is possible to sort thiols according to their superoxide radical quenching ability since they were used at the same concentration (0.3 mM): cys-gly > cys > GSH. The actual scavenging efficiency, however, depends on physiological concentrations of those thiols, which may differ significantly from each other.

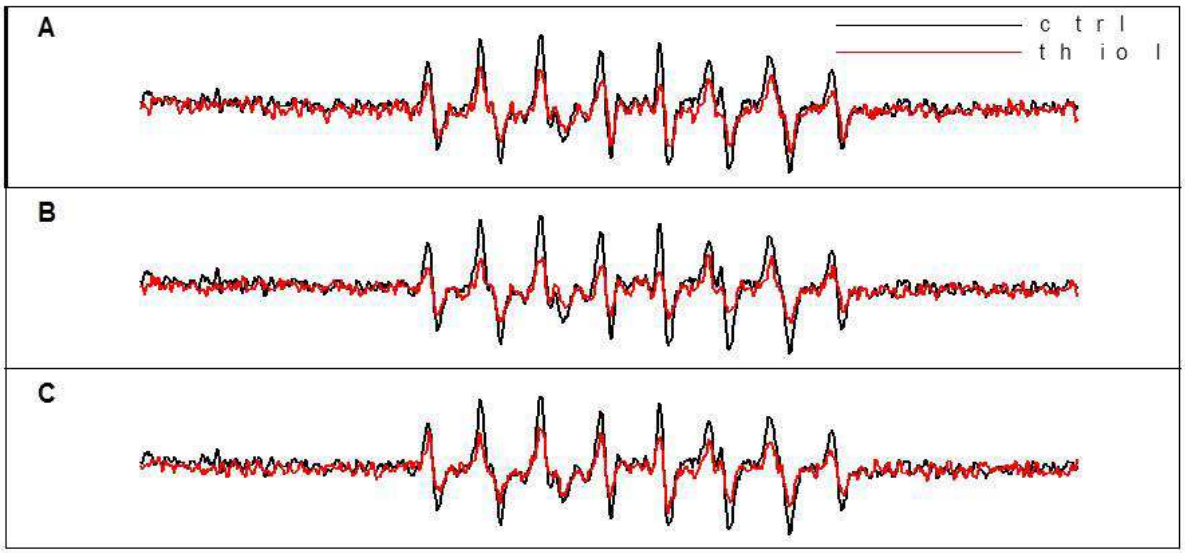


Fig. 3: EPR spectra, showing the in vitro detection of superoxide radical. Control condition is black line meanwhile the red one is after adding 0.3 mM thiols (in A was added cysteine, in B cysteinylglycine and in C glutathione).

Similar observations can be made by the study of hydroxyl radical, •OH (Fig. 4) at lower thiol concentration (0.03 mM). However, in this case the best quenching ability is realised by cys followed by GSH and cys-gly.

It has been reported in literature that cysteine and glutathione are able to form complexes with copper (Carrasco-Pozo *et al.*, 2008; Dokken *et al.*, 2009). Therefore, an in vitro assay at low temperature (125° K) was performed to investigate the interactions occurring between cisteinyl-glycine and copper ions, (Cu^{2+}). When the reaction was carried out in phosphate buffer (Fig. 5A), copper signal was very high but when cysgly was added in ratio 1:1 or 1:3 it disappeared. From this evidence, we could draw the conclusion that this thiol strongly reacts with Cu^{2+}. When water was used instead of buffer the same effect was revealed, but with lower signal intensity (Fig. 5B). However, it is still not possible to conclude if the signal decrease was due to formation of a complex cysgly-Cu^{2+}, or rather to a reduction from Cu^{2+} to Cu^{+}, since the latter ion form does not present a paramagnetic signal.

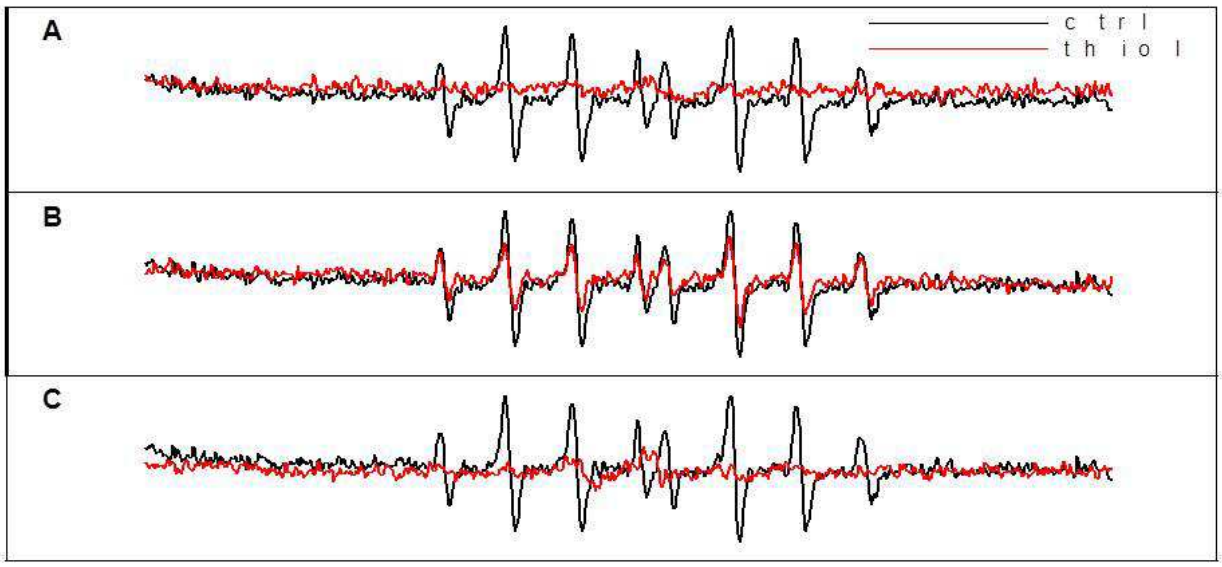


Fig. 4: EPR spectra, showing in vitro detection of hydroxyl radical. Control condition is black line meanwhile the red one is after adding 0.03 mM thiols (in A was added cysteine, in B cysteinylglycine and in C glutathione).

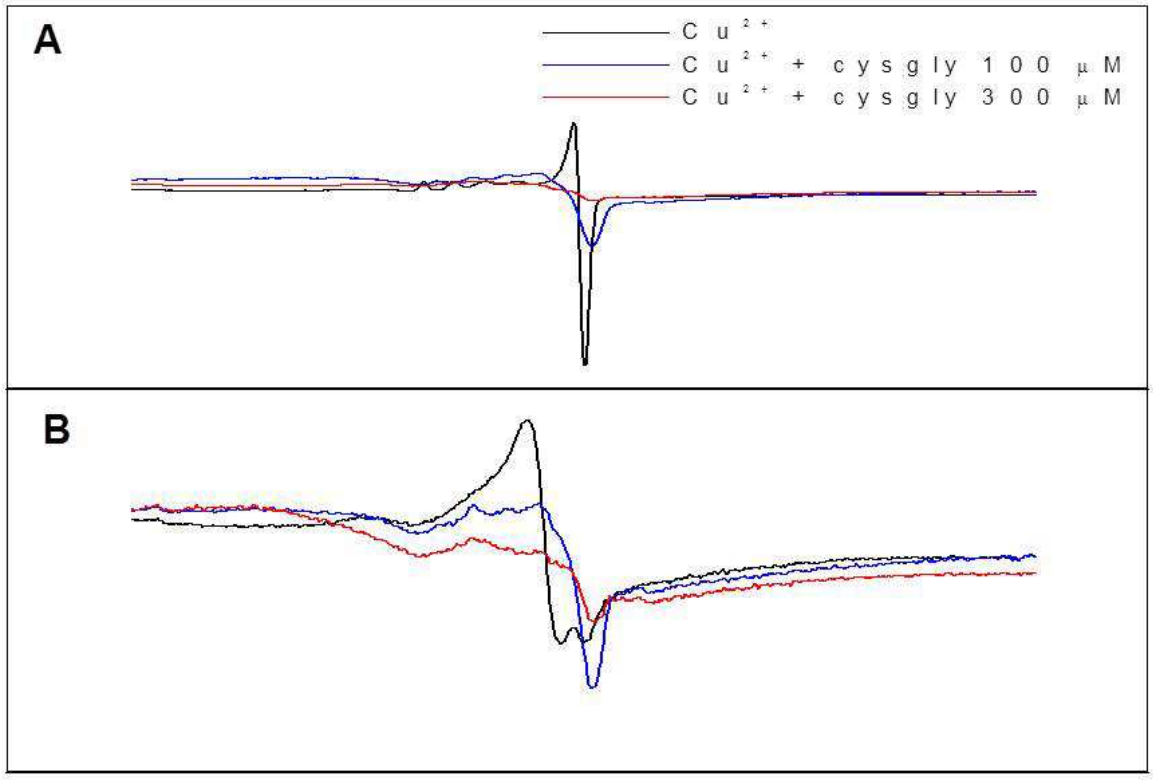


Fig. 5: EPR spectra, showing in vitro at low temperature (125° K): interactions occurring between cisteinyl-glycine and copper ions, (Cu^{2+}). In A the reaction was carried out in phosphate buffer, in B water was used instead of buffer.

In order to understand the modifications occurring in apoplastic space, thiols (at 300 µM final concentration) were added to isolated cell wall fraction. According to in vivo experiments it is possible to infer that paramagnetic signal does not change when glutathione is added (Fig. 6C); moreover, EPR peak height decreases, and signal of Mn^{2+}increases following cys and cysgly addition. So both thiols promote manganese reduction from Mn^{3+} to Mn^{2+}, with cys being more efficient than cysgly (Fig. 6A and 6B). In experiments carried out at increasing cys-gly levels in the essay, the EPR signals were progressively attenuated clearly indicating a concentration-dependent cys-gly quenching power and manganese reduction (Fig. 7).

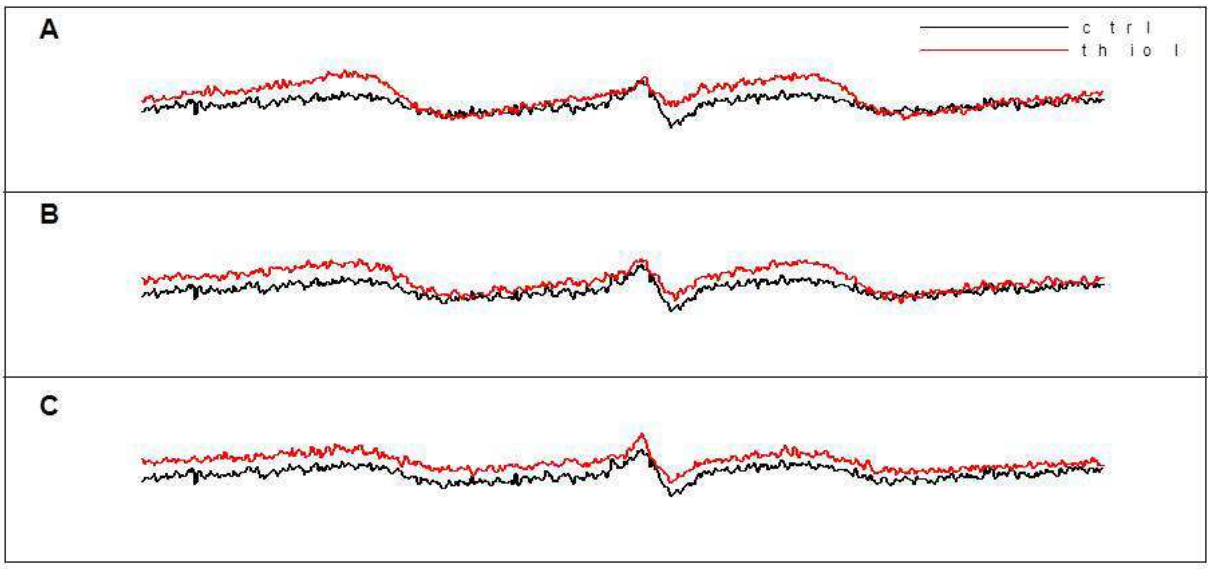


Fig.6: EPR spectra, showing in vivo detection of cell wall isolates (black line) and after adding thiols (red line). Changes in EPR are detected after adding 0.3 mM thiols (red line: in A was added cysteine, in B cysteinylglycine and in C glutathione).

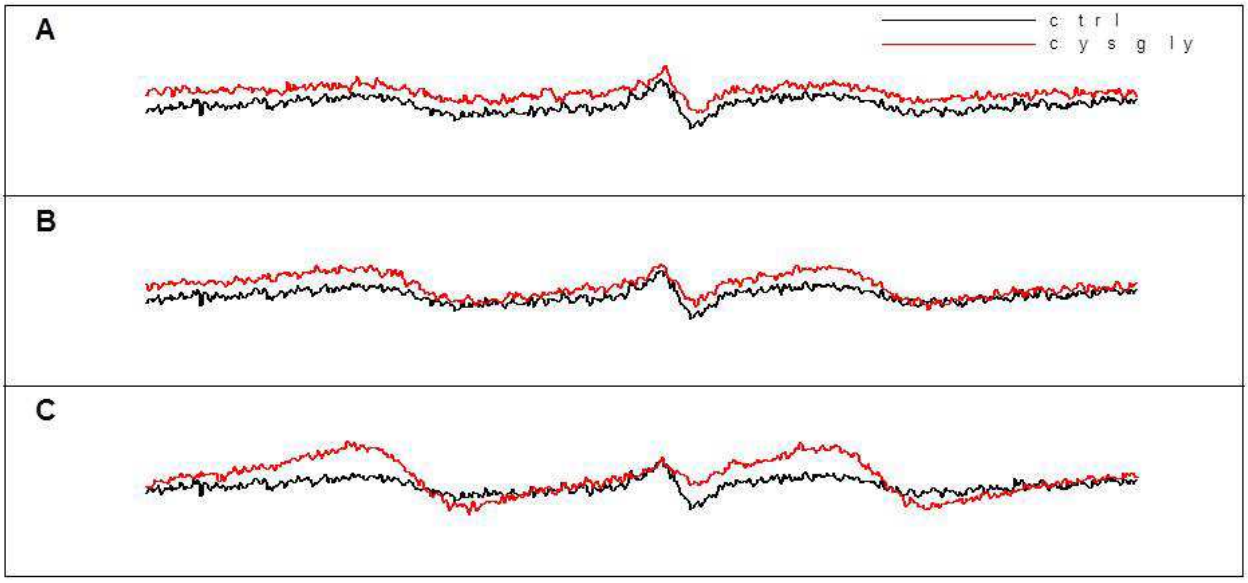


Fig.7 EPR spectra, showing in vivo detection of cell wall isolates (black line) and after adding cysteinyl-glycine at different concentrations (red line: in A cysteinyl-glycine was 30 μM, in B was 300 μM and in C was 1.5 mM).

EPR spectroscopy of cell wall isolate was also performed with the spin-trapping reagent, DEPMPO, to evaluate the detection of •OH and eventually appearance of superoxide radical ($•O_2^-$).

When EPR measurements were done using cell walls isolates, we observed signals from •OH radicals using DEPMPO spin trap. Addition of thiols (Fig. 8) had differential effects on radicals in the cell wall.

- When cysgly was added to the cell wall (Fig. 8B) no differences were detected and the signal was the same as control. However, increasing concentrations of cysteinyl-glycine resulted in higher manganese reduction signals (Fig. 9).
- Glutathione addition induced an increased signal (Fig. 8C) and the adduct may be converted by reduction from $•O_2^-$ to •OH.
- With cysteine addition, the EPR signal results higher than control, but less intense than GSH (fig. 8A), whereas manganese reduction is increased.

It has been reported that prooxidant species, such as superoxide radical and H_2O_2 are produced from the gamma-glutamyl cycle by GSH and cysteine. This prooxidant activity is due to interaction between thiols and metals, thus resulting in modulatory effects on the signal transduction chains (Pompella *et al.*, 2003).

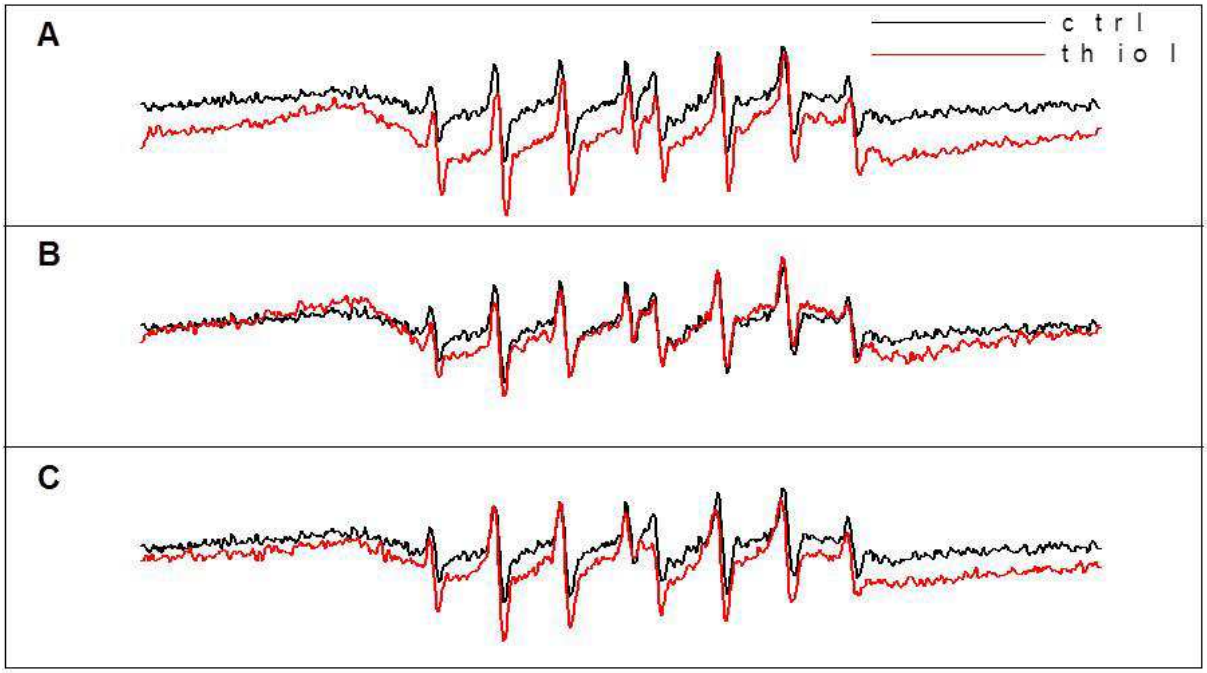


Fig. 8: EPR spectra, in vivo detection of cell wall isolates performed with the spin-trapping reagent, DEPMPO, (black line) and after adding thiols (red line) . Changes in EPR are detected after adding 0.3 mM thiols (red line: in A was added cysteine, in B cysteinylglycine and in C glutathione).

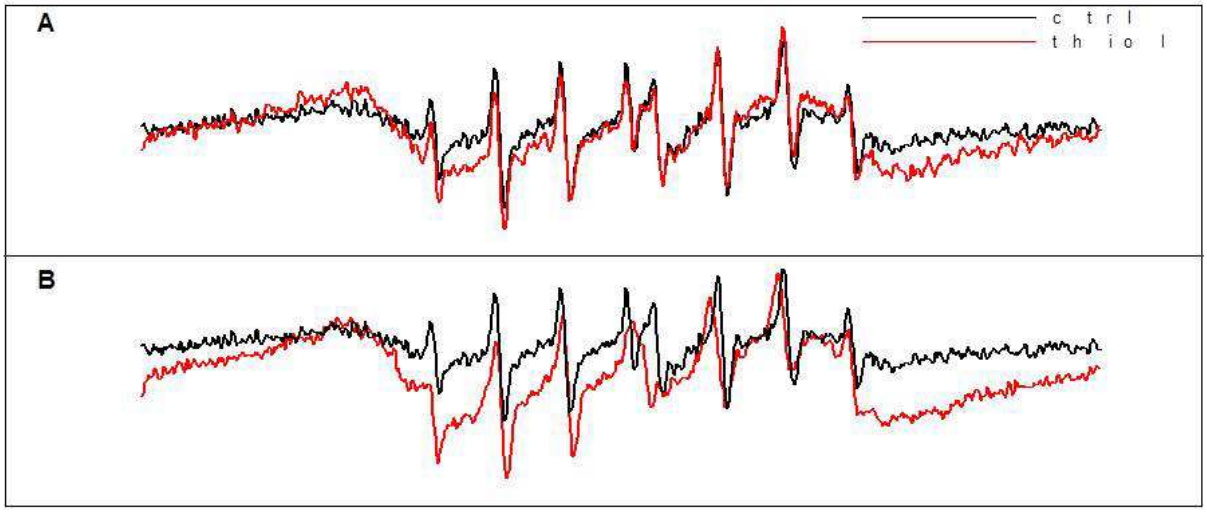


Fig.9 EPR spectra, in vivo detection of cell wall isolates performed with the spin-trapping reagent, DEPMPO, (black line) and after adding cysteinyl-glycine at different concentrations (red line: in A cysteinyl-glycine was 300 μM, in B was 1.5 mM).

Spectroscopy of thiol's interaction with NADH

Thiols interactions with cell wall can cause alterations in the apoplastic space: the - SH moiety is involved in many chemical reactions controlling redox homeostasis, enzyme activity and ROS detoxification (Pivato *et al.*, 2014). In order to demonstrate the redox potential of low molecular weight thiols in apoplast we measured the time courses of the reduction/oxidation of the NADH/NAD^+ couple. In the absence of thiols with cell wall in solution, absorbance decreased rapidly upon NADH addition: this was due to its oxidation to NAD^+. However, without cell wall in cuvette and in the presence of thiols, oxidation did not occur; rather, a non-enzymatic reduction of NADH was observed (Fig. 10). Thiol's ability to reduce NADH is higher in cysteine than cys-gly and glutathione (Fig. 10).This finding is confirmed in literature by Sukalovic *et al.*, 2005; they used cysteine, instead of NADH, as an alternative reducing substrate, to measure oxidative peroxidase activity.

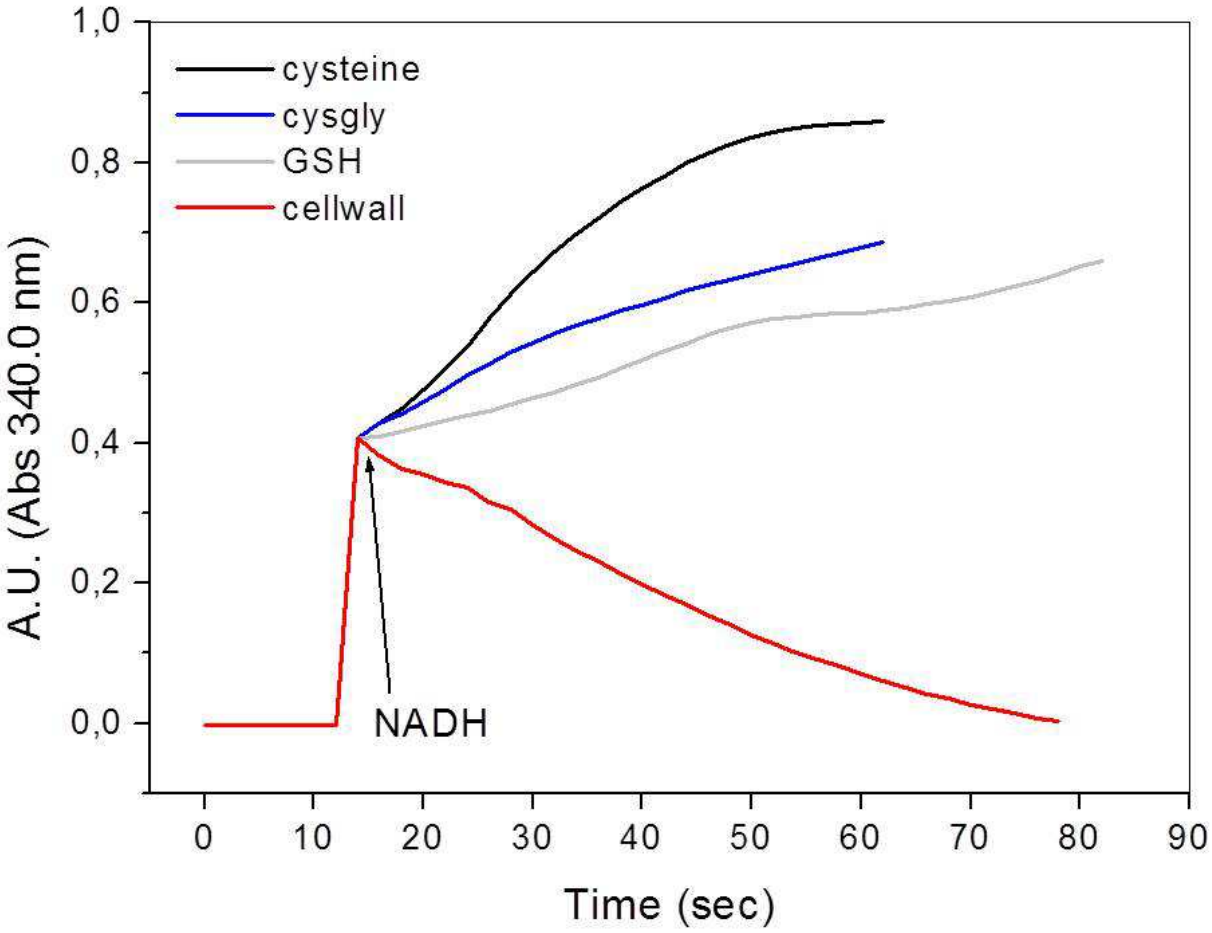


Fig. 10: Time courses of the reduction/oxidation of the NADH/NAD^+ couple.

PODox activity

Thiols' interaction in the cell wall showed different responses depending on the used molecules; glutathione, increased the oxidative activity of peroxidase (Fig. 11B) probably by inducing NADH oxidation, in all the fractions considered: total cell wall extract, ionic and covalent bound proteins. Therefore, it seems to work as a prooxidant molecule; this alternative view was suggested also by Pompella *et al.*, 2003, where glutathione and related thiol metabolites were reported to act as prooxidants in humans.

In our experiments, when cysteinyl-glycine or cysteine were added in the cuvette, a partial inhibition of peroxidase activity, even at low concentrations, was observed (Fig. 11A and Fig 12).

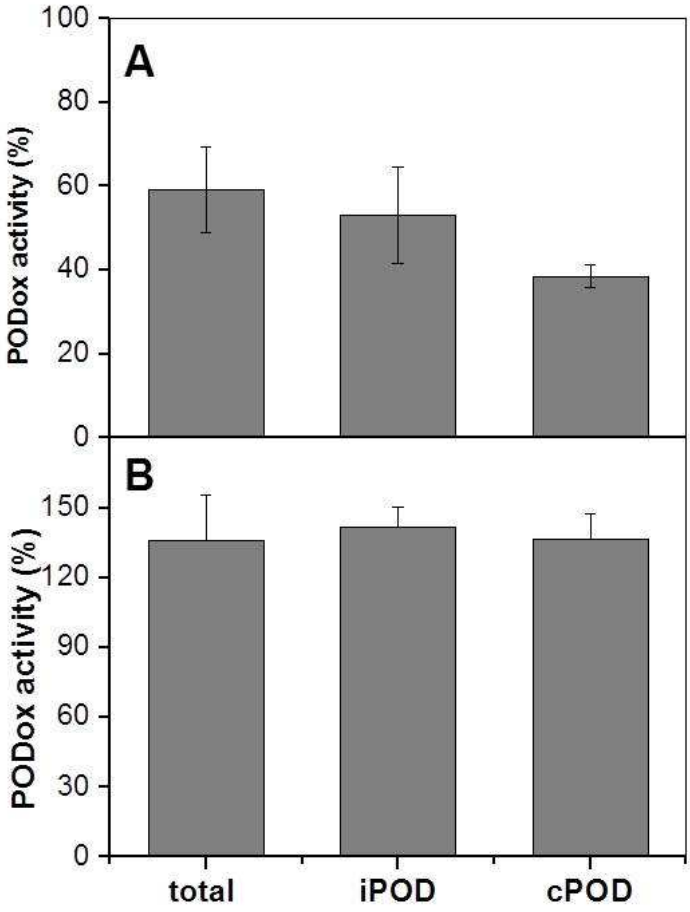


Fig. 11: PODox activity in isolated cell wall: total, ionic and covalent bound fractions. In A activity was measured in presence of cysteinyl-glycine, in B with glutathione.

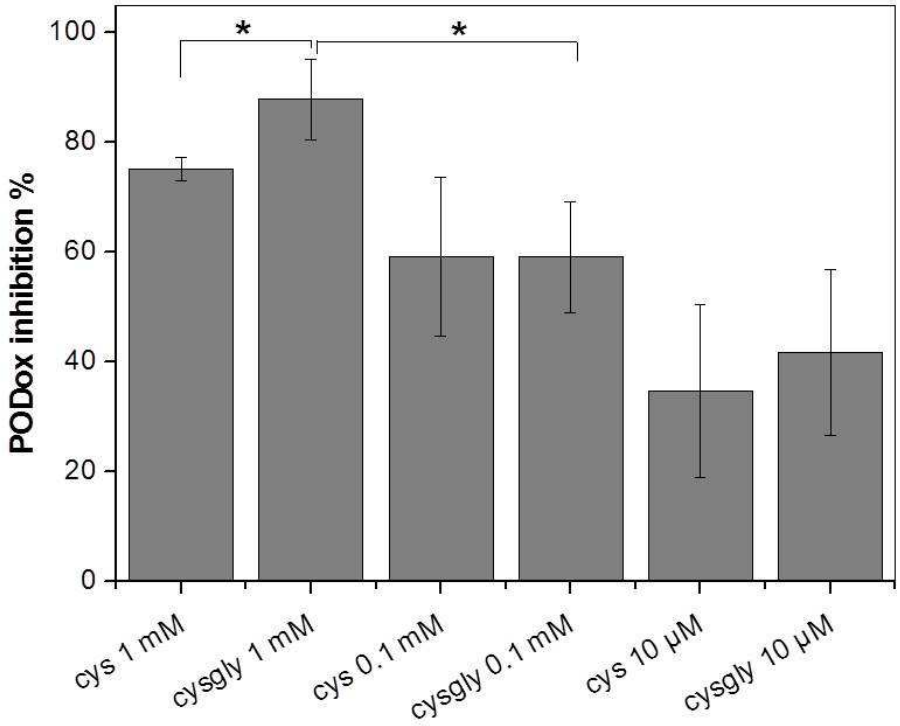


Fig. 12: : PODox activity inhibition in isolated cell wall fractions.

However, if cell walls were incubated with thiols for thirty minutes before measuring activity, no inhibition was detected (Fig. 13A); samples treated with cysteinyl-glycine or glutathione even showed an increased activity. To this regard, the possibility that glutathione is converted to cysgly by cell-wall bound GGT activity should be considered, however.

When cysteine and cysteinyl-glycine are in the apoplastic space, complexes with metal ions may occur (Carrasco- Pozo *et al.*, 2008; Pompella *et al.*, 2003), and as shown by EPR measurements both thiols cause on cell wall a reduction of manganese ions from Mn^{3+} to Mn^{2+}; it is tempting to say that enhanced Mn reduction may prevent inhibition of peroxidase activity.

To validate the observation that thiols are ineffective in inhibiting PODox, incubation was performed also in presence of known inhibitors such as 1 mM H_2O_2 (Fig. 13B), 1 mM Detapac (Fig. 13C) and 1 mM DTT (Fig. 12D). All these compounds caused strong inhibition (approx. 80%) of oxidative peroxidase activity: H_2O_2 because it consumes p-coumaric acid; Detapac is a chelator of 2+ ions, so probably by chelating manganese; and finally dithiothreitol is reported to be an inhibitor of peroxidase activity (Prasad *et al.*, 1995).

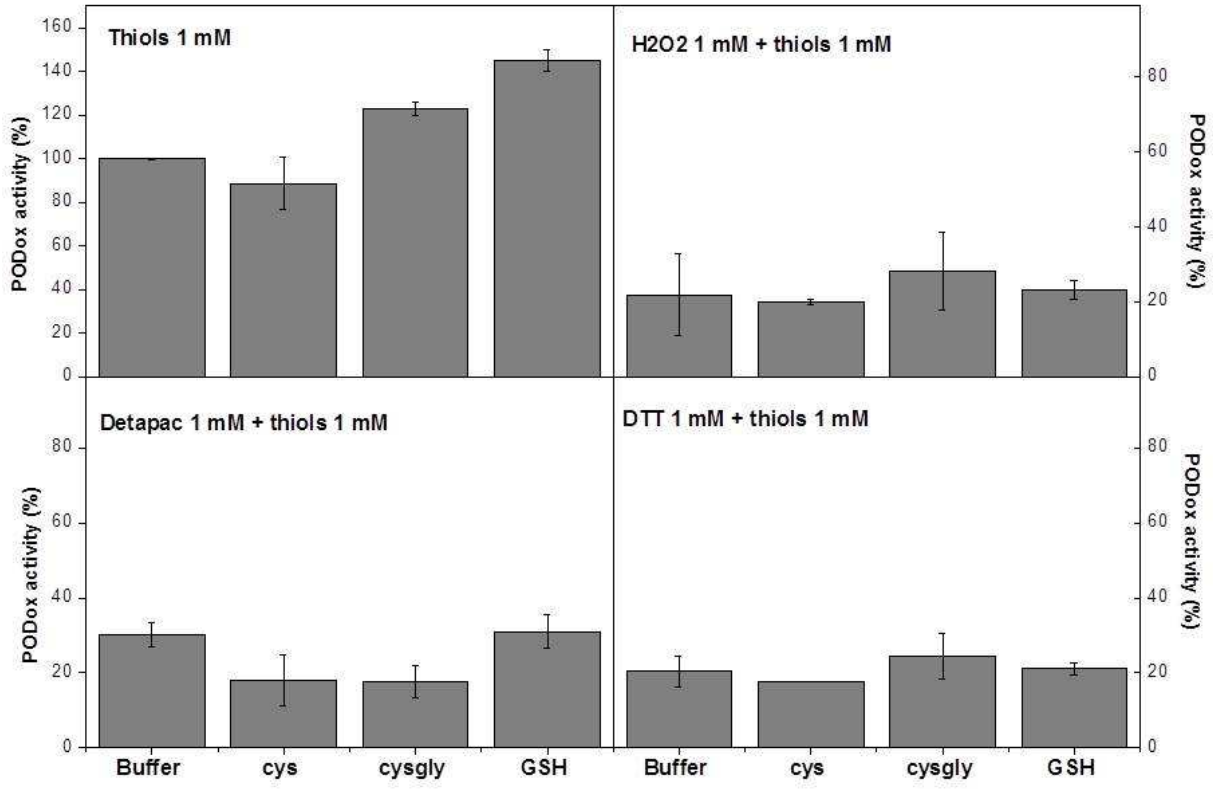


Fig. 13: PODox activity in isolated cell wall fractions. Activity was measured after 30 minutes incubation with thiols (A); thiols and H_2O_2 (B); thiols and Detapac (C); thiols and DTT (D).

Considering the EPR measurement, both cysgly and cys addition yielded an increase in manganese reduction. Under physiological conditions, also formation of metal ions - thiol complexes may result(Dokken *et al.*, 2009). Conversely, metals in peroxidative cycle of peroxidases increase formation of radical •Phe and consequently enzyme activity, so if they are sequestered by thiol complexes POD activity results inhibited (Fig. 14).

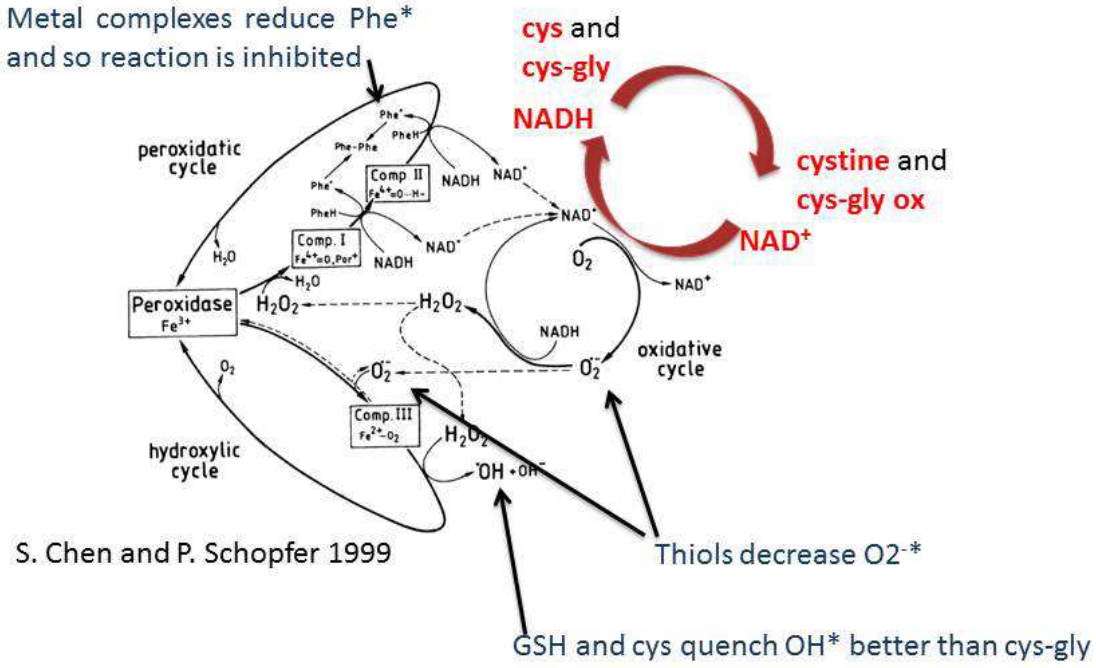


Fig. 14: Schematic overview of sites where thiols modify oxidative peroxidase activity. In blu are evidenced from EPR spectra, and in red from spectrophotometric assay. (Adapted from Chen and Schopfer 1999).

CONCLUSIONS

Overview of obtained results suggest that all LMW thiols used in this study have quenching capacity for apoplastic reactive oxygen species. Glutathione-related metabolites are involved in several apoplastic redox modifications with a variety of molecular targets: by interacting with metal ions, with enzymes and cofactors, and of course by promoting protein S-thiolation. In turn, this may affect physiological functions; for example, thiols mediated redox processes can affect several kinase and phosphatase proteins involved in signal transduction (Matern *et al.*, 2015).

Given the complexity of the interactions among different thiol molecules, competing reactions, metal ion availability and any other intervening factors, assessing their exact role in redox reactions in the apoplastic space, especially under realistic physiological concentrations occurring naturally, is far from being understandable.

However, our findings point to a role, in plant cell wall, of LMW thiols in modulating redox reactions. These molecules are metabolically related to each other in the gamma-glutamyl cycle, which implies that cycling of glutathione between cytosol and apoplastic compartment may be implicated in extracellular redox control.

REFERENCES

Aliaga M.E., López-Alarcón C., Barriga G., Olea-Azar C., Speisky H. (2010). "Redox-active complexes formed during the interaction between glutathione and mercury and/or copper ions".*J Inorg Biochem* 104(10):1084-90. doi: 10.1016/j.jinorgbio.2010.06.006.

Aliaga M.E., López-Alarcón C., García-Río L., Martín-Pastor M., Speisky H. (2012). "Redox-changes associated with the glutathione-dependent ability of the Cu(II)-GSSG complex to generate superoxide".*Bioorg Med Chem* 1;20(9):2869-76. doi: 10.1016/j.bmc.2012.03.027.

Carrasco-Pozo C., Aliaga M.E., Olea-Azar C., Speisky H. (2008). "Double edge redox-implications for the interaction between endogenous thiols and copper ions: In vitro studies". *Bioorg Med Chem* 15;16(22):9795-803. doi: 10.1016/j.bmc.2008.09.068.

Chen S. and SchopferP. (1999). "Hydroxyl-radical production in physiological reactionsA novel function of peroxidase".*Eur J Biochem* 260 pp. 726-735.

del Río L.A. (2015). "ROS and RNS in plant physiology: an overview". *J Exp Bot* 66 (10): 2827-2837. doi: 10.1093/jxb/erv099.

Dixon D.P., Edwards R.(2010)."GlutathioneS-transferases".In*The Arabidopsis Book*. American Society of Plant Biologists, Rockville, MD, doi/10.1199:tab.0131.

Dokken K.M., Parsons J.G., McClure J., and Gardea-Torresdey J.L. (2009). Synthesis and structural analysis of copper(II) cysteine complexes *Inorg Chim Acta*362 pp. 395–401.

Dominici S., Paolicchi A., Corti A., Maellaro E., Pompella A. (2005). "Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyltransferase activity". *Methods Enzymol* 401 pp. 484–501.

Ducic T. and Polle A. (2005). "Transport and detoxification of manganese and copper in plants". *Braz J Plant Physiol*17 pp. 103-112.

Ferretti M., Destro T., Tosatto S.C.E., La Rocca N., Rascio N., Masi A. (2009). "Gamma-glutamyl transferase in the cell wall participates in extracellular glutathione salvage from the root apoplast". *New Phytol* 181 pp. 115–126.

Foyer C.H. and Noctor G. (2009). “Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications”. *Antioxidants & RedoxSignaling* 11(4) pp. 861-905. doi:10.1089/ars.2008.2177.

Foyer C.H., Noctor G. (2011). “Ascorbate and Glutathione: the heart of the redox hub”. *Plant Physiol*155 pp. 2-18.

Hernandez J.A., Ferrer M.A., Jimenez A., Barcelo A.R., Sevilla F. (2001). “Antioxidant systems and $O_2.^-$/ H_2O_2 production in the apoplast of Pea leaves. Its relation with salt-induced necrotic lesions in minor veins”. *Plant Physiol* 127 pp. 817–831.

HigashiY., TakechiK., TakanoH., Takio S. (2015). “Maintenance of Normal Stress Tolerance in the Moss Physcomitrella patens Lacking Chloroplastic CuZn-Superoxide Dismutase”.*AJPS* Vol.06 No.05 Article ID:54601,10 pages doi: 10.4236/ajps.2015.65064

Hu J.F., Li G.F., Gao Z.H., Chen L., Ren H B., Jia W.S. (2005). “Regulation of water deficit-induced abscisic acid accumulation by apoplastic ascorbic acid in maize seedlings”. *J Integr Plant Biol*47 pp. 1335-1344.

Jaspers, P., Kollist, H., Langebartels, C., Kangasjarvi, J. (2005). “Plant responses to ozone,” in *Antioxidants and reactive oxygen species in plants,* ed. N. Smirnoff (Oxford: Blackwell Publishing) pp. 268-292.

Karkonen A. and Fry S.C. (2006).“Effect of ascorbate and its oxidation products on H_2O_2 production in cell-suspension cultures of Picea abies and in the absence of cells”. *J Exp Bot* 57 pp. 1633 – 1644 .

Karuppanapandian T., Moon J.C., Kim C., Manoharan K., Kim W. (2011). “Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms”. *AJCS*5(6) pp. 709-725.

Kukavica B., Mojovic´ M., Vucˇinic´Ž., Maksimovic´ V., Takahama U.and Veljovic´ Jovanovic´ S. (2009).“Generation of Hydroxyl Radical in Isolated Pea Root Cell Wall, and the Role of Cell Wall-Bound Peroxidase, Mn-SOD and Phenolics in Their Production”. *Plant Cell Physiol*50(2) pp. 304–317 doi:10.1093/pcp/pcn199.

Liszkay A., van der Zalm A.and Schopfer P. (2004). “Production of reactive oxygen intermediates (O_2^{-} · $H_2O_2$and ·OH) by maize rootsand their role in wall loosening and elongation growth”. *Plant Physiol*136 pp. 3114 – 3123.

Liu G., Greenshields D.L., Sammynaiken R., Hirji R.N., Selvaraj G., Wei Y. (2007). “Targeted alterations in iron homeostasis underlie plant defense responses”. *J Cell Sci* 15;120 pp. 596-605.

Masi, A., Ghisi, R., Ferretti, M., (2002). “Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L”. *Plant Physiol159* pp. 499-507.

Matern S., Peskan-BerghoeferT., GromesR., Vazquez Kiesel R., and Rausch T. (2015). “Imposed glutathione-mediated redox switch modulates the tobacco wound-induced protein kinase and salicylic acid-induced protein kinase activation state and impacts on defence against”. *J Exp Bot* doi: 10.1093/jxb/eru546.

Ohkama-Ohtsu N., Oikawa A., Zhao P., Xiang C., Saito K., Oliver D. J. (2008). “A gamma-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in *Arabidopsis”*. *Plant Physiol* 148 pp. 1603–1613.

Paolicchi A, Dominici S, Pieri L, Maellaro E, Pompella A.(2002). “Glutathione catabolism as a signaling mechanism”. *Biochem Pharmacol* 64(5–6) pp. 1027–35. Epub 2002/09/06.

Pivato M., Fabrega-Prats M., Masi A. (2014). “Low-molecular-weight thiols in plants: Functional and analytical implications”.*Arch Biochem Biophys* 560 pp. 83–99.

Pompella A., Visvikis A., Paolicchi A., De Tata V., Casini A.F. (2003). “The changing faces of glutathione, a cellular protagonist”. *Biochem Pharmacol* 66(8) pp. 1499–1503.

Prasad T.K., Anderson M.D., and Stewart C.R. (1995).“Localization and Characterization of Peroxidases in the Mitochondria of Chilling-Acclimated Maize Seedlings”.*Plant Physiol* 108 pp.1597-1605.

Pristov J.B., Jovanović S.V., Mitrović A., Spasojević I. (2013). “UV-irradiation provokes generation of superoxide on cell wall polygalacturonic acid”. *Physiol Plant* 148 pp. 574–581.

Rahantaniaina M.S., Tuzet A., Mhamdi A., Noctor G. (2013). “Missing links in understanding redox signaling via thiol/disulfide modulation: how is glutathione oxidized in plants?”*Front Plant Sci* 4:477 10.3389/fpls.2013.00477

Rodriguez-Serano M. , Romero-Puertas M., Zabalza A., CorpasF.J., Gomez M., Del Rio L.A. et al . (2006). “Cadmium effect on oxidative metabolism of pea (Pisum sativum L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo”. *Plant Cell Environ* 29 pp. 1532 ‑ 1544.

Schopfer P., Liszkay A., Bechtold M, Frahy G. and Wagner A. (2002). “Evidence that hydroxyl radicals mediate auxin-induced extension growth”. *Planta* 214 pp. 821 – 828 .

Schopfer P., Plachy C. and Frahry G. (2001). “Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid”. *Plant Physiol* 125 pp. 1591 ‑ 1602.

SharmaP.,Bhushan Jha A., Dubey R.S., and Pessarakli M. (2012).“Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions”.*J Bot* Article ID 217037.

Speisky H., Gómez M., Carrasco-Pozo C., Pastene E., Lopez-Alarcón C., Olea-Azar C. (2008). “Cu(I)-glutathione complex: a potential source of superoxide radicals generation”. *Bioorg Med Chem* 1;16(13):6568-74. doi: 10.1016/j.bmc.2008.05.026.

Speisky H., López-Alarcón C., Olea-Azar C., Sandoval-Acuña C., Aliaga M.E. (2011). “Role of superoxide anions in the redox changes affecting the physiologically occurring cu(i)-glutathione complex”. *Bioinorg Chem Appl* 2011:674149. doi: 10.1155/2011/674149.

Stark A.A, Zeiger E, Pagano D.A. (1993).”Glutathione metabolism by γ-glutamyl transpeptidase leads to lipid peroxidation: characterization of the system and relevance to hepatocarcinogenesis”.*Carcinogenesis* 14 pp. 183–189.

Šukalovic´ V.H.T., Vuletic´ M. and Vucˇinic´ Ž. (2005). “The role of p -coumaric acid in oxidative and peroxidative cycle of the ionically bound peroxidase of the maize root cell wall”. *Plant Sci* 168 pp. 931 – 938.

Urbañski N.K. and Berêsewicz A. (2000). “Generation of •OH initiated by interaction of Fe^{2+} and $Cu^{+}$with dioxygen; comparison with the Fenton chemistry”. *Acta Biochimica Polonica* 47 pp. 951-962.

CONCLUSIONS

CONCLUSIONS

Glutathione is a primary non-proteic antioxidantinvolved in a network of redox reactions for the detoxification of ROS.Its synthesis in the cytosol and degradation in the extracellular space are part of the so called gamma-glutamyl cycle. In this cycle, extracellular glutathione is cleaved by gamma-glutamyltransferase (GGT) to produce cysteinyl-glycine (cys-gly) and glutamate. The significance of the gamma-glutamyl cycle in plants is not fully understood yet, however, glutathione cycling between the symplast and apoplast may represent a way to transfer redox information.

- The loss of function due to the Arabidopsis ggt1 knockout mutation triggers highly specific rearrangements in proteome, which resemble those induced under specific abiotic and biotic stresses. A sort of “alert response” is activated in ggt1 mutant leaves even in the absence of a real environmental threat, suggesting a failure in correct redox sensing.Probably, this can be due to an effect of the altered plasma membrane receptors level and the redox state. (Tolin *et al.*, 2013)
- Oxidative conditions (imposed with UV-B) and apoplastic proteome analysis were used to arrive at a better understanding of the rearrangements in the extracellular compartment. The ggt1 mutant scenario is likely toresult in higher H2O2 levels under physiological conditions, whereas the rise in H2O2 in the wild type is a direct consequence of oxidative stress conditions induced by UV-B radiation. As a signaling molecule, H2O2 may cross membranes in a process facilitated by aquaporins, reaching internal cell compartments and the nucleus, where it can activate defense gene expression.This assumption might explain the “constitutive alert response” effect observed in the previous proteomic analysis of total leaf extracts from ggt1 mutant leaves.(Trentin *et al.*, 2015)
- LMW thiols are metabolically related to each other in the gamma-glutamyl cycle, which implies that cycling of glutathione between cytosol and apoplastic compartment may be implicated in extracellular redox control. In physiological conditions, they are involved in several apoplastic redox modifications, by interacting with a number of molecules and metal ions. (Trentin *et al.*, in preparation)

LIST OF PUBBLICATIONS

LIST OF PUBLICATIONS

A.R. Trentin, G. Arrigoni, Bernd Zechmann A. Masi (2012). “Proteomic analysis of ggt1 mutants indicates the involvement of gamma glutamyl cycle in plant cell redox homeostasis”. VII ItPA National Congress Viterbo, June 12-15, 2012.

A.R. Trentin, D. Prasad, G. Arrigoni A. Masi (2012). “Functional genomic studi by RNAi tecnology and proteomic analisys of Arabidopsis mutants indicates the involvement of gamma-glutamyl transferases in cysteine delivery to the seeds”. VII ItPA National Congress Viterbo, June 12-15, 2012.

S. Tolin, G. Arrigoni, A.R. Trentin, S. Veljovic-Jovanovic, M. Pivato, B. Zechman and A. Masi (2013).“Biochemical and quantitative proteomics investigations in Arabidopsis ggt1 mutant leaves reveal a role for the gamma-glutamyl cycle in plant’s adaptation to environment” Proteomics, 13, 2031–2045.

A.R. Trentin, S. Tolin, M. Pivato, S. Giaretta, M. Fabrega-Prats, M. Syed Muhammad Muntazir, G. Arrigoni, D. Prasad, A.Masi “Functions of the Gamma-Glutamyl Cycle in Plant Metabolism Explored by Proteomic Analysis of Arabidopsis Mutants” VIII ItPA National Congress Padua, June 18th-21st, 2013.

A.R. Trentin, M. Pivato, S. Giaretta, M.Fabrega-Prats, G. Arrigoni, D. Prasad, A. Masi “ggt-assisted glutathione degradation is functional to apoplastic redox control and seed storage proteins accumulation” 11° International POG conference Reactive Oxygen and nitrogen species in plants Warsaw, 17-19 luglio 2013.

Trentin, A.R., Pivato, M., Ebinezer, L.B., Giaretta, S., Prasad, D., Fabrega-Prats, M., Masi, A (2014). “Role of apoplastic GGT1 in plant’s response to UV-B radiation” The final network conference of COST Action FA0906, UV4Growth. Bled, 30 March, 2 April.

Trentin, A.R., Pivato, M., Giaretta, S., Fabrega-Pratz, M., Ebinezer, L.B., Mehdi, M.S., Masi, A. (2014) “Involvement of extracellular glutathione degradation in the response to UV-B radiation in *A. thaliana* wild-type and ggt1 mutant”. 9th International Workshop: Sulfur Metabolism in Plants, 14-17 April

Fabrega-Prats M., Trentin A.R., Masi A., Dall’Acqua S. (2014). "Identification of low molecular weight thiols in plants by fluorescence derivatization and LC-MS/MS" 20th International Mass Spectrometry Conference (IMSC). Geneva, Switzerland, 24-29 August

Trentin A.R., Pivato M., Mehdi M.S., Barnabas L.E., Giaretta S., Fabrega-Prats M., Prasad D., Masi A. (2015). Proteome readjustments in the apoplastic space of *Arabidopsis thalianaggt1* mutant leaves exposed to UV-B radiation. Front Plant Sci. 2015 Mar 24;6:128. doi: 10.3389/fpls.2015.00128.

Masi A., Trentin A.R., Agrawal, G.K., Rakwal R. (2015). "Gamma-glutamyl cycle in plants: a bridge connecting the environment to the plant cell?" Front. Plant Sci., 16 April 2015 | http://dx.doi.org/10.3389/fpls.2015.00252.

Trentin A.R., Sharma N., Giaretta S., Fabrega-Prats M., , Arrigoni G., Masi A. (2015). "gamma-glutamyl cycle and oxidative stress: investigations on apoplastic proteome composition in *ggt1* mutants" 12° International POG conference Reactive Oxygen and nitrogen species in plants Verona, 24-26 giugno 2015.