

DISSERTATION

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Post-translational regulation and evolution of plant γ -glutamate cysteine ligase

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1.1 Summary

Glutamate cysteine ligase (GCL) is catalyzing the rate-limiting step in glutathione (GSH) synthesis. A complex regulation of this enzyme is required to integrate various signals as GSH is fulfilling a plethora of functions in housekeeping metabolism, stress defence, and in the regulation of development. In this thesis the post-translational redox regulation of plant GCL and closely related proteobacterial enzymes was studied.

The crystal structure of *Brassica juncea* GCL (BjGCL) revealed the presence of two intramolecular disulfide bridges. Biochemical analyses of the wild-type enzyme and of mutants lacking cysteines required for the formation of either disulfide bridge showed that both bridges are involved in the *in vitro* redox regulation of BjGCL. One disulfide bridge (CC1) is apparently controlling access to the active site and knock-out results in a slower overall catalysis rate without changes in K_m -values. The second disulfide bridge (CC2) controls the formation of a GCL homo-dimer and reduction of this disulfide bridge leads to monomerization and almost complete deactivation of the enzyme. Sequence analysis showed that only CC2 is conserved in all higher plants while the occurrence of CC1 is restricted to the Rosids clade. Characterization of the redox regulation of GCL from the (non-Rosid) *Nicotiana tabacum* confirmed the presence of only the dimerization-dependent mechanism of redox regulation. Furthermore, it could be shown that feedback-inhibition of plant GCL by GSH is mechanistically independent from redox regulation. A model is presented on how these different mechanisms interact to control GSH synthesis *in vivo*.

Comparative sequence analysis of plant GCL and with related enzymes from proteobacteria revealed that the amino acid residues forming the dimer interface in BjGCL are conserved in higher plants only, while the catalytic residues are highly conserved among all sequences. The characterization of recombinantly produced GCL from *Agrobacterium tumefaciens* and *Xanthomonas campestris* confirmed that these enzymes show kinetics and susceptibility to inhibitors similar to the plant enzyme but completely lack redox regulation and are active as monomers.

In a second project, the influence of soluble thiols on the GSH metabolism of different types of cultured plant cells was studied, revealing a specific induction of GCL expression by cysteine. This observation may hint at a role of GSH synthesis in the control of the cellular concentrations of this amino acid, preventing an accumulation which might lead to oxidative stress.

1.2 Zusammenfassung

Glutamat-Cystein-Ligase (GCL) katalysiert den geschwindigkeitsbestimmenden Schritt in der Synthese von Glutathion (GSH). Eine komplexe Regulation dieses Enzyms, die eine Fülle verschiedener Signale integriert, ist notwendig, da GSH mannigfaltige Funktionen im Haushalts- und Stressstoffwechsel sowie in der Regulation von Entwicklungsprozessen erfüllt. In dieser Arbeit wurde die post-translationale Redox-Regulation pflanzlicher GCL und verwandter proteobakterieller Enzyme untersucht.

Zwei intramolekulare Disulfidbrücken konnten in der GCL von *Brassica juncea* (BjGCL) in der Kristallstruktur identifiziert werden. Eine Beteiligung beider Disulfidbrücken an der *in vitro* Redox-Regulation konnte durch biochemische Analysen am Wildtyp-Enzym und Mutation der Cysteine nachgewiesen werden. Der Knockout einer der Disulfid-Brücken (CC1) führte zu einer Verringerung der Katalysegeschwindigkeit, möglicherweise durch eine Veränderung der Zugänglichkeit des aktiven Zentrums, ohne dabei die K_m -Werte des Enzyms zu beeinflussen. Die andere Disulfidbrücke (CC2) kontrolliert die Bildung eines GCL Homodimers und das Aufbrechen führt zu einer Monomerisierung und zu fast vollständiger Inaktivierung des Enzyms. Sequenzanalysen zeigten, dass nur CC2 in allen höheren Pflanzen konserviert ist, während das Vorkommen von CC1 sich auf die Rosiden beschränkt. Die Charakterisierung der GCL aus (der nicht-Roside) *Nicotiana tabacum* bestätigte das alleinige Vorhandensein des dimerisierungsabhängigen Mechanismus der Redox-Regulation. Darüber hinaus konnte nachgewiesen werden, dass die Inhibition pflanzlicher GCL durch GSH mechanistisch unabhängig von der Redox-Regulation ist. Das mögliche Zusammenwirken der verschiedenen Regulationsmechanismen zur Kontrolle der GSH-Synthese *in vivo* wird in einem Modell dargestellt.

Vergleichende Sequenzanalyse zeigte eine Konservierung der Aminosäurereste, die bei BjGCL die Dimerisierung ermöglichen, nur in höheren Pflanzen, während die katalytischen Reste in allen Sequenzen hoch konserviert sind. Die Charakterisierung der GCL aus *Agrobacterium tumefaciens* und *Xanthomonas campestris* bestätigte, dass diese eine ähnliche Kinetik und Empfindlichkeit gegenüber Inhibitoren zeigen, wie die pflanzlichen Enzyme, aber keinerlei Redox-Regulation aufweisen und als Monomere aktiv sind.

In einem weiteren Projekt wurde der Einfluss löslicher Thiole auf den GSH-Stoffwechsel verschiedener kultivierter Pflanzenzellen untersucht und eine spezifische Induktion der GCL-Expression durch Cystein nachgewiesen. Dies weist auf eine Rolle der GSH-Synthese bei der Kontrolle der zellulären Konzentration dieser Aminosäure hin, deren Akkumulationen zu oxidativem Stress führen könnten.

2 Introduction

The tripeptide glutathione (GSH, γ -glutamylcysteinylglycine) is the most abundant low molecular weight thiol in almost all eukaryotic cells as well as in proteo- and cyanobacteria (Fahey and Sundquist, 1991; Masip et al., 2006). The biological functions of glutathione all depend on the central cysteine, providing the chemical reactivity associated with a reduced sulfur atom. Compared to free cysteine, glutathione is less susceptible to autoxidation in the presence of heavy metals and H_2O_2 (Sundquist and Fahey, 1989). This is probably due to its higher thiol pK_a caused by the vicinity of the SH group to glutamic acid (Spear and Aust, 1994). It has therefore been speculated that evolution of GSH synthesis may have been driven by the need for cells to maintain high intracellular concentrations of reduced sulfur in a form not subject to rapid oxidation (Fahey and Sundquist, 1991). This view is supported by the fact that GSH is the major storage and transport form of reduced sulfur in plants (Noctor and Foyer, 1998; Foyer et al., 2001) and animals (Higashi et al., 1977; Tateishi et al., 1977), making glutathione a central component of eukaryotic sulfur metabolism (see paragraph 2.1).

Besides its prominent role in sulfur metabolism, the reactivity of the glutathione SH group has led to a plethora of other functions, both in housekeeping and stress metabolism. Glutathione is a potent antioxidant and provides one of the three main redox buffers of the eukaryotic cell, acting as a protectant against oxidative stress and as a cofactor for redox active proteins (see paragraph 2.3.1). The nucleophilic properties of the SH group are the basis for glutathione's involvement in the detoxification of xenobiotics and heavy metals (see paragraph 2.3.2). In addition to these metabolic functions glutathione has been found to be a regulator of protein activity, gene expression and development (see paragraph 2.3.3).

While glutathione is not essential for the growth of *Escherichia coli* (Greenberg and Demple, 1986) its multiple roles in eukaryotic metabolism makes it indispensable for the growth and development of plants (Cairns et al., 2006) and animals (Dalton et al., 2000) and reduced capability of glutathione synthesis results in reduced stress tolerance or complete abortion of development (Cobbett et al., 1998; Cairns et al., 2006).

2.1 Glutathione: A central component of cellular sulfur metabolism

Sulfur is taken up by plants primarily in the form of sulfate by the roots via a number of plasma membrane sulfate transporters. The *Arabidopsis thaliana* genome encodes for 12 sulfate transporters which can be divided into four different groups differing in substrate affinity, subcellular localization and expression patterns (The *Arabidopsis* Genome Initiative, 2000). Some of these genes are induced by sulfur starvation (Takahashi et al., 1997). After uptake, sulfur is reduced and integrated into cysteine moiety, the primary product of reductive sulfur assimilation (Figure 2.1). In a first step sulfate is activated by ATP-sulfurylase, producing 5'-adenylylsulfate (APS), which is reduced by APS-reductase to sulfite and AMP using GSH as a cofactor (Bick and Leustek, 1998). Flux analysis has shown that this reaction is limiting sulfur assimilation and therefore may play a key role in the regulation of this pathway (Kopriva et al., 1999; Vauclare et al., 2002). Alternatively APS can be further activated by APS kinase to form 3'-phosphoadenylylphosphate (PAPS) which is required for various sulfation reactions (Varin *et al.*, 1997). Using reduced ferredoxin as electron donor, sulfite is further reduced by sulfite reductase to sulfide, which is then incorporated into cysteine by O-acetylserine (OAS) thiol lyase (OAS-TL). OAS is provided by serine acetyltransferase (SAT), which is generating OAS from acetyl-CoA and serine.

SAT and OAS-TL are forming a regulatory enzymatic complex, where SAT is active in the complex with OAS-TL while the latter, which is present in large excess, is only active in the free state (Bogdanova and Hell, 1997; Wirtz et al., 2001; Berkowitz et al., 2002). While the reduction of sulfate takes place exclusively in plastids (Hawksford and Wray, 2000; Leustek et al., 2000), the enzymes for cysteine synthesis are also found in the cytosol and mitochondria (Wirtz et al., 2004). Experiments, overexpressing inactive SAT in the cytosol of transgenic tobacco, surprisingly led to a stimulation of cysteine synthesis in other compartments, indicating a complex interplay between the different isoforms in the regulation of this reaction (Wirtz and Hell, 2007).

Cysteine is finally incorporated into proteins, GSH or other sulfur-containing molecules. The concentration of free cysteine in the plant cell is kept at a rather constant low level (< 10 μ M), while flux through the cysteine pool is high (Giovanelli et al., 1980).

Sulfate assimilation is regulated metabolically by OAS and soluble thiols. OAS, produced in excess by SAT when sulfide is lacking, acts as a signal of sulfur starvation and leads to an induction of the assimilatory pathway (Smith et al., 1997) and to dissociation of the OAS-TL/SAT-complex (Kredich et al., 1969), reducing its own production. Glutathione, on the other hand, was also shown to act as a signal for the availability of sulfur and as a regulator of sulfur assimilation (Lappartient and Touraine, 1996, 1997). The ratio of sulfate to glutathione in the phloem seems to control sulphate uptake and loading into the xylem (Herschbach *et al.*, 2000).

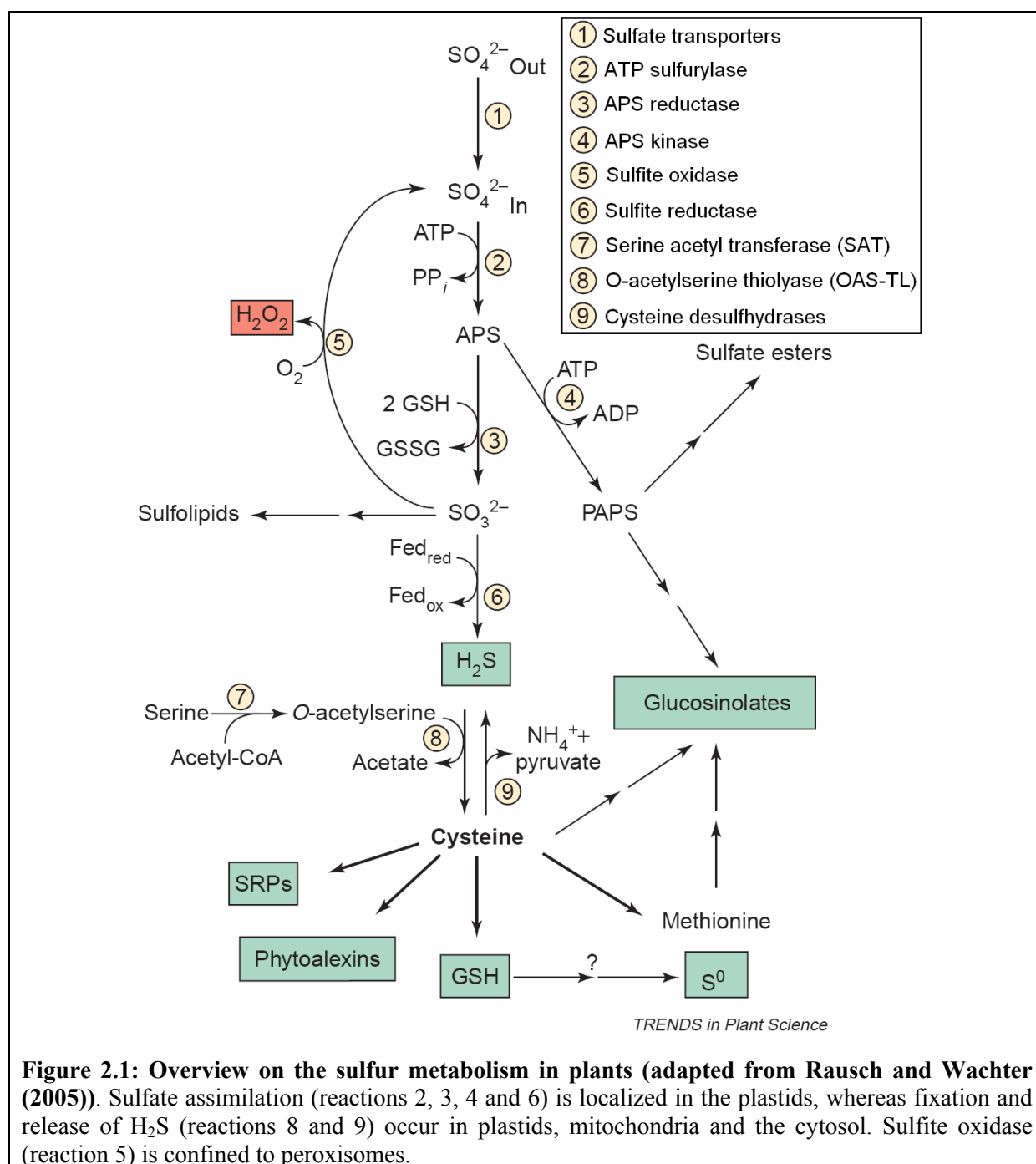


Figure 2.1: Overview on the sulfur metabolism in plants (adapted from Rausch and Wachter (2005)). Sulfate assimilation (reactions 2, 3, 4 and 6) is localized in the plastids, whereas fixation and release of H₂S (reactions 8 and 9) occur in plastids, mitochondria and the cytosol. Sulfite oxidase (reaction 5) is confined to peroxisomes.

2.2 The establishment of glutathione homeostasis

In higher plants glutathione levels have been determined to be in the range of 0.1 to 1.5 mM, primarily in the reduced form (Mullineaux and Rausch, 2005). However, GSH concentrations seem to vary widely between different organs, cell types, developmental stages, and organelles. Recently, *in vivo* labelling of GSH using monochlorobimane (MCB) and microscopic analysis has allowed quantitative estimations of cytosolic GSH concentrations. In poplar, mesophyll and epidermal leaf cells both showed 0.2 to 0.3 mM GSH (Hartmann et al., 2003), whereas in *Arabidopsis* different leaf cell types also showed varying cytosolic GSH levels. While epidermal cells held 0.14 mM GSH and basement cells 0.08 mM, trichomes showed the highest amounts of up to 0.24 mM (Gutierrez-Alcala et al., 2000) and developing root hair and non root hair cells were estimated to hold 2.7 and 5.5 mM GSH, respectively, values similar to those found for *Arabidopsis* suspension culture cells (Meyer and Fricker, 2000; Meyer et al., 2001).

However, probably due to a lack of compatible glutathione-S-transferases, MCB does not allow labelling of organellar GSH pools. Fractionation experiments proposed GSH levels as high as 5 to 20 mM in chloroplasts (Foyer and Halliwell, 1976; Smith et al., 1985; Klapheck et al., 1987), emphasizing the importance of GSH in upholding the plastidic redox state.

Glutathione is involved in the defence of plants against many different forms of stress, which is also reflected by the observation that changes in GSH concentration accompany the adaptation of plants to a variety of unfavourable environmental conditions. Elevated levels of GSH have been found to play a role in the adaptation to such different stress factors as cold (Anderson et al., 1992; Wildli and Lütz, 1996), heat (Nieto-Sotelo and Ho, 1986; Kurganova et al., 1999), salt (Bor et al., 2003), heavy metals (Freeman et al., 2004), and iron deficiency (Zaharieva and Abadia, 2003).

The multiple roles of glutathione in plants require a complex control of glutathione homeostasis, integrating metabolic, developmental and environmental signals. Synthesis, transport, and degradation of glutathione all can contribute in varying proportions to the regulation of GSH levels (Noctor et al., 2002). The synthesis of glutathione has received most attention so far (Rausch et al., 2007), while the molecular characterization of GSH transport and degradation has only recently begun to make significant progress, making it hard to

estimate how strong the contributions of the latter two to the control over GSH concentration are.

Overexpression of the enzymes of GSH synthesis in tobacco provided evidence for the importance of a stringent control over the cellular GSH levels (Creissen et al., 1999). Here the resulting increase in GSH concentration resulted in oxidative stress, probably due to unbalancing of the cellular redox system, and led to light intensity-dependent chlorosis and necrosis. However, similar experiments in poplar, although showing a comparable increase in GSH concentration, did not result in phenotypic effects (Noctor et al., 1998b; Noctor et al., 1998a).

In addition to or instead of glutathione some plants also produce other thiols, where the glycine residue is substituted by different amino acids. In many Fabaceae homoglutathione (γ -glutamylcysteinylalanine) is found (Carnegie, 1963; Klapheck, 1988), while several Poaceae produce hydroxymethylglutathione (γ -glutamylcysteinylserine) or γ -glutamylcysteinylglutamic acid (Klapheck et al., 1992; Meuwly et al., 1993). While these homologs probably can fulfil the same functions as GSH, different ratios of GSH to hGSH in different legume tissues might also indicate specialised functions for either thiol under certain conditions (Matamoros et al., 1999).

2.2.1 Glutamate cysteine ligase is the regulatory step of glutathione synthesis

Glutathione is synthesized enzymatically from the free amino acids in two ATP- and Mg^{2+} -dependent steps. First glutamate cysteine ligase (GCL = GSH1 = γ -ECS, E.C. 6.3.2.2) establishes a peptide bond between the amino group of cysteine and the γ -carboxy group of glutamate, forming γ -glutamylcysteine (γ -EC). In the second step, glutathione synthetase (GSHS = GSH2, E.C. 6.3.2.3) adds a glycine residue to the carboxy-terminus of γ -EC, producing glutathione. While the GCL reaction takes place in plastids, GSHS is found in both plastids and cytosol (See paragraphs 2.2.2 and 2.2.3)

As glutathione synthesis combines precursors from the assimilatory pathways for carbon, nitrogen and sulfur in the form of the amino acids glutamate, cysteine and glycine, the supply of any of these could theoretically regulate the level of GSH. It has been found that primarily sulfur supply does limit the content of GSH in plants, so it can be assumed that the availability of cysteine for the reaction catalyzed by GCL is a strong regulator of GSH synthesis *in vivo* (Meyer and Fricker, 2002; Noctor et al., 2002; Kopriva and Rennenberg,

2004). Furthermore, it has been found that glutathione synthesis may be limited by the photosynthetically active photon flux at low light intensities, possibly due to limitations in the supply of carbon backbones for amino acid synthesis or reduction equivalents necessary for the assimilation of nitrogen and sulfur (Ogawa et al., 2004). As poplar and maize leaves accumulate γ -EC in the dark which is depleted after onset of the photoperiod, it has also been suggested that glycine, produced during photorespiration, could limit GSH synthesis at the reaction catalyzed by glutathione synthetase (Noctor and Foyer, 1998; Masi et al., 2002). However, as light intensity might also influence the cellular redox state and ROS production rates, a more complex mode of light-dependent regulation of GSH synthesis, possibly involving gene expression or post-translational regulation, seems also plausible.

The analysis of transgenic plants strongly indicates that the reaction catalyzed by GCL is limiting glutathione synthesis *in planta* under most conditions. Overexpression of the glutathione synthesis proteins from *E. coli* in poplar resulted in an increase of glutathione content only when GCL was overexpressed (Arisi et al., 1997; Noctor et al., 1998b; Noctor et al., 1998a). No significant differences were observed regarding the overexpression of GCL in the cytosol or the plastids. In both cases glutathione levels were increased up to four fold. Similar results were achieved by overexpression of the *E. coli* genes of GSH synthesis in tobacco (Creissen et al., 1999), whereas overexpression of the endogenous GCL gene in *Arabidopsis* resulted only in an increase in GSH of up to 180 % of wild-type levels only (Xiang et al., 2001). Antisense expression of *GCL* resulted in a significant decrease of GSH levels, accompanied by higher cadmium sensitivity and diminished growth (Xiang *et al.*, 2001).

The decisive role of GCL in glutathione synthesis is further emphasized by the characterization of several *Arabidopsis* mutants with decreased capability to synthesize GSH. The mutant *rml1* (*root meristem less*), which is unable to produce a root meristem (Cheng et al., 1995), shows an exchange of aspartate to asparagine in the position 259 of the GCL protein and has extremely low levels of glutathione (Vernoux et al., 2000). The mutants *cad2-1* (*cadmium hypersensitive*), which has a 6 base pair deletion in *GCL*, and *rax1-1* (*regulator of ascorbate peroxidase2*), showing an arginine to lysine exchange in position 229 of the protein, both show about 40 % of the GCL activity and GSH concentration found in the wild-type (Cobbett et al., 1998; Ball et al., 2004). These mutants showed increased susceptibility to cadmium and changed expression of stress related genes, but showed no phenotype different from the wild-type under non-stress conditions.

Recently the mutant *pad2-1* (*phytoalexin deficient*) could also be mapped to the GCL gene (Parisy et al., 2007). It has been described as being deficient in the phytoalexin camalexin and showing enhanced susceptibility to several pathogens which, however, was not dependent on camalexin deficiency. *pad2-1* was found to cause a serine to asparagine transition in position 298 of the GCL protein, resulting in GSH levels as low as 20 % of that found in the wild-type.

Interestingly, for all these mutants, except for *rax1-1*, increased levels of cysteine were reported, whereas in GCL overexpression experiments no decrease of cysteine concentrations was reported, indicating a complex feedback control of GSH synthesis on the regulation of sulfur assimilation and cysteine synthesis.

2.2.2 Plant glutamate cysteine ligase: Evolutionary relationship and subcellular localization

At the protein sequence level, plant GCL is clearly distinct from its counterparts in animals, fungi, and *E. coli*. Therefore it has been predicted to be structurally unrelated to these (May and Leaver, 1994). However, plant GCL proteins share extensive sequence similarity with those from alphaproteobacteria and some gammaproteobacteria (Copley and Dhillon, 2002). Together they define a group of *GCL* genes (= group 3), separated from animal and fungal *GCL* (= group 1) and most gammaproteobacterial genes (including *E. coli*; group 2), respectively (Figure 2.1). While group 3 also includes cyanobacterial *GCL* genes, these are forming a distinct subgroup and probably are not the predecessors of the plant enzyme (Ashida et al., 2005). Despite the low overall sequence homology between the three groups, Copley and Dhillon (2002) were able to identify three conserved blocks specific for all GCL proteins, indicating a common origin. Based on further similarity searches it has been proposed that GCL and glutamine synthetase proteins, which act as γ -glutamylammonia ligases, actually form a large superfamily of carboxylate-amine/ammonia ligases (Abbott et al., 2001).

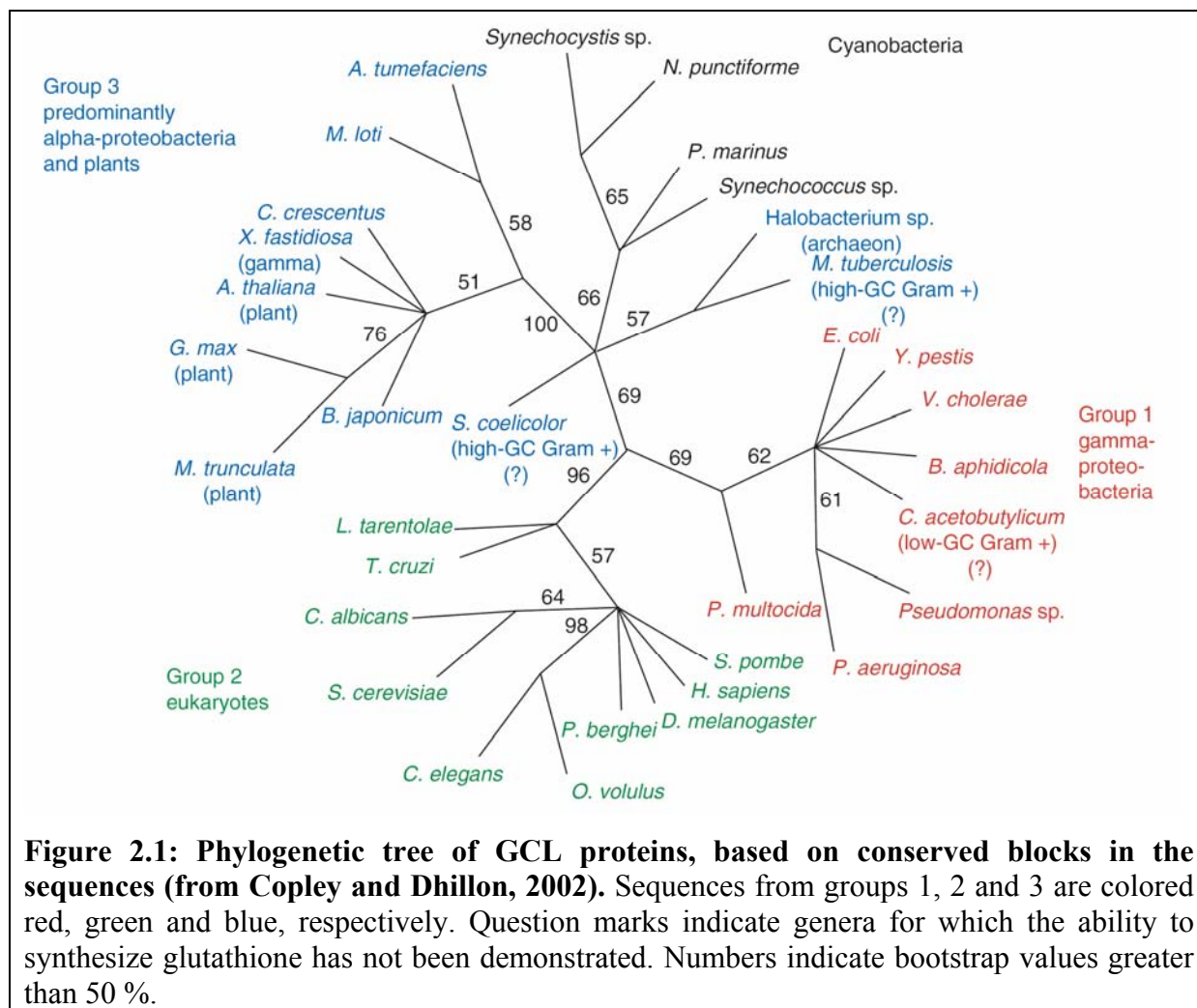


Figure 2.1: Phylogenetic tree of GCL proteins, based on conserved blocks in the sequences (from Copley and Dhillon, 2002). Sequences from groups 1, 2 and 3 are colored red, green and blue, respectively. Question marks indicate genera for which the ability to synthesize glutathione has not been demonstrated. Numbers indicate bootstrap values greater than 50 %.

In *Arabidopsis*, GCL is present as a single gene (May and Leaver, 1994; The *Arabidopsis* Genome Initiative, 2000), while other plant species like *Brassica juncea* can have several isoforms, possibly due to higher ploidy levels (Schäfer et al., 1998). In *A. thaliana* and *B. juncea* GCL is localized exclusively to the plastid, as was shown using in vivo localization studies, immunohistology, and cell fractionation techniques (Wachter et al., 2005). Exclusive plastidic localization of GCL has also been reported for nodules of *Glycine max* (Moran et al., 2000). Earlier cell fractionation experiments detected GCL activity also in cytosolic fractions of *Nicotiana tabacum* (Hell and Bergmann, 1990), *Zea mays* (Rueggsegger and Brunold, 1993) and *Vigna unguiculata* (Moran et al., 2000). However these findings have not been supported by analysis using further methods. While therefore at the moment a cytosolic subpopulation of GCL cannot be excluded for all cases, plastidic localization of plant GCL seems to be predominant or even exclusive in plants.

2.2.3 Regulation of glutamate cysteine ligase activity

The glutamate cysteine ligase reaction is under multiple control, involving expression of the enzyme, supply of its metabolites and feedback inhibition via the redox state and the cellular concentration of glutathione. Increased *GCL* transcript levels have been observed following several kinds of stress treatment like exposure to copper (Schäfer et al., 1997), cadmium, jasmonic acid (Xiang and Oliver, 1998) and chilling (Gomez et al., 2004). Notably, GCL protein amount and extractable activity did not always follow changes in transcript amounts. The observation that GCL protein amount in *Arabidopsis* was significantly enhanced after hydrogen peroxide treatment, while transcript amounts remained unchanged, led to the assumption that the cellular redox state does exert a translational control on GCL expression [May, 1998 #67]. Such a regulation might be controlled by 5'-UTR binding proteins. Interestingly, in *Arabidopsis* and *B. juncea* the GCL genes give rise to two transcript populations, differing in the length of the 5'-UTR but not in the coding region and the ratio of transcript types is influenced by developmental and environmental cues (Wachter et al., 2005). As for both transcripts canonical TATA-boxes are positioned about 30 bp upstream of the transcript starts, it can be assumed that dual, overlapping promoters regulate the expression of different transcripts, potentially affecting the susceptibility to post-transcriptional expression control.

Plant GCL proteins have been characterized by using partially purified enzymes from wheat (Webster and Varner, 1954) and tobacco (Hell and Bergmann, 1990) and recently by using recombinant *Arabidopsis* and *B. juncea* enzymes (Jez et al., 2004). Plant GCL was found to show K_m values for the substrates ATP, cysteine and glutamate that were comparable to those found for animal or bacterial proteins (Table 2.1). As all of these enzymes are inhibited by buthionine sulfoximine, which has been shown to bind to the active site of rat GCL (Orlowski and Meister, 1971), a conserved catalytic mechanism has been proposed (May and Leaver, 1994). In the case of the rat enzyme that the reaction is involving enzyme bound γ -glutamylphosphate as a reaction intermediate (Orlowski and Meister, 1971). A difference between plant and animal GCL, however, is found in the dependence on monovalent cations, as plant GCL shows higher activity in the presence of potassium than in the presence of sodium (Webster and Varner, 1954).

Table 2.1: K_m values of GCL proteins from various organisms (in mM)

	<i>Arabidopsis</i>	<i>B. juncea</i>	<i>N. tabacum</i>	<i>R. norvegicus</i>	<i>C. boidinii</i>	<i>E. coli</i>
L-Cysteine	1.6	0.12	0.19	0.2	0.4	0.09
L-Glutamate	9.1	8.5	10.4	1.4	1.4	0.5
ATP	2.7	1.3	n.d.	0.2	0.2	0.01

K_m values in mM for the GCL enzymes from *Arabidopsis thaliana* (Jez et al., 2004), *Brassica juncea*, *Nicotiana tabacum* (Hell and Bergmann, 1990), *Rattus norvegicus* holoenzyme (Huang et al., 1993a; Huang et al., 1993b), *Candida boidinii* (Dennda and Kula, 1986) and *Escherichia coli* (Watanabe et al., 1986).

Besides a possible regulation of GCL activity by the availability of its substrates, especially cysteine, plant GCL also shows post-translational regulation via the redox state and glutathione concentration. Treatment of GCL protein purified from tobacco or of recombinantly produced *Arabidopsis* GCL, with the reductant dithiothreitol (DTT) led to a significant drop in activity and resulted in a decrease in apparent size of the protein in size exclusion chromatography (Hell and Bergmann, 1990; Jez et al., 2004). Considering the central role of GCL in providing the redox metabolite GSH, such a mechanism could provide an efficient feedback regulation. In case of the rat enzyme redox regulation depends on the association and dissociation of a catalytic and a regulatory subunit, facilitated by formation or reduction of an intermolecular disulfide bridge (Huang et al., 1993a; Huang et al., 1993b). In this case the holoenzyme shows an approximately six-fold lower K_m value for glutamate and lower sensitivity for GSH feedback inhibition.

GCL proteins from various organisms are also inhibited by the presence of GSH. For the recombinantly produced protein from *Arabidopsis* inhibition by GSH has been described as non-competitive, leading to the assumption that it is mechanistically equivalent to redox regulation (Jez et al., 2004). However, for the partially purified GCL protein from tobacco a competitive inhibition towards both glutamate and cysteine has been reported (Hell and Bergmann, 1990). Furthermore analysis of the *B. juncea* protein showed that oxidized GSH (GSSG) and S-methyl-GSH both act as inhibitors comparable in strength to reduced GSH, arguing against a strong contribution of the reductive properties of GSH to the inhibition of plant GCL.

2.2.3 Plant glutathione synthetase: Evolutionary relationship, subcellular localization and regulation

In contrast to GCL, plant glutathione synthetase is closely related to GSHS from other eukaryotes, forming a group distinct from prokaryotic GSHS enzymes (Copley and Dhillon, 2002). In addition to GSHS, in Fabaceae specific homoglutathione synthetases (hGSHS) are found which are derived from GSHS via gene duplication (Moran et al., 2000; Frendo et al., 2001). The concentrations of GSH and hGSH found in the tissues of different Fabaceae seems to be correlated to the expression of these GSHS and hGSHS genes (Matamoros et al., 1999). Whether the synthesis of other GSH homologs is also regulated by GSHS homologs is not yet known, as the formation of GSH homologs can also be facilitated by the action of GSHS proteins with a broad substrate specificity regarding the acyl acceptor (Skipsey et al., 2005) and at least for hmGSH it also seems possible via the substitution of glycine from GSH catalyzed by carboxypeptidase Y (Okumura et al., 2003).

In *Arabidopsis* GSHS is encoded by a single gene showing approximately 40 % identity and 60 % similarity to other eukaryotic GSHS proteins (Ullmann et al., 1996; Wang and Oliver, 1996; The *Arabidopsis* Genome Initiative, 2000). While this gene does encode for a predicted plastidic transit peptide, several transcription initiation sites lead to different transcript populations (Wachter et al., 2005). Most transcripts are lacking the region encoding the transit peptide and therefore result in protein being localized in the cytosol. The ratio of long transcripts, encoding for a plastidic protein, to short transcripts was found to be highest in source leaves and lowest in non-photosynthetic tissue like roots and stem, possibly indicating a tissue-dependence of the localization of GSH synthesis. Cell fractionation experiments confirmed predominant localization of GSHS (and hGSHS) protein in the cytosol with only a minor population in the plastids and possibly in mitochondria (Klapheck et al., 1987; Hell and Bergmann, 1988; Moran et al., 2000).

GSHS purified from tobacco and the recombinantly produced *Arabidopsis* protein have been characterized enzymatically, revealing similar kinetics as found for the animal enzyme (Hell and Bergmann, 1988; Jez and Cahoon, 2004). GSHS is active as a homodimer and studies concentrating on mutant variants of the *Arabidopsis* enzyme recently allowed a detailed characterization of the reaction mechanism of plant GSHS, involving the formation of an acylphosphate intermediate (Herrera *et al.*, 2007).

2.2.4 Transport of glutathione in plants

Long distance transport of GSH occurs in xylem and phloem (Rennenberg et al., 1979) enabling GSH to act as the major transport and storage form of reduced sulfur in plants. However, long distance transport of substantial amounts of S-methylmethionine in the phloem has also been shown (Bourgis et al., 1999).

The need for intracellular GSH transport becomes obvious when considering the localization of GSH metabolizing enzymes. As only plastids and the cytosol are able to synthesize GSH (Wachter et al., 2005), other organelles like mitochondria, the components of the secretory pathway and peroxisomes require GSH import. In addition, the exclusive plastidic localization of GCL means that γ -EC has to be exported from this compartment to feed the GSHS reaction in the cytosol. Export of γ -EC and GSH from the plastid would also act as a mechanism to regulate GCL activity as both substances are able to provide feedback inhibition (Pasternak, 2007). Furthermore, as glutathione reductase genes encode for proteins targeted to the cytosol, plastids and mitochondria (Creissen et al., 1995; The *Arabidopsis* Genome Initiative, 2000), not only the import of reduced GSH but also an efficient export of oxidized GSH from other organelles is necessary.

Kinetic analyses of GSH uptake suggested low- and high-affinity import systems in *Nicotiana tabacum* cells while kinetics measured for protoplasts from *Vicia faba* hinted at a single uptake system for reduced and two systems for oxidized GSH (Schneider et al., 1992). In bean cells GSH uptake was inhibited by GSSG and GSH-conjugates but not by other di- or tripeptides, indicating that a specific uptake system for GSH and its derivatives exists. The first plant GSH transporters were identified based on their homology to a high-affinity GSH transporter from yeast (Bogs et al., 2003; Zhang et al., 2004).

Recently, it could be shown that in GSHS knockout plants γ -EC is accumulating to very high amounts in the cytosol. Furthermore, GSHS expression in either the plastid or the cytosol could rescue the plants from seedling lethality and restore the wild-type phenotype (Pasternak, 2007).

2.2.5 Degradation of glutathione in plants

In mammals GSH is degraded by the sequential action of extracellular γ -glutamyl transpeptidase (GGT) and a membrane bound dipeptidase (Liebermann et al., 1995; Habib et al., 1996) and this pathway has been found to be essential for the reabsorption of cysteine and the degradation of GSH-conjugates (Liebermann et al., 1996). In *Arabidopsis* four GGT isoforms have been identified. GGT1 is localized in the apoplast and accounts for 80 – 99 % of the activity in all tissues except seeds, where the other apoplastic isoform GGT2 provided about 50 % of the activity (Martin et al., 2007; Ohkama-Ohtsu et al., 2007b). *ggt1* mutant plants were described as stunted, early flowering and showing yellowing of leaves and accumulation of GSSG in the apoplastic space, while *ggt2* mutants showed no phenotype (Ohkama-Ohtsu et al., 2007b). It has therefore been assumed that GGT1 is important for preventing oxidative stress by metabolizing extracellular GSSG, while GGT2 might play a role in the import of GSH into developing seeds. GGT3 and GGT4 are localized in the vacuole and are involved in the degradation of GSH conjugates (Grzam et al., 2007; Ohkama-Ohtsu et al., 2007a).

However, to what degree GGTs are involved in GSH degradation in plants is not yet fully understood. γ -EC was found to appear as the degradation product of radio-labelled GSH fed to tobacco cells, indicating a degradation pathway starting with a carboxypeptidase, while further degradation was catalyzed by a γ -glutamyl cyclotransferase, producing 5-oxo-proline (Steinkamp and Rennenberg, 1985). In soybean on the other hand cysteinylglycine was detected as degradation product, indicating a pathway using GGT and a dipeptidase (Bergmann and Rennenberg, 1993). A third possible pathway for GSH degradation, involving a phytochelatin synthetase (PCS) related protein was described for cyanobacteria (Harada et al., 2004) and degradation of GSH conjugates by PCS was also shown to be possible in higher plants (Beck et al., 2003). However, for GSH conjugates import into the vacuole seems to outcompete degradation by PCS (Grzam et al., 2006). To which degree these different pathways contribute to the degradation of underivatated GSH *in planta* and whether there are differences between tissues and developmental stages is unknown so far.

An important factor in the control of GSH degradation seems to be the GSH redox state, as overexpression of GSH reductase led to a significant increase in GSH levels (Foyer et al., 1995). The preferential degradation of GSSG might play a role in the upholding of redox homeostasis, as the accumulation of high amounts of GSSG has been shown to cause oxidative stress (Creissen et al., 1999).

2.3 Stress and housekeeping metabolism – the multiple roles of glutathione

2.3.1 Glutathione as a redox metabolite

2.3.1.1 The basis of glutathione redox chemistry

The role of glutathione as an antioxidant in stress defence and signalling has been extensively studied and discussed in the last years (Foyer and Noctor, 2005a; Mullineaux and Rausch, 2005). As a thiol reductant glutathione can form a dimer (GSSG) by oxidative formation of a disulfide bridge:



This oxidative dimerization is reversible through the action of glutathione reductase (GR, E.C. 1.6.4.2) using NADPH as electron donor. In *Arabidopsis* two genes for glutathione reductase are present. One is encoding for a cytosolic protein while the other encodes for a protein with dual targeting to plastids and mitochondria (The *Arabidopsis* Genome Initiative, 2000; Chew et al., 2003). Glutathione reductase activity was also detected in peroxisomes of pea leaves (Jiménez *et al.*, 1997). All other compartments, especially the apoplast, would require import of GSH and export or degradation of GSSG to balance their redox state.

The Nernst equation allows calculation of the glutathione redox potential, which is dependent on the redox state as well as the total concentration of GSH as it is influenced by oxidized glutathione in first order, while reduced glutathione does so in second order:

$$E_{redox} = E_{redox}^0 - \frac{R \cdot T}{n \cdot F} \cdot \ln \frac{[\text{GSH}]^2}{[\text{GSSG}]}$$

where E_{redox} is the redox potential, E_{redox}^0 the standard redox potential (for GSH/GSSG: $E_{redox}^0 = -240$ mV), R the universal gas constant, ($8.314510 \text{ J K}^{-1} \text{ mol}^{-1}$), T the temperature in Kelvin, n the number of electrons involved (for GSH/GSSG $n = 2$) and F is the Faraday constant $9.6485309 \cdot 10^4 \text{ C mol}^{-1}$.

The amounts of GSH found in eukaryotic cells combined with its standard redox potential of -240 mV (Schafer and Buettner, 2001), which is between the -320 mV of the NAD(P)H/NADP⁺ couple and that of ascorbic acid/dehydroascorbate at -80 mV, render the GSH/GSSG couple one of the three major cellular redox buffering systems.

As a reductant glutathione is involved in several enzymatic reactions such as the reduction of 5'Adenylylsulfate (APS) by APS reductase or glutathione dependent formaldehyde reductase (Giese *et al.*, 1994). It is also acting as an antioxidant, detoxifying reactive oxygen species and is involved in the control of protein redox state.

2.3.1.2 The role of glutathione in the detoxification of reactive oxygen species (ROS)

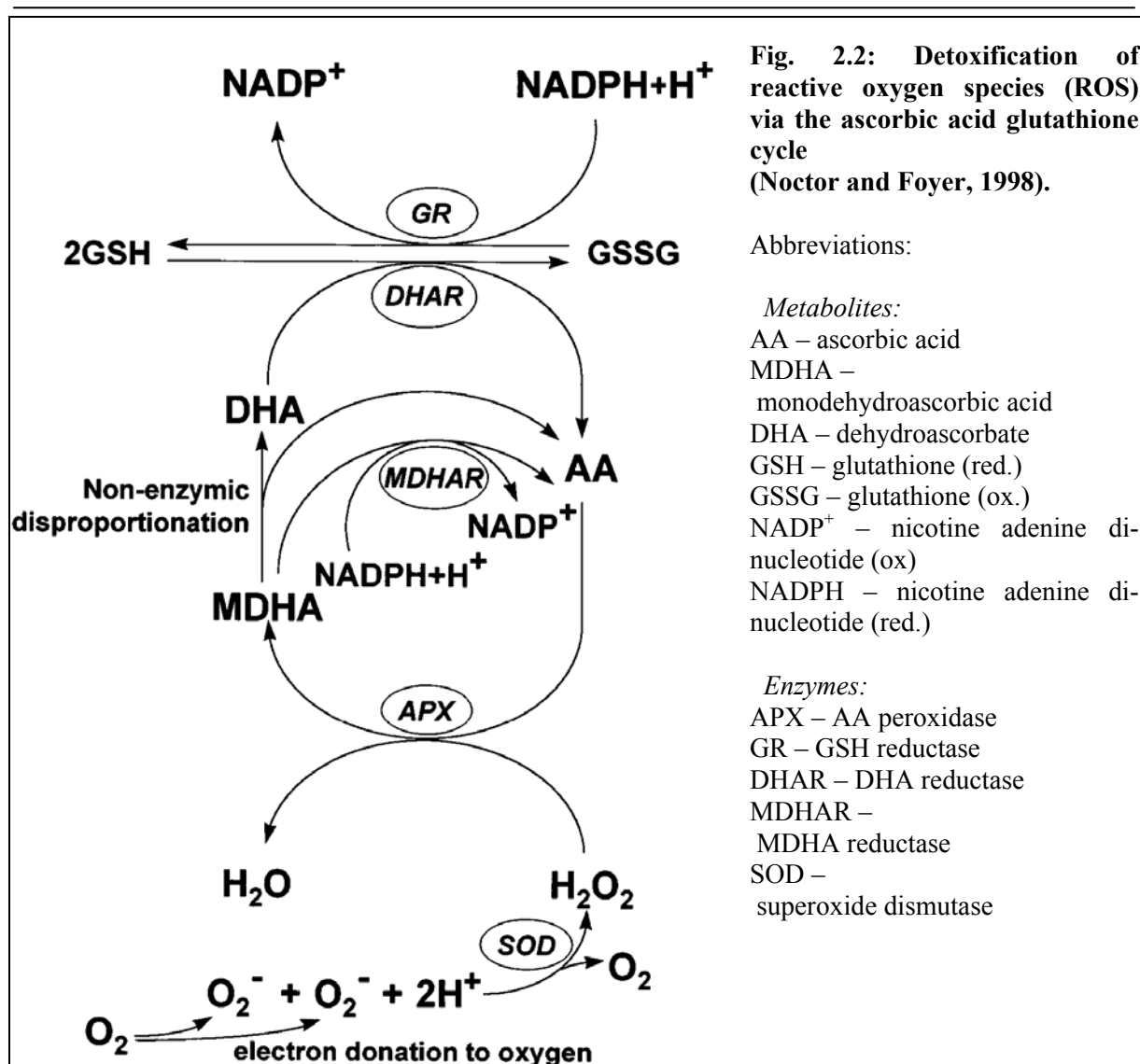
Several reactions in plant metabolism can lead to the formation of reactive oxygen species (ROS), the production of which is further enhanced when the metabolism is disturbed by stress (Alscher *et al.*, 1997; Noctor and Foyer, 1998). While these ROS can have a role in signalling and induction of defence reactions (Vranova *et al.*, 2002; Foyer and Noctor, 2005a), their chemical reactivity threatens to oxidize biologically important molecules like proteins, lipids and nucleic acids, therefore requiring cells to express efficient systems to keep the ROS levels under strict control.

Besides the oxidative reactions in peroxisomes, the largest contribution to ROS production is made by the electron transport chains of mitochondria and plastids. In chloroplasts electrons can be transferred by the Mehler reaction (Mehler, 1951) from photosystem I to molecular oxygen (O₂), resulting in the formation of superoxide anions (O₂⁻). This reaction preferentially takes place when the NADP⁺/NADPH pool is highly reduced due to high light intensities, limiting the availability of the physiological electron acceptor NADP⁺. Superoxide dismutases (SOD, EC 1.15.1.1) convert two superoxide anions to molecular oxygen and hydrogen peroxide (H₂O₂). Both, superoxides and peroxides, can undergo further reactions, resulting in highly reactive compounds like the hydroxyle radical (OH) or lipid peroxides. Several cellular mechanisms exist to detoxify hydrogen peroxide to prevent the formation of more aggressive ROS. Catalases can detoxify hydrogen peroxide by dissociating it to molecular oxygen and water but they show a low substrate affinity and are not found in plastids (Willekens *et al.*, 1995). Therefore other detoxification mechanisms are required in this compartment.

Glutathione can directly react with some toxic oxidants like hydroxyl radicals and peroxyxynitrite (Kalyanaraman et al., 1996; Karoui et al., 1996) and can reduce less reactive oxidants like hydroperoxides with the aid of peroxidases. However, recently the plant genes annotated as glutathione peroxidase due to their homology to animal genes have been shown to actually be thioredoxin dependent peroxidases (Navrot et al., 2006). On the other hand it appears that some glutathione-S-transferases (GSTs, see paragraph 2.3.2.1) are able to reduce peroxides in a glutathione-dependent manner, therefore acting as glutathione peroxidases (Cummins et al., 1999). Furthermore, at least some peroxiredoxins, proteins that can reduce hydrogen peroxide and alkyl peroxides can be regenerated by glutaredoxins, proteins that use GSH as a cofactor to reduce protein disulfide bonds (Rouhier et al., 2001; Rouhier et al., 2002).

Most of the hydrogen peroxide, detoxified in a glutathione dependent manner is initially reduced by ascorbic acid peroxidase (APX, EC 1.11.1.11), resulting in the formation of monodehydroascorbic acid (MDHA) (Noctor and Foyer, 1998). MDHA can be reduced NADPH dependent by monodehydroascorbic acid reductases (MDHAR) or can disproportionate to ascorbic acid (AA) and dehydroascorbic acid (DHA), the latter being reduced to ascorbic acid by the glutathione dependent dehydroascorbic acid reductase (DHAR). As glutathione can be reduced by the NADPH dependent glutathione reductase (GR), the ascorbic acid glutathione cycle connects the three major soluble redox buffering systems of the plant cell to provide an efficient system for the detoxification of ROS (Fig 2.2).

The metabolites and enzymes required for the ascorbic acid-glutathione-cycle have been found in cytosol, plastids, mitochondria and peroxisomes where in *Arabidopsis thaliana* the same isoforms of the enzymes are imported into plastids and mitochondria (Jiménez et al., 1997; Chew et al., 2003). Little is known so far about the transport of glutathione and ascorbic acid between the cellular compartments and about the enzymes providing the peroxisomal activities so that the relative contribution of the different compartments to ROS detoxification remains somewhat unclear. Interestingly, however, it has been found that stress affects the ascorbic acid-glutathione-cycle in the various compartments differently, possibly hinting at a compartment specific role of antioxidant systems in stress defence and signalling (Jiménez et al., 1998; Nishikawa et al., 2003).



Keeping the ascorbic acid pool reduced, glutathione indirectly also acts as a protectant for further antioxidant systems which are reduced by ascorbic acid, like the tocopherol system, which is protecting biological membranes, and the xanthophyll cycle, which is involved in the dissipation of excess energy accumulated in the photosystem during high light conditions. Depletion of GSH in animal cells has been shown to lead to an increased concentration of ROS as well as to lipid peroxidation and DNA damage, emphasizing the importance of glutathione as an antioxidant protecting various cellular systems (Green et al., 2006; Reliene and Schiestl, 2006).

2.3.1.3 The role of glutathione in control of protein redox state

As glutathione is able to undergo redox reactions with the sulphydryl groups of other molecules, most likely it is the reductant to influence the redox state of protein cysteine residues. In animal cells the rate of reduced to oxidized glutathione has been found to be in the range from 30:1 to 100:1 in the whole cell, while the endoplasmatic reticulum (ER) shows a proportion of 3:1 to 1:1 (Hwang et al., 1992). It has been proposed that these differences in the redox state of glutathione are directly responsible for the varying redox state of proteins in these compartments, favouring the formation of disulfide bridges in the ER while keeping cytosolic proteins reduced. While GSH may interact with some protein cysteines or disulfide bridges spontaneously, a specific reduction of protein disulfide bridges can be accomplished by glutaredoxins, which are using glutathione as the electron donor (Morell *et al.*, 1995; Szederkenyi *et al.*, 1997).

The formation of mixed disulfides between glutathione and proteins, referred to as glutathionylation, has only recently become a field of interest. Glutathionylation can occur spontaneously, especially under oxidizing conditions, and it has been estimated that about half of the glutathione in the ER is found in the form of a mixed disulfide with proteins (Bass et al., 2004). De-glutathionylation reactions seem to be dependent on the action of glutaredoxins (Jung and Thomas, 1996; Nulton-Persson et al., 2003).

It has been speculated that glutathionylation could act as a protective mechanism, preventing the irreversible oxidation of cysteine residues. Furthermore, the specific glutathionylation of several proteins has been reported and proposed to play a role in the regulation of these proteins (see paragraph 2.3.3.1). Interestingly, for mammalian 1-Cys peroxiredoxins a mechanism depending on glutathione-S-transferase-mediated glutathionylation has been shown to be required for re-reduction of the enzyme after reacting with peroxides (Noguera-Mazon et al., 2006).

2.3.2 Involvement of glutathione in detoxification reactions

Besides the ability to participate in redox reactions, the sulfhydryl group of glutathione can undergo a number of other chemical reactions, based on its nucleophilic properties. The binding of foreign compounds like xenobiotics or heavy metal ions and the subsequent sequestration of the glutathione complexes is the basis for various detoxification processes.

2.3.2.1 Glutathione S-transferases

Covalent binding of glutathione can happen spontaneously for some reactive electrophilic chemicals like monobromobimane (mBBBr) or 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), both of which can be used for the specific labelling of thiols. For other xenobiotics a detoxification system similar to that described for the animal system is present in plant cells, therefore sometimes referred to as the “green liver” (Sandermann, 1994; Kreuz et al., 1996). Substrates to be detoxified are chemically activated by monooxygenases and subsequently bound to the nucleophilic group of a hydrophilic substance like glutathione, sugars, or amino acids. This does on one hand increase the solubility of the substance, preventing uncontrolled passage of membranes, and on the other hand marks the xenobiotic with a group that can be specifically recognized and transported. The conjugation to glutathione is catalyzed by a group of enzymes referred to as glutathione-S-transferases (GST). These enzymes are expressed in a large number of isoforms, 48 in *Arabidopsis thaliana*, of which several are induced by xenobiotics or other stress factors (Marrs, 1996; Dixon et al., 2002). Finally the conjugates are transported into the vacuole or the apoplast by ATP-dependent transporters of the ABC (ATP binding cassette)-type (Martinoia et al., 1993; Lu et al., 1997; Rea et al., 1998). The transcript levels of these transporters have been found to be up-regulated in parallel to those for GSTs upon exposure to some xenobiotics.

In the vacuole GSH conjugates are quickly degraded by GGTs to cysteinylglycine conjugates and further to the cysteine conjugates (Grzam et al., 2007; Ohkama-Ohtsu et al., 2007a) from which further degradation is presumed to be substrate and species specific (Leustek et al., 2000). Recently, transporters allowing the transport of glutathione conjugates to the apoplast and from cell to cell have been described (Zhang *et al.*, 2004) and experiments conducted on barley roots indicated that long distance transport of GSH conjugates and

exudation from the root might be another way for plants to cope with xenobiotics, being equivalent to animal excretion systems (Schroder et al., 2007). What the relative contribution of deposition and excretion to the detoxification of xenobiotics actually are in living plants remains to be elucidated.

There are several hints that glutathione-S-transferases also fulfil other roles besides the detoxification of xenobiotics. Some plant metabolites like medicarpin, an isoflavone, are subject to GSH-conjugation and transport to the vacuole (Li *et al.*, 1997) and it has been shown that the *bronze-2* mutant of maize is deficient in anthocyanine deposition to the vacuole due to a defect in a GST (Marrs, 1996). This finding is supported by the observation that the accumulation of anthocyanes under high light conditions is GSH dependent (Xiang et al., 2001), indicating that the glutathione-GST system might play an important role in the import of metabolites into the plant vacuole. In addition, GSTs have also been presumed to be involved in glutathionylation reactions, binding GSH to protein cysteines (Noguera-Mazon et al., 2006).

Furthermore some GSTs have been shown to fulfil additional enzymatic functions such as the reduction of peroxides in a glutathione-dependent manner, therefore in fact acting as glutathione peroxidases. This functionality has been found to confer tolerance against chilling and salt in transgenic tobacco (Roxas et al., 1997) and to herbicides in black-grass (Cummins et al., 1999). Another catalytic role that is not involving GSH conjugation has been found for one *Arabidopsis* GST which catalyzes the GSH-dependent isomerization of maleylacetoacetate to fumarylacetoacetate, a step in the pathway of tyrosine degradation (Dixon et al., 2000).

GSTs and GSH conjugates have also been reported to play a role in signalling processes. In parsley it has been shown that the induction of genes encoding for the enzymes of flavonoid biosynthesis by ultraviolet light requires GSH and the expression of a specific GST (Loyall et al., 2000). Furthermore, in onion GSH conjugates have been described as being transported to the nucleus before sequestration to the vacuole, followed by a transient induction of defence reactions (Schröder and Stampfl, 1999). Recently, the electrostatic association of a glutathione S-transferase to the nuclear membrane of rat hepatocytes has been demonstrated, which could play a role in signalling or represent a specific defence mechanism of the nucleus (Stella et al., 2007).

2.3.2.2 Glutathione and heavy metal tolerance

Essential and non-essential heavy metal ions are taken up by plants via the root and can, depending on their cellular concentration, disturb the metabolism by different mechanisms. These include the inactivation of enzymes by displacement of required cations and the catalysis of several noxious chemical reactions, including the production of ROS. Therefore the concentration of heavy metal ion concentrations has to be strictly controlled. One possibility to bind heavy metal ions is provided by the ability of the sulfhydryl group to form metal ion complexes, a property that has led to the trivial name mercapto group for the SH group (Latin: mercurium captare – to grab/catch mercury). Proteins containing several cysteines that are presumed to be involved in metal homeostasis and detoxification are present in plants and animals in the form of metallothioneins (Cobbett and Goldsbrough, 2002). Interestingly, expression of a metallothionein 2 from *Brassica juncea* not only increased copper and cadmium tolerance in *E. coli* and *Arabidopsis*, but inhibited root elongation in *Arabidopsis* seedlings (Zhigang *et al.*, 2006).

Glutathione can also play an important role in the detoxification of heavy metal ions by directly forming complexes as well as in the defence against secondary effects of heavy metal stress by the detoxification mechanism described previously. The production of adequate amounts of glutathione by plants has been shown to be essential for the tolerance of *Arabidopsis* against cadmium (Xiang *et al.*, 2001), where the cadmium sensitive mutant *cad2-1* was found to have lowered glutathione concentrations due to a mutation in the GCL gene (Howden *et al.*, 1995a; Cobbett *et al.*, 1998). In several species of the related genus *Thlaspi* an elevated capacity for glutathione synthesis was shown to be associated with constitutively elevated levels of salicylic acid and was responsible for the high resistance towards nickel, allowing hyper-accumulation of this metal. Also, nickel tolerance of transgenic *Arabidopsis* lines was significantly correlated to the glutathione content (Freeman *et al.*, 2004; Freeman *et al.*, 2005).

Improved complexation of heavy metals is facilitated by the synthesis of metal-chelating glutathione polymers of the structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ with $n = 2\text{-}11$, called phytochelatins (PCs) (Grill *et al.*, 1985). In plant species that contain GSH homologs alternative phytochelatins, substituting the terminal glycine by alanine, serine or glutamate can be produced (Rauser, 1995). The ability to form phytochelatins has also been found in

yeast and some animals, including the nematode *Caenorhabditis elegans* (Cobbett and Goldsbrough, 2002).

The synthesis of phytochelatin from glutathione is catalyzed by phytochelatin synthase (PCS), a γ -glutamylcysteindipeptidyl-transpeptidase (EC 2.2.15) (Grill et al., 1989). The corresponding gene has been identified in *Arabidopsis* by screening mutants for reduced cadmium tolerance, identifying the cadmium sensitive mutant *cad1*, defective in PCS (Ha et al., 1999). The *cad1* mutant is unable to synthesize PCs, resulting in reduced tolerance to cadmium without showing a phenotype under non-stress conditions, indicating that phytochelatin synthesis probably is not involved in non-stress metal homeostasis (Howden et al., 1995b). Interestingly, a second gene showing high homology to PCS and providing PCS activity when expressed in *Saccharomyces*, was identified in *Arabidopsis*. Why this gene cannot rescue the *cad1* phenotype and which role it does play *in vivo* remains unclear (Cazale and Clemens, 2001).

PCS is a cytosolic protein and seems to be constitutively expressed on the transcript level in *Arabidopsis* and *B. juncea* (Ha et al., 1999; Heiss et al., 2003), while the protein amount was reported to increase upon prolonged cadmium treatment (Heiss et al., 2003). The regulation of PCS is thought to be primarily happening on the level of the enzymatic activity, which is dependent on the presence of heavy metal ions (Ha et al., 1999; Heiss et al., 2003). This is probably the case because glutathione-metal ion conjugates serve as the substrate for PC synthesis (Cobbett and Goldsbrough, 2002), which would provide a self-regulatory mechanism as the synthesis of PCs and the resulting depletion of metal ions by complexation would deplete the substrates (Loeffler et al., 1989).

The phytochelatin-metal ion complexes formed in the cytosol are then imported in an ATP-dependent manner into the vacuole (Salt and Rauser, 1995), where the addition of further S^{2-} and metal ions leads to the formation of high-molecular-weight complexes (Rauser, 1995; Cobbett and Goldsbrough, 2002). In addition to direct deposition in the vacuole, it has been shown that PCs can also undergo long-range transport in the plant (Gong et al., 2003), which might be responsible for redistribution of the metal ion load or might play a role in stress signalling.

2.3.3 Glutathione as a regulator of gene expression, protein activity and development

For many cellular or developmental processes a dependence on or regulation by glutathione has been reported. However, it is not in all cases clear whether glutathione is actually acting as a regulator or whether it is rather involved in establishing the metabolic background required for the progression of development.

The *Arabidopsis* mutant *rml1* (*root meristemless1*) which is unable to produce an active postembryonal root meristem was described to contain less than 5 % of wild-type levels of glutathione due to a mutation in the GCL-gene (Cheng et al., 1995; Vernoux et al., 2000), confirming earlier results which indicated that the development of roots in *Arabidopsis* is under redox control via different mechanisms (Sanchez-Fernandez et al., 1997). Recent experiments with *rml1* and T-DNA insertion mutants of *Arabidopsis* have confirmed that glutathione biosynthesis within the embryo is required for normal maturation (Cairns et al., 2006).

It could further be shown that the progression of the cell-cycle from G₁ to S-phase requires a certain level of GSH, which is regulating the expression of several cyclin proteins (Vernoux et al., 2000). In fibroblasts most of the cellular GSH is localized to the nucleus during the S- and M-phase of the cell-cycle, while during G₁ and G₀ phases a major part of the cellular GSH was found in the cytosol (Markovic et al., 2007). In these cells it has also been shown that telomerase is regulated cell-cycle dependent by the redox-state, being most active in its reduced state in the presence of high GSH concentrations (Borras et al., 2004). Together these results indicate that the amount and localization of GSH in the cell might play a crucial role in regulating the progression of the eukaryotic cell cycle.

Other developmental processes that are influenced by glutathione or the ratio of GSH/GSSG are the induction of flowering in *Arabidopsis* and *Eustoma grandiflorum* (Ogawa et al., 2001; Ogawa et al., 2004; Yanagida et al., 2004), the development of trichomes and root hairs (Sanchez-Fernandez et al., 1997), the differentiation of xylem elements (Henmi et al., 2005) and the accumulation of anthocyanins (Xiang et al., 2001).

Evidence is accumulating that glutathione is also involved in the regulation of programmed cell death or apoptosis. As ROS are widely accepted to be an important signal for apoptosis and programmed cell death in various life forms, the antioxidant functions of GSH might act as a modulator of this signalling. It was shown that a glutathione-S-transferase

from tomato can suppress Bax-induced cell death when expressed in yeast, probably due to its action as a glutathione peroxidase, detoxifying hydrogen peroxide (Kampranis et al., 2000). In addition, it has been reported that in cardiomyocytes a decrease of the GSH/GSSG ratio in mitochondria is leading to an increased production of ROS and the opening of membrane ion channels, promoting cell death (Aon *et al.*, 2007). On the other hand an influence of glutathione levels, independent of its redox state, on the decision for cell death accompanying defence reactions in plant has also been reported (Senda and Ogawa, 2004).

The importance of glutathione in the regulation of stress defence is also emphasized by the *rax1-1* mutant of *Arabidopsis thaliana* (Ball et al., 2004), which has significantly reduced glutathione levels due to a mutation in GCL. This mutant shows a constitutive expression of the otherwise stress-induced ascorbate peroxidase 2.

Glutathione is furthermore involved in the regulation interactions between plants and other organisms (Gullner and Kömviés, 2006). Glutathione is involved in the induction of pathogen defence genes (Mou et al., 2003; Senda and Ogawa, 2004) and recently the *Arabidopsis* mutant *pad2-1* (phytoalexin deficient), deficient in the defence molecule camalexin and more susceptible to pathogens, has been identified as a GCL mutant (Parisy et al., 2007). However, as camalexin deficiency alone could not explain the susceptibility towards different pathogens, a role for glutathione in the induction of defence reactions was presumed. In different oat genotypes, resistance to powdery mildew was shown to correlate with an increase of apoplastic GSH and its reduction state (Vanacker et al., 1998).

Furthermore, glutathione is likely to play a role in the defence against secondary effects of infections like ROS production and pathogen-produced xenobiotics and as the sulfur source for the synthesis of defence compounds (Rausch and Wachter, 2005). Surprisingly, the nickel hyperaccumulator *Thlaspi*, which shows constitutively elevated levels of GSH has been found to also show elevated susceptibility to powdery mildew (*Erysiphe cruciferarum*) (Freeman et al., 2005).

The importance of GSH for growth and possibly pathogenesis is hinted at by a high GST-activity of bacteria in the rhizosphere of plants, including the pathogen *Xanthomonas campestris* (Ilio et al., 1993; Zablutowicz et al., 1995) and the identification of a unique glutathione reductase in *Xanthomonas* (Loprasert et al., 2005) which is involved in the defence against oxidative stress.

Glutathione metabolism of plants and bacteria also seems to play an important role in symbiotic relations. *Sinorhizobium* and *Bradyrhizobium* strains without the ability to synthesize glutathione show significantly reduced growth and the former also showed

impaired nodule formation in symbiotic interaction with *Medicago* (Harrison et al., 2005; Sobrevals et al., 2006). Additionally, legumes seem to sustain an active and stress-reactive glutathione or homoglutathione metabolism in nodules to provide a suitable environment for symbiotic bacteria (Iturbe-Ormaetxe et al., 2001; Naya et al., 2007).

2.3.3.1 Mechanisms of glutathione-dependent regulation of proteins and genes

Regulation involving glutathione is most often presumed to be associated with redox regulation, although several cases are known where the total concentration of GSH seems to be the regulating factor. A direct involvement of GSH in the redox regulation of proteins is present in the form of glutaredoxin-mediated reduction of protein disulfide bridges, which is paralleling the reaction catalyzed by thioredoxins which are known to be involved in the redox regulation of various proteins (Vlami-Gardikas and Holgren, 2002). A direct link between glutaredoxin and thioredoxin-mediated protein regulation was found in poplar, where thioredoxin *h4* was found to be reduced not by thioredoxin reductase but by a poplar glutaredoxin (Gelhaye et al., 2003). Reduction of thioredoxins by glutaredoxins was also shown for *Arabidopsis* when the NADPH-dependent thioredoxin reductase genes were knocked out (Reichheld et al., 2007).

In addition to a direct involvement of glutathione in redox regulation of cellular processes, GSH might also play a decisive role in the regulation of ROS signals, which have been described to be involved in the regulation of several processes, including the expression of defence genes and the induction of programmed cell death during hypersensitive response (Foyer and Noctor, 2005b). One example for this kind of involvement of glutathione in ROS signalling might be the prevention of apoptotic cell death by an tomato glutathione-S-transferase/peroxidase expressed in yeast (Kampranis et al., 2000).

Reduced glutathione has been shown to influence the expression of a large number of genes involved in different processes like metabolism, development, and stress defence (Stasolla et al., 2004). Examples include the regulation of superoxide dismutase genes in *Nicotiana tabacum* (Herouart et al., 1993) and the GSH-induced repression of peroxiredoxins (Baier and Dietz, 1997). On the other hand oxidized glutathione has been shown to inhibit translation in eukaryotes by promoting the phosphorylation of initiation factors (Kan et al., 1988).

A possible direct link between the glutathione-mediated redox state and systemic acquired resistance (SAR), which confers immunity to a broad-spectrum of pathogens, has been described recently (Mou et al., 2003). The regulator protein NPR1 was found to be an inactive oligomer, formed through intermolecular disulfide bonds, in the uninduced state. Upon SAR induction by salicylic acid, a biphasic change in cellular redox potential occurs, resulting in the reduction of NPR1 disulfide bonds and subsequent monomerization and accumulation in the nucleus, followed by an activation of defence gene expression. However, for the induction of the pathogenesis-related gene PR-1 a dependence on GSH but not on the redox state of GSH was reported (Senda and Ogawa, 2004), indicating that several mechanistically independent ways of defence gene induction depending on GSH might exist in parallel in plant cells.

In the animal system it has been shown that the activity and nuclear translocation of the transcription factor NF κ B is enhanced by binding of GSH, while GSSG is inhibiting DNA-binding and transactivation (Mihm et al., 1995). It is presumed that glutathione is acting specifically on this transcription factor instead of only providing reductive power, as other antioxidants or thiols could not mimick its effects.

Another possible way for glutathione to directly influence the activity of proteins in a redox-related manner is found in the glutathionylation reaction. A study using biotinylated GSH as a probe detected about 20 proteins to be glutathioylated *in vivo* in *Arabidopsis* cell culture, where signal strength was increased upon hydrogen peroxide treatment (Ito et al., 2003). Glutathionylation has been confirmed for recombinant triose phosphate isomerase (Ito et al., 2003) and A4-glyceraldehyde-3-phosphate dehydrogenase (Zaffagnini et al., 2007) from *Arabidopsis* and for f-type thioredoxin of *Arabidopsis* and spinach (Michelet et al., 2005). In all cases glutathionylation led to an inhibition of enzymatic activity and was reversible by DTT treatment. Notably, reversibility was not given for GSH-independent oxidative inhibition of A4-glyceraldehyde-3-phosphate dehydrogenase (Zaffagnini et al., 2007), indicating that glutathionylation might act as a protective mechanism to prevent irreversible oxidation of cysteine residues.

Interestingly, the targets for glutathionylation in plants identified so far are all involved in plastidic sugar metabolism or its regulation, indicating that glutathionylation plays a role in the repression of sugar metabolism under oxidizing conditions, presenting a counter player to the thioredoxin system, which is activating the Calvin cycle enzymes under reducing conditions.

Another possible involvement of glutathionylation in gene regulation has been found in hamster cells, where glutathionylation of two cysteine residues in the paired domain of the transcription factor Pax-8 can reversibly inhibit DNA-binding under decreasing GSH/GSSG ratios (Cao et al., 2005).

Besides glutathione itself GSH derivatives have also been found to be able to act as signalling molecules as in onion cells GSH conjugates have shown to induce defence reactions (Schröder and Stampfl, 1999). Since glutathione, glutathione S-conjugates and phytochelatins all have been reported to undergo long distance transport in the plant (Foyer et al., 2001; Gong et al., 2003; Schroder et al., 2007), it cannot be excluded that GSH and derivatives might play a role not only in cellular but also in systemic signalling in plants.

3 Results

3.1 The molecular mechanism for the redox regulation of *Brassica juncea* Glutamate cysteine ligase (BjGCL)

3.1.1 The crystal structure of the BjGCL protein shows two disulfide bridges

Glutamate cysteine ligase from *Brassica juncea* (BjGCL, accession number AJ563921) has previously been cloned, expressed recombinantly in *E.coli* and characterized enzymatically (Wachter, 2004). In the course of this thesis, BjGCL protein was produced in large scale under standard conditions and with seleno-methionine labelling (Figure 3.1), to allow crystallization and structure resolution by cooperation partners at EMBL (Hothorn et al., 2006). Crystal structures with bound BSO, mimicking γ -EC, or glutamate, respectively, allowed the identification of amino acids involved in substrate binding and of cysteine residues forming disulfide bridges in the oxidized protein, which might play a role in the redox regulation of the protein. (For description of the crystal structure see Discussion, paragraph 4.1).

The oxidized BjGCL protein shows two intramolecular disulfide bridges (Figure 3.2) and is arranged as a homodimer in the crystal structure. One of the disulfide bridges (CC1, Cys341-Cys356) is located in close proximity to the active site, possibly being involved in the positioning of a β -hairpin structure, while the other one (CC2, Cys178-Cys398) is located in the core of the protein, near the interface structure where two BjGCL monomers contact each other. As previous analysis showed that plant GCL is inhibited by reduction (Hell and Bergmann, 1990; Jez et al., 2004; Wachter, 2004) the possible roles these disulfide bridges might play in the redox regulation of BjGCL were investigated. For this purpose site-directed mutagenesis was combined with biochemical and structural analysis. This approach was chosen to enable separate examination of the two disulfide bridges, simulating the specific reduction of either one by removing one or both of the cysteine residues required for their formation.

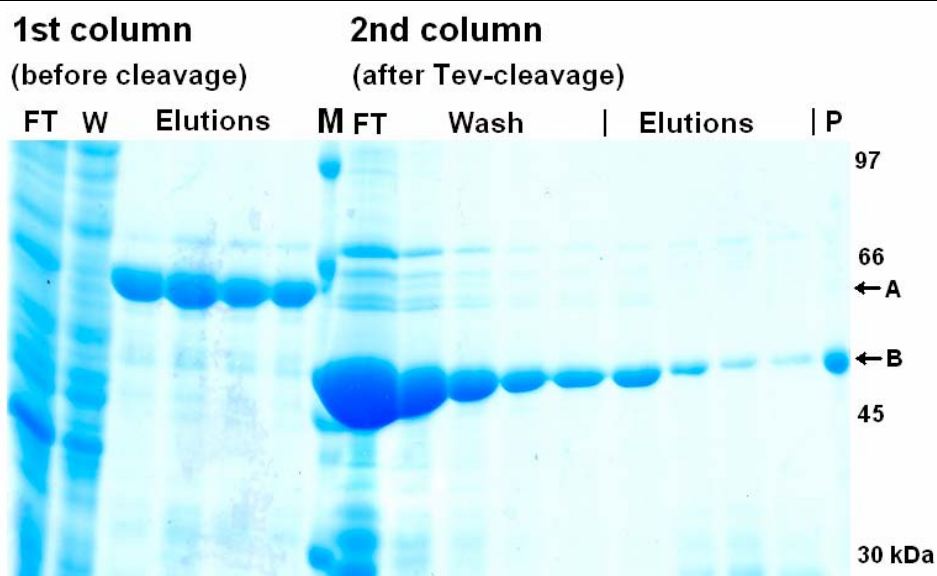


Figure 3.1: Purification of recombinant BjGCL protein from *E.coli* by nickel-affinity chromatography. BjGCL was overexpressed without the transit peptide (residues 66-514) as thioredoxin-6xHis tag fusion protein and purified natively via affinity chromatography. Protein was bound to a Ni^{2+} -NTA column and eluted after several washing steps with imidazole (column 1). The fusion protein was cleaved by TEV protease and the thioredoxin:6xHis tag was separated from BjGCL by binding to a second Ni^{2+} -NTA column. BjGCL:thioredoxin fusion protein is detected at a size of 65 kDa (A), pure BjGCL at 51 kDa (B) and TEV protease at 27 kDa. FT- flowthrough, W – wash, M – low molecular weight marker (Amersham), P – purified recombinant protein (FT and W of second column).

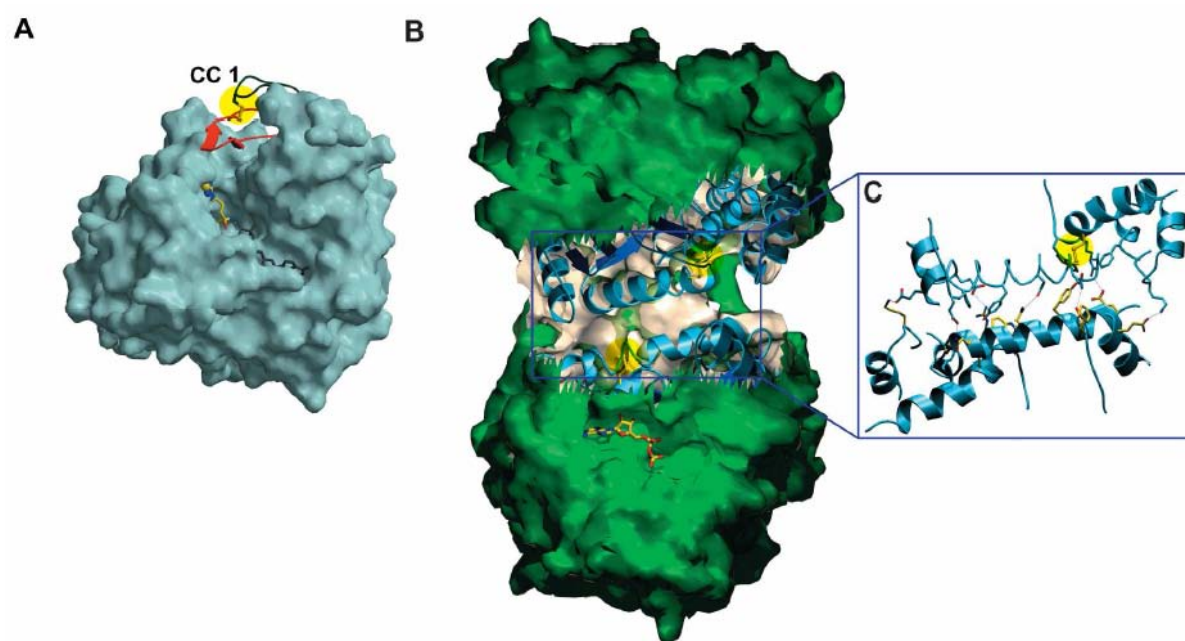


Figure 3.2: The crystal structure of BjGCL. **A** – Surface view of an oxidized BjGCL monomer showing the relative positions of a β -hairpin motif (in red) and the disulfide bond CC1 (Cys341-Cys356, in yellow). BSO bound in the active site is shown in bonds representation (in yellow) along with ADP (in black). **B** – Surface view of the dimer found in crystals grown under non-reducing conditions. At the level of the dimer interface, the surface has been removed to show the contributing helical elements (in blue). The disulfide bond CC2 (Cys178-Cys398) in both molecules is highlighted in yellow, a molecule of ADP is shown in the nucleotide binding site. **C** - Zoomed up view of the dimer interface in the same orientation as seen in B. (from Hothorn et al., 2006)

3.1.2 Knockout of the hairpin disulfide bridge (CC1) affects enzyme activity but not the K_m values of the substrates

BjGCL variants were produced by site-directed-mutagenesis and expressed in *E. coli* to evaluate whether the hairpin disulfide bridge (CC1; Cys341-Cys356) is involved in the redox regulation of BjGCL.

In the mutant proteins, Cys341 was exchanged for serine (C341S), Cys356 for alanine (C356A) or both exchanges were combined (C341S/C356A). All three variants were expressed with efficiencies comparable to the wild-type protein in *E. coli* and were soluble after purification. C341A showed the same elution profile as wild-type BjGCL (BjGCL-wt) in analytical size-exclusion chromatography, corresponding to a dimer in the oxidized state, and a similar distribution of secondary structure content in thermal unfolding experiments by circular dichroism spectroscopy (conducted by M. Hothorn, EMBL, Figure 3.3).

All three mutants showed glutamate cysteine ligase activity, detectable in a coupled enzymatic assay but for all three mutants the specific activity under saturating substrate concentrations was reduced to less than 20 % of that found for the wild-type protein (19 ± 7 % for C341S; 12.6 ± 2.6 % for C356A and 9.4 ± 0.9 % for C341S/C356A).

Michaelis-Menten kinetics of the mutant protein C356A were determined to discern whether this reduction in activity resulted from a reduced reaction velocity or a changed affinity to the substrates. For none of the three substrates cysteine, glutamate and ATP a pronounced change in the K_m value could be detected, showing that in CC1 mutants only the velocity of enzymatic catalysis but not the affinity for the substrates is affected (Table 3.1).

Table 3.1: Comparison of the Michaelis Menten kinetics of BjGCL wild-type and C356A mutant.

kinetic constant	wild-type	C356A
v_{max} (nmol min ⁻¹ mg ⁻¹)	$3\ 336 \pm 114$	407 ± 87^a
K_m (cysteine) (mM)	0.12 ± 0.01	0.18 ± 0.03^a
K_m (glutamate) (mM)	8.5 ± 0.4	8.3 ± 0.5
K_m (ATP) (mM)	1.3 ± 0.2	1.1 ± 0.5

wild-type kinetics according to (Wachter, 2004), kinetics for C356A were determined as described in Material and Methods. v_{max} and K_m are described as means \pm S.D. ($n = 5$).

^a significant difference to the wild-type (Student's t-test, $P < 0.0001$)

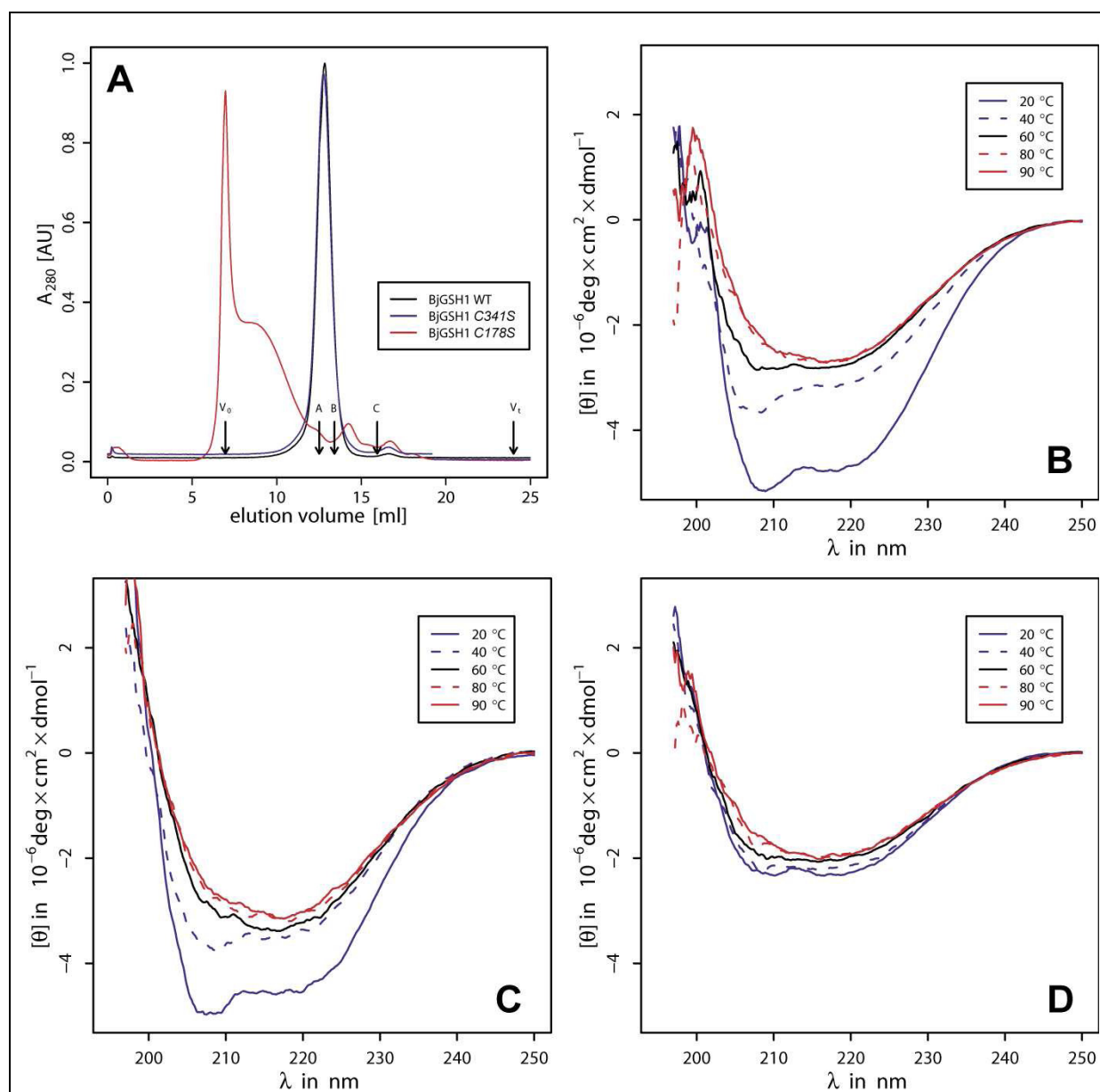


Figure 3.3: Structural analysis of wild-type and mutant BjGCL by analytical size-exclusion chromatography and circular dichroism spectroscopy.

A: 280 nm absorbance trace of analytical size-exclusion chromatography (SD 200 HR10/30; see Methods). Wild-type BjGCL elutes at an apparent size consistent with a dimer (black line) as does the C341S mutant (blue line). The CC2 mutant (C178S) elutes in the void volume (red line). Void (V_0) and total volume (V_t) are shown together with the elution volumes of molecular weight standards (A, aldolase, MW 158 000; B bovine serum albumine, MW 67 000; C, chymotrypsinogen A, MW 25 000).

B, C, D: Far UV circular dichroism melting spectra recorded at 20 (blue line) to 90 °C (red line) of wild-type BjGCL (B), the C341S (C) and the C178S mutant proteins (D) (conducted by M. Hothorn, EMBL)

3.1.3 The Core Disulfide Bridge CC2 of BjGCL mediates redox dependent dimer formation

Overexpression of mutagenised BjGCL protein with knocked-out CC2 disulfide bridge by exchange of Cys178 for serine (C178S), led to protein that appeared to be heavily aggregated in size-exclusion chromatography and was already largely unfolded at room temperature as shown by circular dichroism spectroscopy (Figure 3.3). The residual activity of the soluble fraction of recombinant C178S was approximately 2 % of the wild-type activity, probably due to large amounts of unfolded protein, and did not allow further enzymatic characterization.

To analyze the role of CC2 in the properly folded BjGCL protein, the CC1 mutant proteins C341S and C356A were subjected to treatment with reductants. As these mutant proteins were folded properly and were enzymatically active, they allowed to specifically address the role of CC2 in redox regulation of BjGCL.

Wild-type and C356A proteins were incubated before and during enzymatic assays with different concentrations of the reductants β -mercaptoethanol (β -ME), dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and the resulting change in activity under saturating substrate conditions was determined (Table 3.2 and Figure 3.4).

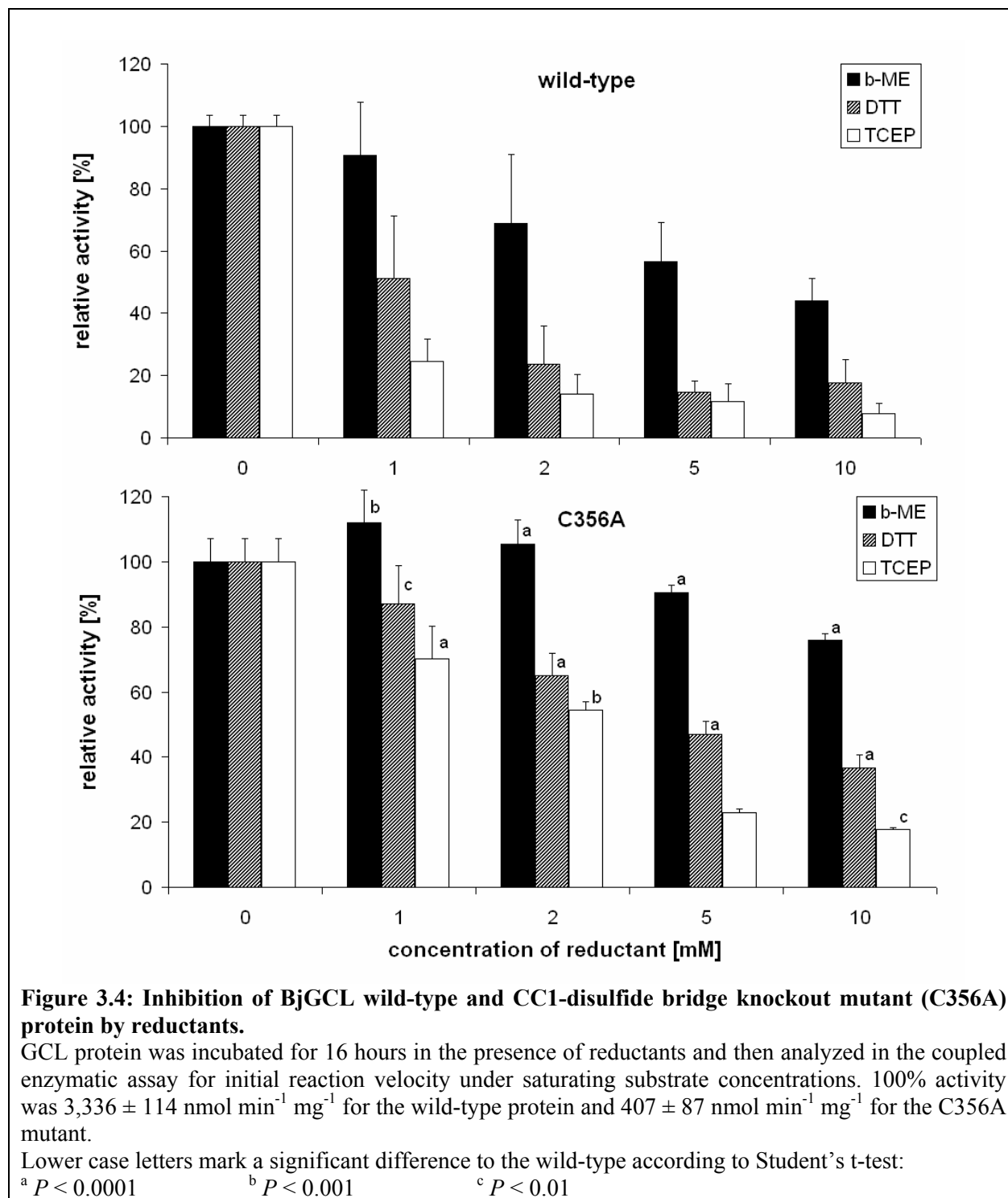
Table 3.2: Inhibition of BjGCL wild-type (wt) and CC1-disulfide bridge knockout mutant (C356A) protein by reductants

Concentration in mM	β -ME		DTT		TCEP	
	wt	C356A	wt	C356A	wt	C356A
	<i>% activity compared to untreated protein</i>					
0	100 \pm 3.4	100 \pm 7.1	100 \pm 3.4	100 \pm 7.1	100 \pm 3.4	100 \pm 7.1
1	90.8 \pm 7.1	112 \pm 9.1 ^b	51 \pm 20	87 \pm 11.8 ^c	25 \pm 17	70 \pm 10.0 ^a
2	68.8 \pm 6.3	105 \pm 7.6 ^a	23 \pm 12	65 \pm 6.7 ^a	14 \pm 22	55 \pm 2.6 ^b
5	56.5 \pm 5.5	90 \pm 2.5 ^a	14.7 \pm 3.6	47 \pm 4.0 ^a	11 \pm 12	23 \pm 1.6
10	44.1 \pm 3.4	76 \pm 1.6 ^a	17.6 \pm 7.6	31 \pm 4.0 ^a	7.7 \pm 6.9	17.7 \pm 0.6 ^c

GCL protein was incubated for 16 hours in the presence of reductants and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations. 100% activity was 3,336 \pm 114 nmol min⁻¹ mg⁻¹ for the wild-type protein and 407 \pm 87 nmol min⁻¹ mg⁻¹ for the C356A mutant.

Lower case letters mark a significant difference to the wild-type according to Student's t-test:

^a $P < 0.0001$ ^b $P < 0.001$ ^c $P < 0.01$



Both proteins showed a concentration dependent decrease in activity when treated with reductants, where the inhibitory effect correlated with the compound's reduction potential. The relative inhibition of activity was significantly stronger in all cases for the wild-type enzyme than for the C356A mutant, indicating that the inhibition by reductants in wild-type BjGCL probably is mediated by both disulfide bridges.

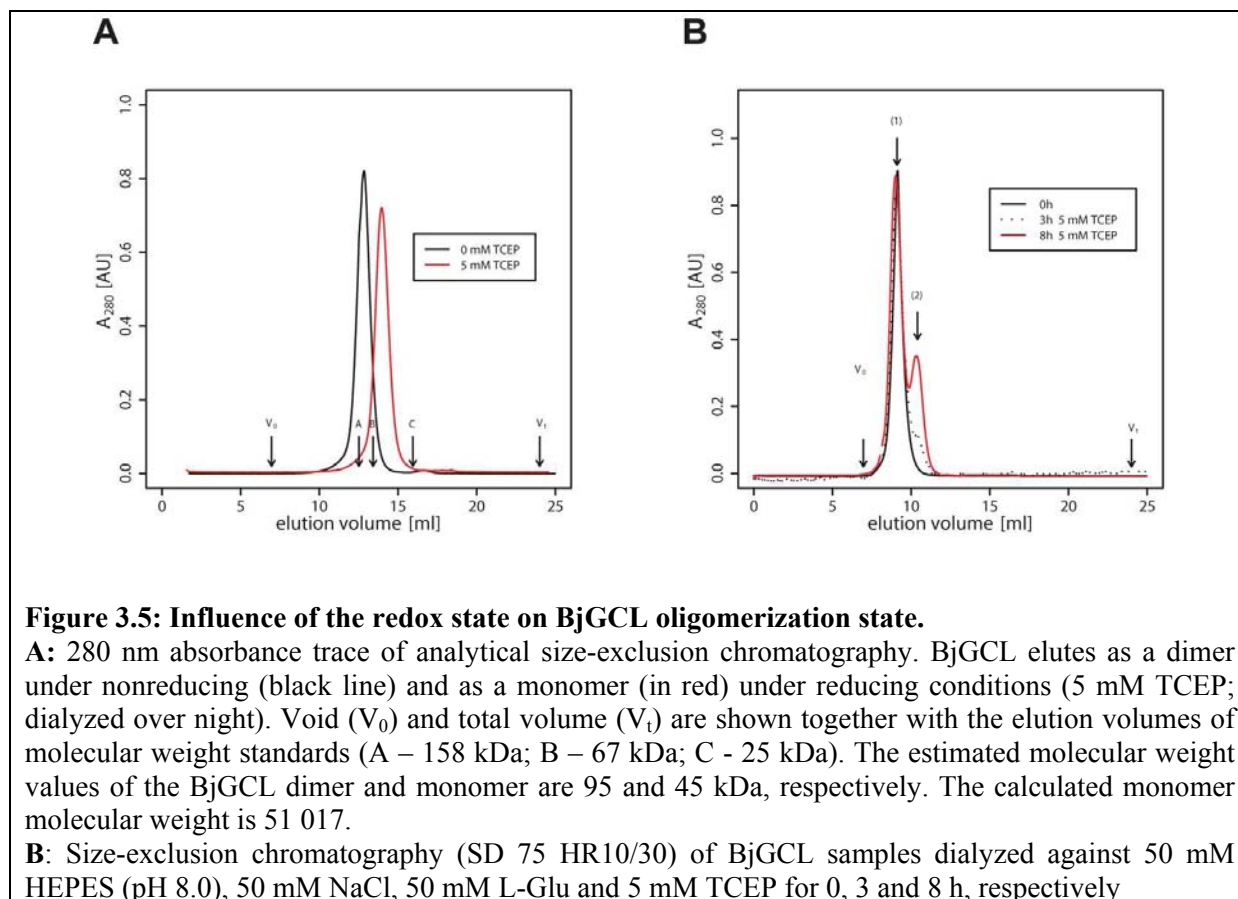
GCL protein from tobacco and *Arabidopsis* was reported to elute differently in size-exclusion chromatography experiments when analyzed in the oxidized or reduced state, where

reduced protein eluted at approximately half the apparent size found for the oxidized protein (Hell and Bergmann, 1990; Jez et al., 2004). This effect was interpreted as a redox-dependent change in protein conformation, possibly resulting in a more compact fold in the reduced state. For BjGCL however, the crystal structure showed that the oxidized protein molecule has a very compact globular structure (Figure 3.2). Notably BjGCL molecules were found to be arranged as dimers with a well-defined interface structure in the crystals, located in close proximity to CC2 (See Figure 3.14 and Discussion paragraph 4.1). This finding proposes the possibility of a dimer to monomer transition to be responsible for the change of apparent size upon reduction. To analyze the effect of reduction on the oligomerization of BjGCL, wild-type and CC1 mutant proteins (C341S and C356A) were reduced by dialysis against a buffer containing 5 mM DTT or TCEP and subjected to analytical size exclusion chromatography.

For all three variants a peak shift upon reduction of the protein could be observed, with the retention times on the column being consistent with oxidized dimeric and reduced monomeric BjGCL protein, respectively (Figure 3.5 A). The change in the elution profile of BjGCL in response to reducing conditions was fully reversible, with the specific activity of the re-oxidized dimeric protein approximately 10-fold higher than for the reduced monomeric protein (1908 ± 38 and 207 ± 64 nmol min⁻¹ mg⁻¹, respectively for wild-type enzyme).

While treatment of BjGCL with DTT led to complete monomerization within one hour, dialysis of BjGCL-wt in a buffer containing 5 mM TCEP resulted in much slower reduction where more and more dimeric GCL dissociated into the reduced monomeric form as a function of time (Figure 3.5 B). The specific activities of dimer and monomer fractions of wt-BjGCL (dimer: $3,336 \pm 114$ nmol min⁻¹ mg⁻¹, dimer + 5 mM TCEP 480 ± 30 nmol min⁻¹ mg⁻¹, monomer + 5 mM TCEP 207 ± 64 nmol min⁻¹ mg⁻¹) suggest that the surface exposed CC1 disulfide was quickly reduced while dimer dissociation occurred on a rather large timescale in the range of several hours.

The reduction of sulfhydryl groups by TCEP requires more space around the target disulfide than reduction by DTT, while TCEP otherwise is the stronger reductant (Cline et al., 2004). While CC2 is located at the surface of the monomeric BjGCL protein, its position near the dimer interface would probably lead to a partial shielding of the disulfide bridge from access by soluble reductants upon the formation of the GCL dimer.



To further analyze the role of the dimer formation for the regulation of BjGCL, the Tyr186 in the center of the dimer interface was mutated to glutamate (Y186E) and the mutant protein expressed in *E. coli*. As found for the CC2 mutant protein, most of the Y186E protein (>95 %) appeared in size-exclusion chromatography to be aggregated. However, a minor population appeared to be correctly folded, where approximately equal amounts of protein eluted at the apparent sizes expected for monomeric and dimeric protein, respectively (Figure 3.6). Despite apparent mis- or unfolding of most of the protein, BjGCL-Y186E did show GCL activity and was partially inhibited by reductants as would be expected, due to reduction of CC1 and dissociation of the remaining dimers following reduction of CC2 (Figure 3.7).

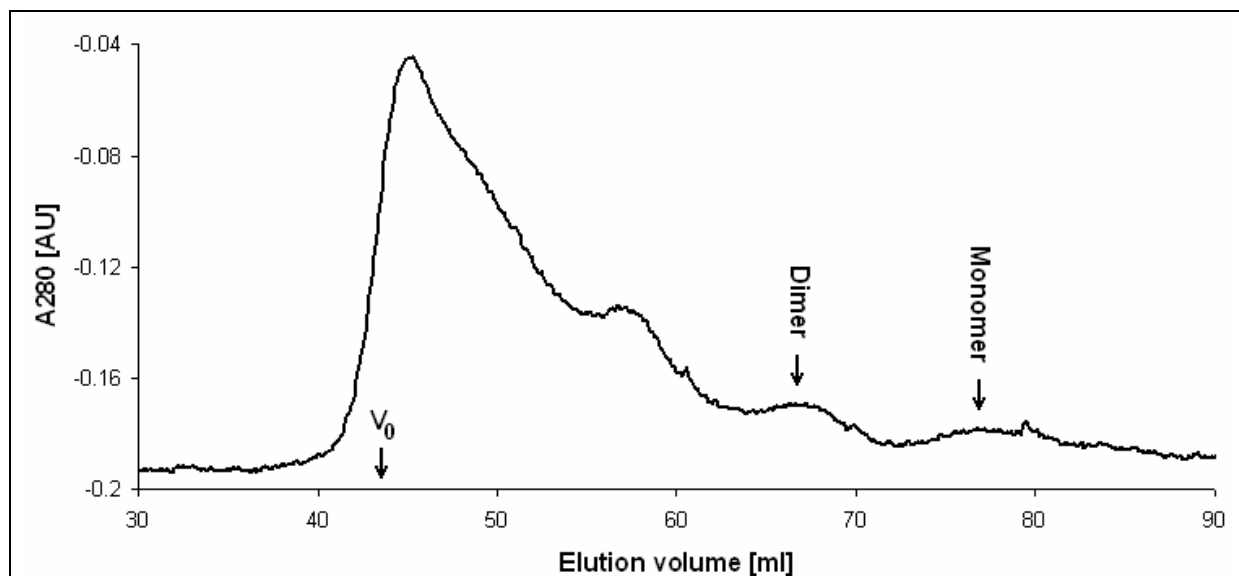


Figure 3.6: Size-exclusion chromatography of BjGCL-Y186E. A_{280nm} profile of size exclusion chromatography on a Superdex 200 column. Peaks of dimeric and monomeric protein are marked by arrows and correspond to 130 and 60 kDa respectively; the molecular weight of monomer BjGCL is predicted to be 51 kDa. V_0 is the column void volume. Estimated from the peak area, about 96 % of the protein was aggregated.

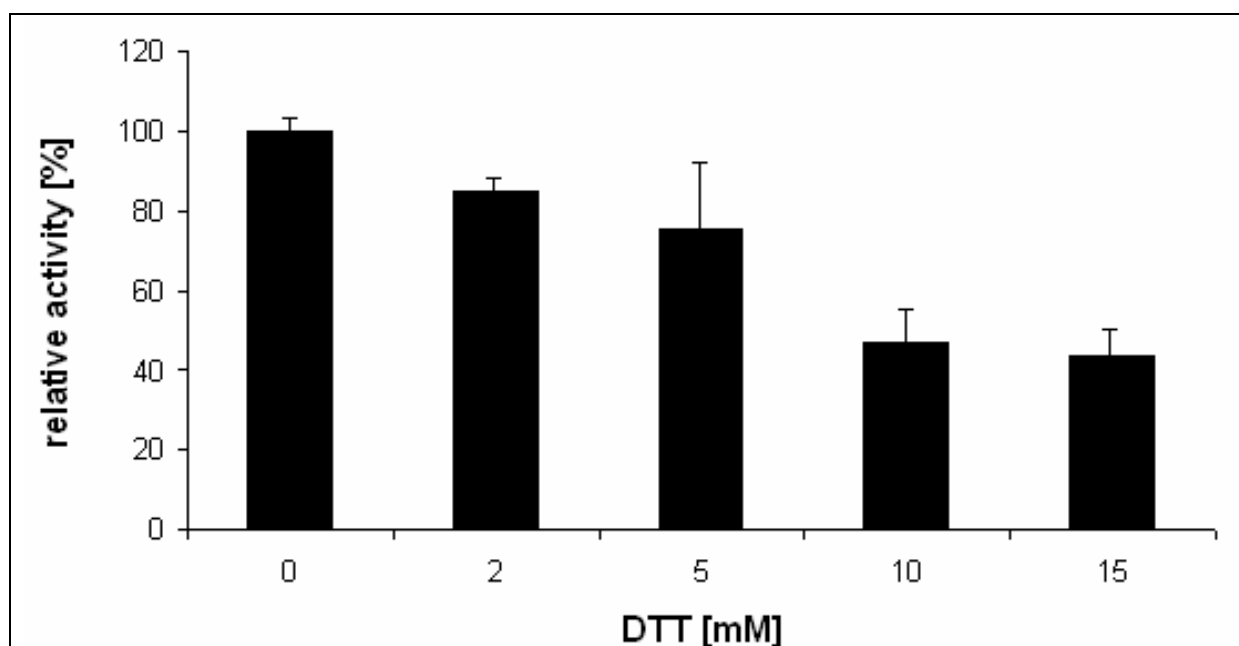


Figure 3.7: Inhibition of BjGCL-Y186E by DTT. GCL protein was incubated for 1 hours in the presence of DTT and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations. 100% activity was $217 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1}$.

The aggregation of both mutant proteins affected in the dimer interface of BjGCL, C178S and Y186E, might point at a role of dimerization in the stabilization of the fold of BjGCL. To test whether BjGCL in the monomeric and dimeric conformation differ in their overall stability, recombinant wild-type protein was incubated for 2 hours with or without 5

Results

mM DTT and then digested by adding proteinase K (GCL/proteinase-ratio: 1/100 w/w) or a crude plastidic protein extract (see Material and Methods, paragraph 5.3.9). After incubation at 25°C the reaction was stopped by denaturing aliquots in protein loading buffer and digestion patterns were analyzed by immunoblotting (Figure 3.8). Proteinase K degradation rapidly led to the appearance of a 48 kDa fragment (“2” in Figure 3.8) while further degradation resulted in a reduction of overall protein amount without further detectable degradation products. At prolonged incubation times, degradation was stronger under reducing conditions, either due to activation of the protease or due to reduced stability of GCL. Degradation by the plastidic extract resulted in the successive appearance of fragments of 45 and 41 kDa (“3a” and “3b” in Figure 3.8). In both experiments no clear differences in the digestion patterns between oxidizing and reducing conditions could be observed, arguing against a prominent conformational change upon reduction of the protein.

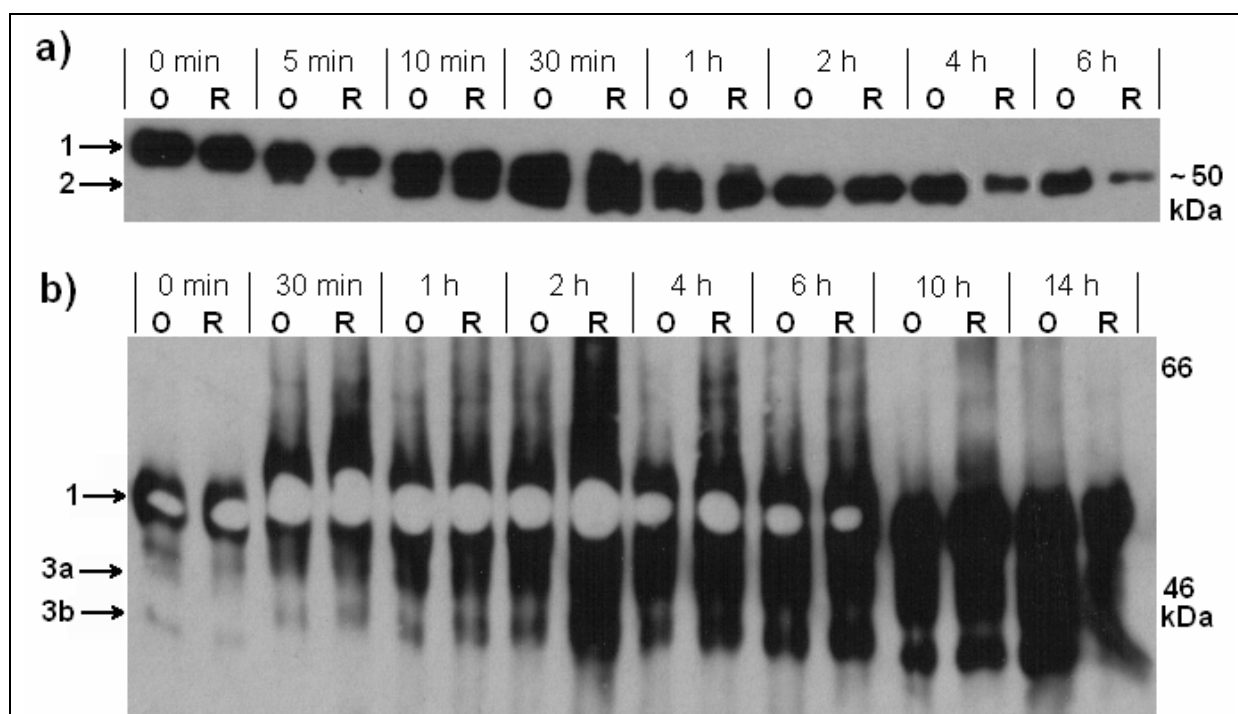


Figure 3.8: Dependence of protease digestion susceptibility of BjGCL on the redox state.

BjGCL wild-type protein with proteinase K (a) or a crude plastidic protease preparation (b) in the oxidized state (O) or in the presence of 5 mM DTT (R). Aliquots were taken after different time points and the reaction was stopped by addition of SDS-PAGE loading buffer and heating to 95°C. GCL protein was separated on 11 % PAA gels and analyzed by immunoblotting. Arrows indicate the positions of undigested protein (1) and prominent digestion fragments (2, 3a, 3b). 0 min indicates recombinant protein preincubated for 2 hours before addition of protease.

3.2 The BjGCL mutant analogous to *rml1* shows normal oligomerization behaviour but is enzymatically inactive

The missense mutant *rml1* (*root meristemless1*) of *Arabidopsis thaliana* GCL has been found to lack detectable GCL activity *in vivo* (Vernoux et al., 2000). In the *rml1* protein Asp259 (Asp250 in BjGCL) is substituted for asparagine, an exchange that, predicted from the structure might influence the binding of the ATP nucleotide.

To analyze the properties of the *rml1* mutant *in vitro*, mutagenized BjGCL, exchanging Asp250 to asparagine, was purified as recombinant protein. The BjGCL-*rml1* protein was apparently folded correctly and eluted in size exclusion chromatography with an apparent size of 86 kDa, hinting at a dimeric state as for the wild-type BjGCL protein (Predicted sizes for dimeric and monomeric BjGCL are 102 and 51 kDa, respectively).

However, in the coupled enzymatic assay BjGCL-*rml1* did show extremely low GCL activity under standard assay conditions or conditions with elevated ATP concentrations up to 100 mM (0.5 and 0.9 nmol min⁻¹ mg⁻¹). It therefore seems that the ability to conduct catalysis is so severely impaired in the *rml1* protein that it can be presumed to be almost completely inactive under all physiological conditions.

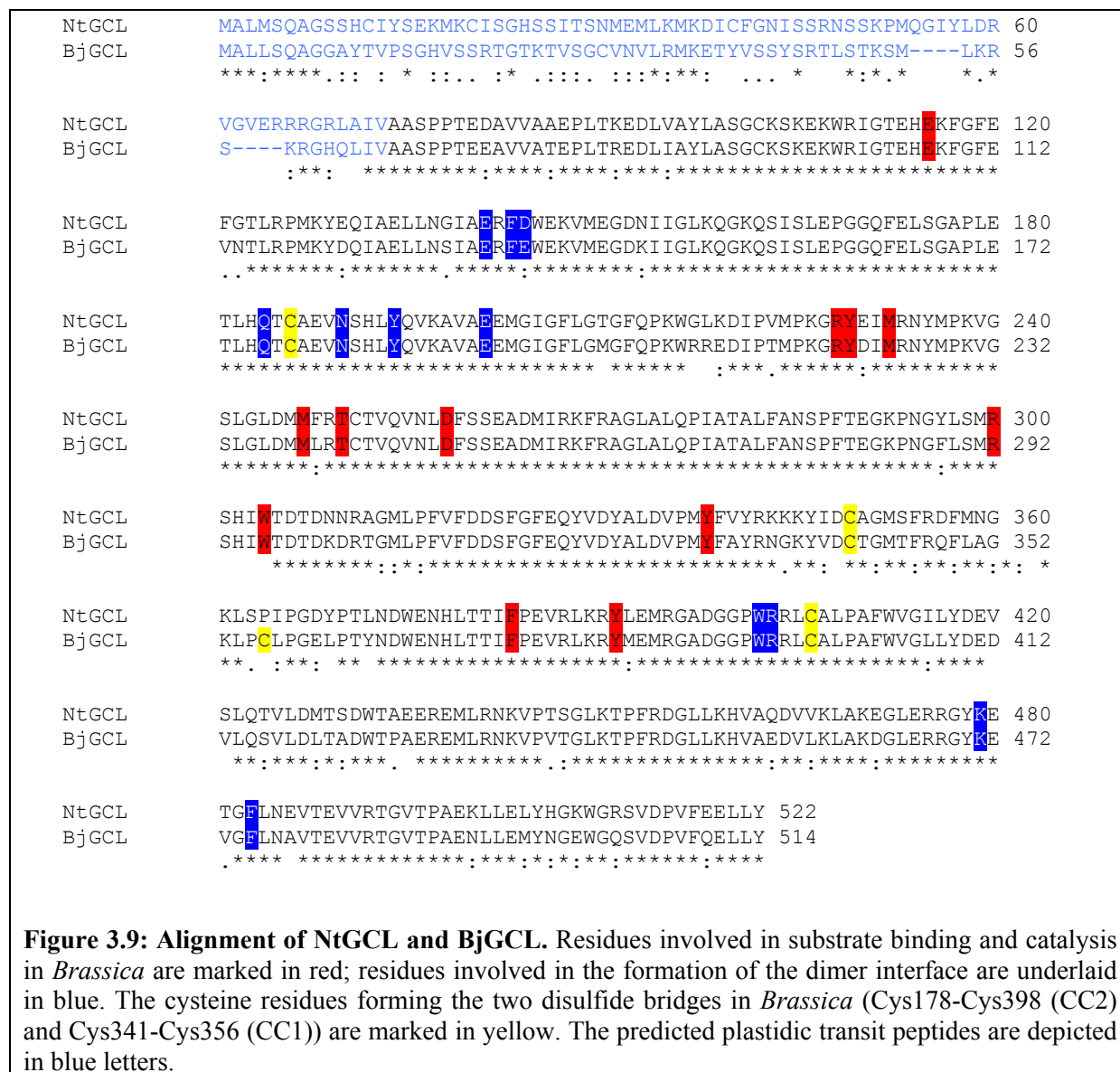
3.3 Sequencing, Cloning and Characterization of *Nicotiana tabacum* GCL

Database research revealed that the cysteine residues involved in the redox-regulation of BjGCL are not fully conserved among plant species (see paragraph 3.5). While all plant genes encode the cysteines forming the core disulfide bridge CC2, most lack the cysteine corresponding to Cys356 in BjGCL that is part of CC1. The *GCL* gene of *Nicotiana tabacum* (*NtGCL*) is found among these sequences and as GCL from tobacco has been previously analyzed as partially purified protein (Hell and Bergmann, 1990), it has been chosen to be studied as a representative of naturally occurring plant GCL proteins lacking CC2.

The full-length sequence of the *NtGCL* mRNA was acquired via rapid amplification of cDNA ends (RACE), based on an EST sequence (BP137080) from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and confirmed by cloning and sequencing of the coding sequence. In contrast to the findings for the GCL genes from *Brassica juncea* and *Arabidopsis thaliana* (Wachter et al., 2005), 5'-RACE of *NtGCL* mRNA yielded only one product, indicating that there is a uniform population of transcripts. The full-length transcript contains a coding sequence of 1566 base pairs, encoding for a protein of 521 amino acid residues, flanked by 5' and 3' UTRs of 240 and 281 base pairs, respectively (see appendix, Figures A1 and A2). The full-length cDNA sequence has been deposited in the NCBI database (accession number DQ444219).

The NtGCL protein encoded by this sequence shows a high homology to BjGCL (79 % identical and 87 % similar residues overall, 87 % identical and 93 % similar residues omitting the transit peptides) and conservation of all amino acid residues identified in BjGCL to play a role in catalysis (Figure 3.9). The residues required for formation of the dimer interface are also found in NtGCL, except that Glu136 is exchanged for an aspartate, which can be considered a conservative exchange. In addition, the cysteines involved in the formation of CC2 are conserved, whereas BjGCL-Cys356 is substituted by a proline residue, preventing the formation of CC1. The NtGCL protein sequence does encode for a putative transit peptide (residues 1 – 45), which is predicted *in silico* to enable plastidic targeting (targetP).

Results



Due to difficulties in producing sufficient amounts of stable, active recombinant NtGCL protein, a complete enzymatic characterization was not conducted. The highest specific activity measured for a NtGCL preparation was at approximately $1,400 \pm 300$ nmol $\text{min}^{-1} \text{mg}^{-1}$, roughly half of the activity found for wild-type BjGCL but three to four times that of BjGCL mutants lacking the hairpin disulfide bridge.

NtGCL activity was inhibited in a concentration dependent manner by buthionine sulfoximine (BSO), DTT and glutathione (Table 3.3, Figures 3.10, 3.13 and 3.19). Inhibition of NtGCL by the reductant DTT was found to be following a dose-response curve very similar to that found for the BjGCL-hairpin mutant C356A (Figure 3.10). As for BjGCL, incubation of NtGCL with DTT resulted in a monomerization of the dimeric protein (Figure

3.11). These results indicate that the mechanism of redox regulation, depending on the core disulfide bridge (CC2) in *Brassica* GCL, is also found in *Nicotiana* GCL.

Table 3.3: Effects of different inhibitors on the activity of *Nicotiana tabacum* GCL

Concentration in mM	BSO	DTT	GSH
	% activity compared to untreated protein		
0	100 ± 7.8	100 ± 7.8	100 ± 7.8
1	n.d.	89.1 ± 7.0	80.6 ± 9.4
2	63.6 ± 11.5	58.4 ± 7.3	63.0 ± 17.3
5	74.7 ± 4.5	35.1 ± 2.3	33.7 ± 11.2
10	59.6 ± 12.4	31.8 ± 3.7	18.0 ± 6.4
15	39.6 ± 1.0	n.d.	n.d.

GCL protein was incubated for 1 hour in the presence of inhibitors and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations. 100% activity was $1,400 \pm 300 \text{ nmol min}^{-1} \text{ mg}^{-1}$.

n.d. – activity not determined for this concentration of inhibitor

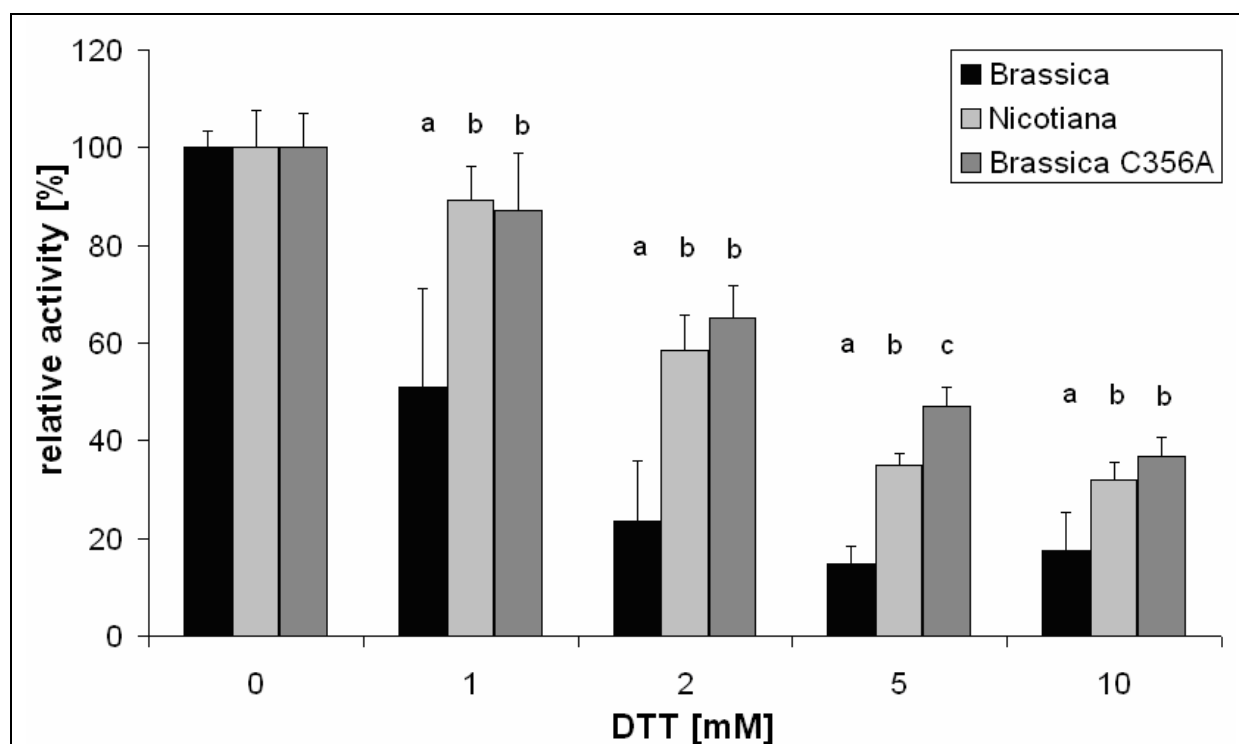


Figure 3.10: Sensitivity of *Nicotiana tabacum* GCL towards reductants compared to *Brassica juncea* GCL wild-type and CC1 mutant. GCL protein was incubated for 1 hour in the presence of DTT and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations. was $3,336 \pm 114 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for the *Brassica* wild-type protein, $1,400 \pm 300 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for the *Nicotiana* protein and $407 \pm 87 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for the C356A mutant. Different lower case letters mark significant differences, according to Student's t-test ($P < 0.001$)

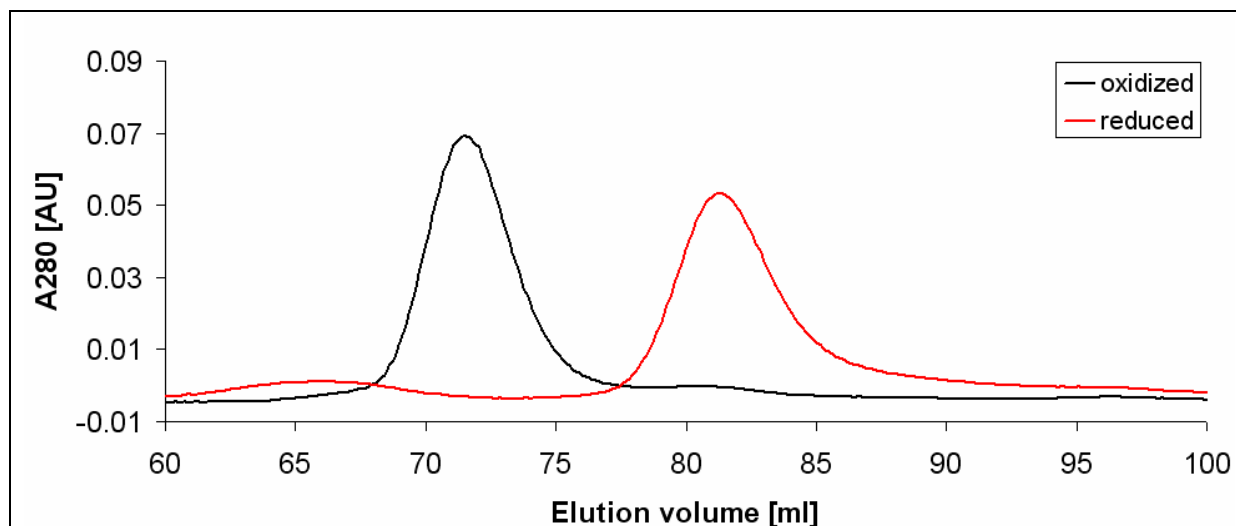


Figure 3.11: Dependence of the oligomerization state of *Nicotiana tabacum* GCL on the redox environment. A_{280nm} profile of size exclusion chromatography of NtGCL protein in the oxidized (black) and reduced (red, preincubation with 5 mM DTT) state. GCL preparations were separated by gel filtration via FPLC on a Superdex 200 column. Peaks of oxidized and reduced NtGCL correspond to 96 and 45 kDa respectively; the molecular weight of monomer NtGCL is predicted to be 51 kDa.

In contrast to the exclusive plastidic localization of BjGCL (Wachter et al., 2005), cellular fractionation experiments revealed plastidic and cytosolic GCL activities in tobacco (Hell and Bergmann, 1990). To elucidate whether the putative plastidic transit-peptide of NtGCL actually confers plastidic localization, the full-length cDNA was cloned into the vector pK7FWG2, enabling the expression *in planta* with GFP fused to the protein's C-terminus. Transient expression of NtGCL:GFP in *Nicotiana tabacum* via leaf infiltration of *Agrobacterium*, resulted in GFP fluorescence colocalizing with plastidic autofluorescence (Figure 3.12), while fluorescence in other compartments or the cytosol was not detectable. This experiment verifies that the N-terminal peptide of NtGCL does act as a functional plastidic transit peptide.

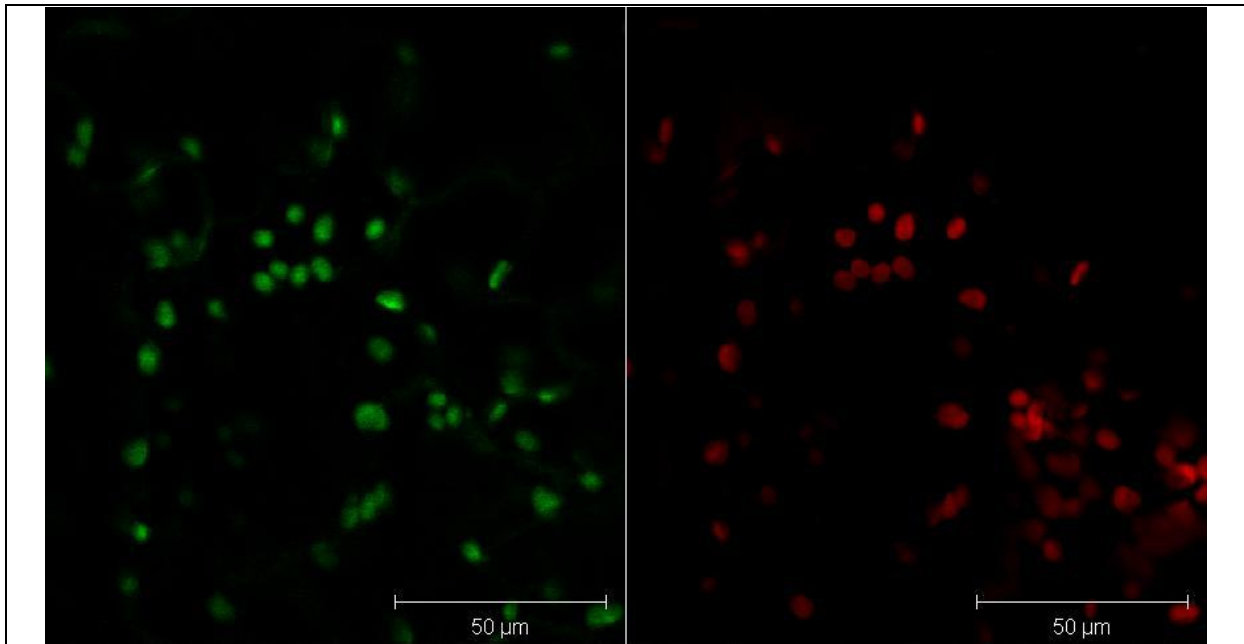


Figure 3.12: Subcellular localization of *Nicotiana tabacum* GCL. NtGCL:GFP fusion protein was transiently overexpressed in tobacco leaves via *Agrobacterium* leaf infiltration. GFP fluorescence (green, left) and plastidic autofluorescence (red, right) were detected via confocal laser scanning microscopy.

3.4 Redox and GSH feedback regulation of plant GCL are mechanistically independent

Glutathione has been reported to inhibit GCL from *Nicotiana tabacum* as a competitive inhibitor (Hell and Bergmann, 1990) and as a non-competitive inhibitor of *Arabidopsis thaliana* GCL (Jez et al., 2004). In the latter case, it has been speculated that inhibition by GSH acts via the same mechanism as inhibition by other reductants. Previous analysis of BjGCL on the other hand had shown that the enzyme was also inhibited by oxidized GSH (GSSG) and S-methyl-glutathione, both without reductive capability, to a similar extent as by reduced GSH (Wachter, 2004).

Based on the characterization of the molecular basis for redox regulation in BjGCL and on protein variants showing different dose-response curves when treated with reductants (BjGCL-C356A and NtGCL), a closer analysis of the relation of GSH and reductant inhibition of plant GCL was possible.

Brassica wild-type and mutant proteins lacking the hairpin disulfide bridge (CC1) as well as NtGCL were inhibited by the addition of glutathione to the assay medium (Table 3.4). Interestingly, the dose-response curves for inhibition by GSH were almost identical for all these proteins (Figure 3.13) while dose response curves for inhibition by β -ME, DTT and TCEP differed significantly with the *Brassica* wild-type enzyme being more susceptible to reductants than the other two proteins (Figure 3.10). This strongly indicates that CC1 is not involved in the inhibition of plant GCL by glutathione.

Table 3.4: Inhibition of BjGCL wild-type, CC1-disulfide bridge knockout mutant (C356A) and NtGCL by glutathione

[GSH]	BjGCL-wt	C356A	NtGCL
<i>mM</i>	<i>% activity compared to untreated protein</i>		
0	100.0 ± 3.4	100.0 ± 7.1	100.0 ± 7.8
1	88.5 ± 13.3	100.2 ± 21.1	80.6 ± 9.4
2	62.2 ± 10.4	87.6 ± 16.8	63.0 ± 17.3
5	56.0 ± 9.2	45.5 ± 13.3	33.7 ± 11.2
10	29.1 ± 3.9	17.9 ± 9.3	18.0 ± 6.4

GCL protein was incubated for 1 hour in the presence of glutathione and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations.

Differences between GCL proteins were in no case significant (Student's t-test, $P < 0.01$)

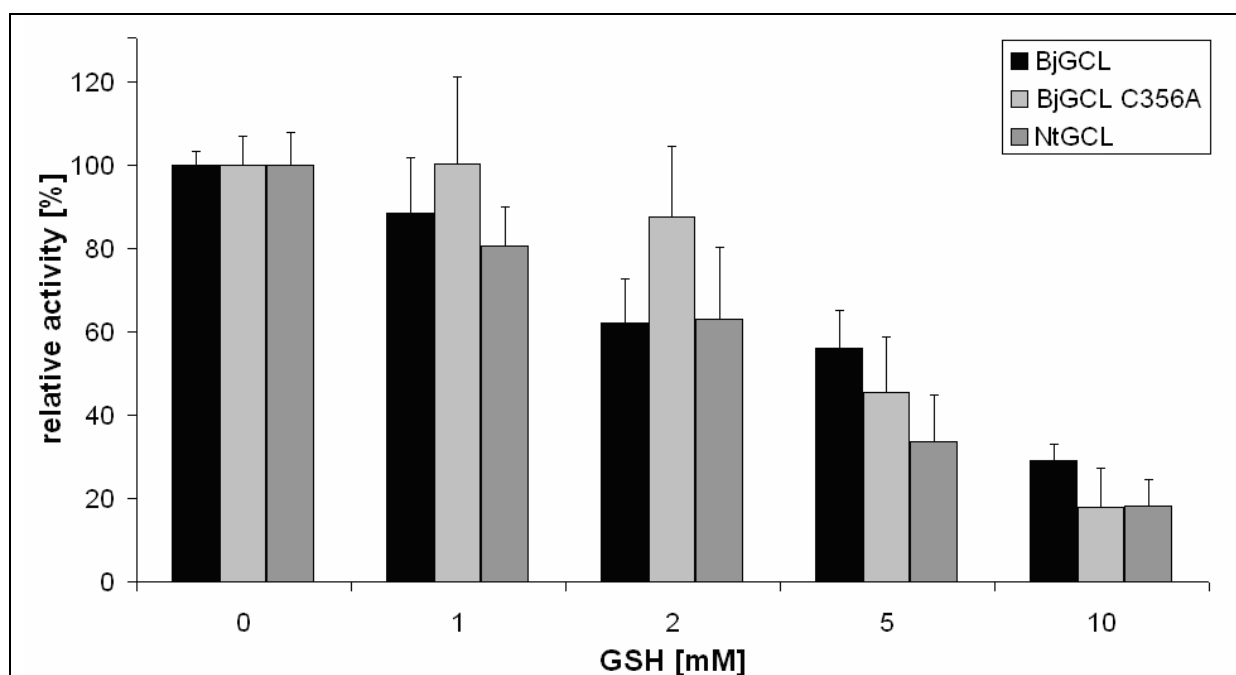


Figure 3.13: Feedback inhibition of plant GCL by glutathione. Comparison of the influence of GSH on the activity of *Brassica juncea* GCL, *Brassica juncea* GCL with knocked out hairpin disulfide bridge CC1 (C356A) and *Nicotiana tabacum* GCL. GCL protein was incubated for 1 hour in the presence of glutathione and then analyzed in the coupled enzymatic assay for initial reaction velocity under saturating substrate concentrations. Differences between proteins were in no case significant (Student's t-test, $P < 0.01$)

BjGCL wild-type protein was also incubated in buffer containing 10 mM GSH and analyzed by size exclusion chromatography to show whether the CC2-dependent dimer to monomer transition upon reduction is involved in GSH feedback inhibition. No shift from monomer to dimer could be observed after short incubation times of 1 to 2 hours and even after incubation for 20 hours only a minor part of the protein was found to be in the monomer conformation (Figure 3.14). As for the reduction by TCEP this observation could be explained by blocked access to CC2 due to the dimer formation. Significant inhibition of BjGCL by GSH on the other hand was observed immediately after addition of GSH to the assay medium.

Together these data suggest a mechanism of inhibition by GSH, not depending on reduction of either of the two disulfide bridges in BjGCL, and therefore not directly related to redox regulation of the protein.

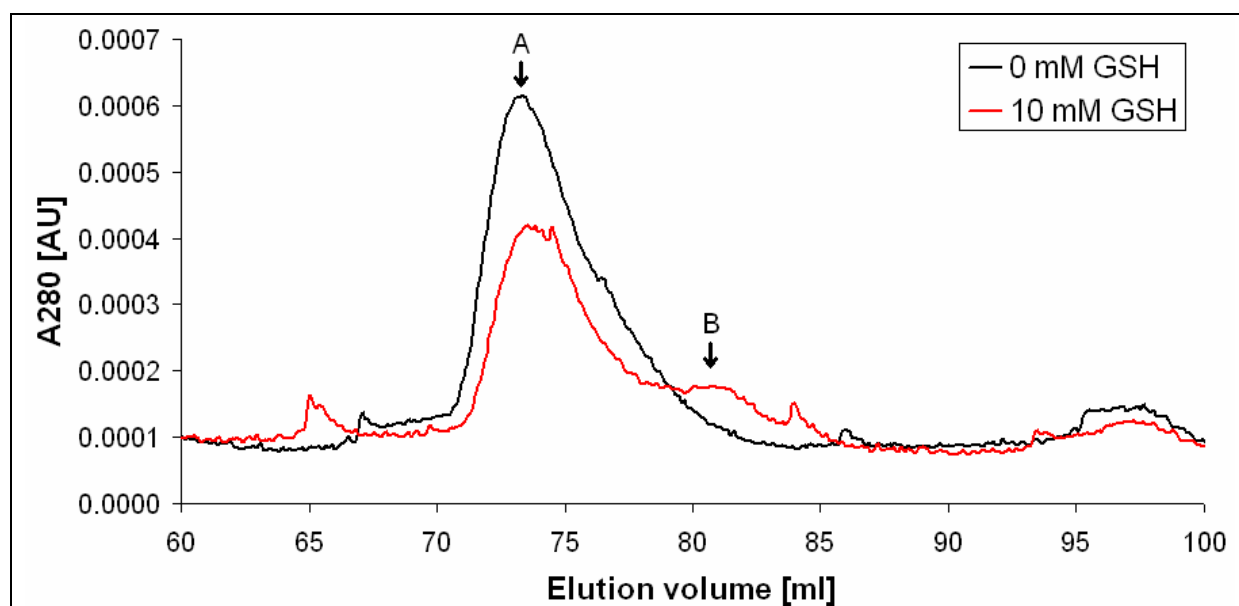


Figure 3.14: Influence of glutathione on the oligomerization state of BjGCL. A_{280nm} profile of size exclusion chromatography of BjGCL protein pretreated for 20 hours with 0 or 10 mM GSH. GCL preparations were separated by gel filtration via FPLC on a Superdex 200 column. The peak of untreated BjGCL corresponds to 83 kDa, representing dimeric protein. After treatment with 10 mM GSH a minor part of the protein population is found at an apparent size of 47 kDa (indicated by the arrow). The molecular weight of monomer BjGCL is predicted to be 50 kDa.

3.5 Conservation of sequence motifs among plant and proteobacterial GCL proteins.

To assess whether and to which degree the regulatory mechanisms found for plant GCL are conserved among all group 3 GCL proteins, extensive database research was conducted. Using the BjGCL protein sequence as input, a BLAST (Basic Local Alignment Search Tool)-search directed against the NCBI database (<http://www.ncbi.nlm.nih.gov/>) identified 63 sequences annotated as full-length group 3 GCL sequences from different taxa (13 plants, two green algae, 40 alphaproteobacteria, 6 gammaproteobacteria and 2 unclassified proteobacteria, status: June 2007). To include more plant sequences into the study, additional searches directed against the EST databases were conducted, allowing the identification of further plant GCL sequences from which 9 additional full-length GCL sequences could be assembled. 3 sequences from *Physcomitrella patens* and 4 more green algal sequences were acquired from the “DOE Joint Genome Institute”-database (www.jgi.doe.gov) and the sequence for *Vitis vinifera* was acquired from Genoscope (<http://www.genoscope.cns.fr>). Additional partial plant GCL sequences were used for the analysis of the distribution of the disulfide bridge CC1. (The accession numbers of all sequences analyzed are listed in the appendix Tables A1 and A2).

To analyze the conservation of sequence motifs in plant and proteobacterial GCL, all full-length sequences were aligned and the conservation of residues involved in catalysis or redox regulation in BjGCL was analyzed (Representative alignment of selected sequences in Figure 3.15, complete alignment in the Appendix, Figure A3, Conservation matrices for interface residues in Appendix, Tables A3 and A4).

A phylogenetic tree of group 3 GCL sequences was constructed by the neighbor joining method, including the plant and proteobacterial sequences described above, five cyanobacterial sequences and GCL from *Mycobacterium tuberculosis* (Figure 3.16). Most phylogenetically recognized groups were recovered within the tree and had high bootstrap support. However, the relationships between the groups were not resolved or only poorly supported. The GCL sequences from green algae were found in one clade with the sequences from embryophytes but this association was not supported well.

Results

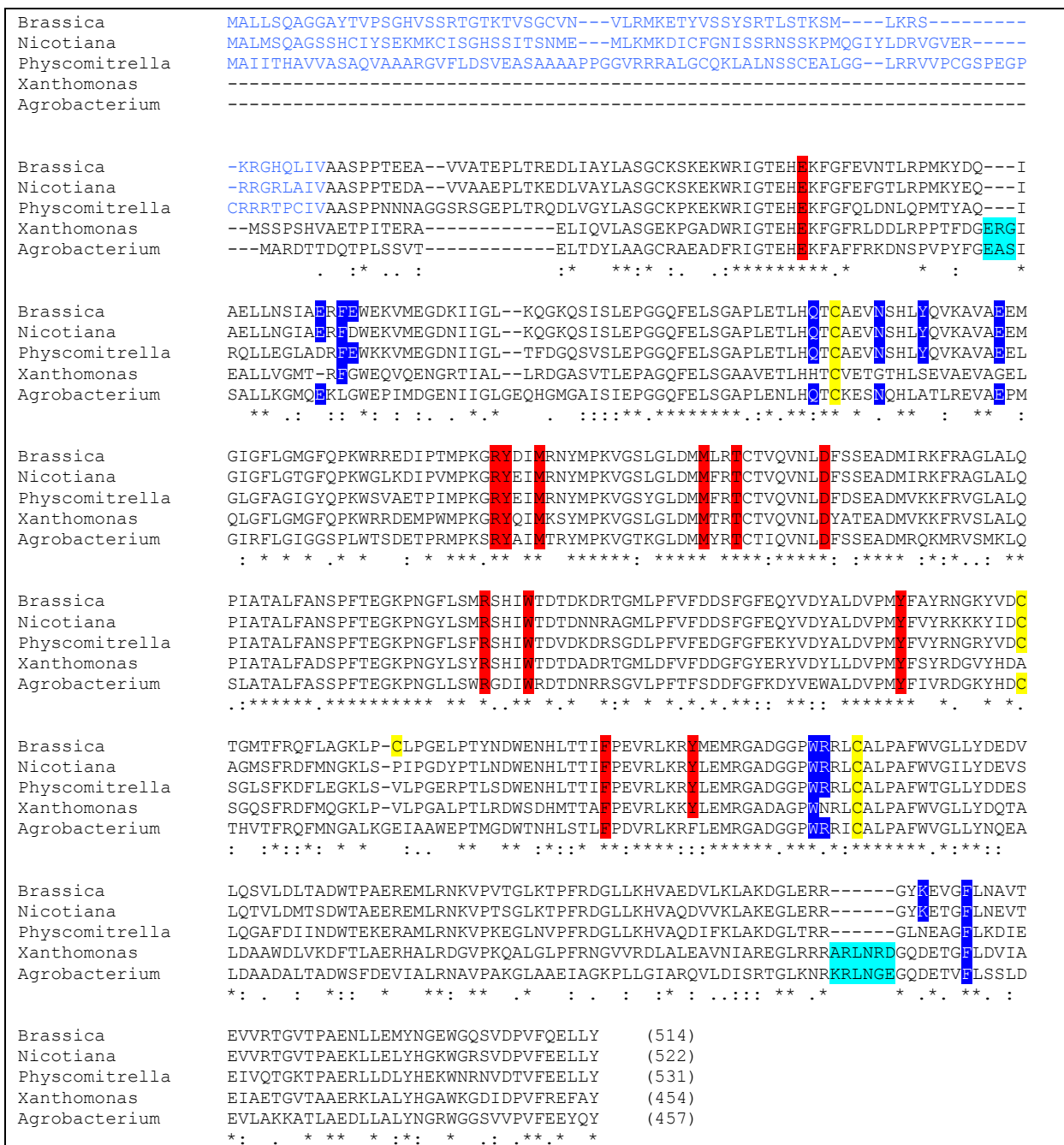
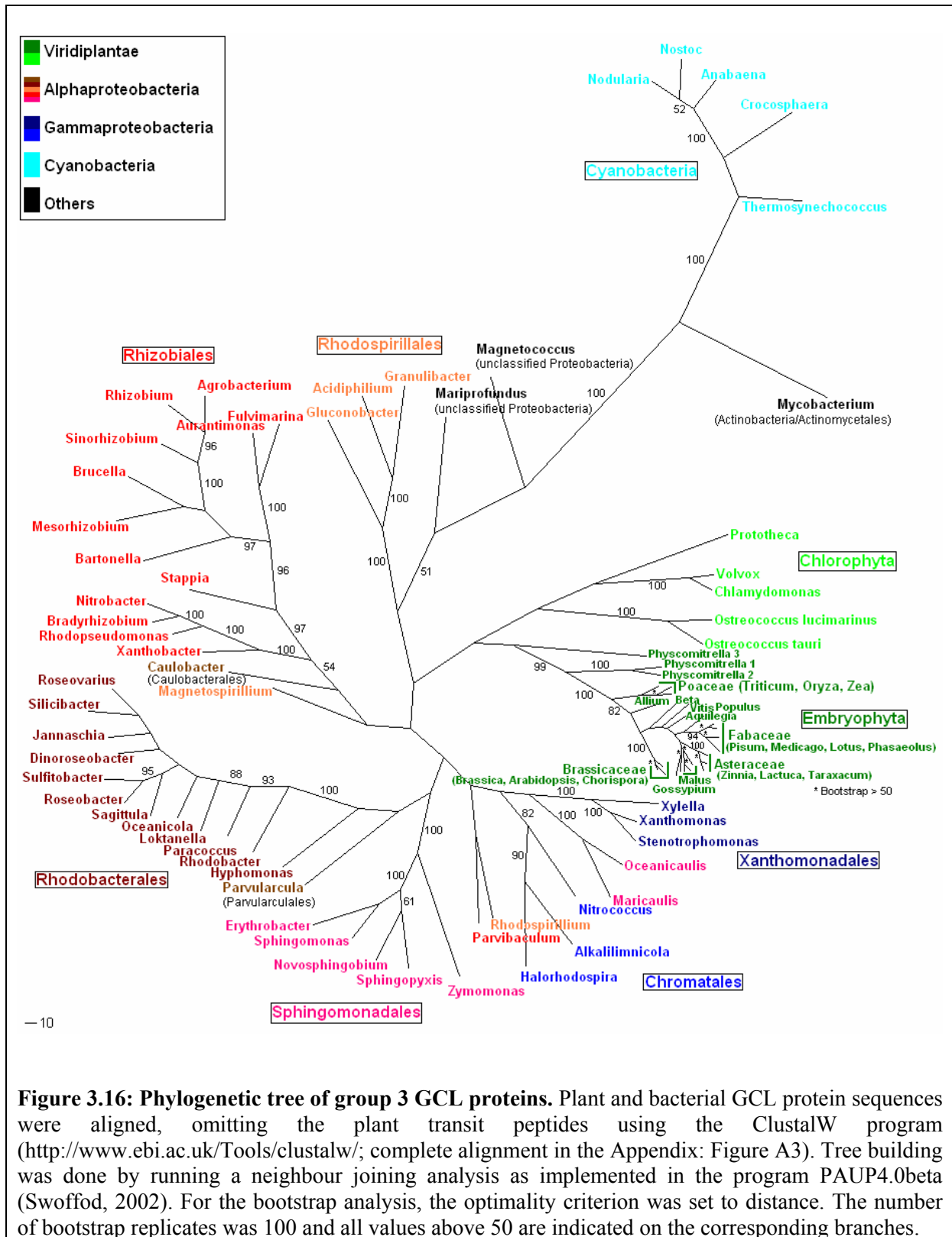


Figure 3.15: Alignment of GCL protein sequences from the vascular plants *Brassica juncea* (CAD91712) and *Nicotiana tabacum* (ABD98695), the moss *Physcomitrella patens* (assembled and translated from BJ964968, BY949421, BQ826821 and BJ976182), and the proteobacteria *Agrobacterium tumefaciens* (NP_353679) and *Xanthomonas campestris* (NP_638742). Residues involved in substrate binding and catalysis in *Brassica* are marked in red, residues involved in the formation of the dimer interface are highlighted in blue, and cysteine residues forming the two disulfide bridges in *Brassica* (CC1: Cys341-Cys356; CC2: Cys178-Cys398) are marked in yellow. Two insertions found in proteobacterial proteins are highlighted in light blue. The predicted plastid transit peptides of plant genes are depicted in blue letters. Numbers at the end of each sequence show the number of total amino acid residues.

Results



3.5.1 The catalytic residues identified in BjGCL are highly conserved among plants and proteobacteria

Twelve amino acid residues have been identified to be involved in substrate binding in the BjGCL protein (Hothorn et al., 2006). Arg220, Tyr221, Met224, Met239, Tyr330 and Phe375 are involved in cysteine binding, Glu107, Thr242, Arg292 and Trp296 bind glutamate and Asp250 and Tyr383 are presumed to be involved in binding of ATP.

All of these residues are highly conserved among all group 3 GCL sequences analyzed, with only very few minor, conservative substitutions (Table 3.5). This indicates that the catalytic mechanism of group 3 GCL proteins has been highly conserved during evolution.

Table 3.5 Conservation of catalytic residues in plant and proteobacterial GCL enzymes.

Residue ^{a)}	binds	Plants	Green Algae	Alpha- proteobacteria	Gamma- proteobacteria	Others
E107	Glu	27/27	2/3	40/40	6/6	2/2
R220	Cys	27/27	4/4	40/40	6/6	2/2
Y221	Cys	27/27	4/4	40/40	6/6	2/2
M224	Cys	25/27 (2xIle)	4/4	40/40	6/6	2/2
M239	Cys	27/27	4/4	40/40	6/6	2/2
T242	Glu	27/27	3/4 (1xSer)	37/40 (3xSer)	6/6	2/2
D250	ATP	27/27	4/4	40/40	6/6	2/2
R292	Glu	27/27	4/4	40/40	6/6	2/2
W296	Glu	27/27	4/4	40/40	6/6	2/2
Y330	Cys	26/27 (1xLeu)	4/4	39/40 (1xLeu)	6/6	2/2
F375	Cys	27/27	4/4	40/40	6/6	2/2
Y383	ATP	27/27	4/4	12/40 (28xPhe)	3/6 (3xPhe)	2/2

^{a)} numbering of residues according to *Brassica* GCL

Ratios indicate how many GCL sequences are conserved in the respective amino acid position (based on the BjGCL sequence) as compared to the total number of examined sequences. Sequences were retrieved from a BLAST search directed against the NCBI database (<http://www.ncbi.nlm.nih.gov/>), and all sequences with more than 50% amino acid identity to BjGCL were included. Additional plant sequences were assembled from EST sequences as described in Material and Methods.

3.5.2 The residues involved in redox regulation of BjGCL are conserved only among plant GCL sequences

Of the cysteine residues forming the two disulfide bridges in BjGCL three are conserved among all higher plants, while Cys356 is absent in most, indicating that the hairpin disulfide bridge CC1 cannot be formed in those proteins (Table 3.6). Mapping the occurrence of both CC1 cysteines to a phylogenetic tree of the angiosperms (AngiospermPhylogenyGroup, 1998, 2003) revealed that Cys356 is confined to sequences of species from the rosids clade, indicating a single origin for the mutation allowing the formation of CC1 instead of several independent incidents of convergent evolution (Figure 3.17).

Structural analysis of the homodimer interface of BjGCL (Hothorn et al., 2006) predicted that 11 amino acid residues are involved in establishment of the dimer contact, i.e. Glu133, Phe135, Glu136, Gln176, Asn182, Tyr186, Glu193, Trp394, Arg395, Lys471 and Phe475 (Figure 3.18; numbering according to BjGCL). In particular, several salt bridges (Glu133/Arg395, Glu193/Lys471, Glu136/Asn176) and aromatic amino acid side chains (Phe135, Tyr186, Trp394, Phe475) contribute to a zipper-like interface.

Parallel to the conservation of the cysteine residues involved in the formation of the core disulfide bridge CC2, the residues making up the dimer interface are also highly conserved among all higher plants, with only a few conservative exchanges in some sequences (Table 3.6, complete conservation matrix in Appendix Table A3). This also holds true for the most of the GCL sequences from non-angiosperms available in the databases (i.e. the moss *Physcomitrella patens* (sequences #1 and #3), the fern *Ceratopteris reinhardii* and the gymnosperm *Picea glauca*, Appendix Figure A4). However, one of the three GCL proteins encoded for in the genome of *Physcomitrella patens* is not only lacking the cysteines for CC2 but also shows very low conservation of the interface residues (Table 3.7). Interestingly, the same correlation is found among the green algal sequences. Here the GCL proteins from *Chlamydomonas reinhardii* and *Volvox carteri* show conservation of CC2 cysteines and interface residues while the proteins from *Ostreococcus* and *Prototheca wickerhamii* not only lack those cysteines but also show no conservation of the interface residues (Table 3.7, Figure A4). Considered together, these observations indicate that the biochemical basis for the redox regulation by reversible dimerization observed in BjGCL and NtGCL is highly conserved among terrestrial plants and conservation of the dimer interface residues is coupled to the occurrence of the CC2 cysteines.

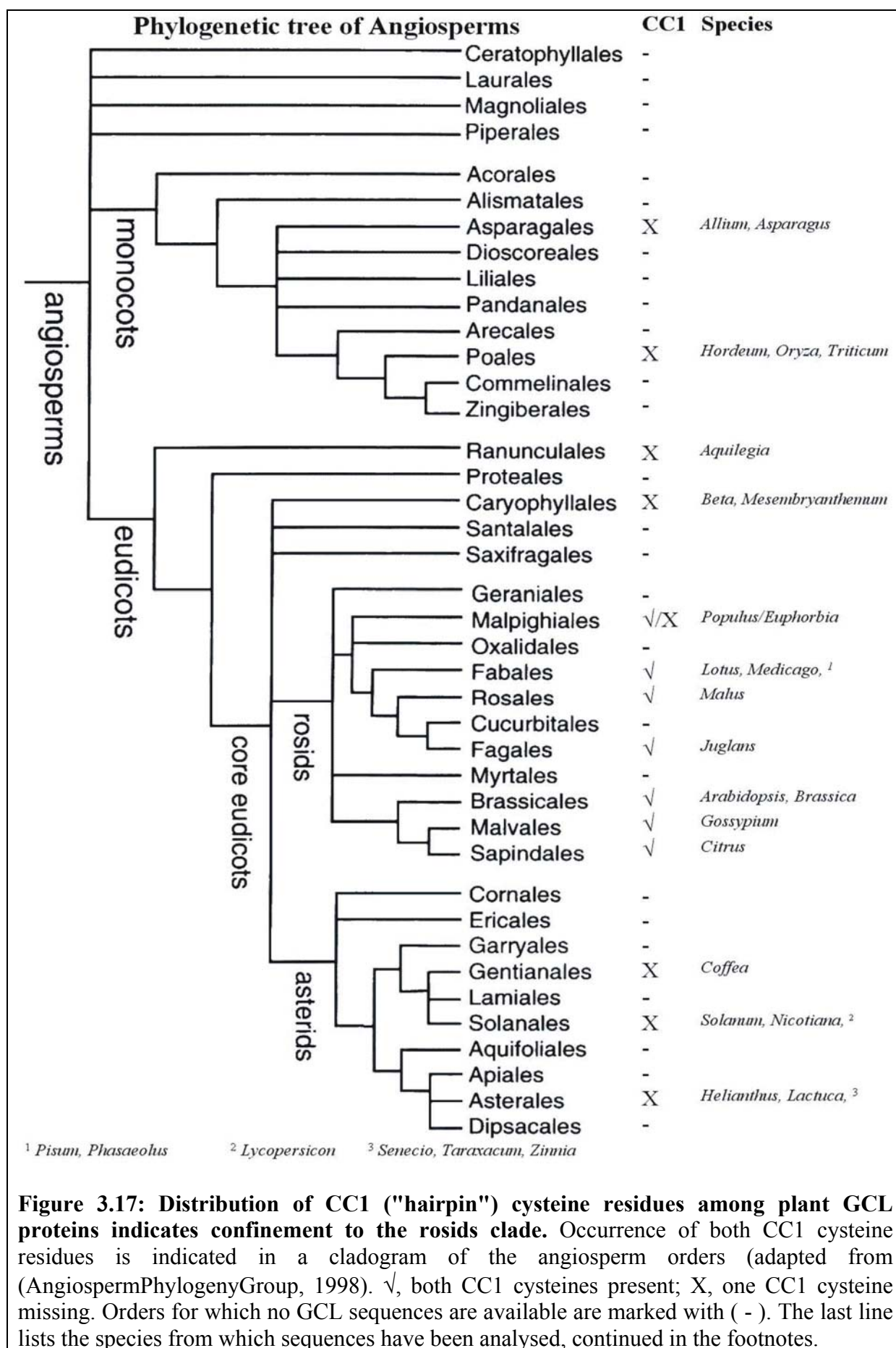


Figure 3.17: Distribution of CC1 ("hairpin") cysteine residues among plant GCL proteins indicates confinement to the rosids clade. Occurrence of both CC1 cysteine residues is indicated in a cladogram of the angiosperm orders (adapted from (AngiospermPhylogenyGroup, 1998). √, both CC1 cysteines present; X, one CC1 cysteine missing. Orders for which no GCL sequences are available are marked with (-). The last line lists the species from which sequences have been analysed, continued in the footnotes.

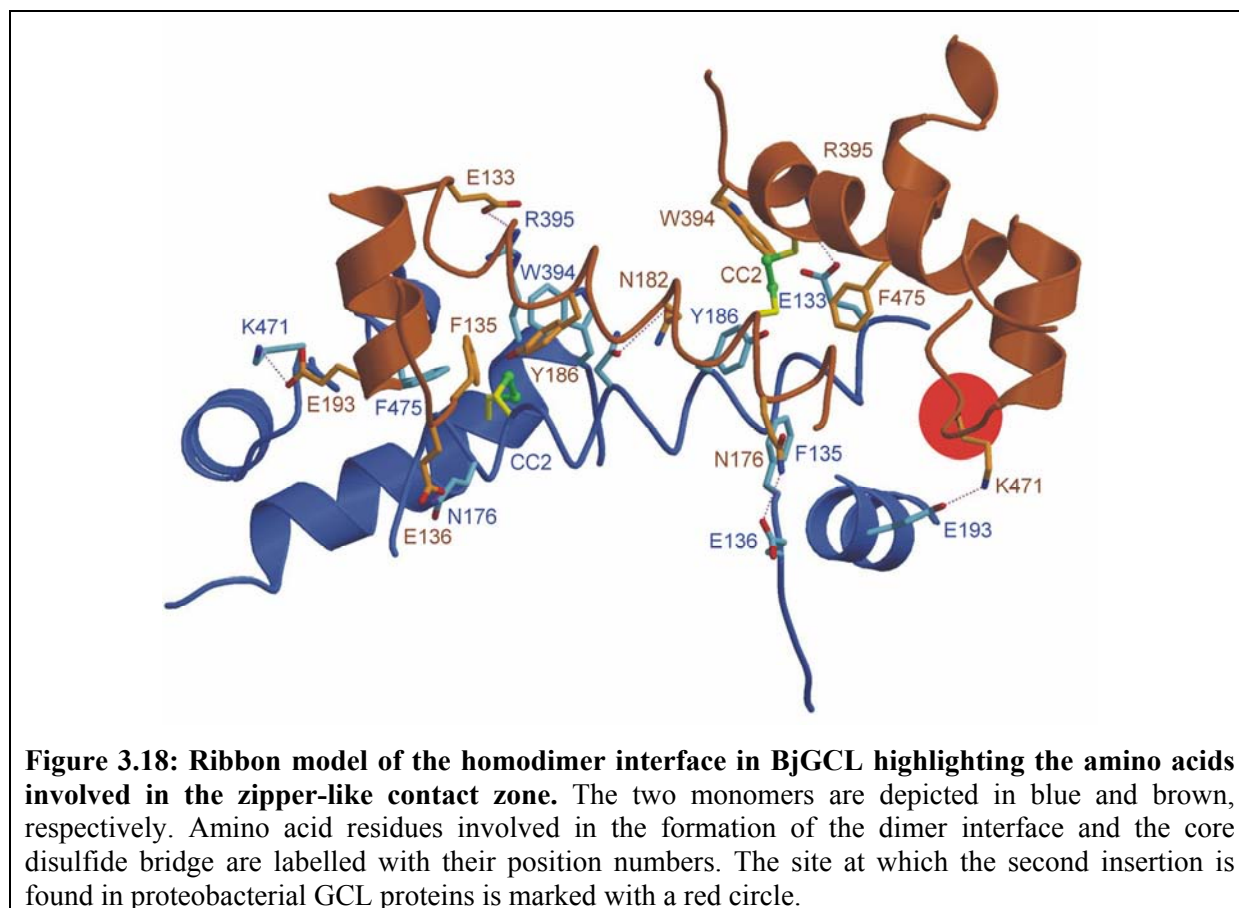


Table 3.6: Conservation of residues involved in redox regulation.

Disulfide bridge residues						
Residue ^{a)}	bridge	Plants	Green Algae	Alpha-proteobacteria	Gamma-proteobacteria	Others
C178	CC2	25/25	2/4	29/40	5/6	1/2
C341	CC1	25/25	0/4	5/40	1/6	1/2
C356	CC1	11/25	0/4	0/40	0/6	0/2
C398	CC2	25/25	2/4	32/40	6/6	2/2
Interface residues						
Residue ^{a)}	Plants	Green Algae	Alpha-proteobacteria	Gamma-proteobacteria	Others	
E133	24/27 (2xAsp)	1/3 (1xAsp)	2/40 (10xAsp)	0/6	0/2(1xAsp)	
F135	26/27 (1xTyr)	1/3	16/40	6/6	0/2	
E136	5/25 (21xAsp)	2/3	0/40 (1xAsp)	0/6	1/2	
Q176	26/27	1/4	21/40	4/6	0/2	
N182	26/27	2/4	20/40	1/6	1/2	
Y186	26/27	2/4	0/40	0/6	0/2	
E193	26/27	2/4 (1xAsp)	13/40 (14xAsp)	0/6 (1xAsp)	0/2	
W394	26/27	3/4	34/40	5/6	1/2	
R395	26/27	2/4	20/40	1/6	0/2	
K471	24/27 (2xAsn)	0/4 (2xAsp)	0/40	0/6	0/2(1xAsn)	
F475	26/27	2/4 (2xTyr)	26/40	5/6	2/2	

^{a)} numbering of residues according to *Brassica GCL*

Ratios indicate how many GCL sequences are conserved in the respective cysteine residue (based on the BjGCL sequence) as compared to total number of examined sequences. Sequence acquisition as described for Table 3.5.

Results

Table 3.7: Conservation of CC2 cysteines and dimer interface residues in plants and green algae

Clade	Species	Localization ^{a)}	CC2 cysteines	Interface residues
Angiosperms	24 different ^{b)}	Plastid	Yes	9.2; 10.1 or 11
Gymnosperms	<i>Picea glauca</i>	n.d.	Yes	10.1
Bryophyts (Mosses)	<i>Physcomitrella patens</i> #1 ^{c)}	Cytosol	Yes	8.2
	<i>Physcomitrella patens</i> #2 ^{c)}	Mitochondrion	No	2
	<i>Physcomitrella patens</i> #3 ^{c)}	Mitochondrion or Plastid	Yes	8.2
Pteridophytes (Ferns)	<i>Ceratopteris richardii</i>	n.d.	Yes	8.1; 2 n.d.
Chlorophytes (Green Algae)	<i>Chalmydomonas rheinhardii</i>	Plastid	Yes	8
	<i>Ostreococcus lucimarinus</i>	Mitochondrion	No	2.1
	<i>Ostreococcus tauri</i>	n.d.	No	1.2
	<i>Prototheca wickerhamii</i>	Plastid	No	3.1
	<i>Volvox carteri</i>	n.d.	Yes	6; 3 n.d.

Numbers indicate how of the eleven dimer interface residues are conserved in the respective GCL sequences. Digits in front of the dot indicate conservation while numbers behind the dot indicate conservative exchanges. Sequence acquisition as described for Table 3.5. Accession numbers for all sequences are listed in the Appendix, Table A1

n.d. – not determined because of truncated sequence

^a as predicted by targetP

^b see text

^c The *Physcomitrella* genome encodes for 3 *GCL* genes PhyPa1_1:70546 (#1), PhyPa1_1:146491 (#2) and PhyPa1_1:173526 (#3); DOE Joint Genome Institute (www.jgi.doe.gov)

Among proteobacterial GCL proteins, the cysteine residues corresponding to CC2 (but not CC1) are widely conserved whereas amino acid residues forming the dimer interface in BjGCL are not (Table 3.6, full conservation matrix in the Appendix, Table A4). Among all proteobacterial GCL sequences analyzed, the enzyme of *Agrobacterium tumefaciens* (AtuGCL) shows the highest similarity in the dimer interface region with 7 out of 11 positions conserved. However, in AtuGCL, as in all other proteobacterial GCL sequences, the residues involved in formation of two out of the three salt bridges found in BjGCL are not conserved (Glu193/Lys471 and Glu136/Asn176; see above). Also, among the aromatic residues, Tyr186, which is in close proximity to CC2, is absent in all proteobacterial GCL proteins. Furthermore, when compared to their plant counterparts, proteobacterial GCL sequences show two insertions of, in most cases, three and six additional amino acids, following residues 123 and 468, respectively (Figure 3.18; numbering according to BjGCL). While the first of these insertions is located in a loop region distant from the active site and dimer interface in BjGCL, the second insertion is positioned in proximity to the dimer-forming residues (i.e. Lys471 and Phe475 in BjGCL).

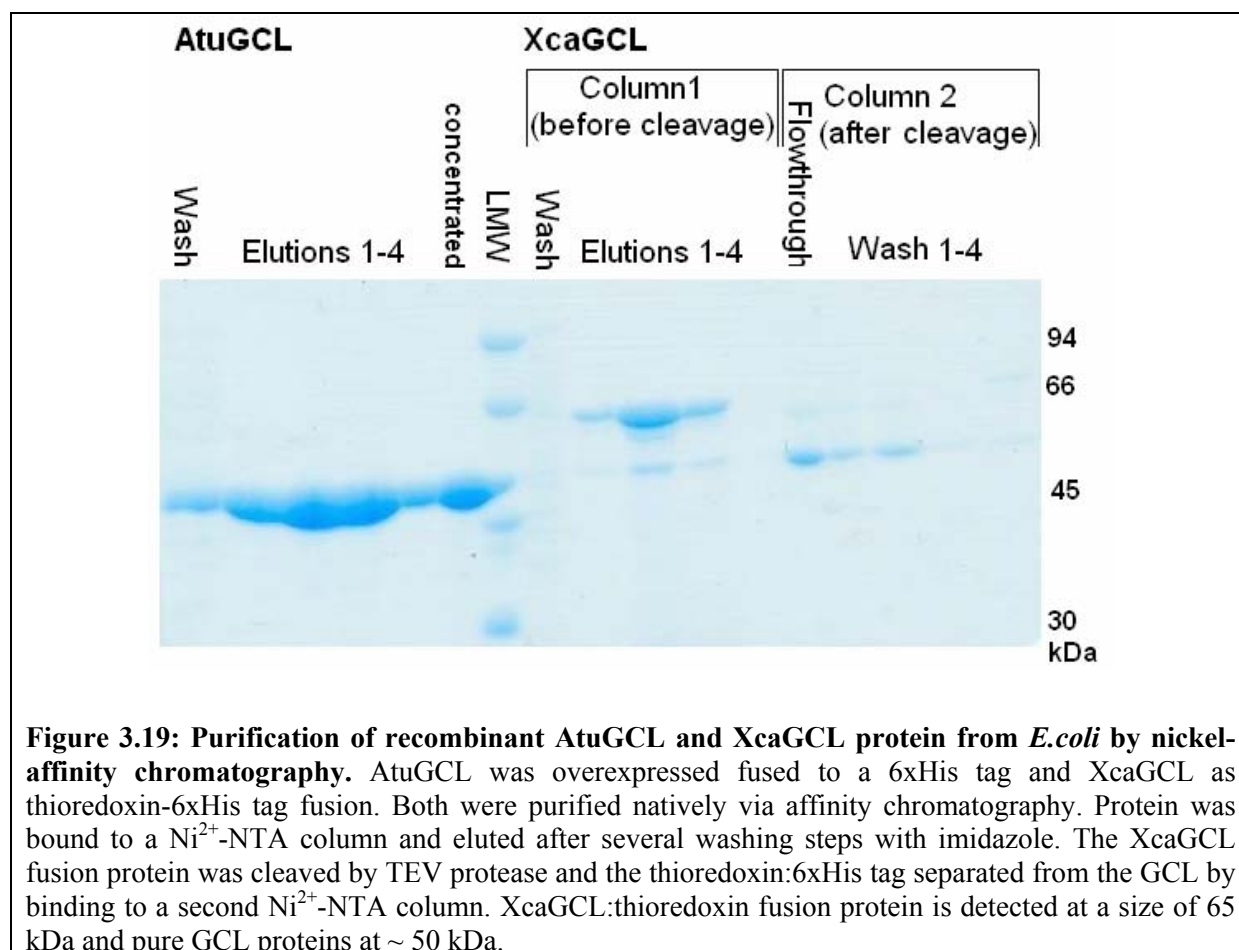
Based on these sequence comparisons it was assumed that homodimerisation is confined to plant GCL enzymes. Conversely, as CC2 appears to be conserved in

proteobacterial GCL proteins, a regulatory function independent of GCL dimerisation could not be excluded. To analyze whether these predictions hold true, the GCL enzymes from *Agrobacterium tumefaciens* and *Xanthomonas campestris* were cloned, expressed in *E. coli* and characterized.

3.6 Cloning and characterization of proteobacterial GCL homologues

The ORFs coding for the GCL of the alphaproteobacterium *Agrobacterium tumefaciens* and of the gammaproteobacterium *Xanthomonas campestris* were cloned, and the corresponding proteins overexpressed in *E. coli* as described in the Material and Methods section. *Xanthomonas* GCL (XcaGCL) was chosen as it is the proteobacterial enzyme with the highest sequence similarity to plant GCLs, showing 61% conserved and 74% similar residues when compared to BjGCL. *Agrobacterium* GCL (AtuGCL) is the proteobacterial gene with the highest number of homodimer interface residues conserved (7 out of 11, see above). At the protein level, AtuGCL exhibits 56% identity and 69% similarity to BjGCL (Figure 3.15).

Recombinant XcaGCL protein was expressed as TrxA:6xHis:GCL fusion in the *E. coli* strain Rosetta gami DE3 and purified after cleavage from TrxA, while AtuGCL was expressed and purified as fusion to 6xHis only (Figure 3.19).



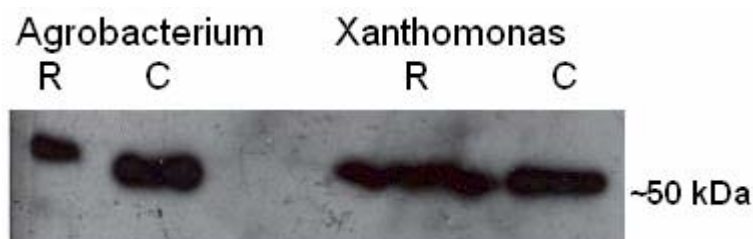


Figure 3.20: Detection of native and recombinant γ -GCS protein from *Agrobacterium* and *Xanthomonas*. Appropriate amounts of protein extract from bacterial culture (C) and recombinant protein overexpressed in *E.coli* (R) have been separated by SDS-PAGE and γ -GCS was detected using an antiserum raised against BjGCL. Predicted sizes for GCL proteins are: AtuGCL: native – 51 076 Da; recombinant – 52 474 Da; XcaGCL: native – 50 950 Da; recombinant – 51 325 Da.

Recombinant proteobacterial GCL proteins were detectable in a Western Blot using antibodies directed against BjGCL and had the same electrophoretic mobility as proteins detected in lysates from *Agrobacterium* and *Xanthomonas* cultures. As the gene encoding for XcaGCL is annotated starting with a TTG, while the next upstream ATG would result in a protein of about 55 kDa, this does verify cloning of the correct open reading frame (Figure 3.20).

Activities of recombinant GCL enzymes were characterized in the coupled spectrophotometric assay and the reaction product γ EC was also independently identified by HPLC after derivatisation with monobromobimane to confirm γ -glutamylcysteine ligase activity. Specific activities, turnover numbers and K_m values for cysteine, glutamate and ATP, respectively, were comparable to those found for plant GCL enzymes although the K_m value for glutamate was about four times lower for the proteobacterial proteins (Table 3.8). In addition, proteobacterial GCL was inhibited by buthionine sulfoximine in a similar fashion as plant GCL (Figure 3.21). Both proteins were also inhibited by addition of glutathione to the assay, and dose response curves matched those found for plant GCL (Figure 3.22). It is noteworthy that this inhibition of GCL activity was not dependent on the reducing power of GSH as S-methyl-GSH caused a similar inhibition (data not shown). Together these results indicate a mechanism of catalysis highly conserved among group 3 GCL proteins as predicted from the sequence comparison.

Both proteobacterial GCL proteins showed an approximately 4-5 fold higher specific activity in a buffer containing potassium chloride compared to sodium chloride (Figure 3.23). The same has been observed for the plant GCL, while animal GCL enzymes respond in reverse fashion (Webster and Varner, 1954; Davis et al., 1973).

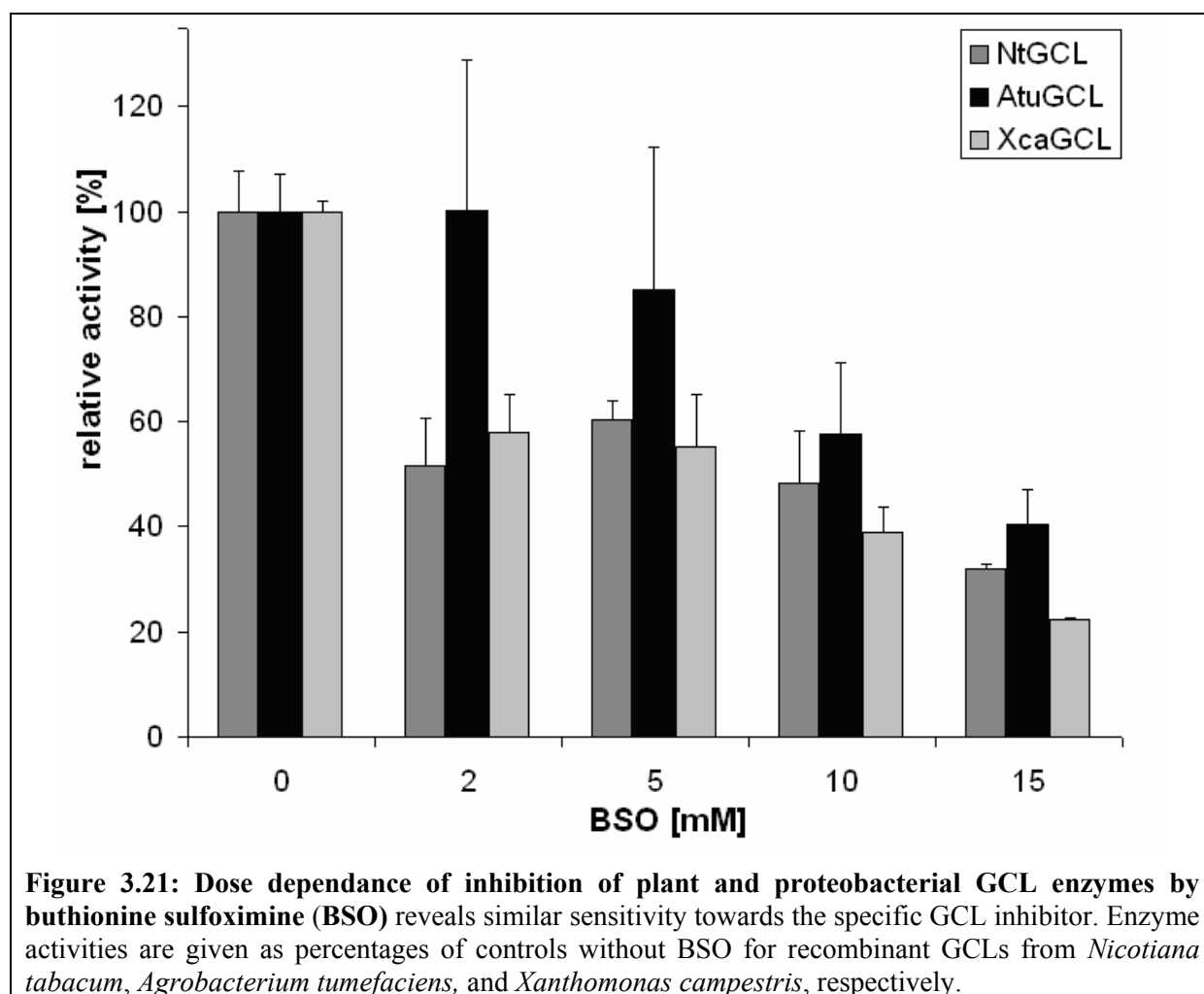
Results

Table 3.8: Comparison of enzyme activities, turn over numbers, and substrate affinities for recombinant GCL enzymes from *Agrobacterium tumefaciens*, *Xanthomonas campestris* and *Brassica juncea*.

	<i>Agrobacterium</i>	<i>Xanthomonas</i>	<i>Brassica</i> ^{*)}
Specific activity (nmol min ⁻¹ mg ⁻¹)	2585 ± 186 ^a	2405 ± 45 ^a	3336 ± 114 ^b
Turnover number (sec ⁻¹)	7920 ± 570 ^a	7350 ± 140 ^a	10190 ± 350 ^b
K_m (cysteine) (mM)	0.14 ± 0.01 ^a	0.07 ± 0.02 ^b	0.12 ± 0.01 ^a
K_m (glutamate) (mM)	1.9 ± 0.2 ^a	2.3 ± 0.4 ^a	8.5 ± 0.4 ^b
K_m (ATP) (mM)	0.59 ± 0.06 ^a	3.1 ± 0.7 ^b	1.3 ± 0.2 ^c

^{*)}Data for BjGCL according to Hothorn *et al.* 2006.

Different lower case letters mark significant differences between enzymatic characteristics ($P < 0.0001$)



Results

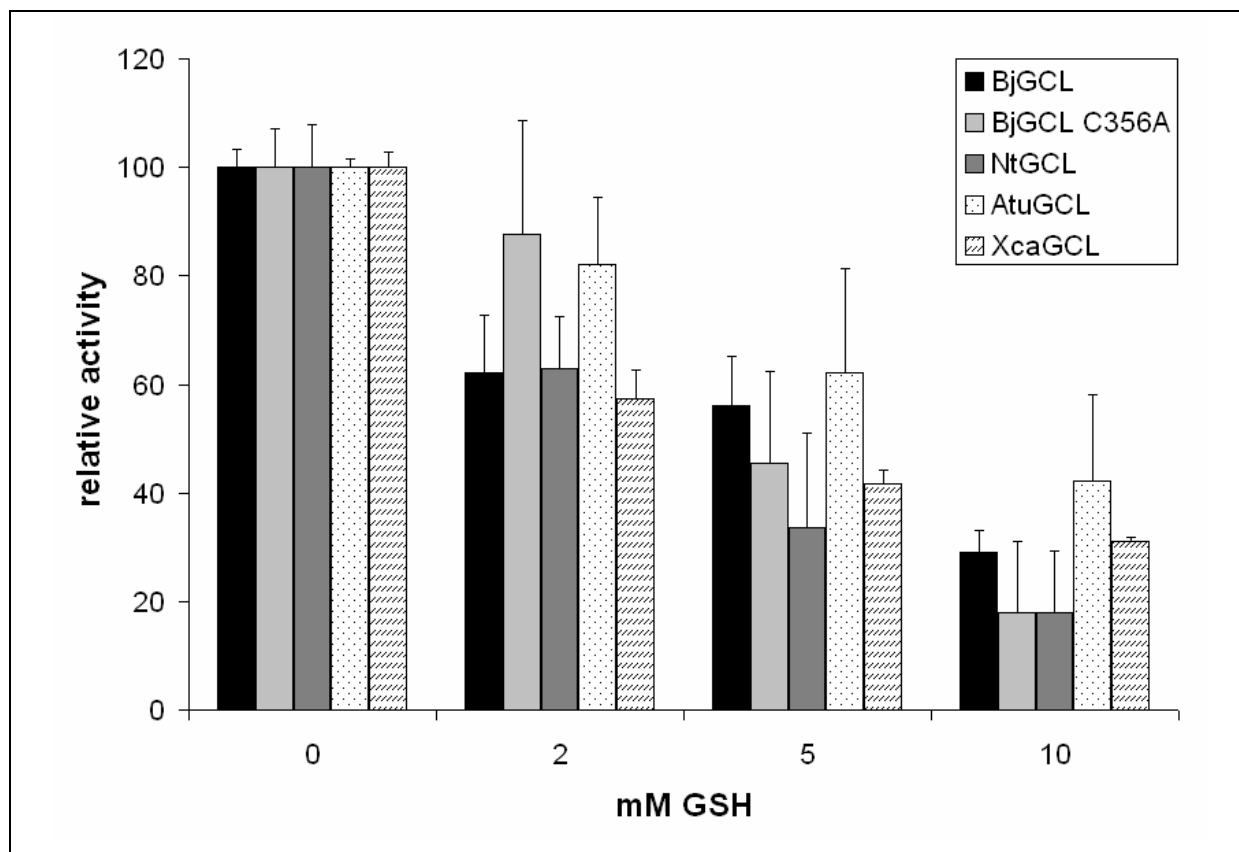


Figure 3.22: Dose dependence of inhibition of plant and proteobacterial GCL enzymes by glutathione indicates comparable feedback inhibition. GSH effects on activities of recombinant wild-type GCLs from *Nicotiana tabacum* and *Brassica juncea*, as compared with the corresponding mutant in CC1 (*Brassica* C356A) and proteobacterial GCLs from *Agrobacterium tumefaciens* and *Xanthomonas campestris*, respectively. Enzyme activities are given as percentages of control activity in buffer without GSH.

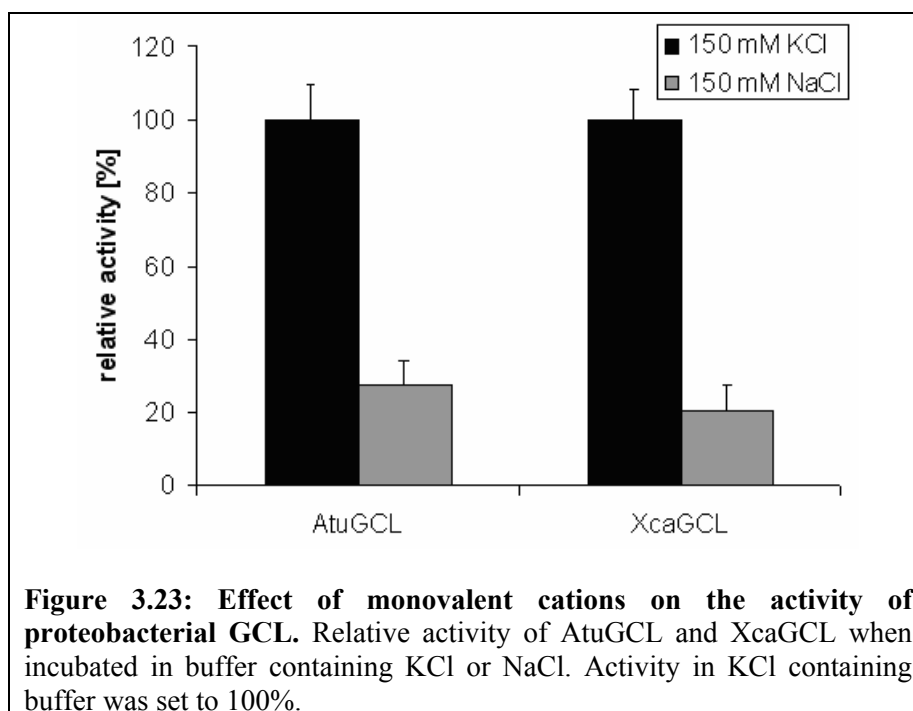


Figure 3.23: Effect of monovalent cations on the activity of proteobacterial GCL. Relative activity of AtuGCL and XcaGCL when incubated in buffer containing KCl or NaCl. Activity in KCl containing buffer was set to 100%.

3.6.1 Proteobacterial GCL proteins are not inhibited by reduction and are functional as monomers

As both proteobacterial GCL proteins analyzed show conservation of the cysteines required for formation of the core disulfide (CC2) bridge in BjGCL, the effect of reductants on the fold and oxidation state of AtuGCL and XcaGCL was examined. Non-reducing SDS-gel electrophoresis of AtuGCL pre-treated with oxidizing or reducing (5 mM DTT) conditions showed a shift in retention times between treatments, as did BjGCL-C341S (mutant lacking the CC1 disulfide bridge). Both proteins showed a slightly smaller apparent size when oxidized, possibly caused by a more compact conformation due to formation of a disulfide bridge (Figure 3.25).

Initial mass spectrometry analysis (conducted at the ZMBH, AG Ruppert) also showed that only one cysteine residue in XcaGCL was accessible to derivatization with iodoacetamide in the oxidized state, while all three were derivatized after reduction of the protein (data not shown). This might indicate that two cysteines, probably those corresponding to the CC2 cysteines of BjGCL, are forming a disulfide bridge in the oxidized state.

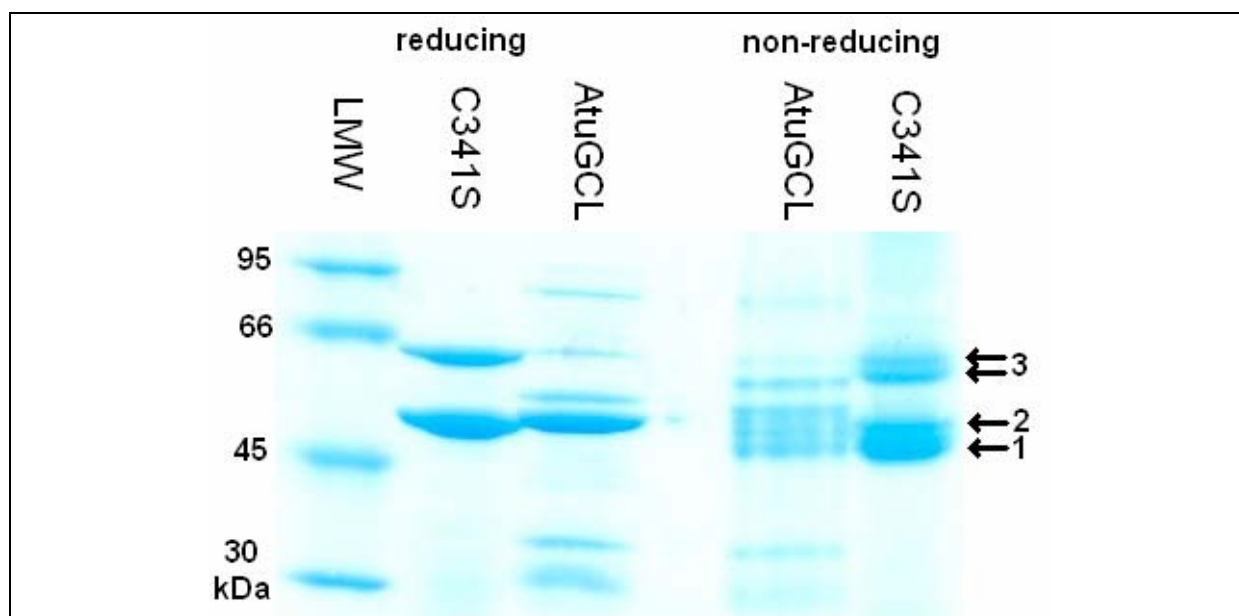


Figure 3.25: Non-reducing SDS-gel electrophoresis of AtuGCL and BjGCL-C341S. Protein was pretreated with 5 mM DTT (reduced) or loaded as purified (oxidized) and run after denaturing in non-reducing sample buffer. BjGCL protein is detected at ~ 50 kDa as a single band under reducing conditions (1) while an additional slightly higher band is detected under non-reducing conditions (2). Some uncleaved BjGCL-C341S:Trx fusion protein can be detected at ~ 65 kDa (3). The fusion protein is also detected as a double band under non-reducing conditions.

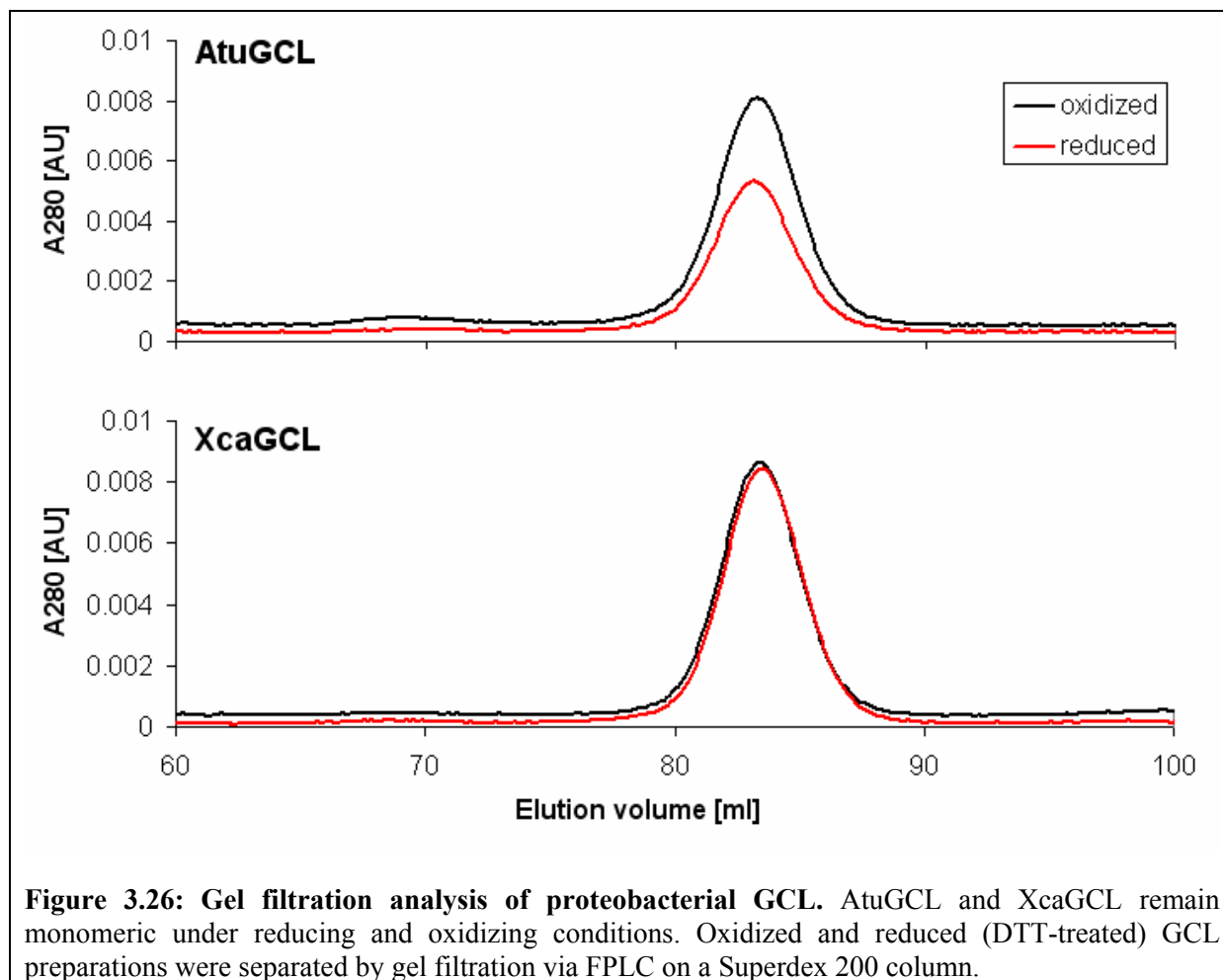


Figure 3.26: Gel filtration analysis of proteobacterial GCL. AtuGCL and XcaGCL remain monomeric under reducing and oxidizing conditions. Oxidized and reduced (DTT-treated) GCL preparations were separated by gel filtration via FPLC on a Superdex 200 column.

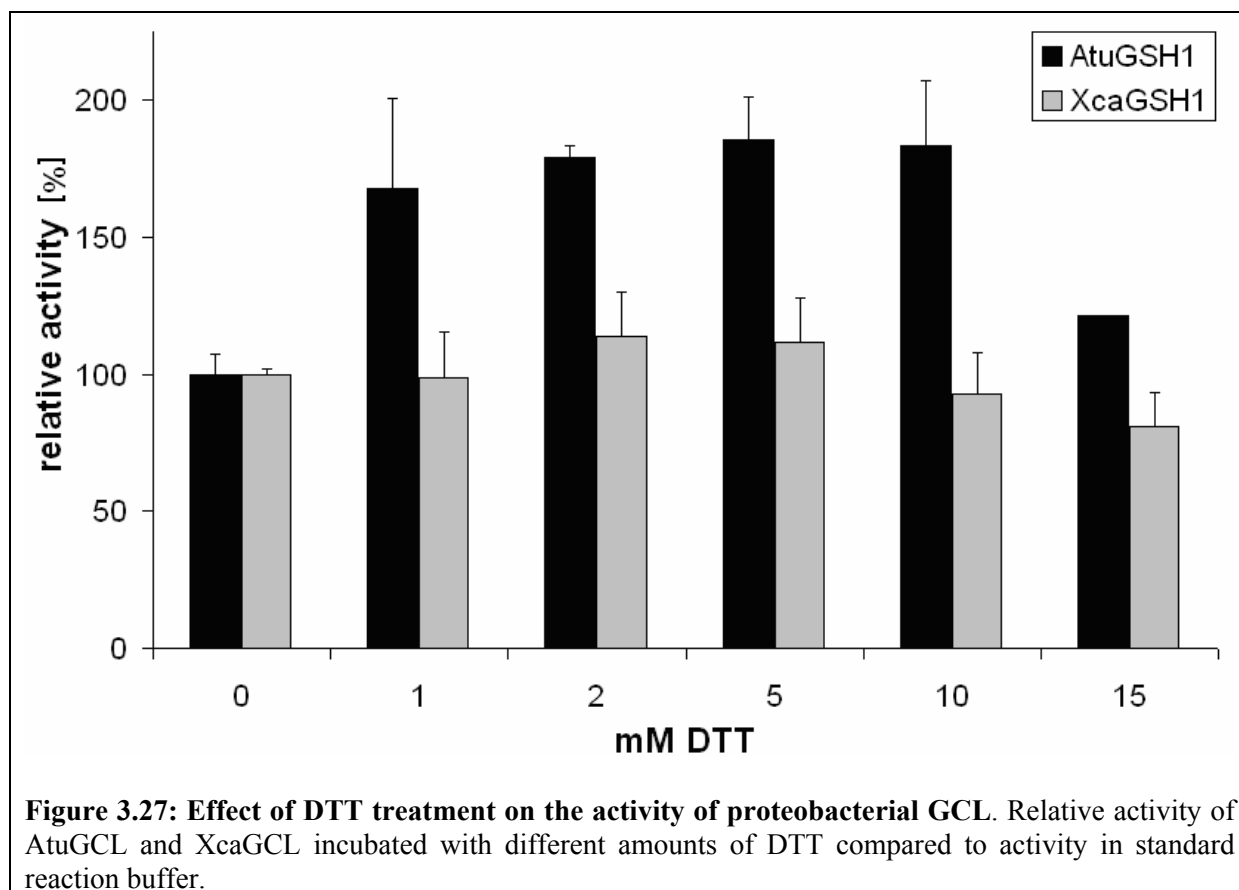
To determine whether proteobacterial GCL enzymes form homodimer complexes like their plant counterparts, and, if so, whether this is also affected by the redox state, recombinant GCL proteins were incubated under reducing (5 mM DTT) or oxidizing conditions, and their apparent sizes were subsequently analysed via FPLC. AtuGCL and XcaGCL eluted at a volume corresponding to their monomeric sizes, regardless of their pre-treatment, indicating that no dimer was formed under oxidizing conditions (Figure 3.26).

To assess whether the activity of proteobacterial GCL enzymes is affected by *in vitro* reduction as plant GCL proteins are, AtuGCL and XcaGCL enzymes were treated with different concentrations of DTT and analyzed in the coupled enzymatic assay under saturating substrate conditions (Figure 3.27). While XcaGCL was rather insensitive to DTT, the AtuGCL enzyme exhibited a 60 to 80% increase in activity. However, the degree of activation was not reproducible in all recombinant enzyme preparations. When AtuGCL activity was increased by DTT treatment, this had no effect on K_m values for its substrates. As recombinant GCL proteins were formed in an oxidizing cytosol (see above), it cannot be excluded that because of the presence of additional Cys residues the AtuGCL enzyme was partially

Results

misfolded due to the formation of illegitimate disulfide bridges and became activated upon DTT treatment. As the stimulatory effect of DTT varied between different enzyme preparations, it was not further studied and assumed an artefact.

In summary, these results demonstrate that the redox response of GCL activity and dimerisation are unique features of plant GCLs, despite the high similarity in protein sequence with their proteobacterial counterparts.



3.6.2 *Agrobacterium* and *Xanthomonas* show an active glutathione metabolism

To allow comparison of the enzymatic characteristics measured *in vitro* to the *in vivo* thiol metabolism of the two bacterial species, the thiols of bacteria growing under standard conditions have been extracted, derivatized with Monobromobimane (mBBr) and analyzed via HPLC (Figure 3.24). Comparison of bacterial extracts to standards of pure cysteine, γ -glutamylcysteine and GSH allowed identification and quantification of bacterial thiols. *Agrobacterium* showed 39.1 ± 11.2 nmol/gFW cysteine and 191 ± 61 nmol/gFW GSH with an average GSH/cys ratio of 4.86 ± 0.49 ($n = 4$). *Xanthomonas* showed 1317 ± 795 nmol/gFW cysteine and 453 ± 254 nmol/gFW GSH with an average GSH/cys ratio of 0.39 ± 0.14 ($n = 4$). In both cases total cellular cysteine and glutathione amounts were considerably higher than the total amounts found in uninoculated medium (Table 3.9), indicating that the bacteria actively synthesized these thiols and did not only take them up. γ -Glutamylcysteine concentrations were below the level of detectability in both species. Comparison to thiol concentrations found in other bacteria showed that the amounts of cysteine found in the two species are well within the range found in *E. coli* (Wheldrake, 1967), while GSH concentrations were among on the lower end of what was found in other aerobically grown bacteria (Fahey et al., 1978).

Based on an assumed even distribution within proteobacterial cells, intracellular GSH levels are estimated to be in the range of 0.2 to 0.5 mM. Thus under *in vivo* conditions a direct feed back inhibition of proteobacterial GCL by GSH appears unlikely. Under the same assumption cysteine concentrations would be approximately 0.04 mM for *Agrobacterium*, about one third the K_m (cysteine) of AtuGCL, and 1.3 mM for *Xanthomonas*, corresponding to 20 times the K_m (cysteine) of XcaGCL. This might indicate that cysteine availability is limiting for glutathione synthesis in *Agrobacterium* under standard culture conditions but not in *Xanthomonas*. As cysteine availability in the medium used for *Xanthomonas* culture was lower than in the medium used for *Agrobacterium* this might hint to a more efficient sulphur assimilation metabolism in the first (Table 3.9).

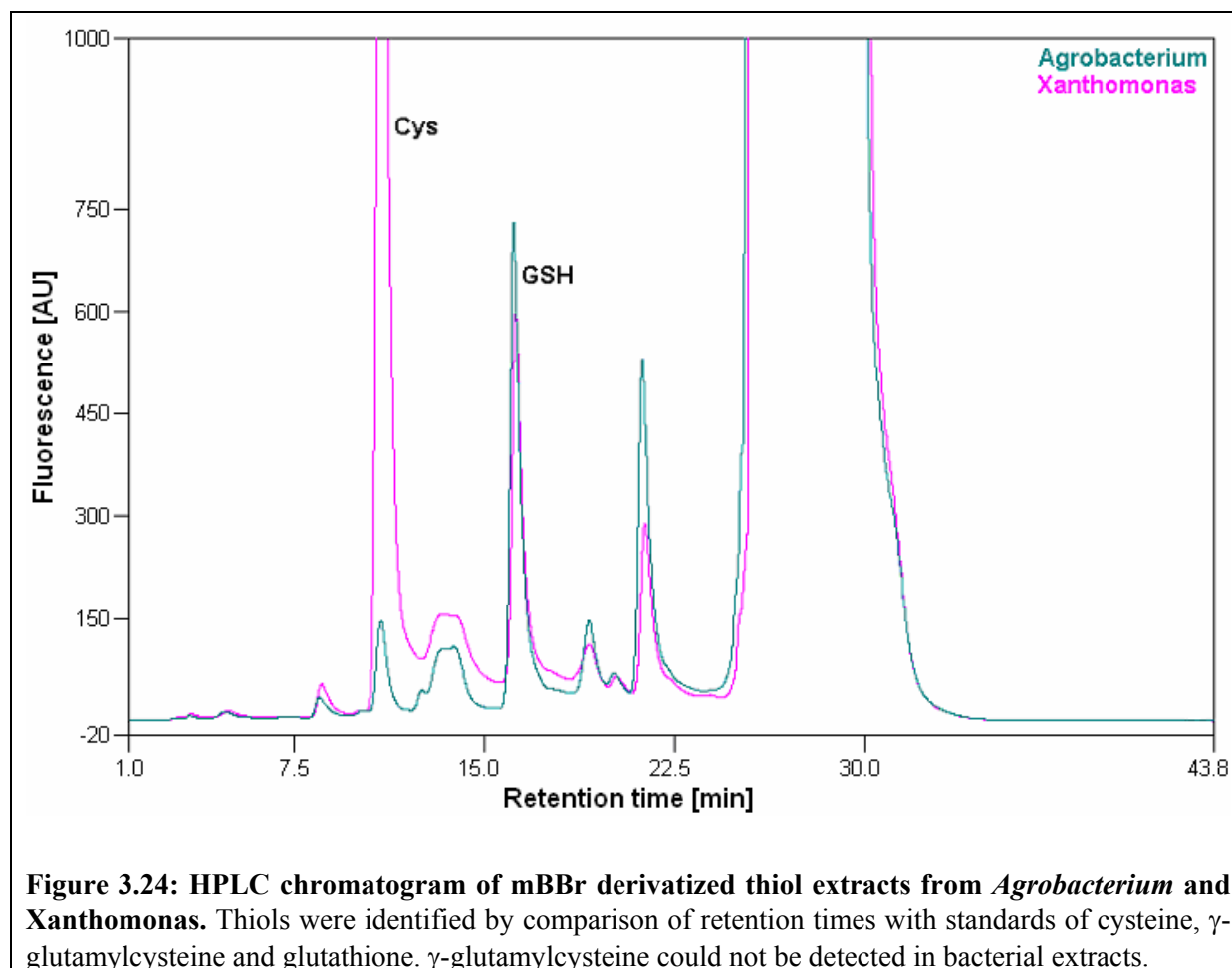


Figure 3.24: HPLC chromatogram of mBBBr derivatized thiol extracts from *Agrobacterium* and *Xanthomonas*. Thiols were identified by comparison of retention times with standards of cysteine, γ -glutamylcysteine and glutathione. γ -glutamylcysteine could not be detected in bacterial extracts.

Table 3.9: Analysis of thiol content in proteobacterial culture

		<i>Agrobacterium</i>	<i>Xanthomonas</i>
Cysteine concentration in bacteria	[μ M]	~ 40 ^{a)}	~ 1300 ^{a)}
Cysteine concentration in uninoculated medium	[μ M]	0.021 ± 0.005	< 0.001
Glutathione concentration in bacteria	[μ M]	~ 200 ^{a)}	~ 450 ^{a)}
Glutathione concentration uninoculated medium	[μ M]	0.045 ± 0.001	0.293 ± 0.005
Total cysteine in bacteria from 50 ml medium	[nmol]	1.56 ± 0.32	19.6 ± 9.5
Total cysteine in 50 ml uninoculated medium	[nmol]	0.15 ± 0.04	< 0.01
Total glutathione in bacteria from 50 ml medium	[nmol]	7.60 ± 1.89	6.83 ± 2.80
Total glutathione in uninoculated medium	[nmol]	0.313 ± 0.004	2.05 ± 0.04
GSH/Cys in bacteria		4.86 ± 0.49	0.39 ± 0.14
GSH/Cys in un inoculated medium		~ 3.2	> 500

Thiols were measured from pelleted bacterial culture or uninoculated medium by derivatization with monobromobimane and separation by HPLC.

^{a)} thiol concentrations in bacteria were estimated by assuming a volume of 1 ml per g fresh weight

3.7 The expression of plant GCL is affected by the availability of soluble thiols

Beside post-translational regulation via substrate availability, redox state, and GSH feedback, plant GCL activity is also influenced by the control of protein expression (See paragraph 2.2.2.1). A possible connection between a GCL substrate and the expression of the protein has been found in previous analyses which had shown that the expression of GCL in *Beta vulgaris* hairy roots is enhanced upon feeding of cysteine or glutathione to the culture (Gromes, 2004; Müller, 2006). Interestingly, an increased expression has been found for the endogenous protein as well as for transgenically expressed *Brassica* GCL which are distinguishable due to a slightly different apparent size in the immunoblot, hinting at a possible posttranscriptional mechanism of expression regulation.

To exclude that the effects observed are specific for the hairy root system, feeding experiments have been repeated with suspension cell cultures from *Arabidopsis* and *B. vulgaris*. Upon addition of 1 mM cysteine or GSH both species showed an induction of GCL expression in immunoblots (Figure 3.28). The induction expression was stronger after GSH feeding and in *B. vulgaris* cell culture was less pronounced than in hairy roots, possibly due to a stronger GCL expression in cell culture under control conditions.

In all types of cell culture addition of either thiol led to an increase in both cysteine and GSH concentrations (Figure 3.29). However, thiol concentrations were significantly higher in suspension culture cells than in hairy roots, before and after addition of cysteine or glutathione. While feeding experiments had little influence on the redox state of glutathione in *Beta* hairy root or suspension cell culture both types of feeding did result in an increase of GSH oxidation in *Arabidopsis* suspension cell culture.

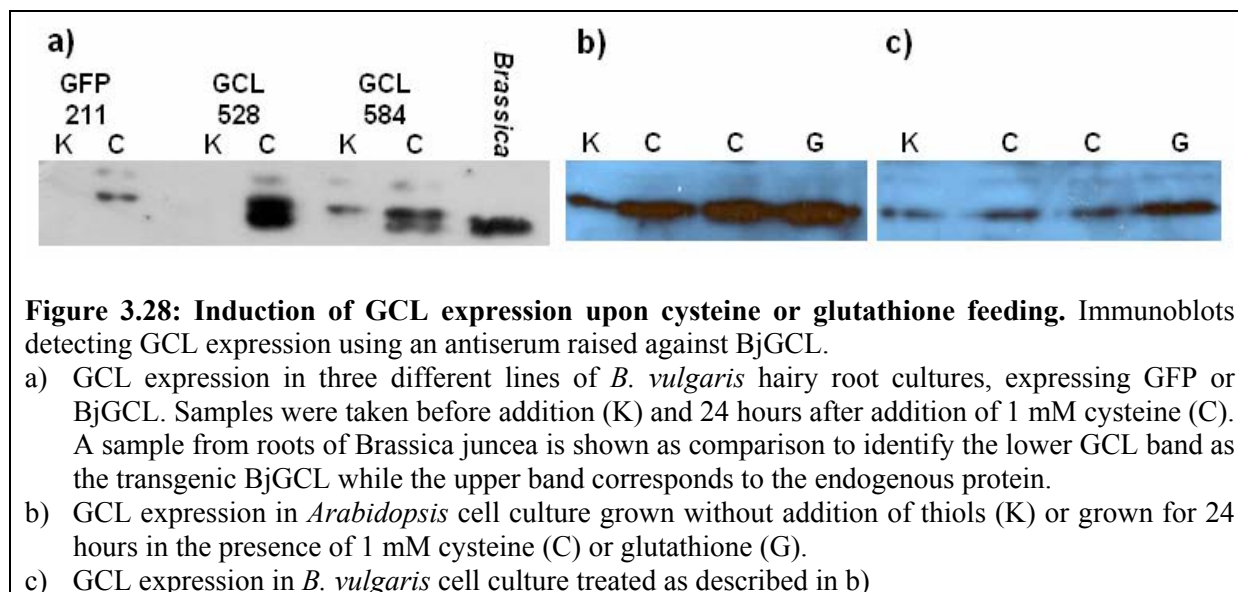


Figure 3.28: Induction of GCL expression upon cysteine or glutathione feeding. Immunoblots detecting GCL expression using an antiserum raised against BjGCL.

- a) GCL expression in three different lines of *B. vulgaris* hairy root cultures, expressing GFP or BjGCL. Samples were taken before addition (K) and 24 hours after addition of 1 mM cysteine (C). A sample from roots of *Brassica juncea* is shown as comparison to identify the lower GCL band as the transgenic BjGCL while the upper band corresponds to the endogenous protein.
- b) GCL expression in *Arabidopsis* cell culture grown without addition of thiols (K) or grown for 24 hours in the presence of 1 mM cysteine (C) or glutathione (G).
- c) GCL expression in *B. vulgaris* cell culture treated as described in b)

Further experiments were conducted to evaluate whether the effects of cysteine or glutathione feeding are specific for these thiols or dependent on a possible influence of such feeding on the redox state or sulfur metabolism of the culture. *B. vulgaris* hairy root culture was chosen for these experiments as the effect on GCL expression was most pronounced in this system. Sulfate feeding was performed to supply additional sulfur in a non-reducing form, providing 3 mM additional sulphate, therefore doubling the normal concentration found in the medium. To analyze whether feeding of a reducing thiol not related to cysteine affected GCL expression and glutathione synthesis, dithiothreitol (DTT) was added to the culture. Hydrogen peroxide feeding at 5 mM was conducted to analyze the effect of oxidative stress. All feeding experiments were analyzed for GCL expression and thiol concentration after 24 hours (Figure 3.30).

Hydrogen peroxide feeding was the only feeding that resulted in a visible change in the phenotype of hairy roots, resulting in a strong browning of the culture (Figure 3.30 d), associated by a mild increase in the rate GSH oxidation from $6.5 \pm 3.6 \%$ to $9.2 \pm 3.1 \%$ ($n = 5$). Neither sulphate, nor DTT or H_2O_2 feeding led to a general strong increase of GCL expression as found for cysteine feeding (Figure 3.30 a-c) and only DTT feeding led to a significant change in glutathione concentration, resulting in an approximately 6-fold increase. This effect might be due to incorporation of the reduced sulfur of DTT into GSH or due to a stabilization of GSH under reducing conditions as described for GSH reductase overexpressing plants (Foyer et al., 1995).

Results

These results show that the effect of cysteine and GSH feeding on the expression of GCL is specific for cysteine-related thiols, where the induction of expression following GSH feeding might be caused by elevated intracellular cysteine levels due to GSH degradation.

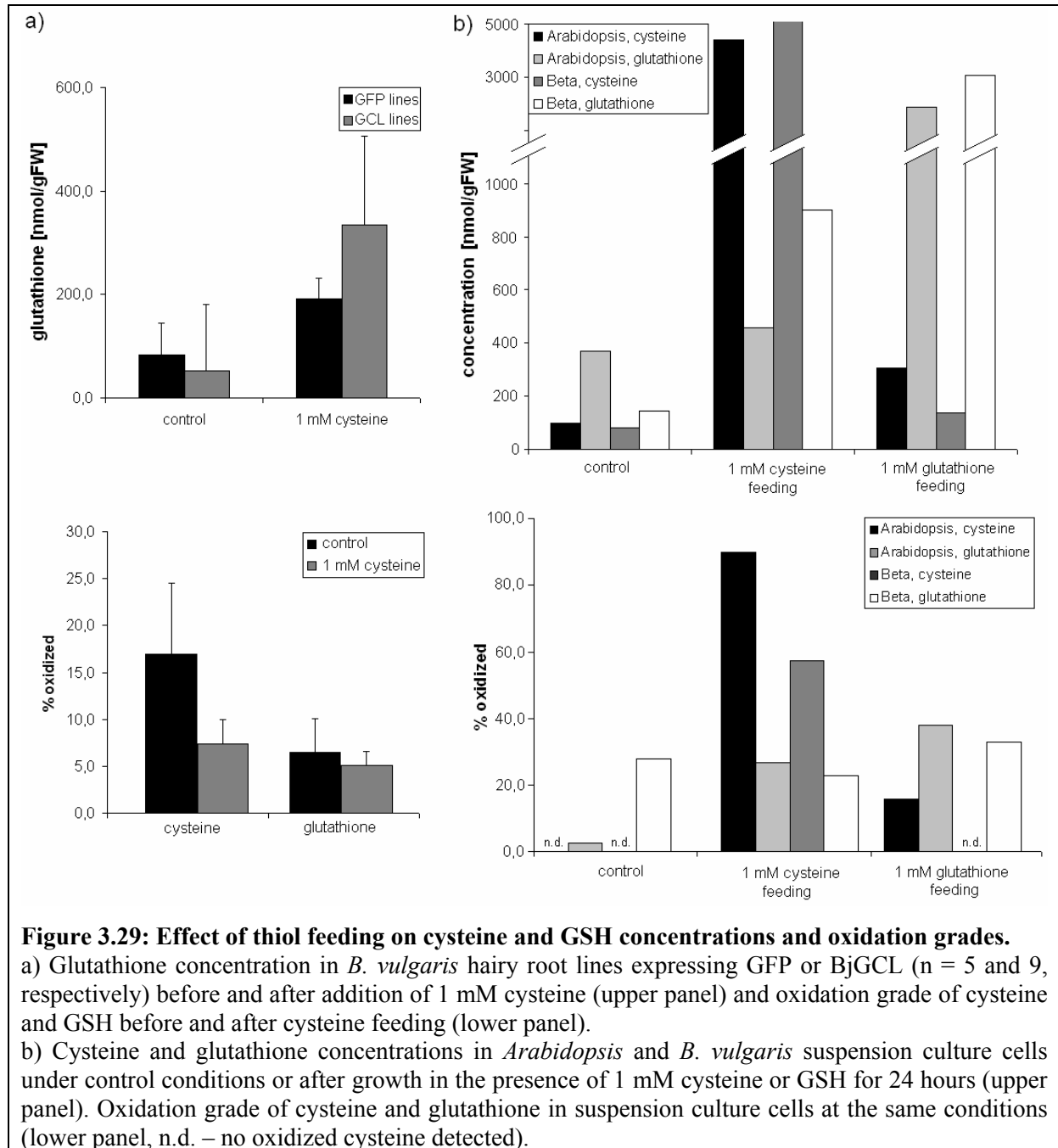


Figure 3.29: Effect of thiol feeding on cysteine and GSH concentrations and oxidation grades.

a) Glutathione concentration in *B. vulgaris* hairy root lines expressing GFP or BjGCL (n = 5 and 9, respectively) before and after addition of 1 mM cysteine (upper panel) and oxidation grade of cysteine and GSH before and after cysteine feeding (lower panel).

b) Cysteine and glutathione concentrations in *Arabidopsis* and *B. vulgaris* suspension culture cells under control conditions or after growth in the presence of 1 mM cysteine or GSH for 24 hours (upper panel). Oxidation grade of cysteine and glutathione in suspension culture cells at the same conditions (lower panel, n.d. – no oxidized cysteine detected).

Results

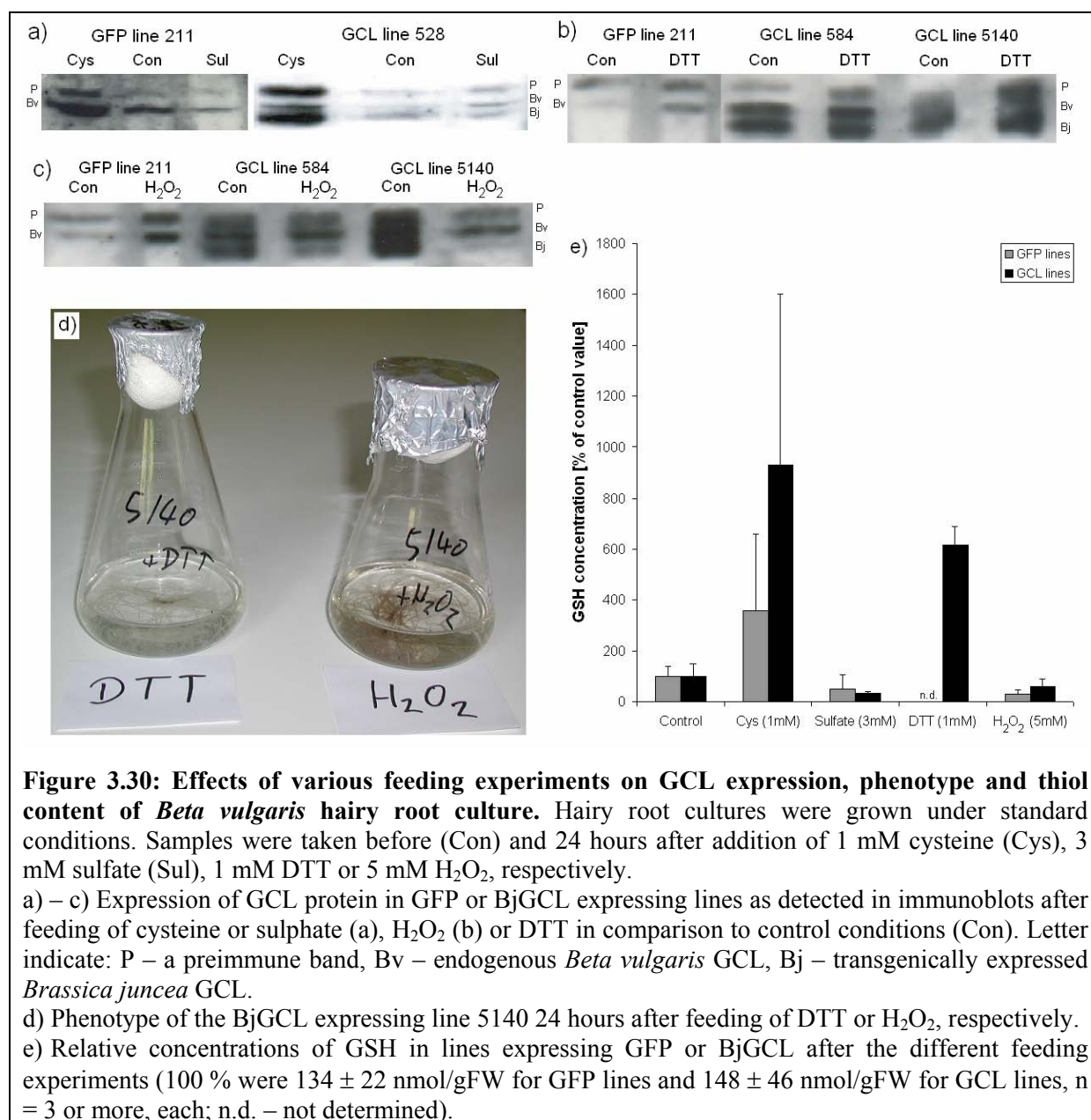


Figure 3.30: Effects of various feeding experiments on GCL expression, phenotype and thiol content of *Beta vulgaris* hairy root culture. Hairy root cultures were grown under standard conditions. Samples were taken before (Con) and 24 hours after addition of 1 mM cysteine (Cys), 3 mM sulfate (Sul), 1 mM DTT or 5 mM H₂O₂, respectively.

a) – c) Expression of GCL protein in GFP or BjGCL expressing lines as detected in immunoblots after feeding of cysteine or sulphate (a), H₂O₂ (b) or DTT in comparison to control conditions (Con). Letter indicate: P – a preimmune band, Bv – endogenous *Beta vulgaris* GCL, Bj – transgenically expressed *Brassica juncea* GCL.

d) Phenotype of the BjGCL expressing line 5140 24 hours after feeding of DTT or H₂O₂, respectively.

e) Relative concentrations of GSH in lines expressing GFP or BjGCL after the different feeding experiments (100 % were 134 ± 22 nmol/gFW for GFP lines and 148 ± 46 nmol/gFW for GCL lines, n = 3 or more, each; n.d. – not determined).

4 Discussion

Considering the plethora of functions glutathione is fulfilling in plant metabolism it is obvious that complex regulation of its synthesis is required to integrate metabolic, developmental and environmental signals on one side and to provide the necessary amounts of glutathione under different conditions during plant life on the other side. As the reaction of glutamate cysteine ligase (GCL) is limiting for GSH synthesis under most conditions (Noctor et al., 1998b; Noctor et al., 1998a), it is likely that most of this control is happening at the level of the reaction catalyzed by this enzyme. While GCL expression is regulated to some degree by environmental factors on transcript and protein levels (Schäfer et al., 1997; Xiang and Oliver, 1998), several studies indicate that post-translational regulation of GCL activity might play an important role in controlling GSH synthesis (May et al., 1998).

On one hand it has been shown that GCL activity can increase with unchanged expression level (May et al., 1998) and on the other hand even strong overexpression of plant GCL usually results in only moderate effects on the GSH content of plants (Xiang et al., 2001; Wachter, 2004). Furthermore, enzymatic characterization of GCL enzymes from different plants have shown that the K_m values for cysteine are in a range similar to the cellular conditions and that GCL enzymes are highly sensitive to changes in the redox environment, showing a strongly enhanced activity in the oxidized state (Hell and Bergmann, 1990; Jez et al., 2004; Wachter, 2004). Substrate and redox control therefore might both play a prominent role in regulating GSH synthesis, emphasizing the importance to elucidate the underlying molecular mechanisms.

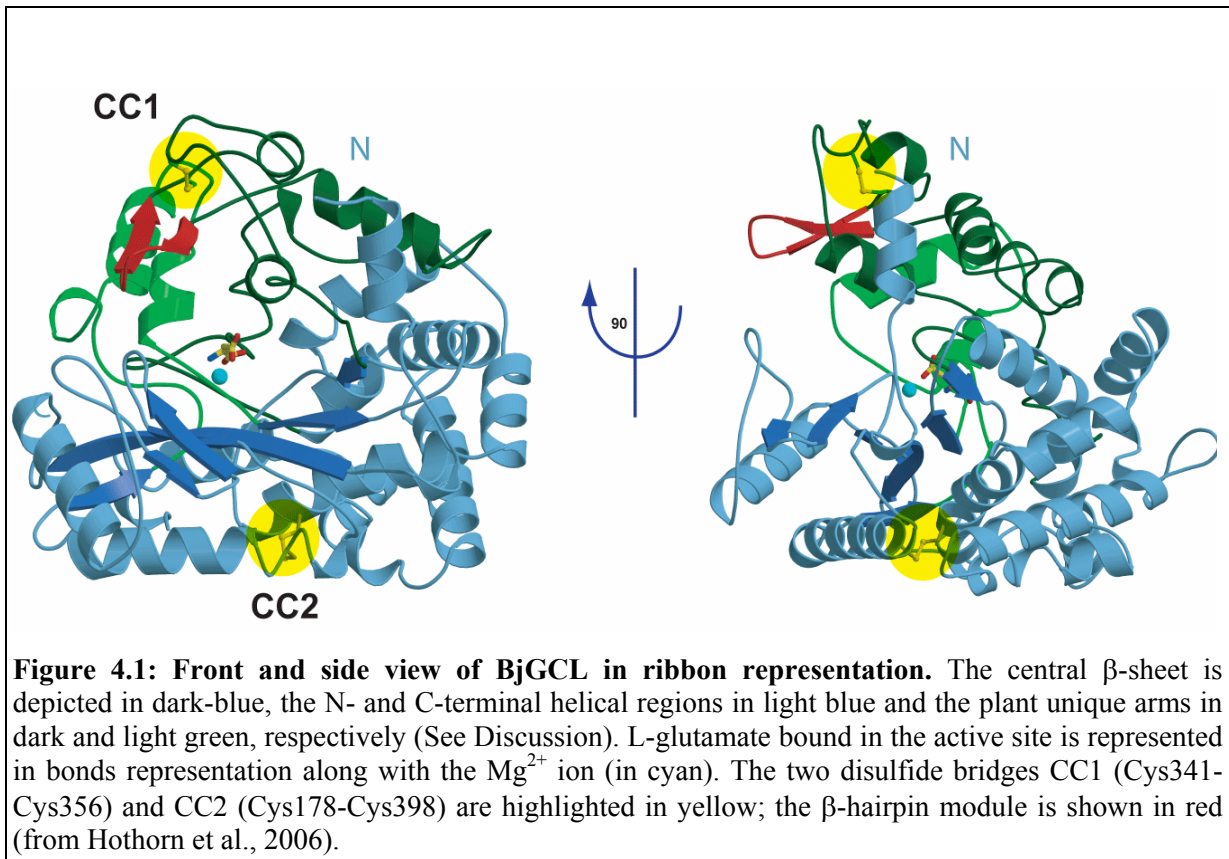
In the course of this thesis the redox regulation of recombinantly purified GCL from *Brassica juncea* (BjGCL) and *Nicotiana tabacum* (NtGCL) was characterized, based on biochemical analyses and the structure determination of the BjGCL protein. In addition, the evolution of the regulatory mechanisms of plant GCL was investigated by *in silico* analysis of plant and proteobacterial GCL homologs and by characterization of the GCL proteins from *Agrobacterium tumefaciens* (AtuGCL) and *Xanthomonas campestris* (XcaGCL). These comparative analyses allowed discerning which of the features found for BjGCL are common to all group 3 GCL proteins, specific for plant GCLs, or only found in a few closely related proteins, thereby providing new insight into the evolution of the central protein of GSH synthesis.

4.1 The Crystal Structure of *Brassica juncea* GCL reveals unique features compared to the *Escherichia coli* enzyme

Based on protein preparations provided during this and a previous thesis (Wachter, 2004), the crystal structure of *Brassica juncea* GCL was determined by collaborators at the EMBL (Hothorn et al., 2006). Two crystal forms were solved, showing the protein bound to BSO or glutamate, respectively, allowing the identification of the active site of the enzyme.

Despite the low sequence homology, BjGCL shows significant structural similarity to the *Escherichia coli* enzyme (May and Leaver, 1994; Hibi et al., 2004). The central part of BjGCL is a bowl-like structure composed of a six-stranded anti-parallel β -sheet, flanked by helical regions, and superimposes well with the *E. coli* counterpart (shown in blue in Figure 4.1).

Analysis of the crystal structure of BjGCL also allowed identification of the active site, which is located in a solvent accessible cavity, formed by two arm-like structures, flanking the central β -sheet, which are specific for the plant enzyme (shown in green in Figure 4.1). Interestingly, the residues making substrate contact in the active site were found to be highly conserved between BjGCL and the *E. coli* enzyme. Three glutamate residues (Glu107, Glu159, Glu165), one of which is substituted by an aspartate residue in *E. coli*, bind one Mg^{2+} ion. Glutamate is coordinated by Arg292 along with Thr242 (Ile146 in *E. coli*) while additional contacts are made by Trp296 in BjGCL which are not found in the *E. coli* enzyme. The position of the cysteine binding pocket could be deduced from the position of the aliphatic side chain of BSO bound to the enzyme and is formed by several hydrophobic residues reaching the alkyl chain of BSO from four directions. This region also contains a β -hairpin structure (shown in red in Figure 4.1) located near the active site and stabilized by a disulfide bridge (Cys341-Cys356: CC1).



The crystal structure of BjGCL also allows interpretation of the molecular mechanisms leading to reduced GSH levels in several *Arabidopsis* GCL mutants. In the *rm11* mutant Asp250 (corresponding to Asp259 in BjGCL) is substituted for asparagine, leading to an almost complete loss of GCL activity and GSH synthesis *in vivo* (Vernoux et al., 2000). Comparison of the *E. coli* GCL structure with that of BjGCL revealed that this residue is probably involved in binding of the adenine nucleotide and the recombinantly produced *rm11* protein showed no detectable GCL activity, even under elevated ATP levels, indicating the crucial role of this residue in catalysis.

In the *rax1-1* mutant Arg229 (Arg220 in BjGCL) is exchanged for lysine (Ball et al., 2004). This arginine residue is located at the proximal side of the cysteine binding pocket and may be important for recognition of the sulfhydryl-group of cysteine. This was confirmed by enzymatic characterization of the recombinantly produced *rax1-1* protein, which showed a 5-fold higher K_m value for cysteine (Hothorn et al., 2006).

The mutant *cad2-1* is caused by a six base-pair deletion in the *GCL* gene, affecting the residues 220-222 (211-213 in BjGCL). These residues are located in a loop-region and their deletion most likely alters the position of residues involved in substrate binding (e.g. R220, T242 in BjGCL) and may thus explain the altered GCL activity *in planta*. Recently, the GCL

mutant *pad2-1* has been shown to exhibit a S298N (Ser289 in BjGCL) substitution (Parisy et al., 2007). Again, this mutation is located close to the cysteine binding site.

Together these mutations emphasize the role of cysteine in the regulation of GCL activity, where changes in the affinity for this substrate result not only in significantly reduced GCL activity but also in strongly lowered GSH levels *in vivo*.

Noteably, while the *E. coli* protein was found to be monomeric, BjGCL proteins were arranged as dimers in the crystals (Figure 4.2) and show a well defined interface structure made up by 11 amino acid residues (Glu133, Phe135, Glu136, Gln176, Asn182, Tyr186, Glu193, Trp394, Arg395, Lys471 and Phe475; Figure 3.14. In particular, several salt bridges (Glu133/Arg395, Glu193/Lys471, Glu136/Asn176) and aromatic amino acid side chains (Phe135, Tyr186, Trp394, Phe475) contribute to a zipper-like interface. The whole interface appears to be stabilized by a disulfide bridge not present in the *E. coli* enzyme (Cys198-Cys398: CC2), linking two helices from the C- and N-terminus of the protein. Size-exclusion chromatography confirmed a dimer status of soluble oxidized BjGCL.

4.2 The redox regulation of BjGCL is dependent on two disulfide bridges

Redox regulation of plant GCL has been described previously and was found to be associated with a decrease of apparent size for the reduced protein, interpreted as a change in conformation (Hell and Bergmann, 1990; Jez et al., 2004). The crystal structure of BjGCL showed two disulfide bridges that might play a role in this process, possibly involved in positioning of a β -hairpin structure about 20 Å away from the active site (CC1) and in stabilizing the central structure of GCL, including the formation of the dimer interface (CC2). Recently, the presence of the corresponding disulfide bridges has been confirmed for GCL from *Arabidopsis* (AtGCL) by mass spectrometry of protease processed protein and site-directed mutagenesis (Hicks et al., 2007).

Site-directed mutagenesis and biochemical analysis showed that both of these disulfide bridges contribute to redox regulation of BjGCL *in vitro*. Knockout of CC1 led to a decrease of protein activity whereas substrate affinities were not affected. This could possibly be explained by a changed positioning of the β -hairpin structure upon loss of CC1 and a resulting hindrance of access to the active site, slowing down binding of substrates and release of the product. Dose response curves for BjGCL-C356A or NtGCL, both lacking the ability to form CC1, reveal a significant decrease in the susceptibility to treatment with reductants, compared to the wild-type BjGCL enzyme, confirming a contribution of CC1 to the *in vitro* redox regulation of the latter. However, in AtGCL, knockout of CC1 led to a decrease in activity of only 50 % in contrast to the 80 % decrease found for BjGCL. Also, the knockout did not lead to a significant change in midpoint potential of the protein, indicating no contribution to redox sensitivity. The different contributions of CC1 to *in vitro* redox regulation might, at least partially, depend on the different assay conditions applied in the two studies. While in this work a buffer containing KCl was used to determine the activity of BjGCL, a NaCl containing buffer was used for AtGCL (Jez et al., 2004; Hicks et al., 2007), despite the fact that sodium ions inhibit plant GCL (Webster and Varner, 1954). The preference for potassium over sodium ions was also confirmed for BjGCL (A. Wachter, personal communication). As plant tissues contain potassium ions and not sodium ions as the major monovalent cation, it is likely that the observations made for BjGCL are closer to the conditions found *in vivo*,

Interestingly, one of the cysteine residues involved in formation of CC1, corresponding to Cys356 in BjGCL, is not conserved in GCL enzymes from species outside

the Rosids clade while the other one is (Figure 3.17). Whether the ability to form CC1 does improve regulatory control of GSH synthesis in Rosids cannot be estimated yet, but the significant reduction of activity in BjGCL-CC1 mutants does imply that formation of CC1 is required for full activity of these enzymes *in vivo*. However, *Nicotiana tabacum* GCL (NtGCL) is lacking CC1 and does show a specific activity significantly higher than that of BjGCL-CC1-knockout enzyme, implying that a blockage of the active site is not occurring to the same degree as found for the latter.

Production of recombinant BjGCL protein with knocked-out CC2 cysteines leads to misfolded protein with minimal residual activity, whereas reduction of correctly folded wild-type or CC1 mutant protein does not result in aggregation or precipitation. Re-oxidation of these proteins leads to regeneration of enzymatic activity, indicating that CC2 is not required for the structural integrity after initial folding. As BjGCL is a nuclear-encoded plastidic protein (Wachter et al., 2005) and import into the chloroplast does require unfolding and refolding of the protein in two compartments with a reducing environment (Soll and Rien, 1998), it is likely that folding of BjGCL *in planta* is regulated differently than in *E. coli* Rosetta gami cells which have an oxidizing cytosol. However, it should be noted that production of correctly folded BjGCL was also possible from the *E. coli* strain B834 with a reducing cytosol, which was used for production of selenomethionine-labelled protein.

The effect of reduction of CC2 on plant GCL activity was investigated using mutant BjGCL without CC1 and NtGCL. Treatment of these proteins with reductants leads to a strong inhibition of enzymatic activity, along with a reduction of apparent molecular weight in size-exclusion chromatography to half the apparent size of the oxidized protein. The same observation has been described previously for partially purified GCL from *Nicotiana* and for the recombinant *Arabidopsis* enzyme (Hell and Bergmann, 1990; Jez et al., 2004). To decide whether this change in apparent size is caused by a different oligomerization state or by a massive change in conformation, ESI-TOF analysis has been conducted for the *Arabidopsis* enzyme and the detection of only very small amounts of dimeric protein has led the authors to favor the conformational change hypothesis (Jez et al., 2004).

However, the results obtained during this thesis, based on the crystal structure of BjGCL, strongly support the interpretation that the change in apparent size of plant GCL upon reduction is caused by the monomerization of the oxidized dimeric protein.

As described in the previous chapter, oxidized BjGCL is arranged as dimers in the crystal and shows a well defined dimer interface structure, consisting of 11 amino acid residues and stabilized by a disulfide bridge (CC2). However, in contrast to the heterodimer

found for animal GCL, this homodimer is based on non-covalent bonds and therefore is likely to break up under denaturing conditions like the 50 % methanol buffer used for the ESI-TOF analysis of the *Arabidopsis* enzyme, providing an explanation for the low amount of dimer found in this analysis. Interestingly, the determination of the molecular weight of GCL extracted from *Arabidopsis* chloroplasts by colorless native gel electrophoresis point to a dimeric state (Peltier et al., 2006).

Furthermore, the oxidized protein shows a compact globular structure and not the rod-like shape proposed in the conformational change hypothesis. Proteolytic processing of BjGCL also did not show differences in the digestion pattern of oxidized and reduced GCL that would strengthen the interpretation of a strong conformational change or hint at a hinge region.

The importance of the interface residues is further emphasized by their high conservation among higher plant GCL sequences along with complete conservation of the CC2 cysteines. Also, sequences from *Physcomitrella* or green algae that lack CC2 show low conservation for the interface residues as do proteobacterial sequences, where the resulting proteins are not inhibited by reductants or change their apparent size upon reduction. This correlation of conservation between CC2 and interface residues supports the assumption of a shared role for both in redox regulation.

Further support for the dimerization interpretation comes from the observation that TCEP is apparently reducing CC2 much slower than the weaker reductant DTT. As TCEP requires a larger space than DTT to efficiently reduce disulfide bonds (Cline et al., 2004), this would indicate a shielding of CC2 in the oxidized protein which is most easily explained by the formation of a dimer (Figure 4.2).

What the exact role of dimerization is in the redox regulation remains unknown but it can be speculated that the formation of CC2 in the core of the plant GCL enzyme has a stabilizing effect on the dimer interface as well as on the conformation of the active site. Association of two oxidized monomers would possibly strengthen the overall structure and, by shielding of CC2 from reductants, stabilize the oxidized form of GCL under *in vivo* conditions.

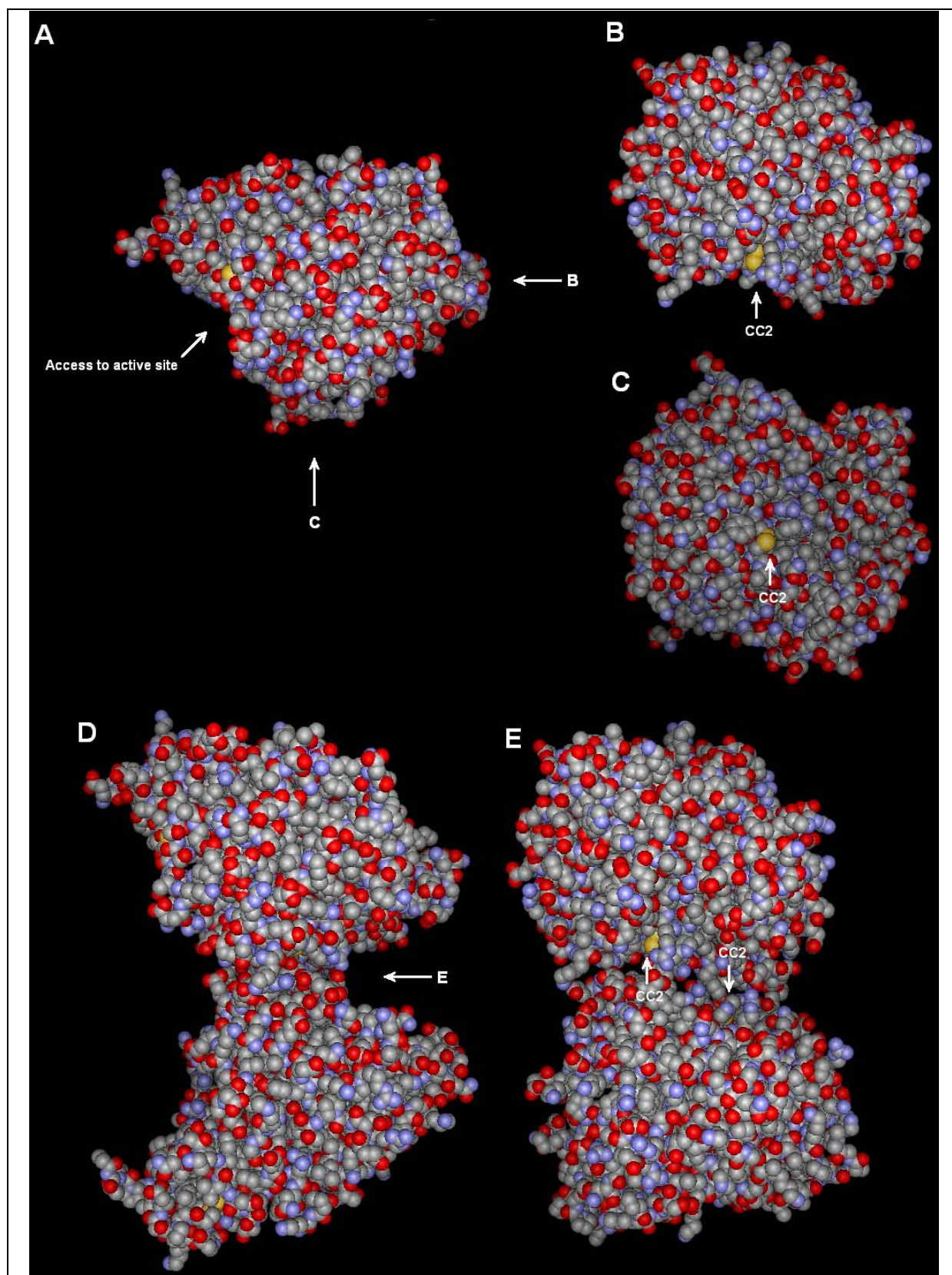


Figure 4.2: Accessibility of CC2 in the BjGCL monomer and dimer. Three-dimensional model of BjGCL with space filling model for atoms, gray = carbon, red = oxygen, blue = nitrogen, yellow = sulfur; A – side view of the BjGCL monomer; B and C – as A, turned 90°, viewing direction as indicated by the arrows in A; D – side view of the BjGCL dimer; E as D, turned 90°, viewing direction as indicated by the arrow in D. In the dimer the access to CC2 from the direction shown in C is blocked.

4.2.1 Several lines of evidence point to a role of GCL redox regulation *in vivo*

On theoretical ground, redox regulation of GCL would be an efficient and elegant way to integrate environmental signals into the regulation of GSH synthesis. Reduced BjGCL re-oxidizes and re-dimerizes spontaneously upon dialysis in buffer without additional oxidants. It is likely, that oxidation of plant GCL *in vivo* can also be caused by molecular oxygen or reactive oxygen species (ROS), especially under conditions of stress, where ROS accumulate. Reduction of BjGCL on the other hand requires strong reductants and is not efficient in the presence of GSH alone. It can therefore be assumed that *in vivo* reduction of plant GCL is either a very slow process or dependent on proteinaceous co-factors, such as a thioredoxin or glutaredoxin.

At the moment a direct evidence for an *in vivo* role of GCL redox regulation is still lacking, but several observations strongly support such a possibility. First of all, both cysteines of CC2 as well as the amino acid residues forming the dimer interface are highly conserved among all higher plant and at least some green algal GCL sequences. Even considering the overall high homology of plant GCL proteins, it seems unlikely that these residues, positioned at the surface of the monomeric protein, would be conserved by chance, especially when considering that they are obviously not required for catalysis as shown by the fact that they are not conserved among proteobacterial group 3 GCL proteins. The easiest explanation, why evolutionary pressure favours conservation of these residues in plants, is that they are involved *in vivo* in the same redox-dependent regulatory mechanism as observed *in vitro*.

Several observations made by other groups strengthen the assumption that the redox regulation mechanism described above is active *in vivo*. Fast increases of GCL activity in *Arabidopsis thaliana* cell culture, which were not accompanied by changes in the transcript amount, indicate an efficient posttranscriptional regulatory mechanism (May et al., 1998) as does the observation that plant GCL alone does not fully complement a GCL deficient yeast strain, probably due to the lack of an activating factor (May et al., 1998). Redox regulation could account for both observations as oxidation of GCL upon stress could lead to a 5 to 10-fold increase in activity without changes in the protein amount, while efficient reduction of heterologous GCL in the yeast cytosol would partially inactivate plant GCL.

Recently, Hicks et al. (2007) determined the redox midpoint potential of *Arabidopsis* GCL to be at -318 mV at pH 7.0 and at -365 mV at pH 7.9 for the wild-type enzyme with

little changes for CC1 mutants. This puts GCL in the same range as described for other redox-active proteins (from -290 mV to -330 mV) (Hutchinson and Ort, 1995; Hirasawa et al., 1999). Interestingly, the significantly lower midpoint potential found at a higher pH would indicate a lower susceptibility of GCL to reduction under conditions of active photosynthesis, possibly explaining the observation that GCL activity is correlated to photosynthetic activity (Ogawa et al., 2004). Hicks et al. (2007) were also able to extract a reduced and an oxidized form of GCL from roots of *Arabidopsis* seedlings, finding similar amounts of both under non-stress conditions while treatment of the plants with hydrogen peroxide, cadmium, BSO, or menadione led to a shift towards the oxidized form within one to four hours. However, as these experiments were conducted on non-reducing but denaturing gels, they do not tell anything about the *in vivo* oligomerization state of *Arabidopsis* GCL as non-covalent dimers would certainly be broken up by the treatment with SDS.

The main difficulty in proving the existence of a dimerisation dependent redox regulation *in vivo* by protein extraction methods lies in the requirement to extract protein from plant chloroplasts without disturbing the redox state and the non-covalent dimerisation. As initial trials to do so have failed in our lab, non-invasive approaches to analyze *in vivo* redox-regulation and dimerization might be necessary to achieve conclusive results. These questions might be approached by techniques like FRET-analysis or by analysing the ability of *GCL*-genes mutated in cysteine or interface residues to complement GCL-knockout lines.

4.4 Cysteine and glutathione regulate the activity of plant GCL via multiple mechanisms

In addition to redox regulation, metabolic regulation has a strong impact on glutathione synthesis. The K_m value of plant GCL for cysteine is close to the assumed physiological concentrations of this amino acid (Hell and Bergmann, 1990; Hothorn et al., 2006) and a rather mild (3-fold) reduction of the K_m for cysteine in the *rax1-1* mutant protein seems to be sufficient to result in reduced GSH levels *in vivo* (Ball et al., 2004; Hothorn et al., 2006). As sulfur supply has been found to limit the content of GSH in plants, it can be assumed that cysteine levels exert a rather stringent control on GCL activity and therefore overall GSH synthesis *in vivo* (Meyer and Fricker, 2002; Noctor et al., 2002; Kopriva and Rennenberg, 2004).

The other metabolite likely to influence GCL activity *in vivo* is glutathione itself. GSH has been described as an inhibitor for plant GCL but different mechanisms have been proposed as biochemical analyses indicated a competitive (Hell and Bergmann, 1990) or non-competitive mechanism of inhibition (Jez et al., 2004). Analyses of the dose dependent inhibition of different plant GCL variants by GSH, and the observation that GSH does not reduce CC1 and CC2 in BjGCL efficiently, indicate that feedback regulation of plant GCL by GSH is largely independent from redox regulation *in vitro*. This conclusion is supported by the finding that BjGCL is inhibited in a very similar way by oxidized GSH or S-methyl GSH, where in both cases the redox active sulfhydryl-group is blocked (Wachter, 2004; Pasternak, 2007). One reason why GSH is not able to efficiently reduce BjGCL might be steric. As access to CC2 appears to limit the efficiency of reduction for TCEP, this might also be the case for GSH. Crystallographic analysis has shown that GSH actually can bind in the active site of BjGCL and it is therefore likely that GSH is acting as competitive inhibitor (Esther Lenher, personal communication). However, it cannot be excluded that *in vivo* GSH is involved in the redox regulation of GCL, as proteinaceous cofactors like glutaredoxins might lead to an efficient reduction if they are able to facilitate access of GSH or redox-active cysteine residues to CC2.

Interestingly, the induction of GCL expression in hairy roots and suspension cell culture after cysteine or GSH feeding reveals another mechanism by which soluble thiols may influence GSH synthesis. Treatment with DTT does lead to a rise in GSH levels, possibly due

to reduction of the GSH pool, following a similar mechanism as observed upon GSH reductase overexpression (Foyer et al., 1995), while hydrogen peroxide or sulfate feeding result only in minor changes of GSH concentration. Notably, none of these treatments prominently affects GCL expression. This shows that induction of GCL expression is specific for cysteine or GSH feeding and is not dependent on a change of the cellular redox state or the overall sulfur availability. It can be assumed that the induction observed upon GSH feeding actually is a reaction to the increased levels of intracellular cysteine, caused by GSH uptake and degradation. A clear differentiation between intra- and extracellular thiols is, despite washing of the cells before extraction, very difficult in these experiments. Considering that GSH is the major transport form of reduced sulfur in plants, and therefore probably taken up more rapidly than cysteine, GSH feeding might actually lead to a higher intracellular cysteine concentration and therefore result in the stronger induction of GCL expression observed for suspension cultured cells.

Notably, in *Beta vulgaris* hairy roots, GCL expression is not only increased for the endogenous protein but also for heterologous BjGCL, expressed under control of the CaMV 35S promoter. As the construct used for transformation of these cultures includes the complete 5'UTR of *BjGCL*, this might be explained by a regulatory event either at the level of translation or in a change of the transcript or protein stability.

GSH metabolism has been proposed to have originally evolved to provide a soluble thiol less reactive and potentially toxic than cysteine (Fahey and Sundquist, 1991). It is intriguing to interpret the induction of GCL expression after cysteine feeding as part of a system to balance the intracellular content of free cysteine. This hypothesis may be supported by the observation that *Arabidopsis* plants with lowered GCL activity due to mutations were also reported to show up to five-fold increased concentrations of cysteine (Cobbett et al., 1998; Vernoux et al., 2000; Parisy et al., 2007).

Interestingly, the feeding of reduced or oxidized GSH to whole *Arabidopsis* plants had no effect on the expression of the endogenous GCL gene (Xiang and Oliver, 1998). A possible reason for this may be a lower rate of GSH uptake or degradation for whole plants when compared to heterotrophic cultured cells, resulting in a weaker impact of GSH feeding on intracellular cysteine content. On the other hand, increased expression of the GCL gene has been found for the *rml1* mutant of *Arabidopsis*, which does show a very low GSH content (Wachter, 2004), indicating a feedback mechanism correlating GCL expression to the cellular GSH content. In yeast, GCL expression is negatively affected by high GSH levels via regulation by the Met4 transcription factor (Wheeler et al., 2002) but an analogous system in

plants has not yet been characterized. Summarizing, it appears that the intracellular concentrations of both GSH and cysteine affect the expression and activity of GCL via several independent mechanisms.

4.5 The combination of redox and metabolite regulation allows an efficient control of glutathione levels

Combining the observations described above, a model can be proposed for the *in vivo* regulation of plant GSH synthesis (Figure 4.3). As a nuclear encoded gene, GCL is translated in the cytosol and subsequently imported into the plastid. Proteins are imported into the plastid unfolded, assisted by cytosolic and plastidic chaperones (Soll and Rien, 1998). Therefore, newly synthesized GCL will probably arrive in this compartment in the reduced, less active form. Under non-stress conditions most of the GCL pool will remain in the reduced state, possibly maintained by the action of thioredoxins or glutaredoxins. The remaining GCL activity is then regulated by the ratio of cysteine to glutathione. A high availability of reduced sulfur in the form of cysteine supports a high GCL activity, resulting in the production of GSH as a non-toxic, exportable form of reduced sulfur. An additional activation of GSH synthesis will occur under conditions of increased GSH demand in other organelles, cells, or organs, leading to an increased GSH export and reducing the feedback inhibition exerted by this metabolite. Low supply of reduced sulfur or high amounts of GSH, independent of its redox state, on the other hand will inhibit further GSH synthesis, preventing potential disturbances in the cellular redox poise as observed upon overexpression of *E. coli* GCL in tobacco (Creissen et al., 1999).

It is noteworthy that the different localization of GCL and GSHT, with the first exclusively in the plastid and the latter mainly in the cytosol (Wachter et al., 2005), will have a direct impact on the regulation of GSH synthesis. The export of γ -EC to the cytosol and the exchange of GSH between both compartments allows regulation of the feedback inhibition exerted by these metabolites on GCL in the plastid, possibly integrating signals from outside this organelle.

Under stress conditions, the occurrence of elevated ROS concentrations and an overall more oxidative chloroplast stroma will lead to oxidation of GCL, providing a fast mechanism to activate GSH synthesis. Dimerization results in a shielding of CC2 from access of reductants and will therefore stabilize the more active, oxidized form even under conditions when the overall redox potential might favor reduction of the disulfide bridge. This stabilizing role of the dimer might also explain why Hicks et al. (2007) detected a considerable amount of oxidized protein even under non-stress conditions. Under conditions of GSH depletion, caused by increased export, GST-activity, or phytochelatine synthesis, the metabolic level of GCL control would further increase GCL activity. However, an accumulation of oxidized GSH as well as a drop in cysteine concentrations would counteract this activating mechanism, preventing a complete depletion of reduced sulfur or an overshooting production of glutathione. Upon cessation of the stress conditions the changes of redox and metabolite states might deactivate GCL activity again. However, due to the slow reduction of GCL, a considerable deactivation would probably occur only some time after the cessation of the stress condition, resulting in increased GSH levels and possibly contributing to hardening of the plant against repeated stress conditions.

While these mechanisms of post-translational regulation allow a quick adjustment of GSH synthesis rate to the conditions prevailing in the plastid, expression regulation of GCL allows the integration of signals from other compartments, developmental stages and long-term stress conditions. This might explain the observation that treatment of *Brassica juncea* plants with cadmium led to a notable increase in GCL expression only after prolonged exposure of at least three days (Schäfer et al., 1997; Wachter et al., 2005). However, a fast increase in GCL expression within a time frame of few hours was observed for oxidative and heavy metal stress (Xiang and Oliver, 1998), as well as for cysteine feeding (Müller, 2006), showing that expression of GCL is actually regulated at a variety of time frames.

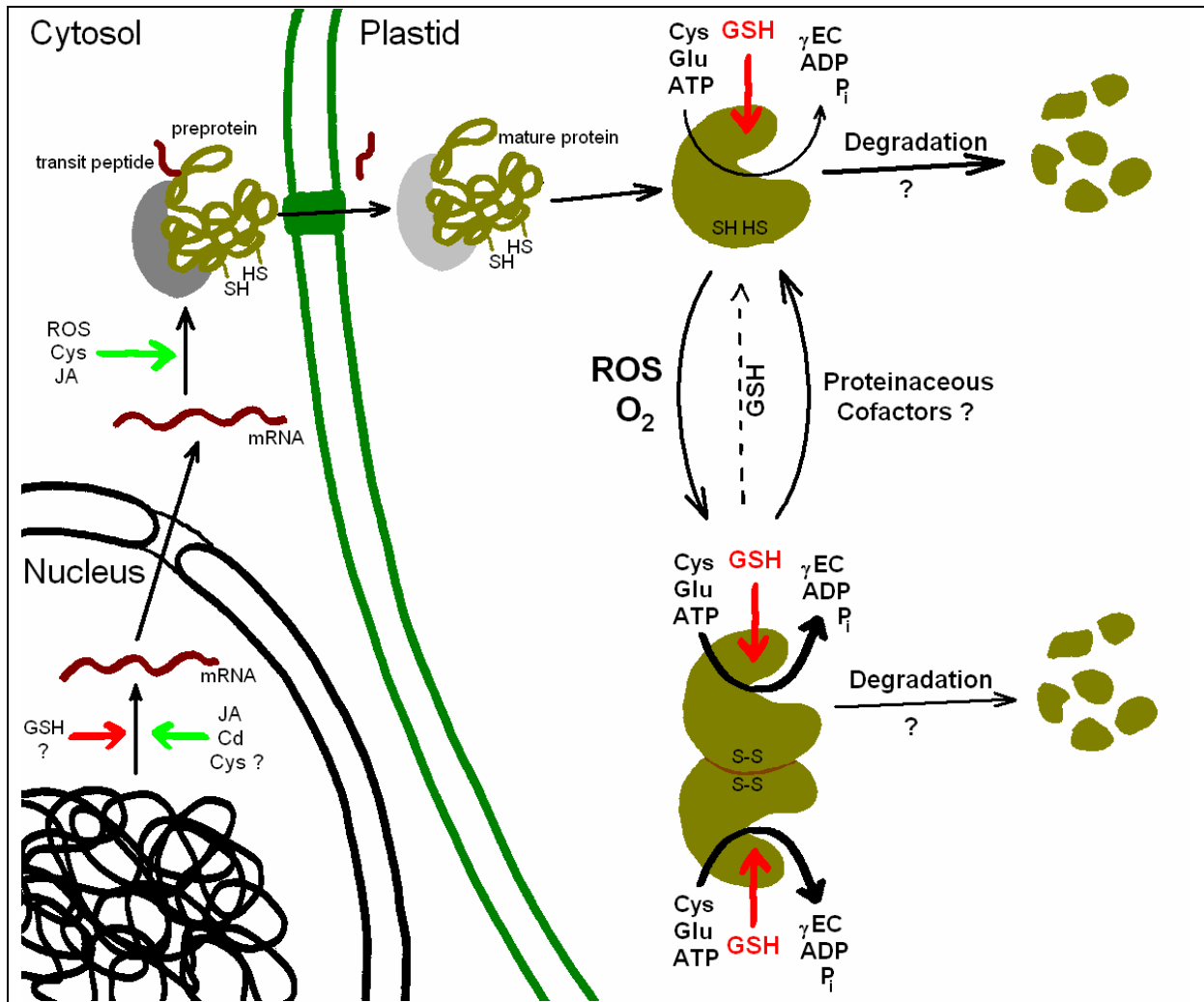


Figure 4.3: Model for the regulation of plant GCL activity. Green arrows indicate activation while red arrows indicate inhibition. GCL expression is controlled on the levels of transcription and translation by metabolic, developmental and stress signals and the resulting protein is imported into the plastid in the reduced state assisted by cytosolic and plastidic chaperones (grey). After final folding the redox state of GCL is controlled by the level of ROS and possibly the action of proteinaceous cofactors, while the enzymatic reaction is influenced by the availability of substrates and GSH. Which role turnover of GCL plays and whether it is influenced by the redox state is unknown. However, a role of oxidized CC2 in stabilizing the structure of the folded protein is hinted at by the aggregation of recombinantly produced BjGCL without this disulfide bridge.

4.6 Proteobacterial glutathione biosynthesis is not subject to redox control

The catalytic residues identified in BjGCL are highly conserved not only among plant proteins but also among their proteobacterial homologs. Comparing the enzymatic characteristics of recombinant *Agrobacterium* and *Xanthomonas* GCL with those of the *Brassica* enzyme reveals similar kinetic properties, susceptibility to inhibition by GSH and the same preference for potassium over sodium ions.

These observations support a conservation of the catalytic mechanism among group 3 GCL proteins. However, while bacterial GCL show a specific activity and K_m values for cysteine and ATP similar to those found for plant GCL, the K_m values for glutamate are about four times lower than for BjGCL. This might indicate an adaptation to cellular glutamate concentrations or a more prominent role of this amino acid in the regulation of GSH synthesis in bacteria.

It is noteworthy that, although the cysteine residues forming CC2 in BjGCL are conserved in many bacterial proteins and preliminary data suggest that a disulfide bridge is present in recombinant AtuGCL and XcaGCL, neither of these proteins shows an inhibition of enzymatic activity upon reduction. Both proteins also do not undergo dimerization and are active in a monomeric state under oxidizing as well as under reducing conditions. Sequence analysis shows that in contrast to the conservation of CC2 cysteines, bacterial GCL proteins do not show a stringent conservation of the dimer interface residues and show an insertion of six amino acids near the interface region. This suggests that redox susceptibility is not conferred by the presence of CC2 alone, but probably also requires structural features and the dimerization mechanism which are exclusive to plant GCL. Interestingly, AtuGCL and XcaGCL both are inhibited by glutathione in a manner very similar to plant proteins, providing further evidence that feedback-inhibition of GCL by GSH is independent from redox regulation.

Analysis of the thiol concentrations from cultured *Agrobacterium* and *Xanthomonas* shed further light on the regulation of glutathione biosynthesis in these bacteria. As in neither of the species γ -glutamylcysteine could be detected, it appears that, as in plants, the reaction catalyzed by GCL is the limiting factor for glutathione synthesis. The GSH concentration, which could affect GCL activity via feedback regulation, was found in the range of 0.2 to 0.5 mM under standard culture conditions in both bacteria, far too low to cause efficient

inhibition. It therefore appears that GCL activity in these proteobacteria is mainly regulated by protein amount and substrate availability.

Cysteine concentrations in *Agrobacterium* were found to be as low as one third of the K_m value of AtuGCL (~0.04 mM and 0.14 mM, respectively), therefore probably exerting a rather stringent control of GCL activity. In contrast, very high amounts of cysteine could be detected in *Xanthomonas*, reaching up to 20-fold the K_m value of XcaGCL (1.3 mM and 0.07 mM, respectively). As the medium used for the growth of *Xanthomonas* shows a very low concentration of free cysteine (below the detection limit of 0.001 μ M), these measurements indicate an active sulphur metabolism in *Xanthomonas*, either synthesizing cysteine by assimilation of sulphate or massively releasing sulphur from organic compounds like GSH or proteins. Consequently, it appears that in *Xanthomonas* the amount of expressed GCL protein would pose as the bottleneck of GSH synthesis. A difference in sulphur metabolism between the two bacterial species is also reflected in the proportion of cysteine to glutathione detected. While in *Agrobacterium* the concentration of GSH surpassed that of cysteine about 5-fold, in *Xanthomonas* GSH concentrations reached only about 40 % of the values found for cysteine.

However, it should be noted that analyses of the thiol metabolism of bacteria have revealed high variations in the concentrations of intracellular cysteine and GSH, depending on species and culture conditions (Wheldrake, 1967; Fahey et al., 1978). By which factors GSH synthesis is limited in proteobacteria would therefore probably vary depending on a number of conditions. Understanding the role of cysteine and possibly glutamate availability, as well as that of GCL expression, will therefore certainly require further research.

4.7 The Evolution of Plant GCL can be traced by comparison of biochemical analysis and *in silico* data

4.7.1 Plants acquired their GCL genes via endosymbiosis or lateral gene transfer

The *GCL* genes of bacteria and eukaryotes fall into three distinct groups, representing gammaproteobacteria (group 1), animals and fungi (group 2), and alphaproteobacteria and plants (group 3). Although the phylogenetic relationship between the groups cannot be resolved reliably, the identification of conserved blocks among all three types indicates a common origin (Copley and Dhillon, 2002). This is also supported, at least for group 1 and group 3 *GCL* genes, by the high structural similarity of BjGCL to the *E. coli* enzyme as well as the apparent conservation of the catalytic mechanism. Further similarity searches have led to the suggestion that GCL enzymes are related to glutamine synthetases which catalyze the ligation of ammonium to the γ -carboxy group of glutamate (Abbott et al., 2001). It has been proposed that the *GCL* gene first evolved in cyanobacteria, as these were the first photosynthetically active cells and therefore probably needed an efficient defence against ROS (Copley and Dhillon, 2002). The γ -EC produced by GCL could have acted as an antioxidant as it still does in some halobacteria (Sundquist and Fahey, 1989). From here lateral gene transfer would have brought the gene to proteobacteria and eukaryotes.

Group 3 *GCL* genes are widely spread among alphaproteobacteria and are well conserved. It therefore seems reasonable to assume that the gene has a single origin for all alphaproteobacteria and then evolved into the “typical” group 3 gene within this group. The related *GCL* genes in the gammaproteobacterial Xanthomonadales and Chromatales have probably been acquired from alphaproteobacteria by lateral gene transfer.

Another question of interest is, how plants acquired the group 3 *GCL* gene. The most obvious way would be by gene transfer from the alphaproteobacterial ancestor of the mitochondrion or during the fusion of a proteobacterium with the predecessor of the eukaryotic cell (Gray et al., 1999; Rivera and Lake, 2004). However, both of these events occurred early in eukaryotic evolution. So it remains puzzling that plant GCL is from a different type than the GCL proteins found in other eukaryotes belonging to group 2 (May and Leaver, 1994; Copley and Dhillon, 2002). These genes are found not only in animals and fungi but also in the Bacillariophytes *Thalassiosira pseudonana* and *Phaeodactylum*

tricornutum (Protein Ids Thaps3:13064 and Phatr2:27240, respectively, DOE Joint Genome Institute; www.jgi.doe.gov) and in the red alga *Cyanidioschyzon merolae* (LocusCMG141C, *Cyanidioschyzon merolae* Genome Project; <http://merolae.biol.s.u-tokyo.ac.jp/>). As so far no eukaryotes with both types of genes are known, it cannot be judged whether a common ancestor held two types of GCL genes – group 2 from the host cell and group 3 from the mitochondrion. If this had been the case, plants would have retained the group 3 genes, possibly because they were the only eukaryotes integrating this gene into the nuclear genome, while in all other eukaryotes the group 2 gene prevailed.

Another possible way of acquisition of group 3 GCL could be the lateral gene transfer from a free living bacterium to an early plant. Since highly homologous genes are found throughout the plant kingdom, including the green algae, this transfer would have to have happened before the first land plants appeared in the Silurian age, approximately 425 million years ago (Lang, 1937).

From which alphaproteobacterial group exactly plant GCL originates remains uncertain. The available sequence data do not give strong support for either theory of gene acquisition. However, as lateral gene transfer between bacteria and eukaryotes is considered to be an extremely rare event, a mitochondrial heritage of plant GCL seems more likely at this point.

4.7.2 Redox regulation of plant GCL evolved in green algae, possibly in parallel to the plastidic localization of the enzyme

While several proteobacterial GCL proteins contain the cysteines required for the formation of CC2, they show low conservation of the dimer interface residue found in BjGCL and are not susceptible to redox regulation. On the other hand, all higher plant GCL proteins show an almost complete conservation of these residues, so it can be assumed that redox regulation, depending on CC2 and dimerization, is functional in all of these species. The same is found for the proteins of *Chlamydomonas* and *Volvox*, showing that this type of regulation probably evolved in the green algae. As both of these species belong to the Chlamydomonales, which are not considered to be direct predecessors of higher plants (Chapman and Buchheim, 1992), a broader conservation of these features among green algae appears likely. In contrast, one GCL isoform from *Physcomitrella* (#2 according to Tables 3.7 and A1) as well as the GCL proteins from the green algae *Ostreococcus* and *Prototheca* lack not only the CC2 cysteines, but also show very low conservation of the dimer interface residues.

Why some primitive plants retain GCL genes without redox regulation remains unclear. However, *Prototheca* is a non-photosynthetically active, parasitic green alga (Leimann et al., 2004) and *Ostreococcus* is the smallest known eukaryote (Courties et al., 1994). It therefore cannot be excluded that features found in these untypical organisms are a consequence of specialization. In *Physcomitrella* only one of three GCL isoforms (#2) appears to lack redox regulation. It would be interesting to know whether these isoforms differ in expression or subcellular localization.

The role of the second redox regulation mechanism found *in vitro* for *Brassica* GCL involving CC1 remains unclear. Both cysteines required for this mechanism are found only in those sequences from plants belonging to the Rosids clade. Therefore, this mechanism probably originated in a single event and might have proved beneficial under selective pressure.

In comparison to plant GCL with only the core disulfide bridge, wildtype *Brassica juncea* GCL showed a stronger inhibition in the presence of DTT. This might indicate that the selective benefit of the hairpin mechanism lies in the ability to more stringently regulate GCL activity depending on the redox environment. However, redox titration conducted with *Arabidopsis* GCL mutants did not provide evidence for a strong contribution of CC1 to redox regulation of this protein (Hicks et al., 2007). Further research, preferably involving *in vivo*

studies on plants expressing GCL mutants with or without the CC1 bridge, will be necessary to conclusively judge whether this second mechanism does contribute significantly to redox regulation *in planta*.

It is striking that eukaryotes apparently evolved mechanisms for the redox regulation of GCL on two independent occasions – the mechanism depending on intramolecular disulfide bridges in the group 3 proteins of plants and the mechanism depending on an intermolecular disulfide bridge in group 2 GCL (Huang et al., 1993a; Huang et al., 1993b). In both cases redox regulation allows for a quick upregulation of GSH synthesis under conditions where oxidative agents are found in compartments with an otherwise reducing redox potential.

Several reasons might exist why this additional level of regulation is required in eukaryotic cells. Obviously, eukaryotic redox metabolism is much more complex than the prokaryotic one, as eukaryotic cells contain different compartments with different states of redox balance. Glutathione is believed to play an essential role in upholding these redox potentials (Schafer and Buettner, 2001). Overexpression of the *E. coli* GCL, lacking redox regulation, and the resulting increase in GSH content in tobacco led to unbalancing of the cellular redox state, resulting in visible stress symptoms (Creissen et al., 1999). Much larger variations of the cellular GSH and cysteine levels could be observed in *E. coli* under different conditions of sulfur availability without apparent negative effects (Wheldrake, 1967; Fahey et al., 1978) and in contrast to animals and plants *E. coli* is viable without the capability of GSH synthesis (Greenberg and Demple, 1986; Dalton et al., 2000; Cairns et al., 2006). These observations might indicate that eukaryotes are more susceptible to disturbances of the redox potential caused by deregulated GSH synthesis.

Beside its direct involvement in redox balance, GSH fulfils a plethora of functions in eukaryotes, including its involvement in such delicate processes as the regulation of gene expression, development, and protein activity (Mihm et al., 1995; Vernoux et al., 2000; Ball et al., 2004; Markovic et al., 2007; Parisy et al., 2007). A feedback regulation via the redox state, probably mediated by glutaredoxins, might provide a more efficient mechanism to control GSH levels than the direct competitive inhibition which depends on the levels of competing substrates.

Finally, redox feedback regulation might be especially efficient in eukaryotes as it provides a possibility to respond very fast to stress signals within the compartment where GSH synthesis takes place, circumventing the complex eukaryotic expression machinery.

Interestingly, the subcellular localization of GCL apparently differs between plants and other eukaryotes. While animal GCL is localized to the cytosol, all higher plant GCL sequences contain a presumed transit peptide ending in the highly conserved cleavage site IV/AASP, and in most cases this is predicted to facilitate import into the plastid (Appendix, Table A5). Exclusive plastidic localization was confirmed for GCL from *Brassica* and *Arabidopsis* (Wachter et al., 2005), tobacco (this work), and sugar beet (Müller, 2006). Sulfur is assimilated in the plastid (Leustek et al., 2000) and cysteine synthesis can take place here (Wirtz and Hell, 2007). Together with the production of ATP via photosynthesis and glutamate via nitrogen assimilation, the plastid might provide the most favourable environment for γ -glutamylcysteine synthesis in terms of substrate availability. Furthermore, as the Mehler reaction is one of the major sources of ROS in the plant cell, an active GSH metabolism in this compartment is required to cope with this type of stress (Noctor and Foyer, 1998). Plastids also hold a number of thioredoxins and glutaredoxins, possibly able to efficiently transmit redox signals to the GCL enzyme (Foyer and Noctor, 2003; Lemaire, 2004). It therefore could be speculated that the redox regulation of plant GCL evolved in parallel to its plastidic localization.

However, it should be noted that one of the GCL sequences from *Physcomitrella* (#1 according to Tables 3.7 and A1) apparently lacks a transit peptide (Table 3.7; Appendix, Figure A4), while showing conservation of the CC2 cysteines and dimer interface residues, and the predictions are not clear for the proteins from green algae.

5 Material and Methods

5.1 Plant and Bacterial Culture

5.1.1 Plant material and Plant Cell Cultures

Tobacco plants (*Nicotiana tabacum* L. cv SNN) were grown on soil under standard greenhouse conditions.

Beta vulgaris hairy root cultures, transformed with either *GFP* or *BjGCL* were provided by the RooTec AG (Witterswill). Cultures were grown in 3.2 g/l Gamborg B5 medium (Serva) with 3 % sucrose and, in the case of plate culture 0.8 % plant agar (Duchefa), pH 5.8. Plate cultures were incubated in the dark at 22 °C and liquid cultures at 25 °C and 90 rpm shaking. Hairy root cultures were transferred to new plates once per month or grown for one to two weeks in liquid culture for feeding experiments.

Suspension cell cultures were incubated at 25 °C and 90 rpm shaking and transferred to new medium once per week. Approximately 5 g cells were incubated in 100 ml medium.

Medium for *Arabidopsis thaliana*: 4.3 mg/l MS medium (Serva), 20 g/l sucrose, 1 mg/l 2,4 D, 1 ml/l vitamin stock solution (per 50 mg: 5 g inositol, 25 mg nicotinic acid, 25 mg pyridoxine, 25 mg thiamine), pH 5.7

Medium for *Beta vulgaris*: 3.2 g/l Gamborg B5 medium (Serva), 0.5 g/l caseine hydrolysate, 20 g/l sucrose, 0.2 mg/l kinetin, 0.5 mg/l NAA, 0.5 mg/l IAA, 2 mg/l 2,4 D), pH 5.5

5.1.2 Bacterial strains

For plant transformation and cloning of the *AtuGSH1* gene *Agrobacterium tumefaciens* str. C58C1 carrying the T_i-plasmid pGV2260 was used.

For all cloning purposes *Escherichia coli* strain XL1 blue (Stratagene) was used. Genotype: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F'proAB lacI^q ZΔM15 Tn10(Tet^r)]^c

For production of recombinant protein the following strains were used:

- B834 [DE3] (Novagen, Madison, USA), methionine-deficient. Genotype: F⁻ *ompT hsdSB*(r_B⁻ m_B⁻) *gal dcm met*
- M15 [pREP4] (Qiagen, Hilden, Germany), reducing cytoplasm. Genotype: *NaI^S, Str^S, Rif^S, Thi⁻, Lac⁻, Ara⁺, Gal⁺, Mit⁻, F⁻, RecA⁺, Uvr⁺, Lon⁺*
- Rosetta gami [DE3] (Novagen, Madison, USA), oxidizing cytoplasm and pRARE-plasmid coding for tRNAs rare in *E.coli*. Genotype: *Δara-leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL F0[lac+(lacIq)pro] gor522 ::Tn10 trxB ::kan pRARE*

For cloning of the *XcaGSH1* gene *Xanthomonas campestris* pv. *Campastris* str. ATCC 33913 was acquired from the German collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany).

5.1.2.1 Bacterial culture media and growth conditions

Glucose Yeast Extract-medium (*Xanthomonas* culture):

20 g/l glucose, 10 g/l yeast extract, 20 g/l CaCO₃ (precipitates); for plate cultures 17 g/l agar was added to the medium. *Xanthomonas campestris* was grown at 28 °C and, in case of liquid culture, 200 rpm shaking.

Low Salt Luria Bertani (LS-LB)-medium (*E.coli* culture):

10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0; for plate cultures 20 g/l agar was added to the medium. After autoclaving, appropriate antibiotics were added (see 4.1.2 and 4.2.1, the strain specific antibiotics were used for production of competent cells only). *Escherichia coli* was grown at 37 °C and, in case of liquid culture, 200 rpm shaking.

Medium A (Minimal medium for production of Se-Met labelled protein)

For 1 l: 100 ml M9 medium (10x), 10 ml trace element solution (100x), 20 ml 20% (w/v) glucose, 1 ml 1 M MgSO₄, 0.3 ml 1 M CaCl₂, 1 ml Biotin (1 mg/ml), 1 ml Thiamin (1 mg/ml)

M9 medium (10x), for 1 l: 80 g Na₂HPO₄, 40 g KH₂PO₄, 5 g NaCl, 5 g NH₄Cl

Trace element solution (100x), for 1 l: 5 g EDTA, 0.83 g FeCl₃ x 6 H₂O, 84 mg ZnCl₂, 13 mg CuCl₂ x 2 H₂O, 10 mg CoCl₂ x 6 H₂O, 10 mg H₃BO₃, 1.6 mg MnCl₂ x 6 H₂O

SOC-medium (Recovery of bacteria after transformation by electroporation):

20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2,5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 10 mM glucose, pH 7.0

Terrific Broth (TB)-medium (Overexpression of recombinant proteins in *E.coli*):

For 3 l the following components were autoclaved separately and then mixed:

300 ml TB-A: 0.17 M KH₂PO₄ (6.9 g), 0.72 M K₂HPO₄ (49.2 g)

2,700 ml TB-B: 36 g tryptone, 72 g yeast extract, 12 ml glycerol

Escherichia coli were grown at 37 °C and 200 rpm shaking. After induction of overexpression incubation temperature was reduced to 28 °C.

YEB-medium (*Agrobacterium* culture):

1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0.493 g/l MgSO₄ x 7 H₂O, pH 7.5; for plate cultures 20 g/l agar was added to the medium. After autoclaving, appropriate antibiotics were added (see 4.1.2 and 4.2.1). *Agrobacterium tumefaciens* was grown at 28 °C and, in case of liquid culture, 200 rpm shaking.

5.1.2.1.1 List of Antibiotics used

Table 5.1: Antibiotics used in this work

Antibiotic	Abbreviation	Concentration	Solvent	Selection
Ampicillin	Amp	100 µg/ml	Water	Plasmids (see 4.2.1)
Carbenicilline	Carb	50 µg/ml	Water	Ti-Plasmid pMP90
Chloramphenicol	CM	34 µg/ml	Ethanol	pRARE plasmid
Rifampicin	Rif	50 µg/ml	Methanol	Agrobacterium (genomic)
Spectinomycine	Spec	100 µg/ml	Water	pK7FWG2 plasmid
Tetracycline	Tet	12.5 µg/ml	70 % Ethanol	E.coli XL1 blue

5.1.2.1.2 Preparation of Glycerol Stocks

For long term storage of bacterial strains glycerol stocks were prepared by adding 200 μ l of glycerol to 800 μ l of liquid culture. Immediately after mixing the glycerol stock was shock frozen in liquid nitrogen and afterwards stored at -80°C .

5.1.2.2 Production of Competent Cells for Electroporation

For the preparation of electrocompetent bacterial cells bacteria 50 ml overnight culture was diluted in 1 l of standard medium with appropriate antibiotics (see 4.1.2.1) and incubated until reaching an OD_{600} of 0.7. The culture was cooled on ice and centrifuged for 15 min at 4000 rpm and 4°C . The pellet was washed two times in cold water and once in 40 ml of 10 % glycerol and finally resuspended in 4 ml of 10 % glycerol. Aliquots of 50 μ l were frozen in liquid nitrogen and stored at -80°C .

Transformation efficiency was determined by transformation with 10 pg of pUC19 vector and counting of the resulting colonies. Competent cells had transformation efficiencies of at least 2×10^8 cfu/ μ g DNA.

5.1.2.3 Transformation of bacteria

For transformation by electroporation 50 μ l of competent bacteria (see 5.1.2.2) were thawed on ice and 0.5 to 1 μ l of plasmid or ligation reaction was added. The sample was electroporated in a Gene Pulser II (Bio Rad) at 1.6 kV, 200 W and 25 μ F. Immediately afterwards the bacteria were taken up in 1 ml SOC medium (see 5.1.2.1) and incubated for 1 hour at 37°C (*Escherichia coli*) or for 2 hours at 28°C (*Agrobacterium tumefaciens*) before plating on appropriate medium with antibiotics. In case of selection for ampicillin-resistance this incubation was omitted.

5.2 Nucleic Acid Methods

5.2.1 List of Plasmids

Tab.5.2 of the basic plasmids used in this work, including references and applications. The constructs created from these are described in the appropriate sections. See Paragraph 5.1.2.1.1 for antibiotic abbreviations.

Tab. 5.2: Plasmids used in this work

Plasmid	Resistance	Provider/Reference	Application
pCR2.1	Amp/Kan ^a)	Invitrogen	TA Cloning
pDONR201	Kan	Invitrogen	Entry vector for GATEWAY cloning
pETM-20	Amp	(Hothorn et al., 2003)	Protein Overexpression
pK7FWG2	Spec/Kan ^b)	(Karimi et al., 2002)	Binary vector for plant transformation
pQE30	Amp	QIAGEN	Protein Overexpression

^a bacterial ampicillin and kanamycin resistance

^b bacterial spectinomycin resistance, plant kanamycin resistance

5.2.2 List of Oligonucleotides

All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany) and dissolved in water or TE buffer for a final concentration of 100 mM and stored at -20°C. Restriction sites are underlined.

Tab. 5.3 Oligonucleotides used in this work. Restriction sites (Res. Site) are underlined, bases changed in comparison to the wild type are printed in bold italics for mutagenesis primers.

Nr.	Name	Sequence	Res. Site
BjGCL cloning, sequencing and mutagenesis			
<i>B1</i>	BjGSH1-C341S	5'-CGGCAAATACGTT <i>TGACTCT</i> ACTGGAATGACATTTTCG-3'	HincII
<i>B2</i>	BjGSH1-C341S_R	5'-CGAAATGTCATTCCAGTAG <i>GAGTCAAC</i> GTATTTGCCG-3'	HincII
<i>B3</i>	BjGSH1-C356A	5'-GCTGGAAAGCTTCCT <i>GCTCTCCC</i> GGGTGAACTGCCTAC-3'	SmaI
<i>B4</i>	BjGSH1-C356A_R	5'-GTAGGCAGTTCAC <i>CCCGGG</i> GAGAG <i>GC</i> CAGGAAGCTTTCCAGC-3'	SmaI
<i>B5</i>	BjGSH1-Y186E	5'-CTGAAGTCAACTCACAC <i>CTCGAG</i> CAGGTCAAAGCTGTCGCT-3'	XhoI
<i>B6</i>	BjGSH1-Y186E_R	5'-CAGCGACAGCTTTGACCTG <i>CTCGAG</i> GTGTGAGTTGACTTCAG-3'	XhoI
<i>B6</i>	BjGSH1-D250N	5'-GTACTGTT <i>CAGGTTAA</i> CCTGAAT TTTAGCTCAGAAGCTGATATG-3'	HincII
<i>B7</i>	BjGSH1-D250N_R	5'-CATATCAGCTTCTGAGCTAAA AT <i>TCAGGTTAA</i> CCTGAACAGTAC-3'	HincII
NtGSH1 RACE, sequencing and cloning			
<i>N1</i>	NtGSH15'RACE	5'-AACAATTGCCAAGCGTCCTCGCCTA-3'	-
<i>N2</i>	NtGSH15'RACEnested	5'-TGCTCGTGATGCTGCTGTGTCACT-3'	-
<i>N3</i>	NtGSH13'RACE	5'-TAGGCGAGGACGCTTGGCAATTGTT-3'	-
<i>N4</i>	5'NtGSH1otAflIII	5'-ACGT <i>ACATGTGGG</i> CTGCAAGTCCTCCAACAG-3'	AflIII
<i>N5</i>	3'NtGSH1XhoI	5'-ACGT <i>CTCGAGT</i> CAGTAGAGAAGCTCCTCAAAG-3'	XhoI
<i>N6</i>	5'GATEWAYNtGSH1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGA TGGCCTTGATGTCTCAGGCAG-3'	-
<i>N7</i>	3'GATEWAYNtGSH1	5'-GGGGACCAGTTTGTACAAGAAAGCTGGGTTAGTAG AGAAGCTCCTCAAAG-3'	-
<i>N6</i>	NtGSH1seq	5'-CTGGAACCTGGTGGTCAGTT-3'	-
Cloning and sequencing of proteobacterial GCL			
<i>P1</i>	5'AtuGSH1_BamHI	5'-CATGGATCCATGGCACGCGACACGAC-3'	BamHI
<i>P2</i>	3'AtuGSH1_KpnI	5'-CATGGTACCTCAATACTGATATTCCTCGAA-3'	KpnI
<i>P3</i>	5'XcGSH1_NcoI	5'-CATCCATGGATATGTCGAGTCCCAGCCACGTT-3'	NcoI
<i>P4</i>	3'XcGSH1_XhoI	5'-CAT <i>CTCGAG</i> CCGCACGGCTCACTGACT-3'	XhoI

5.2.3 DNA Methods

5.2.3.1 Extraction of Genomic DNA from Bacteria

Bacterial DNA was extracted according to (Maloy, 1990) 1.5 ml of bacterial culture was pelleted by centrifugation, resuspended in 467 μ l of TE buffer and incubated for 1 h with 30 μ l 10 % SDS and 3 μ l of 20 mg/ml proteinase K. Proteins were extracted twice with phenol/chloroform and the DNA from the aqueous phase was precipitated with 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The pellet was washed in 70 % ethanol and resuspended in TE buffer.

5.2.3.2 Extraction of Plasmid DNA from Bacterial Culture

Plasmids were extracted from bacterial culture using the Plasmid Miniprep kit (Machery/Nagel) following manufacturers instructions or according to the following protocols.

1-5 ml of overnight culture was pelleted (1 min at 15,000 x g) and resuspended in 125 μ l GTE buffer (50 mM glucose, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). 250 μ l of lysis buffer was added (0.1M NaOH, 1% SDS, always prepared fresh) and the sample mixed by inverting 6 to 8 times. To neutralize the sample and precipitate proteins and genomic DNA 185 μ l KAcetate (3 M, pH 4.3) was added and the sample mixed again by inverting. After centrifugation (15 min, 15,000 x g, 4 °C) the supernatant was transferred to a new Eppendorf tube and mixed with 1 volume Isopropanol. The plasmid DNA was pelleted by centrifugation (15 min, 15,000 x g, 4 °C) and washed with 750 μ l of ethanol (70 %). After 3 min of centrifugation the supernatant was discarded, the pellet was dried and finally resuspended in 25 μ l water or TE buffer or directly in a restriction reaction master mix (See 5.2.3.6).

For quick plasmid preparation for PCR checks 50 μ l overnight culture or part of a bacterial colony resuspended in 50 μ l TE was mixed with equal volume of chloroforme/isoamylalcohol (24:1) by vigorous vortexing. After centrifugation (10 min, 15,000 x g) 1 μ l of the aqueous phase was used as PCR template.

5.2.3.3 Determination of Nucleic Acid Concentrations

DNA or RNA concentration was determined measuring the absorbance at 260 and 280 nm. According to Lambert-Beer's law ($E = \epsilon * c * d$, with $\epsilon_{DNA} = 20 \mu\text{l}/\mu\text{g cm}$, $\epsilon_{RNA} = 25 \mu\text{l}/\mu\text{g cm}$). Purity was controlled by calculation the ratio of OD_{260}/OD_{280} which ideally should be 1.8 to 2.0.

5.2.3.4 Nucleic Acid Gel Electrophoresis

5.2.3.4.1 Agarose Gel Electrophoresis

For agarose gel electrophoresis DNA samples were mixed with ¼ volume of 5x DNA loading buffer (5x TAE, 50 % glycerol, OrangeG) and run on gels of 0.7 to 1.5 % agarose in TAE. Gels were run at 70 to 90 V and afterwards stained in 1 mg/l ethidiumbromide and documented with a HeroLab E.A.S.Y. RH-3 system.

50x TAE buffer: 121 g/l Tris-Base, 68 g/l NaAcetate x 3 H₂O, 9.3 g/l EDTA, pH 7.2

5.2.3.4.2 Polyacrylamide Gel Electrophoresis

Small sized DNA fragments were analyzed by native PAGE on gels of 11.25 % polyacrylamide. Gels were run at 200 to 230 V and documented as described in paragraph 4.2.3.4.1

Recipe per gel:

3 ml water, 2 ml buffer (1.5 M Tris-Base, pH 8.8), 3 ml acrylamide solution (29.2 % (w/v) acrylamide, 0.8 % bisacrylamide), 30 µl 10 % ammoniumperoxidosulfate, 15 µl TEMED

Native running buffer: 3.6 g/l Tris-Base, 14.4 g/l glycine, pH 8.6

5.2.3.5 Polymerase Chain Reaction

For the specific amplification of DNA polymerase chain reaction was performed using JumpStart Taq (Sigma-Aldrich) as a non-proof-reading polymerase or Vent Polymerase (New England Biolabs) or Phusion Polymerase (Finnzymes, purchased from New England Biolabs) as proof-reading polymerases. PCR samples were prepared as follows (all amounts given in µl):

Tab. 5.3: Preparation of PCR samples

Polymerase	Stock	JumpStart Taq	Vent Polymerase	Phusion
Buffer ^{a)}	10x	5	5	5
MgSO ₄ ^{b)}	50 mM	-	1	-
Primer (left)	50 mM	1	1	1
Primer (right)	50 mM	1	1	1
dNTP solution	10 mM each ^{c)}	1	1	1
Polymerase	as supplied	0.25	0.25	0.25
Template	varying	varying	varying	varying
Water	bidest	ad 50	ad 50	ad 50

^{a)} buffers were used as supplied with the polymerase

^{b)} Mg²⁺ was already included in JumpStart Taq and Phusion polymerase buffers

^{c)} dATP, dCTP, dGTP and dTTP

PCR was conducted according to the following program:

Initial denaturing phase (1x):

2 to 5 min 94 °C (98 °C for Phusion polymerase)

PCR cycles (35x):

30 s 94 °C (15 s 98 °C for Phusion polymerase)

30 s 52 °C (Temperature varied depending on primers)

varying 72 °C

(time: 1 min/kB for Taq and Vent, 30 s/kB for Phusion polymerase)

Final elongation Phase (1x):

5 to 10 min 72 °C

5.2.3.5.1 PCR-based Site Directed Mutagenesis

The protocol used for site-directed mutagenesis was based on the QuickChange mutagenesis kit (Stratagene, La Jolla, CA).

A plasmid containing the gene of interest was amplified by PCR, using two complementary primers including the site to be mutagenized, an additional silent mutation introducing a restriction site for check-digestion of the resulting plasmid and 15 to 20 perfectly matching bases up- and down-stream of the mutagenesis site. PCR was performed with elongation times allowing complete amplification of the plasmid. After PCR, the sample was incubated digested with 1 µl of DpnI at 37°C for 1 hour to digest the methylated template plasmid. Afterwards competent cells were transformed from the sample.

5.2.3.5.2 Purification of PCR or restriction digested DNA fragments

DNA was purified using the NucleoSpin® Extract II Kit (Machery-Nagel) for direct purification or the QIAquick® Gel extraction kit (QIAGEN) according to the provider's instructions.

5.2.3.6 Restriction digestion

All restriction digests were conducted with enzymes from New England Biolabs in the recommended buffers. 2 to 3 units per µg DNA and enzyme were used and incubated for 1 to 16 hours at the recommended temperature and fragments purified as described under 5.2.3.3.1.

5.2.3.7 Ligation of DNA fragments

For the ligation of fragments into plasmids 1 µl of plasmid (100 ng) was mixed with 1 µl T4-Ligase (NEB), 1 µl buffer, a three to tenfold molar excess of insert and the sample filled to 10µl with water. For ligation of PCR fragments equimolar concentrations of both fragments were used. Ligation reactions were incubated at 100 cycles of 30 s at 4 °C and 30 s at 30 °C each, followed by a denaturing phase of 65 °C for 30 min.

For direct cloning of PCR fragments the TA-cloning kit (Invitrogen) was used according to the supplier's instructions.

5.2.3.8 Cloning of DNA fragments by GATEWAY® cloning

For recombination-based cloning of PCR fragments the GATEWAY®-system (Invitrogen) was used according to the supplier's instructions, where all samples were prepared with one fourth of the volume given proposed in the manual.

PCR-fragments containing the appropriate recombination sites were purified by PEG-MgCl₂ as described in the GATEWAY® manual.

5.2.3.9 DNA Sequencing

All sequencing reactions were conducted as Extended Hot-Shot® sequencing by the SeqLab Sequencing Laboratories (Göttingen, Germany).

5.2.3.10 Cloning of GSH1 Genes from different organisms

5.2.3.10.1 Cloning and Mutagenesis of *Brassica juncea* GCL

BjGCL (acc AJ563921) had previously been PCR-amplified using sense primer 5'ACTGCCATGGGGGCGGCGAGTCCTCCCAC-3' and anti-sense primer 5'-TAAGTCGACTCAGTAAAGCAGTTCCTGGAA-3', thereby omitting the predicted plastidic transit peptide (residues 1-65). Subsequently, the NcoI/SalI-excised fragment was ligated into vector pETM20 (Wachter, 2004).

Site-directed mutagenesis was performed using the QuickChange Mutagenesis kit (Stratagene) according to the manufacturers instructions. For the mutant BjGCL-C341S primers B1 and B2 were used, for BjGCL-C356A primers B3 and B4, BjGCL-C341S/C356A was created using primers B3 and B4 on the BjGCL-C341S mutant. BjGCL-Y186E was created using primers B5 and B6 and BjGCL-D250N with primers B7 and B8.

5.2.3.10.2 Cloning of *Nicotiana tabacum* GCL

RNA from *Nicotiana tabacum* cultivar SNN was prepared using the RNeasy kit (Qiagen, Hilden, Germany). Full length *NtGSH1* for overexpression of the corresponding protein was amplified using primers N4 and N5, digested with AflIII and XhoI, and ligated into the pETM-20 vector. For transient overexpression in tobacco, *NTGSH1* was amplified using primers N6 and N7 and cloned via Gateway cloning (Invitrogen) into the vector pK7FWG2 (Karimi et al., 2002) to produce a N-terminal fusion to the gene coding for enhanced GFP.

The full-length mRNA sequence of *NtGSH1* was deposited in the database as accession number DQ444219.

5.2.3.10.3 Cloning of proteobacterial GCL genes

The *Agrobacterium tumefaciens* γ -GCL (*AtuGCL*) gene was PCR amplified according to the NCBI database entry AE009034 (bases 651-2024, complement), using the primers P1 and P2. The product was purified, digested with *Bam*HI and *Kpn*I and cloned into the pQE-30 vector (Qiagen, Hilden, Germany) to produce a protein fused to a 6xHis-tag.

The *Xanthomonas campestris* γ -GCL (*XcaGCL*) gene was PCR amplified according to the NCBI database entry AE012458 (bases 3888-5252, complement), using the primers P3 and P4, substituting the wildtype start-TTG by ATG. The product was purified, digested with

NcoI and *XhoI* and cloned into the pETM-20 vector (Hothorn et al., 2003) to produce a protein fused to thioredoxin and a 6xHis-tag for increased solubility.

5.2.4 RNA Methods

5.2.4.1 Extraction of Total RNA from Plant Tissue

RNA from *Nicotiana tabacum* cultivar SNN was prepared using the RNeasy kit (Qiagen, Hilden, Germany).

5.2.4.2 Reverse Transcription for cDNA Production

To produce cDNA the Omniscript® RT Kit (QIAGEN) was used according to the provider's instructions.

5.2.4.3 Rapid Amplification of cDNA Ends (RACE)

cDNA for RACE-PCR was produced and the 5'- and 3'-ends of the NtGSH1-gene were amplified using the GeneRacer kit (Invitrogen).

For the *NtGSH1* gene primers (N1) and (N2) were generated based on the EST sequence BP137080 and used for 5' RACE as gene specific first PCR and nested PCR primers, respectively. For 3' RACE the reverse complements of the same primers were used. PCR was conducted as described under 5.3.2.2 and the resulting amplicate cloned into the pCR2.1 vector (Invitrogen) as described under 5.3.2.5.

5.3 Protein Methods

5.3.1 Production of Recombinant Protein in *E. coli*

Recombinant protein was expressed in *E. coli* Rosetta gami DE3 (Novagen, Madison, WI, oxidizing cytosol) or *E. coli* M15[pREP4] (Qiagen, Hilden, Germany, reducing cytosol).

Cells grown to an OD_{600nm} of 1.5 were induced with 1 mM isopropyl-b-D-thiogalactopyranoside in Terrific Broth at 28 °C for 18 h. Pelleted cells were resuspended in lysis buffer (50 mM Na-Phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 50 mM glutamate, 25 µg/mL DNase I) and lysed with an EmulsiFlex-C5 (Avestin Inc., Ottawa, Canada). The suspension was centrifuged at 22,000 x g for 30 min and purified by Ni²⁺ affinity chromatography (Qiagen, Valencia, CA). The column was washed and the protein eluted by applying increasing concentrations (10 to 200 mM) of imidazole. For BjGCL NtGCL and XcaGCL the eluted fusion protein was concentrated, dialyzed against 50 mM Na-Phosphate (pH 8.0), 200 mM NaCl and cleaved with recombinant tobacco etch virus (TEV) protease for 12 h at 4 °C. GCS was separated from the 6xHis tagged protease and thioredoxin

by a second Ni²⁺ affinity step. Pure recombinant GCS was dialyzed against 20 mM HEPES (pH 8.0) and 50 mM glutamate.

5.3.1.1 Production of Seleno-methionine-labelled Protein

Seleno-methionine-labelled protein was produced in the methionine heterotrophic *E. coli* strain B834(DE3). Bacteria transformed with the pETM-20::BjGSH1 construct were incubated over night in 5 ml Medium A plus 50 µg/ml methionine at 37 °C, 200 rpm shaking. This culture was diluted in 1 l Medium A plus 50 µg/ml methionine and grown to an *OD*₆₀₀ of 0.6. Cells were pelleted by centrifugation and resuspended in Medium A without methionine. After incubation for 4 hours at 37 °C 50 µg/ml seleno-methionine was added and after another 30 min incubation expression of the recombinant protein was induced by addition of 1 mM IPTG at 28 °C. After induction purified as described under 5.3.1.

5.3.2 Preparation of Soluble Protein from Plant Tissue

For the extraction of soluble protein from plant tissue 50 to 250 mg of frozen ground tissue was mixed with 3.75 µl/mg of extraction buffer (10 mM HEPES/KOH, 250 mM sorbitol, 10 mM MgCl₂, 10 mM KCl, 1 mM PMSF, pH 7.1) by vigorous vortexing. After centrifugation (15,000 x g, 30 min, 4 °C) the supernatant was transferred to a new reaction tube. For gel electrophoresis the sample was mixed with 1/3 volume of 4x reducing or non-reducing loading buffer (Roti-Load, Roth) and denatured by heating to 94 °C for 7 min.

5.3.2.1 Crude plastid preparation for protease assay

200 mg of *Arabidopsis* leaves were ground in 1 ml of extraction buffer (10 mM HEPES/KOH, 250 mM sorbitol, 10 mM MgCl₂, 10 mM KCl, pH 7.1) and centrifuged for 2 min at 2,000 x g. The supernatant was transferred to a new tube and centrifuged for 2 min at 6,000 x g and the pellet resuspended in 200 µl of extraction buffer + 0.1 % Triton X-100.

5.3.3 Protein Extraction from Bacteria

For crude protein extraction from *Agrobacterium* and *Xanthomonas* 1 ml of bacterial liquid culture was pelleted by centrifugation (1 min, 15,000 x g), the pellet resuspended in 50 µl loading buffer (Roti-Load, Roth) and denatured by heating to 94 °C for 7 min.

5.3.4 Determination of Protein Concentration

Protein concentration was determined using the method of Bradford (Bradford, 1976). Protein-Assay solution (Bio-Rad) was diluted five-fold with water and 0.8 ml were mixed with appropriate amounts of protein solution. After 5 to 10 min incubation the *OD*₅₉₅ was measured and the protein concentration calculated from comparison to a standard curve generated with bovine serum albumine (BSA).

5.3.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein was analyzed electrophoretically by discontinuous SDS-PAGE (Laemmli, 1970). The stacking gel was prepared with 4.5 % (w/v) and the separating gel with 11 % (w/v) acrylamide. Gels were run at 100 V in the stacking and at 200 to 230 V in the separating gel. Gels were either directly stained with Coomassie Brilliant Blue or used for Western blotting. Samples for SDS-PAGE were denatured by addition of a 4x concentrated SDS-sample buffer containing a reducing agent (Roti-Load1, Roth) and boiling for 5 min at 95 °C. For the conservation of disulfide bridges, the non-reducing Roti-Load2 was used.

5.3.6 Analysis of protein by Immunoblotting

Immunoblotting was performed using the semidry procedure and a 1:5,000 dilution of an antiserum raised against *Brassica juncea* GSH1-1 (Wachter et al., 2005). 20 µl of total bacterial protein extract and adequately diluted recombinant protein were loaded on a 11 % SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane (Immobilon; Millipore, Billerica, MA, USA) at 3.5 mA/cm² and 15 V for 45 min. After blocking with 5 % low fat milk powder in TBST for 1 h, the membrane was incubated with the primary antiserum in a 1:10,000 dilution in 5 % BSA (in TBS) at 4 °C for 12 h. Immunoblots were developed with anti-rabbit IgG-horse radish peroxidase conjugate (Sigma-Aldrich, Munich, Germany) and subjected to enhanced chemiluminescence detection (Super Signal West Dura; Pierce, Rockford, IL, USA), according to the manufacturer's protocol.

TBS (10x): 58.4 g/l NaCl, 24.2 g/l Tris-base, pH 7.5 (HCl)

TBST (10x): As TBS plus 5 g/l Tween-20

5.3.7 Enzymatic Characterization of GCL Protein

Pure samples of recombinant protein were analysed in a coupled enzymatic assay as described (Abbott et al., 2001). A standard reaction mixture (0.5 ml) contained 100 mM Tris (pH 8.0), 150 mM KCl, 20 mM MgCl₂, 10 mM L-cysteine, 20 mM L-glutamate, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 5 units type II rabbit muscle pyruvate kinase and 10 units type II rabbit muscle lactic dehydrogenase (chemicals and enzymes were purchased from Sigma, St. Louis, MO). Reactions were initiated by addition of GCL (100–400 ng). The resulting decrease of OD_{340nm} was followed and steady-state kinetic parameters were determined by initial velocity experiments. For determining K_m , substrate concentrations were varied from 0.2 to 20 mM (cysteine), 0.5 to 25 mM (glutamate) or 1 to 10 mM (ATP) while keeping the other component concentrations constant. Kinetic parameters were calculated to fit data to $v = [S]/(K_m + [S])$ using the Solver function of Microsoft Excel.

5.3.8 Analysis of Protein Folding and Oligomerization State

5.3.8.1 Size-exclusion chromatography

Analytical size-exclusion chromatography for BjGCL was performed at EMBL using a Superdex 200 HR 10/30 column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated in

50 mM HEPES (pH 8.0), 50 mM NaCl and 50 mM L-glutamate. Fifty micro liters of the sample (10 mg/mL) were loaded onto the column and elution at 0.8 mL/min was monitored by ultraviolet absorbance at 280 nm.

For reduction experiments, half of the C341S or C356A mutant protein from one preparation was loaded on the column equilibrated in 50 mM HEPES (pH 8.0), 50 mM NaCl and 50 mM L-glutamate, whereas the other half was extensively dialyzed over night against 50 mM HEPES (pH 8.0), 50 mM NaCl, 50 mM L-glutamate and 5 mM TCEP or 5mM DTT, respectively and then applied to the column equilibrated in the reducing buffer. For re-oxidation the reduced protein was dialyzed over night against 50 mM HEPES (pH 8.0), 50 mM NaCl and 50 mM L-glutamate and then reapplied to the column equilibrated in the oxidizing buffer.

Size exclusion chromatography for other GCL proteins was conducted at HIP. Protein was run on a Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 50 mM HEPES, 1 mM EDTA, pH 8.0 at 1 ml/min and protein in the flowthrough detected via its absorption at 280 nm.

5.3.8.2 Circular dichroism (CD) spectroscopy

CD spectroscopy was conducted at EMBL and was performed on a temperature controlled J-710 spectropolarimeter (Jasco, Easton, MD) at a cell-path of 0.2 cm and at a sensitivity of 50 mdeg using protein samples at a concentration of 0.25 mg/mL, dialyzed against 5 mM Na Pi (pH 8.0), 5 mM NaCl.

5.3.9 Protease stability assay

For the protease stability assay, 50 µg of recombinant BjGCL were incubated in 2 ml of buffer (100 mM Tris, 150 mM KCl, 20 mM MgCl₂, 5 mM ATP, pH 8.0) with or without 5 mM DTT for 2 hours. Digestion was started by addition of with 0.5 µg of proteinase K (Sigma) or 50 µl of crude plastid preparation (see 5.3.2.1) and the reaction was stopped by denaturing aliquots in sample loading buffer (Roti Load, Roth).

5.4 Other methods

5.4.1 Extraction and analysis of thiols

5.4.1.1 Thiol extraction from plant tissue

For thiol extraction approximately 30 mg of ground plant material was taken up in 1 ml of extraction buffer (0.1 M HCl, 1 mM EDTA, 4 % PVP), vortexed and centrifuged at 15,000 x g and 4 °C for 30 min. For derivatization, 100 µl of the supernatant was mixed with 100 µl of CHES-buffer (500 mM, pH 9.4), 20 µl monobromobimane (30 mM, in acetonitril) and 20 µl DTT (10 mM). After 15 min of incubation at room temperature in the dark, the reaction was stopped by addition of 800 µl acetic acid (10 %).

5.4.1.2 Thiol extraction from bacteria

Thiol determination was conducted as described (Newton et al., 1996) with minor changes. 15 to 50 µg of frozen bacteria were dissolved by vortexing in 0.5 ml of warm (60 °C) aqueous solution of 50 % acetonitrile, 20 mM Tris-HCl, pH 8.0, 4 mM monobromobimane (mBBR, Fluka, St.Gallen, CH) and 5 mM dithiothreitol. The mixture was incubated for 20 min at 60 °C in the dark, then proteins were precipitated by addition of 2.5 µl of 2.5 M HClO₄ and centrifugation at 15,000 x g for 10 min. Samples were stored at – 20 °C.

5.4.1.3 HPLC analysis of derivatized thiols

Derivatized thiols were analyzed by reverse phase HPLC (Kontron Instruments 322 pump system/360 autosampler) on a C-18 column (Nova-Pak; pore size 4 µm). Separated fluorescent thiol-bimane conjugates were detected with 380 nm excitation at an emission wavelength of 480 nm on an attached fluorescence detector (FP1520-S Intelligent Fluorescence Detector, Jasco). Samples were in a gradient of 10 % to 90 % Methanol, 2.5 % acetic acid pH 4.3. Data acquisition and processing were performed by Kroma System2000 software (Kontron). Identification of peaks and quantification was conducted by comparison to pure standards of derivatized cysteine, γ-glutamylcysteine and glutathione.

5.4.2 Statistical analysis

For statistical analysis of data Student's t-test was conducted. *t* was calculated as:

$$t = \frac{m_1 - m_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

(*m*: mean values, *σ*: standard deviations, *n*: number of replicates)

Significance of differences was determined by comparing *t* to the quantile *u_α* with results considered as significant with a probability of error (*P*) smaller than 1-*α* for *t* > *u_{1-α}* for one-sided hypothesis (*m*₁ > *m*₂ or *m*₁ < *m*₂) or *t* > *u_{1-α/2}* for two-sided hypothesis (*m*₁ ≠ *m*₂) (Tab. 5.X).

Tab. 5.X: Quantiles of the standard distribution function

<i>α</i>	<i>u_α</i>	<i>α</i>	<i>u_α</i>
0.9999	3.7190	0.9900	2.3263
0.9995	3.2905	0.9500	1.6449
0.9990	3.0902	0.9000	1.2816
0.9950	2.5758	0.5000	0.0000

Adapted from (Elpelt and Hartung, 2004)

5.4.3 Sequence analysis of plant and bacterial GCL

For computational analysis of GCL genes, sequences were retrieved via from a BLAST search directed against the NCBI database (<http://www.ncbi.nlm.nih.gov/>, ; Altschul et al.,

1997) and all sequences with more than 50% identity to *Brassica juncea* GSH1 (40 alpha-proteobacterial, 6 gamma-proteobacterial, 2 other proteobacterial and 13 plant sequences, including NtGCL) were included into further analysis. In case of several entries for one genus only the one with the highest homology to BjGCL was used for further analysis. To allow the analysis of more plant GSH1 sequences an additional BLAST search was conducted directed against the plant EST sequence database. From the results of this search 11 additional full-length sequences could be assembled. For a complete list of sequences used see Tables A1 and A2 in the appendix.

GCL sequences were aligned on the protein level using the ClustalW algorithm (Chenna et al., 2003) and residues important for catalytic function were identified by comparison to *Brassica juncea* GCL (Hothorn et al., 2006).

For prediction of GCL localization, plant GCL protein sequences were analyzed using the targetP program (Emanuelsson et al., 2000).

Tree building was done by running a neighbour joining analysis as implemented in the program PAUP4.0beta (Swoffod, 2002). For the bootstrap analysis the optimality criterion was set to distance. The number of bootstrap replicates was 100. Trees were unrooted.

6 Abbreviation Index

(not including SI units, chemical elements, nucleic acid bases and amino acids)

AA	Ascorbic acid
ABC	ATP binding cassette
At	<i>Arabidopsis thaliana</i> (genes or proteins)
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
Amp	Ampicillin
APS	5'Adenylylsulfate
APX	Ascorbic acid peroxidase
ATP	Adenosine-5'-triphosphate
Atu	<i>Agrobacterium tumefaciens</i> (genes or proteins)
AU	Arbitrary units
Bj	<i>Brassica juncea</i> (genes or proteins)
BLAST	Basic Local Alignment Search Tool
β-ME	β-mercaptoethanol
Bp	Base pairs
BSA	Bovine serum albumine
BSO	Buthionine sulfoximine
Bv	<i>Beta vulgaris</i> (genes or proteins)
$c(x)$	concentration of substance x
<i>cad</i>	cadmium sensitive (<i>Arabidopsis</i> PCS and GCL mutants)
CaMV	cauliflower mosaic virus
Carb	Carbenicillin
cDNA	complementary DNA
cfu	colony forming unit(s)
CHES	2-(Cyclohexylamino)ethansulfonic acid
CLSM	Confocal Laser Scanning Microscope/Microscopy
CM	Chloramphenicol
C-terminal	Carboxy-terminal
d	diameter
DHA	Dehydroascorbic acid
DHAR	DHA reductase
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide (dATP, dCTP, dGTP and/or dUTP)
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ϵ_x	specific extinction coefficient of substance x
E	Extinction
E_{redox}	redox potential
E^0_{redox}	standard redox potential
EC	Enzyme Commission number
EDTA	Ethylenediamine tetraacetic acid
EMBL	European Molecular Biology Laboratory

Abbreviation Index

ER	Endoplasmatic Reticulum
EtBr	Ethidium bromide
<i>F</i>	Faraday constant
Fig.	Figure
x g	multiple of standard terrestrial gravity (9.81 m/s ²)
GCL	γ -Glutamylcysteine Ligase
γ EC	γ -Glutamylcysteine
GFP	Green Fluorescent Protein
GR	Glutathione reductase
GS	Glutathione Synthetase
GSH	Glutathione (reduced)
GSH1	γ -Glutamylcysteine Synthetase (= GCL)
GSH2	Glutathione Synthetase (= GS)
GSSG	Glutathione disulfide (oxidized)
GST	Glutathione S-transferase
HEPES	N-(2-Hydroxyethyl)piperazine-2-ethanesulfonic acid
HPLC	High pressure liquid chromatography
IPTG	Isopropylthiogalactoside
Kan	kanamycin
kB	kilo base pairs
kDa	kilo Daltons
K_m	Michaelis Menten constant
LMW	Low molecular weight protein marker (Amersham)
LS-LB	low salt Luria-Bertani medium
M	molar (1 M = 1mol/l)
mBBr	Monobromobimane
MCS	multiple cloning site
MDHA	Monodehydroascorbic acid
MDHAR	MDHA reductase
mRNA	messenger RNA
NAD(P) ⁺	Nicotine Adenine Dinucleotide (Phosphate), oxidized
NAD(P)H	Nicotine Adenine Dinucleotide (Phosphate), reduced
NCBI	National Center for Biotechnology Information
n.d.	not detected <i>or</i> not determined (see specific description)
NEB	New Englang Biolabs (corporation)
N-terminal	Amino-terminal
NTP	Nucleotide Triphosphate (ATP, CTP, GTP and/or TTP)
$OD_{x\ nm}$	Optical Density at x nm wavelength
<i>P</i>	probability of error (statistical analysis)
PAA	Polyacrylamide
<i>pad2-1</i>	phytoalexin-deficient (GSH1 mutant)

Abbreviation Index

PAGE	PAA gel electrophoresis
PC	phytochelatine
PCR	Polymerase Chain Reaction
PCS	Phytochelatine Synthase
pH	negative decadic logarithm of $[H^+]$
P_i	inorganic phosphate
PMSF	Phenylmethanesulfonylfluoride
psi	pounds per square inch
<i>R</i>	universal gas constant
RACE	Rapid Amplification of cDNA ends
<i>rax1-1</i>	regulator of APX (GSH1 mutant)
Rif	Rifampicin
<i>rm11</i>	root meristem less (GSH1 mutant)
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RT	room temperature
RT-PCR	Reverse transcription followed by PCR
SAT	Serine Acetyl Transferase
<i>S.D.</i>	Standard deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
Strep	Streptomycine
<i>T</i>	temperature in Kelvin
Tab.	Table
TAE	Tris acetate EDTA buffer
TB	Terrific Broth medium
TBS	Tris buffered saline buffer
TBST	TBS + Tween
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TE	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5)
TEMED	N,N,N',N'Tetramethylethylamine
Tet	Tetracycline
TEV	tobacco etch virus
T_i	tumor inducing (plasmid)
Tris	2-amino-2-hydroxymethyl-1,3-propanediol (trishydroxymethylaminomethane)
TP	targeting peptide
UTR	untranslated region
Vol.	volume
wt	wildtype
w/v	weight per volume (where 1 % w/v is 10 g/l)
Xca	<i>Xanthomonas campestris</i> (genes or proteins)

7 Literature

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6 Appendix

6.1 Sequence data for NtGCL

Figure A1: Full-length sequence of the *NtGCL* mRNA. The coding sequence is printed bold. The full length cDNA sequence has been deposited in the NCBI database as accession number DQ444219.

GAAATTTTTCTTTCAATCTGCTTCAGTACTCACTCCCAAGGCTCAACAGAATAAAAAGAAAGGTAAAAAAAAGCA
 AAGAAATTAAGACATGGCAATCAAAGAGTTGTAGCCGCCCCAGCAGCCAAAAAATTATTTTTGTGACACCCAA
 TTACTATATATATCTTTGAAAACTTCCCTCAATTTCCAGTCCAAACCTACAAAATTGTCTGTTTTCTTTTTACTGA
 GCCAAGGCACAAAGAATGGCCTTGATGTCTCAGGCAGGTTCTTACATTGCATTTACTCTGAAAAGATGAAATGT
ATAAGTGGACACAGCAGCATCACGAGCAATATGGAGATGCTCAAAATGAAGGACATATGCTTTGGCAATATTTCT
TCACGGAATTCCTCAAAACCAATGCAAGGGATTTACTTTGGACCGCGTTGGGGTGAACGTAGGCGAGGACGCTTG
GCAATTGTTGCTGCAAGTCTTCCAACAGAGGATGCCGTTGTTGCAGCAGAGCCATTAACAAAAGAAGACCTTGTA
GCTTATCTTTGCTTCTGGATGCAAAATCCAAGAAAAATGGAGGATAGGCACTGAACATGAAAAGTTTGGTTTCGAG
TTTGGAACTCTGCGACCCATGAAGTATGAACAAATAGCTGAATTACTAAATGGTATTTGCCGAGCGATTTGATTTGG
GAAAAAGTAATGGAGGGTGACAACATTATTGGCTTGAAACAGGGAAAGCAAAGCATATCACTGGAACCTGGTGGT
CAGTTTTGAGCTTAGTGGTGCACCCTTGAAACACTGCATCAAACTTGTGCAGAGGTTAATTCACATCTTTACCAG
GTTAAAGCTGTTGCAGAGGAAATGGGAATTGGATTCTTAGGAACTGGATTCCAGCCAAAGTGGGGGCTGAAAGAT
ATACCAGTAATGCCGAAGGGGAGATACGAGATTATGAGAAATTACATGCCCAAAGTTGGCTCACTTGGACTAGAT
ATGATGTTTGAACATGCACTGTTTCAAGTAAATCTGGACTTCAGTTCTGAAGCTGACATGATCAGAAAAGTTTCGT
GCTGGTCTTGCCTTGCAGCCTATTGCTACAGCTCTCTTTGCTAATTCACCTTTCCTGAAGGAAAACCTAATGGT
TATCTCAGCATGAGAAGCCACATTTGGACCGATACAGATAATAACCGTGCCGGGATGCTTCCCTTCGTCTTTGAT
GACTCTTTTGGGTTTGGAGCAGTATGTGGATTATGCACCTTGACGTCCCTATGTATTTTGTCTATCGGAAGAAG
TATATTGATTGTGCTGGAATGTCTTTCCGGGACTTCATGAATGGAAAACCTTCCCTTATTCCTGGCGACTACCTT
ACTCTTAATGATTGGGAGAATCACCTCACAACAATATTTTCTGAGGTGAGACTCAAAAAGATATCTGGAATGCGA
GGTGCCGATGGAGGACCTTGGAGAAGGTTGTGTGCATTGCCTGCATTCTGGGTGGGTATACTCTACGATGAGGTG
TCTTTGCAAACCGTTTTGGATATGACGTCTGATTGGACTGCAGAAGAAAGAGAGATGTTGAGGAATAAGGTGCCA
ACCAGCGGTTTGAAGACACCATTTGAGATGGATTGCTTAAGCATGTTGCTCAAGATGTTGTCAAGTTGGCAAAG
GAAGGCTTGGAGAGAAGAGGCTATAAGGAAACAGGATTTTTGAACGAAGTAACCGAGGTAGTCAGAACAGGTGTA
ACACCAGCTGAGAAGCTCCTGGAATTGTACCATGGGAAGTGGGGACGAAGCGTGGATCCAGTCTTTGAGGAGCTT
CTCTACTGAAGTTATTTGAGAGTGCATTTCAATCCAGTCATTTCTATGTTGTAATGATTTCTCAATTTCTTTT
 TGAGAGTTATATCTGCGAATTTTGTCTATGATGAGACAAATAATCTAGCTAGATTAAGATCTGTTACATCTATCA
 TATGGCATGAAACGTCATATGTAGCTCTCACCTTGGATCTTATCAATCTGCTTCCAAATAGCAATTTTATACACT
 GGGACATACGTTTTTCCAGAAGAGTAACATAGCCATGCAAAATGAAATAAATGAACTTTGGCTGAAAAAAAAA
 AAAAAAAAAAAAAA

Figure A2: Full-length sequence of the NtGCL protein. The plastidic transit peptide as determined by TargetP prediction is underlined.

MALMSQAGSSHCIYSEKMKCISGHSSITSNMEMLKMKDICFGNISSRNSSKPMQGIYLDRVG
VERRRGR LAI VAAS PPTEDAVVAAEPLTKEDLVAYLASGCKSKEKWRIGTEHEKFGFEFGTL
 RPMKYEQIAELLNGIAERFDWEKVMEDNI IGLKQKQSI SLEPGGQFELSGAPLETLHQTC
 AEVNSHLYQVKAVAEEMGIGFLGTGFQPKWGLKDI PVMPKGRYEIMRNYPKVGSLGLDMMF
 RTCTVQVNLDFSSEADMIRKFRAGLALQPIATALFANS PFTEGKPNGYLSMRSHIWTDTDNN
 RAGMLPFVFD DSFGFEQYVDYALDVP MYFVYRKKKYIDCAGMSFRDFMNGKLSPI PGDYPTL
 NDWENHLTTIFPEVRLKRYLEM RGADGGPWRRLCALPAFWVGILYDEVSLQTVLDMTSDWTA
 EEREMLRNKVPTSG LKTPFRDGLLKHVAQDVVKLAKEGLERRGYKETGFLNEVTEVVRTGVT
 PAEKLLLELYHGK WGRSVDPVFEELLY

6.2 GCL sequences used for *in silico* analysis

Table A1: Plant GCL sequences

Species	Family/Order	Accession number(s) ^{a)}
<i>Allium cepa</i>	Alliaceae/Asparagales	AAL61610
<i>Aquilegia formosa x Aquilegia pubescens</i>	Ranunculaceae/Ranunculales	DR913245 - DR918827 - DR918828
<i>Arabidopsis thaliana</i>	Brassicaceae/Brassicales	AAN28909
<i>Beta vulgaris</i>	Chenopodiaceae/Caryophyllales	none ^{b)}
<i>Brassica juncea</i>	Brassicaceae/Brassicales	CAA71801
<i>Chorisporea bungeana</i>	Brassicaceae/Brassicales	ABM46854
<i>Citrus sinensis</i>	Rutaceae/Sapindales	CF838565 - CX049544 - DR908254 - CX049543
<i>Gossypium raimondii</i>	Malvaceae/Malvales	CO112812 - CO116286 -CO130243 - CO112811
<i>Lactuca perennis</i>	Asteraceae/Asterales	DW091328 - DW094108 - DW087296
<i>Lotus japonicus</i>	Fabaceae/Fabales	AAO27827
<i>Lycopersicon esculentum</i>	Solanaceae/Solanales	AAB71230
<i>Malus domestica</i>	Rosaceae/Rosales	CO066139 - CO867638 -EB148218 - CN995967 - DR998671
<i>Medicago trunculata</i>	Fabaceae/Fabales	AAC82334
<i>Nicotiana tabacum</i>	Solanaceae/Solanales	ABD98695
<i>Oryza sativa</i>	Poaceae/Poales	CAD48599
<i>Phaseolus vulgaris</i>	Fabaceae/Fabales	AAF22136
<i>Physcomitrella patens 1</i>	Funariaceae/Funariales ^{c)}	Phypa1_1:70546 ^{d)}
<i>Physcomitrella patens 2</i>	Funariaceae/Funariales ^{c)}	Phypa1_1:146491 ^{d)}
<i>Physcomitrella patens 3</i>	Funariaceae/Funariales ^{c)}	Phypa1_1:173526 ^{d)}
<i>Pisum sativum</i>	Fabaceae/Fabales	AAF22137
<i>Popula trichocarpa x deltoids</i>	Salicacaceae/Malpigiales	DT503226 - DT504859 - DT506856
<i>Solanum tuberosum</i>	Solanaceae/Solanales	BQ045799 - CV302325 - BM111889
<i>Taraxacum officinale</i>	Asteraceae/Asterales	DY841453 - DY826048 - DY841453
<i>Triticum aestivum</i>	Poaceae/Poales	CJ560929 - CJ670946 - CK204295
<i>Vitis vinifera</i>	Vitaceae/Rhamnales	GSVIVP00000418001 ^{e)}
<i>Zea mays</i>	Poaceae/Poales	CAC83005
<i>Zinnia elegans</i>	Asteraceae/Asterales	BAD27390
Green Algae		
<i>Chlamydomonas reinhardtii</i>	Chlorophyceae/Chlamydomonales	Protein ID: 181975 ^{d)}
<i>Ostreococcus lucimarinus</i>	Prasinophyceae/Mamiellales	XP_001418312
<i>Ostreococcus tauri</i>	Prasinophyceae/Mamiellales	Ostta4:15784 ^{d)}
<i>Prototheca wickerhamii</i>	Chlorellaceae/Chlorellales	AY616091
<i>Volvox carteri</i>	Chlorophyceae/Chlamydomonales	Volca1:104352 ^{d)}

^{a)} according to NCBI, in case of more than one accession number given, the full-length sequence was assembled from EST sequences ^{b)} Gromes, 2004 and Müller, 2006

^{c)} belongs to the Bryophyta (Mosses) and has three *GCL* genes

^{d)} DOE Joint Genome Institute (www.jgi.doe.gov) ^{e)} Genoscope (www.genoscope.cns.fr)

Table A2: Proteobacterial GCL sequences

Species	Accession	Species	Accession
α-Proteobacteria			
<i>Acidiphilium cryptum</i>	ZP_01144393	<i>Agrobacterium tumefaciens</i>	NP_531356
<i>Aurantimonas sp.</i>	ZP_01227851	<i>Bartonella quintana</i>	CAF25799
<i>Bradyrhizobium sp.</i>	ZP_00861439	<i>Brucella melitensis</i>	AAL53770
<i>Caulobacter sp.</i>	EAU09715	<i>Dinoroseobacter shibae</i>	ZP_01583544
<i>Erythrobacter litoralis</i>	YP_457472	<i>Fulvimarina pelagi</i>	ZP_01438843
<i>Gluconobacter oxydans</i>	AAW61713	<i>Granulibacter bethesdensis</i>	YP_746008
<i>Hyphomonas neptunium</i>	YP_760091	<i>Jannaschia sp.</i>	YP_511851
<i>Loktanella vestfoldensis</i>	ZP_01003184	<i>Magnetospirillum magneticum</i>	YP_423386
<i>Maricaulis maris</i>	ZP_01395103	<i>Mesorhizobium loti</i>	BAB53101
<i>Nitrobacter hamburgensis</i>	YP_575610	<i>Novosphingobium aromaticivorans</i>	YP_496228
<i>Oceanicaulis alexandrii</i>	ZP_00953035	<i>Oceanicola batsensis</i>	ZP_00998061
<i>Paracoccus denitrificans</i>	ZP_00631260	<i>Parvibaculum lavamentivorans</i>	ZP_01659605
<i>Parvularcula bermudensis</i>	ZP_01018087	<i>Rhizobium etli</i>	YP_468341
<i>Rhodobacter sphaeroides</i>	ZP_00915169	<i>Rhodopseudomonas palustris</i>	NP_946175
<i>Rhodospirillum rubrum</i>	YP_425661	<i>Roseobacter denitrificans</i>	YP_684207
<i>Roseovarius nubinhibens</i>	ZP_00959350	<i>Sagittula stellata</i>	ZP_01746232
<i>Silicibacter pomeroyi</i>	AAV96849	<i>Sinorhizobium medicae</i>	ZP_01414144
<i>Sphingomonas sp.</i>	ZP_01302692	<i>Sphingopyxis alaskensis</i>	YP_617319
<i>Stappia aggregata</i>	ZP_01548031	<i>Sulfitobacter sp.</i>	ZP_00953876
<i>Xanthobacter autotrophicus</i>	ZP_01195801	<i>Zymomonas mobilis</i>	AAF18278
γ-Proteobacteria			
<i>Alkalilimnicola ehrlichei</i>	ZP_00864771	<i>Halorhodospira halophila</i>	ZP_01151267
<i>Nitrococcus mobilis</i>	ZP_01126885	<i>Stenotrophomonas maltophilia</i>	ZP_01645073
<i>Xanthomonas campestris</i>	AAM42666	<i>Xylella fastidiosa</i>	AAF84237
Unclassified Proteobacteria			
<i>Magnetococcus sp.</i>	EAN29119	<i>Mariprofundus ferrooxydans</i>	ZP_01451910

6.3 Conservation matrices

Table A3. Conservation matrix for the dimer interface residues in plant GCLs.

Brassica residue	E133	F135	E136	Q176	N182	Y186	E193	W394	R395	K471	F475	Residues conserved
<i>Allium</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Aquilegia</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Arabidopsis</i>	1	1	1	1	1	1	1	1	1	1	1	11
<i>Beta</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Brassica</i>	1	1	1	1	1	1	1	1	1	1	1	11
<i>Chorispora</i>	1	1	1	1	1	1	1	1	1	1	1	11
<i>Citrus</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Gossypium</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Lactuca</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Lotus</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Lycopersicon</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Malus</i>	1	0.1	0.1	1	1	1	1	1	1	1	1	9.2
<i>Medicago</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Nicotiana</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Oryza</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Phasaeolus</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Physcomitrella 1</i>	0.1	1	0	1	1	1	1	1	1	0.1	1	8.2
<i>Physcomitrella 2</i>	0	1	1	0	0	0	0	0	0	0	0	2
<i>Physcomitrella 3</i>	0.1	1	0	1	1	1	1	1	1	0.1	1	8.2
<i>Pisum</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Populus</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Solanum</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Taraxacum</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Triticum</i>	1	1	1	1	1	1	1	1	1	1	1	11
<i>Vitis</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
<i>Zea</i>	1	1	0.1	1	1	1	1	1	1	1	1	11
<i>Zinnia</i>	1	1	0.1	1	1	1	1	1	1	1	1	10.1
conserved in species	24	26	5	26	26	26	26	26	26	24	26	
Exchanges	2xD	1xY	21xD	-	-	-	-	-	-	2xN	-	
Green algae												
<i>Chlamydomonas</i> *	0	1	1	0	1	1	1	1	1	0	1	8
<i>Ostreococcus</i>	1	0	0	1	0	0	0	0	0	0	0.1	2.1
<i>Prototheca</i>	0.1	1	1	0	0	0	0.1	1	0	0	0.1	3.1
<i>Volvox</i> *	?	?	?	0	1	1	1	1	1	0	1	6/3?

This table lists conservation of the residue in comparison to BjGCL as 1, conservative exchanges as 0.1, and non-conservative exchanges as 0, ? indicates that the database entry for the *Volvox* GCL appears to be truncated. The total number of residues conserved per protein is highlighted in grey (right), as is the number of proteins in which each residue is conserved (below). Conservative exchanges are listed in the bottom row.

* Note that *Chlamydomonas* and *Volvox* GCL have the CC2 cysteines

Table A4. Conservation matrix for the dimer interface residues in proteobacterial GCLs. (next page)

This table lists conservation of the residue in comparison to the plant BjGCL enzyme as 1, conservative exchanges as 0.1 and non-conservative exchanges as 0. The total number of residues conserved per protein is highlighted in grey (right), as is the number of proteins in which each residue is conserved (below). Conservative exchanges are listed in the bottom row. γ -proteobacterial species are marked by an asterisk following the name; all other species belong to the α -proteobacteria.

Appendix

Table A4. Conservation matrix for dimer interface residues in proteobacterial GCLs.

	E133	F135	E136	Q176	N182	Y186	E193	W394	R395	K471	F475	Residues conserved
Alkalilimnicola *	0	1	0	1	0	0	0	1	0	0	1	4
Halorhodospira *	0	1	0	0	0	0	0	1	1	0	0	3
Nitrococcus *	0	1	0	1	1	0	0.1	1	0	0	1	5.1
Stenotrophomonas *	0	1	0	1	0	0	0	1	0	0	1	4
Xanthomonas *	0	1	0	0	0	0	0	1	0	0	1	3
Xylella *	0	1	0	1	0	0	0	0	0	0	1	3
Magnetococcus #	0.1	0	0	0	1	0	0	0	0	0	1	2.1
Mariprofundus #	0	0	1	0	0	0	0	1	0	0.1	1	3.1
Acidiphilium	0	0	0	0	0	0	1	0	0	0	0	1
Agrobacterium	1	0	0	1	1	0	1	1	1	0	1	7
Aurantimonas	0	0	0	0	1	0	0	1	1	0	1	4
Bartonella	0	0	0	0	0	0	0	1	0	0	1	2
Bradyrhizobium	0	0	0	1	0	0	0	1	0	0	0	2
Brucella	0	0	0	1	1	0	1	1	1	0	1	6
Caulobacter	0	1	0	0	0	0	0.1	1	0	0	0	2.1
Dinoroseobacter	0.1	0	0	0	1	0	1	1	1	0	1	5.1
Erythrobacter	0	1	0	1	0	0	1	1	0	0	1	5
Fulvimarina	0	0	0	0	1	0	0	1	1	0	1	4
Gluconobacter	0	0	0	0	0	0	0	0	0	0	0	0
Granulibacter	0	0	0	1	0	0	0	0	0	0	0	1
Hyphomonas	0.1	1	0	0	0	0	1	1	0	0	1	4.1
Jannaschia	0.1	1	0.1	1	1	0	0.1	1	1	0	1	6.3
Loktanelia	0	1	0	1	1	0	0.1	1	1	0	1	6.1
Magnetospirillum	0	0	0	1	0	0	0	1	1	0	0	3
Maricaulis	0	1	0	1	1	0	0.1	1	0	0	1	5.1
Mesorhizobium	0	0	0	1	1	0	1	1	1	0	1	6
Nitrobacter	0	0	0	1	0	0	0	1	0	0	0	2
Novosphingobium	1	0	0	1	0	0	0.1	1	0	0	0	3.1
Oceanicaulis	0	1	0	0	1	0	0.1	1	0	0	1	4.1
Oceanicola	0.1	0	0	1	1	0	0.1	1	1	0	1	5.2
Paracoccus	0	1	0	1	1	0	0.1	1	1	0	1	6.1
Parvibaculum	0	1	0	1	0	0	0	0	1	0	0	3
Parvularcula	0	0	0	0	0	0	1	1	0	0	1	3
Rhizobium	0	0	0	1	1	0	1	1	1	0	1	6
Rhodobacter	0.1	1	0	0	0	0	1	0	0	0	0	2.1
Rhodopseudomonas	0	0	0	1	0	0	0	1	0	0	0	2
Rhodospirillum	0	1	0	0	0	0	0	1	0	0	1	3
Roseobacter	0.1	1	0	0	1	0	0.1	1	1	0	1	5.2
Roseovarius	0.1	0	0	0	1	0	0.1	1	1	0	1	4.2
Sagittula	0.1	0	0	1	1	0	0	1	1	0	1	5.1
Silicibacter	0.1	0	0	0	1	0	0.1	1	1	0	1	4.2
Sinorhizobium	0	0	0	1	1	0	1	1	1	0	1	6
Sphingomonas	0	1	0	1	0	0	0.1	1	0	0	0	3.1
Sphingopyxis	0	1	0	1	0	0	0	1	0	0	1	4
Stappia	0	1	0	0	0	0	1	1	1	0	0	4
Sulfitobacter	0.1	0	0	0	1	0	0.1	1	1	0	1	4.2
Xanthobacter	0	0	0	0	1	0	1	1	0	0	1	4
Zymomonas	0	1	0	0	0	0	0.1	0	0	0	0	1.1
conserved in species	2	22	1	25	22	0	13	40	21	0	33	
conservative	11xD		1xD		15xD			1xN				

Description see previous page

Arabidopsis	-----AASP
Chorispora	-----AASP
Brassica	-----AASP
Pisum	-----AASP
Medicago	-----AASP
Lotus	-----AASP
Phaseolus	-----AASP
Populus	-----AASP
Lactuca	-----AASP
Taraxacum	-----AASP
Zinnia	-----AASP
Lycopersicon	-----AASP
Solanum	-----
Nicotiana	-----AASP
Citrus	-----AASP
Gossypium	-----AASP
Malus	-----TASP
Aquilegia	-----AASP
Vitis	-----AASP
Beta	-----AASP
Allium	-----SASP
Oryza	-----AASP
Zea	-----AASP
Triticum	-----AASP
Physcomitrella1	-----MRTGIGKGE-----VIGSP
Physcomitrella2	-----MGLFSKALGGLRRVPCGSPGEGPCRRRTPCIVAASP
Physcomitrella3	-----
Volvox	-----
Chlamydomonas	-----MALASGVGRRQHVSASPSRSRGVPSPR-----LSPVH
Prototheca	MSCMLEKHWDAGSAAPSVSTTRPHRSAPVEVETGPVPPINRVLFYIHIMC
Ostreococcus_lucimarinus	-----MTATTARGDALRVGTATRRGRRATRRATT-----
Ostreococcus_tauri	-----
Xanthomonas	-----MS
Stenotrophomonas	-----MRCKAPSPTSPATVQRVLPPLS
Xylella	-----MS
Oceanicaulis	-----MS
Maricaulis	-----MS
Alkalilimnicola	-----MS
Halorhodospira	-----MS
Nitrococcus	-----MT
Rhodospirillum	-----MS
Parvibaculum	-----MS
Sphingopyxis	-----MREMS
Novosphingobium	-----MS
Sphingomonas	-----MS
Erythrobacter	-----MS
Zymomonas	-----MS
Roseobacter	-----MS
Sulfitobacter	-----MS
Sagittula	-----MS
Silicibacter	-----MS
Roseovarius	-----MS
Jannaschia	-----MS
Dinoroseobacter	-----MS
Oceanicola	-----MS
Loktanella	-----MS
Paracoccus	-----MS
Rhodobacter	-----MS
Parvularcula	-----MT
Hyphomonas	-----MT
Caulobacter	-----MA
Bradyrhizobium	-----MA
Nitrobacter	-----MA
Rhodopseudomonas	-----MLPAAASTTSRRPIPRASSGLTMA
Xanthobacter	-----MA
Mesorhizobium	-----MA
Brucella	-----MA
Rhizobium	-----MA
Agrobacterium	-----MA
Sinorhizobium	-----MA
Bartonella	-----MA
Aurantimonas	-----MA
Fulvimarina	-----MA
Stappia	-----MA
Magnetospirillum	-----MLSRIIPPMMSA
Acidiphilium	-----MSN
Granulibacter	-----MSN
Gluconobacter	-----MPPEGDRIVEPATVQSLSNNEFRPRPNAMSN
Mariprofundus	-----MN
Magnetococcus	-----MCEKFEYVSACCRRAMTAPLLAPITAFVPLAKAFKS
Nostoc	-----
Nodularia	-----
Anabaena	-----
Crocospaera	-----
Thermosynechococcus	-----
Mycobacterium	-----

6.4 Alignment of plant and bacterial GCL sequences

Figure A3 (this pages and following pages): Alignment of plant and proteobacterial GCL protein sequences, including all sequences included in Tables A1 and A2 and listed on the next page. Plant transit peptides excluded.

Appendix

Arabidopsis	PTEEAV--VATEPLTREDLIAYLASGCKTKDKYRIGTEHEKFGFV----
Chorispora	PTEEAV--VATEPLTREDLIAYLASGCKSKDKYRIGTEHEKFGFV----
Brassica	PTEEAV--VATEPLTREDLIAYLASGCKSKEKWRIKTEHEKFGFV----
Pisum	PTEDAV--VATEPLTKQDLIDYLASGCKPKDKWRIKTEHEKFGFEL----
Medicago	PTEDAV--VATEPLTKQDLIDYLASGCKTKDKWRIKTEHEKFGFEL----
Lotus	PTEDAV--VATEPLTKQDLVDYLASGCKPKQNWRIKTEHEKFGFEL----
Phaseolus	PTEDAV--VATDPLTKQDLVDYLASGCKPREKWRIKTEHEKFGFEL----
Populus	PTEDAV--VATEPLTKEDLIRYLASGCKPKENWRIKTEHEKFGFEL----
Lactuca	PTEDAV--VATEPLTKEDLVGYLASGCKPKENWRIKTEHEKFGFEL----
Taraxacum	PTEDAV--VATDPLTKEDLVGYLASGCKPKENWRIKTEHEKFGFEL----
Zinnia	PTEDAV--VATEPLTKEDLVGYLASGCKPKENWRIKTEHEKFGFEL----
Lycopersicon	PTEDAV--VAAEPLTKEDLVGYLASGCKSKEKWRIKTEHEKFGFEL----
Solanum	-----AAEPLTKEDLVGYLASGCKTKEKWRIKTEHEKFGFEL----
Nicotiana	PTEDAV--VAAEPLTKEDLVGYLASGCKSKEKWRIKTEHEKFGFEL----
Nicotiana	PTEDAV--VVTEPLTKEDLVGYLASGCKPKEKWRIKTEHEKFGFEL----
Citrus	PTEDAV--IATEPLTKQDLVDYLASGCKSKEKWRIKTEHEKFGFEL----
Gossypium	PTEDAV--IATEPLTKEDLVGYLASGCKSKEKWRIKTEHEKFGFEL----
Malus	PTEDAV--IATEPLTKEDLVGYLASGCKSKEKWRIKTEHEKFGFEL----
Aquilegia	PTEDAV--IATEPLTKEDLVGYLASGCKSKEKWRIKTEHEKFGFEL----
Vitis	PTEDAV--VATEPLTREDLIGYLASGCKPKEKWRIKTEHEKFGFEL----
Beta	PTEDSV--VAADPLTKEDLVNYLASGCKPKQKWRIKTEHEKFGFEL----
Allium	PTEDAV--VMAEPLTKEDLVGYLASGCKTKDKWRIKTEHEKFGFEL----
Oryza	PTEEAV--QMTEPLTKEDLMAYLVSGCKPKENWRIKTEHEKFGFEL----
Zea	PTEEAV--QMTEPLTKEDLVGYLASGCKPKENWRIKTEHEKFGFEL----
Triticum	PTEEAA--KLTEPLTKEDLVGYLASGCKPKENWRIKTEHEKFGFEL----
Physcomitrella1	PSNDAG--SYGPELTRQDLVGYLASGCKPKEKWRIKTEHEKFGFEL----
Physcomitrella2	PNNNAGGSRSGEPLTRQDLVGYLASGCKPKEKWRIKTEHEKFGFEL----
Physcomitrella3	-----DLVDFLRSCKPKSEWRIGTEHEKLGFLQ----
Volvox	-----
Chlamydomonas	ANAPAVAEFRTEPLKQELVDYLYKSGCRPRSAWRIKTEHEKLGFLN----
Prototheca	TDKVAIPVGAAPHLLTLEDLVNLRKGGCKPRSSWRIGTEAREVGVRLR----
Ostreococcus_lucimarinus	TTIRAAATAPARAVTRECFAVFRGCKPREAFRIKTEHEKFGYDE----
Ostreococcus_tauri	-----RIGTEHEKLGFLD----
Xanthomonas	SPSHVAET---PI TERAELIQVLASGEKPGADWRIKTEHEKFGFRL----
Stenotrophomonas	SPSHVADT---PITDRSQLVAELIASGEKPRQWRIKTEHEKFGFRL----
Xylella	SPSHITET---PITNRAQLVEVLASGEKPSQWRIKTEHEKFGFRL----
Oceanicaulis	GTYNAGGEG--APIESKAQLIGHIAGEKPKDQWRIKTEHEKFGFAL----
Maricaulis	GTYNPGGEG--APIESLDQLTAYFAAGEKPDFAFRIGTEHEKFGFAL----
Alkalilimnicola	APDEAAVA--EPVTRKQALVDWVAAGCKPPEDWRIKTEHEKFGVHR----
Halorhodospira	G-VSPQPG--EPVTDVAQLAEHLRGRSPPQWRIKTEHEKFGVHHL----
Nitrococcus	QAKASVSE--EPITNPRQLVYLVYLRGSKAEAHWRIKTEHEKFGFRL----
Rhodospirillum	SLTDASILS--APLT--RDDLVAHMAGGSRPKADWRIKTEHEKYAFTI----
Parvibaculum	ILVAG---P-EPITDRDLVAALERGNPKSDWRIKTEHEKFPFCV----
Spingopyxis	TRTASDRHD--PVIEHRDQLAAPMADGCKPKERWRIKTEHEKFGVYR----
Novosphingobium	TRQVSDRND--PLIESRDMVLVAPMOKGEKPSAWRIKTEHEKFGVYRT----
Spingomonas	TRTDSAGHD--PVIESRDQLIAAFKAGEKPKDRWRIKTEHEKFGVYDR----
Erythrobacter	TREDSGAD--PIIESRDQLVAPMAGEKPKDRWRIKTEHEKLVYKR----
Zymomonas	TRQTSSSQN--HPIESRDLLRIFQAGEKPKAQWRIKTEHEKLVYK----
Roseobacter	IPQSGG---GPITSYDQVMVGYLASGCKPKEDWRIKTEHEKFGYCK----
Sulfitobacter	IPQSGG---GPISYDQVLVNYLADGCKPKEDWRIKTEHEKFGYCK----
Sagittula	IPQSGG---GPIESFDQMDYLASGCKPKEDWRIKTEHEKFGYCK----
Silicibacter	IPQSGG---GPIERHEQLAEYLASGCKPKSEWRIGTEHEKFGFCK----
Roseovarius	IPQSGG---GPIEHHSQALAEYLSGCKPRDAWRIKTEHEKFGYCR----
Jannaschia	IPQSGG---GPIEHHSQALAEYLSGCKPKADWRIKTEHEKFGFCR----
Dinoroseobacter	IPQSGG---GPIESRAQLAEYLSGCKPKADWRIKTEHEKFGYCK----
Oceanicola	IPQSGG---GPIERPEQLAEYLSGCKPKEDWRIKTEHEKFGYCR----
Loktanelia	IPQSGG---GPIEHHDQLAEYLSGCKPKADWRIKTEHEKFGYCA----
Paracoccus	IPQSGG---GPIERPEQLAEYLSGCKPRESWRIKTEHEKFGYRH----
Rhodobacter	IPQSGG---GPIENFEQLAEYLSGCKPASDWRIGTEHEKFGYLT----
Parvularcula	QLDASAREDLPIEDVAALSAYLASGCKPVAEWRIKTEHEKFGFLT----
Hyphomonas	TPTSDIDEASPRISGKHELVYLEGGSKPASDWRIGTEHEKFGFIW----
Caulobacter	EAMSQE---RPLTLDLITAYFAQGSKPKAEFRVGAHEKFGFYL----
Bradyrhizobium	RDQIDM---TPLQSRDELVAWLEAGVKPPSAYRIKTEHEKTPFTL----
Nitrobacter	RDQIDM---TPLQSRNELVAWLEAGVKPASEFRIGTEHEKTPFTL----
Rhodopseudomonas	RDQLDM---TPLNSRDELVAWLEAGVKPASEFRIGTEHEKTPFTL----
Xanthobacter	RDTLDA---TPIAGRHELVWMEQGSKPERAFRIKTEHEKTPFTL----
Mesorhizobium	RDTTDF---RPIEGVDELVDHLAEGNKPRDKWRIKTEHEKFPFYV----
Brucella	RDTTDE---TALTSVEELATYLAGGCKPKDAWRIKTEHEKFPFYT----
Rhizobium	RDTTDQ---TPLSSVQELTDYIAAGNKPPERFRIGTEHEKFAFFR----
Agrobacterium	RDTTDQ---TPLSSVTELTLDYLAAGCRAEADFRIGTEHEKFAFFR----
Sinorhizobium	RDTTDQ---TPVTSVAELTAYLASGCKPKENFRIGTEHEKFAFFK----
Bartonella	LDTTDE---SEVYNLDSLVYFQGGCKAENDWRIGTEHEKFPFYI----
Aurantimonas	RDTTDL---TPVTSVEDLTAHLATGEKPSQFRIGTEHEKFGFYT----
Fulvmarina	RDTNSA---TPIGSIDDLTAYLKSGEKPSDFRIGTEHEKFAFYL----
Stappia	RDTVDS---TPIETVADLAATLEEGCKPKEKFLIGTEHEKFGFCL----
Magnetospirillum	PAKPSG---EPIAGKHQVLEYSLESGCKPKDAWRIKTEHEKFGFSL----
Acidiphilium	PGEADATPIDPAQPAARQLADWFAAGCKPPADFRIGTEHESFGFRR----
Granulibacter	PGDADTTPAT---TTRQLADYLAAGCRAEADFRIGTEHEKFGFSL----
Gluconobacter	PGTNTASIE---SRAQLVEAIARGCKPQSEWKIGTEHEKFGFVLPAAH----
Mariprofundus	RPLTSS---DFAAFVAVGCKPRNEWRIKTEHEKIGFCM----
Magnetococcus	WDSMSAIDLATPVTRFDQLVTYLEAGCTPKEQWRIKTEHEKFGFRK----
Nostoc	-----MVLKLG--FEIEIYGTGPQGEIVGLSD----
Nodularia	-----MYTGTGPQGEIVGLSD----
Anabaena	-----MVLKLG--FEIEIYGTGPQGEIVGLSD----
Crocospaera	-----MTRCLKG--VEVEMYGTGPQGEIVGLSD----
Thermosynechococcus	-----MLSKG--FEVELYTGKPTGEIVGLSD----
Mycobacterium	-----MPARRSAARIDFAGSPPRTLVGEWEFALVDSQT----

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences. In addition to the sequences from Tables A1 and A2 the following more distant type 3 GCL sequences are included: *Anabaena variabilis* (YP_323696), *Ostreococcus lucimarinus* (XP_001418312), *Nodularia spumigena* (ZP_01629821), *Crocospaera watsonii* (ZP_00516735), *Thermosynechococcus elongatus* (BAC07874), *Mycobacterium tuberculosis* (YP_001281721)

Appendix

Arabidopsis	---NTLRPMKYD-----QIAELLNG---IAERFEWEKVMEGDKI IGLKQ
Chorispora	---NTLRPMKYD-----QIAELLNS---IAERFEWEKVMEDDKI IGLKQ
Brassica	---NTLRPMKYD-----QIAELLNS---IAERFEWEKVMEGDKI IGLKQ
Pisum	---GSLRPVKYE-----QIAELLNA---IAERFDWEKIMEGDKI IGLKQ
Medicago	---GSLRPMKYE-----QISELLNG---IAERFDWDKIMEGDNI IGLKQ
Lotus	---GSLRPMKYE-----QIAELLNG---IAERFDWDKIMEGDKI IGLKQ
Phaseolus	---GSLRPMKYE-----QIAELLNG---IAERFDWDKIMEGDKI IGLKQ
Populus	---GTLRPMKYE-----QIAELLHG---IAERFDWDKIMEGDTI IGLKQ
Lactuca	---KTLRPMKSYG-----QIADLLNA---ISERFDWEKVMEGDNI IGLKQ
Taraxacum	---KTLRPMKSYV-----QIADLLNA---ISERFDWEKVMEGENI IGLKQ
Zinnia	---KTLRPMTYE-----QIAHLLNA---ISERFDWEKVMEGDNI IGLKQ
Lycopersicon	---GTLRPMKYD-----QIADLLNG---IAERFDWEKVMEGDKI IGLKQ
Solanum	---GTLRPMKYD-----QIADLLNG---IAERFDWEKVMEGDKI IGLKQ
Nicotiana	---GTLRPMKYE-----QIAELLNG---IAERFDWEKVMEGDNI IGLKQ
Citrus	---GTLRPMKYE-----QIAELLNS---IAERFDWEKVMEGDYI IGLKQ
Gossypium	---KTLRPMKYE-----QIAELLNG---ISERFDWEKVMEGDKI IGLKQ
Malus	---KTLRPIKYE-----QIAELLNG---ISERYDWEKVMEGDKI IGLKQ
Aquilegia	---DTLRPMKYE-----QIANLLYG---LAERFDWEKIMEGDYI IGLKQ
Vitis	---RTLPRMRYE-----QISELLYG---VSEFWDKIMEGDNI IGLKQ
Beta	---DTLRPMKYE-----QIAELLNG---IAERFDWDKVMEGDYI IGLKQ
Allium	---NTLRPMTYE-----QIAYLLNG---IAERFDWEKVMEGDYI IGLKQ
Oryza	---DTLRPIKYD-----QIRDILNG---LAERFDWDKIVEENNV IGLKQ
Zea	---DTLRPLKYD-----QIRDILNG---LAERFDWDKIMEKNNV IGLKQ
Triticum	---ETLRPITYD-----QISAILNG---LSEFWDKIMEENHVI IGLKQ
Physcomitrella1	---DNLQPMTYP-----QIKQLLEG---LADRFEWQVRMEGDNI IGLTTL
Physcomitrella2	---DNLQPMTYA-----QIRQLLEG---LADRFEWKVMEGDNI IGLTTL
Physcomitrella3	---DTLKRMTFD-----QISKLLDG---MASRFGYERVMEGENI IGLSK
Volvox	-----MEDGRI IGVSL
Chlamydomonas	---ADNSRMNYD-----QIAQVLRK---LEARFGWEPIMEEGRI IGVQL
Prototheca	---LTPCPATHQ-----QISDLLTV---IHDSNGWEYI KEGEAKI GLAH
Ostreococcus_lucimarinus	---ATKRMDYP-----VVRHVLSS---LVERHGWTPI NEADNI IGCTK
Ostreococcus_tauri	---ATLRMDYP-----VVRHVLTSS---LVDRYGWAPI YEGENI IGCTK
Xanthomonas	---DDLRPPTFD---GER-GIEALLVG---MTR-FGWEQVQENGTI ALLR
Stenotrophomonas	---DDLRPPTFE---GER-GIEALLNG---LVR-FGWAPVQENGTI ALLR
Xylella	---DDLHPPTFD---DER-GIEALLTG---MTR-FGWTPVQEHGRTI ALLR
Oceanicaulis	---KDHALPYE---GEGPSVRKLLG---LTR-FGWQAI EENGKPVALKR
Maricaulis	---GDHTPLPFR---AEGPSVEKMLSG---LER-FGWQPVREGGELI ALKR
Alkalilimnicola	---GDLSPVPYQ---GER-GIGQFLAR---LQR-FGWAPVHEGEHI IALKK
Halorhodospira	---EDFSPVPIA---GER-GIGAFLEG---LVR-FGWQPVHEDGRVIALKR
Nitrococcus	---ADRRPVSYA---GEA-GIGAFLEA---MTA-FGWEPLLEEGQI ALQR
Rhodospirillum	---ADGRPLTYE---GPH-GVRALLEG---LTR-FGWSPVYEGETVIALTC
Parvibaculum	---HNRTPIPYG---GEH-GIRAMLEG---LTR-FGWEPMEGENI IGLES
Sphingopyxis	---SDHRAPSYD---EPG-GIHDLIMA---LTR-FGWEPI EGGKVI ALAG
Novosphingobium	---DDRRAPSYT---EAG-GIRDLLMA---LTE-YGWSPIMEGDNI IAMSG
Sphingomonas	---SDHHAPSYE---EKG-GIHALLLG---LTR-FGWEPPVYEGGKI IALSG
Erythrobacter	---DGFRAPSYD---EDG-GIRDILLE---MQQ-FGWQPVVEEDGKVI ALKG
Zymomonas	---QNHQAPSYE---EKG-GICDLLQG---FTR-FGWQPI YENDKI IGLSG
Roseobacter	---DTLLPLPYE---GDRSIRAMLEG---LRDTFGWAPVSEDGKI IGLEK
Sulfitobacter	---ETLLPLPYE---GDCSIRAMLEG---LRDGHGWDPVEEGKLI GLVK
Sagittula	---DTLNPLPFE---GERSIRAVLEG---LRDRHGWAPVEEGGHI IGLTK
Silicibacter	---DTLKLPPFE---GTRSI VAVLEG---LRDRHGWAEMREGGNI IGLQK
Roseovarius	---DSLPLPYE---GERSVLAVLEG---LRDVHGWAPVEEKLI GLEK
Jannaschia	---DTLQPIPYE---GARSVRVAVLEG---LRDRFDWTPVMEGGHI IGLEK
Dinoroseobacter	---DTLKLPPYD---GTRSIKAMLEG---LRDRFGWAPVMEAGNI IGLEK
Oceanicola	---DTLQPIPYD---GERSVLTVLEG---LRDGHGWAPLEEGGKLI GLTK
Loktanelia	---DTLQPLPYD---GARSIKAVLEA---LHSRFAWDRVEEDGKI IGLTK
Paracoccus	---ADLMLPYPYAPAGQPSVKAMLEG---LQARFGWTPVLEAGHI IGLER
Rhodobacter	---SGLAPLPYE---GEASVRVAVLEG---LRDRFGWTPVMEKDAI IGLSR
Parvularcula	---DSYAPLPFS---GERSIEAVLHR---LSAQYGWTPLEHGDAI VALKK
Hyphomonas	---DGLKPLPYE---GPASILAMLEG---LRDRFAWEPVLEGGYI IGLK
Caulobacter	---KDHTVPVYE---GDRGIHALTG---LQR-FGWKPVMEGEVI IGLER
Bradyrhizobium	---DGHRPVPYE---GPRGIGALLEG---MKLLLGWEPIMERGNI IGLYD
Nitrobacter	---QGHDPVPYE---GSRGIGALLEG---MKLLLGWEPIMEQGNI IGLHD
Rhodopseudomonas	---DGHPVPYE---GARGIGALLEG---MQILLGWEPIMEGPHI IGLHD
Xanthobacter	---GRHEPVPYE---GPKGIRKLLG---MRSLLGWEPIMEGPTI IGLAD
Mesorhizobium	---DGNAPVPYQ---GERGIRALLEG---MQSKLGDWPI I DDGRI IGLVE
Brucella	---ANNSPVPYQ---GPRGIKAI LEG---MQARLGWEPIMDEGNI IGLVE
Rhizobium	---ADNSPVPYF---GDASI SALLTG---LQKKS GWEPIL DGGNI IGLAE
Agrobacterium	---KDNSPVPYF---GEASI SALLKG---MQEKL GWEPIM DGENI IGLGE
Sinorhizobium	---ADNSPVPYF---GEASIQALLNG---MAQRNGWEPIMDEGNV IGLAE
Bartonella	---DDFRPVPYE---GSRGIRALLEG---MQKALGWKPI LDGNI IGLVG
Aurantimonas	---EDLSPVPYE---GERGIGKLLER---MQALS GWEA I DDGRI IGLGF
Fulvmarina	---DDLSPVPYE---GDRGI AKI LEG---LKALSGWEA I EDDGRI IGLFN
Stappia	---NELTPVPIY---GEKVEAITG---MEKLIHWERIEDAGKI IGLAD
Magnetospirillum	---DDLKVPVYQ---GDKGIKAMLDG---LVG-LGWEPVFEEDGNI IALHD
Acidiphilium	---DNFAPPYPA---PDGIRAVLE---SVAAAEGIEPI LDAGNPI GLKG
Granulibacter	---TDLAAPPYDG---PAGIAAILEGLHTAKAAEGWTP I LDRGHSI GLKG
Gluconobacter	EGREPLSPPPYE---PRGIGALLE---KLQGPDWAPIMDGENI IGLKG
Mariprofundus	---DTFRPIPYD---GERSIRAVLER---LAGGEWIVNENGGPI IALKN
Magnetococcus	---RDLTPVPIYEG---EAGIGRLLNS---MADRYGWQRVEEDGHVIALIK
Nostoc	-----KIVAALDG-----FMREPDSRNVEYITQ
Nodularia	-----KIVASLDG-----FVREPDSRNVEYITD
Anabaena	-----KIVTQLDG-----FVREPDSRNVEYITTE
Crocospaera	-----RISKDLNG-----FVREPDSRNVEYITTA
Thermosynechococcus	-----RIVKDLPG-----FVREPDSRNVEFTTP
Mycobacterium	-----RDLNS-----EATAVIAEIGENPRVHKELL

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Chorispora	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Brassica	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Pisum	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Medicago	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Lotus	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Phaseolus	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Populus	E----KQISLLEPGGQFELSGAPVETLHQTCAEVNSHLYQVKVAEEMGI
Lactuca	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Taraxacum	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Zinnia	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Lycopersicon	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Solanum	G----KQISLLEPGGQFELSGAPVETLHQTCAEVNSHLYQVKVAEEMGI
Nicotiana	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Citrus	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Gossypium	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Malus	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Aquilegia	G----KQSVSLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Vitis	G----DQRLSLEPGGQFELSGAPLETLHQPCEVNSPLYQVKVAEEMGI
Beta	G----NQNISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Allium	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Oryza	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Zea	G----KQISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Triticum	G----KQNISLLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Physcomitrella1	D----GQVSVLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Physcomitrella2	D----GQVSVLEPGGQFELSGAPLETLHQTCAEVNSHLYQVKVAEEMGI
Physcomitrella3	D----GASVSVLEPGGQFELSGAPLKDVAHQRELDTHLEQVNIIGKELGL
Volvox	D----GQSVTLEPGGQFELSGAPVDTIHKTCAEVNSHLYQVKVAEEMGI
Chlamydomonas	D----GQSVTLEPGGQFELSGAPVETIHKTCAEVNSHLYQVKVAEEMGI
Prototheca	A----NETVTLEPGGQFELSGAPLKLHVAQETKEHLIQVRAACDKVGI
Ostreococcus_lucimarinus	D----KQSVTLEPGGQFELSGAPLENLTQTDEEVRWHIETVNALAREKGH
Ostreococcus_tauri	D----KQSVTLEPGGQFELSGAPLENLRQTDEEIRWHIETVTALAREKGH
Xanthomonas	D---GA-SVTLEPAGQFELSGAAVETLHHTCVETGTHLSEVAEVALQQL
Stenotrophomonas	D---GA-SVTLEPAGQFELSGAALETIHQTCVETGTHLNEVAQVAGELQL
Xylella	G---DT-SITLLEPAGQFELLAGAPLETIHQTHIETETHLREVNEIANTLQL
Oceanicaulis	E---GG-SITLLEPGGQFELSGAPLENLHETCREVNTHLREVREVADEVGA
Maricaulis	D---GA-SITLLEPGGQFELSGAPLETIHQTCAEVNSHLEVRVAEIGA
Alkalilimnicola	D---KG-SITLLEPGGQFELSGAPLENIHQSCREVEHLRQVDEVARELDL
Halorhodospira	E---GGASVSVLEPGGQFELSGALETLHEACVEVREHLQVDTVARELQI
Nitrococcus	G---AA-ATLLEPAGQFELSGAPLETLHQSCEEVNAHLREVRVADELGL
Rhodospirillum	P---TMGSVTLLEPGGQFELSGALETIVHGTCCVETTHRNLKIVGGELGI
Parvibaculum	GN-GERGNISLLEPGGQFELSGALETLHQTCSLHDHLAQVKEVGAEHGI
Sphingopyxis	S---DG-TVSVLEPAGQFELSGAPLENLHQTCAETGRHLKQVKEVGAELGL
Novosphingobium	P---DG-AVSVLEPAGQFELSGAPLENLHQTCAETGRHLAQVKTIGDRRLGL
Sphingomonas	S---DG-TISLLEPAGQFELSGAPLENLHQTCAETGRHLQVVKYVGDQLGL
Erythrobacter	E---DG-AVSVLEPAGQFELSGAPLENLHQTCAETGRHLQVKAIEKCGV
Zymomonas	D---DG-ATSVLEPAGQFELSGAPRSTIHESYDEICRHIQQTQEVGDELGL
Roseobacter	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNHLREVKEVADRIGV
Sulfitobacter	D---GANVSVLEPGGQFELSGAPVETIHETCDEVNHLREVRDVARIGA
Sagittula	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNHLREVKGVADELGV
Silicibacter	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNHLREVKDIADKIGV
Roseovarius	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNAHLRDVVDKDIADKIGV
Jannaschia	D---GANVSVLEPGGQFELSGAPLETVHQTCEVNDHLRQVREVADIIGV
Dinoroseobacter	N---GANVSVLEPGGQFELSGAPLETIHETCDEVNHLREVQDVAAEKIGV
Oceanicola	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNEHLADVVDKDIADKIGV
Loktanelia	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNSHLAEVKAVSDEIGV
Paracoccus	D---GANVSVLEPGGQFELSGAPLETIHETCDEVNSHLAEVKAVADDLGA
Rhodobacter	E---GANVSVLEPGGQFELSGAPLETVHETRAELSHLREVAEVAEPLGV
Parvularcula	E---GASITLLEPGGQFELSGAPLVSLHETCNEVHTHLAEVKAEAPLGI
Hyphomonas	D---GASVSVLEPGGQFELSGAPLKS IHETCDEVGRHLTEVREIAEPLGI
Caulobacter	N---GANVSVLEPGGQFELSGAPLLTMHDI CEETGQHLDEVKTVADELGL
Bradyrhizobium	V---TGGAISLLEPGGQFELSGAPVETVHQTSELMAHLAQVREIATPLGI
Nitrobacter	V---TGGAISLLEPGGQFELSGAPVETVHQTQAEALAAHLAQVKEIATPLGI
Rhodopseudomonas	V---TGGAISLLEPGGQFELSGAPLETVHQTAEALMAHLAQVREIATPLGI
Xanthobacter	V---TGGAISLLEPGGQFELSGAPVSTIHETCAELNAHLAQVREIATPLGI
Mesorhizobium	P---TGQGAISLLEPGGQFELSGAPLETIHQTCRENAHLAQVREIATPLGI
Brucella	P---TGQGAISLLEPGGQFELSGAPLETIHQTCRENAHLAQVREIATPLGI
Rhizobium	Q---NGMGAISLLEPGGQFELSGAPLETIHQTCRESNQHLATLREIATPLGI
Agrobacterium	Q---HGMGAISLLEPGGQFELSGAPLENLHQTCKESNQHLATLREIATPLGI
Sinorhizobium	P---SGNGAISLLEPGGQFELSGAPLENLHQTCKESNQHLAVLREIATPLGI
Bartonella	S---VDQGAISLLEPGGQFELSGAPLKTIGHTYCEVMEHLALKKISGPLGI
Aurantimonas	S---EGQGAISLLEPGGQFELSGAPLETIHETCRESNAHLAQVRSVAKELGI
Fulvmarina	D---EEGGAISLLEPGGQFELSGAPLKTIHETCRESNKHLAEVRSIAKELGI
Stappia	D---RGGGAISLLEPGGQFELSGAPLDNLHLTCREANQHLADVRRVAEPLGI
Magnetospirillum	Y---CG-AAISLLEPGGQFELSGAPLETIHETCRESNAHLAQVRSVAKELGI
Acidiphilium	R---GFSISLLEPGGQFELSGAPLASHIETKREIDAHIARVHRIGALGL
Granulibacter	PSSSLGASLLEPGGQFELSGAPLTLNHLQTRQEFQIHFAELHRVAAPLGL
Gluconobacter	QNAQAGRAISLLEPAGQFELSGAPVVS IQTEAEMQAHFQVGRPAEELGL
Mariprofundus	G---MASVTLLEPGGQFELSGAPLDS IHATCLETTGHLKALKTI SAELRDI
Magnetococcus	D---GASVTLLEPGGQFELSGAPLQTIHETQDEINEHLHLQELGQDMDM
Nostoc	P-----SQNYENLLCALLRPRRLRNYLNRLDNYTLIPGS
Nodularia	P-----LHRYENLLCALLRPRRLRNYLNRLDNYTLIPGS
Anabaena	P-----LHSYESLLCALLRPRRLRNYLNRLDNYTLIPGS
Crocospaera	P-----LSCYDRLLCALVRPRQNLRAYLQKIGNYTIIPGS
Thermosynechococcus	P-----VYLYDQALCDLLRPRFRRLRAYLQSLGDLTLVPGS
Mycobacterium	R-----NTVEIVSGICECTAEAMQDLRDTLGPARQIVRDRGM

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	GFLGIGFQP--KWRREDIPMPK-----GRYDIMRNYMPKV--GTLGLDM
Chorispora	GFLGIGFQP--KWRREDIPMPK-----GRYDIMRNYMPKV--GSLGLDM
Brassica	GFLGMGFQP--KWRREDIPTMPK-----GRYDIMRNYMPKV--GSLGLDM
Pisum	GFLGIGFQP--KWERKDI PMMPK-----GRYEIMKRYMPKV--GSLGLDM
Medicago	GFLGIGFQP--KWERKDI PMMPK-----GRYEIMKRYMPKV--GSLGLDM
Lotus	GFLGIGFQP--KWGLKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Phaseolus	GFLGIGFQP--KWGIEDI PVMPK-----GRYDIMRNYMPKV--GSLGLDI
Populus	GFLGIGFQP--KWVLKDI PMPK-----GRYDIMRKYMPQS--GSLGLDM
Lactuca	GFIGIGFQP--KLERKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Taraxacum	GFIGIGFQP--KLERKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Zinnia	GFIGIGFQP--KWERKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Lycopersicon	GFLGTGFQP--KWGLKDI PMPK-----GRYEIRNYMPKV--GSLGLDM
Solanum	GFLGTGFQP--KWGLKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Nicotiana	GFLGTGFQP--KWGLKDI PVMPK-----GRYEIMRNYMPKV--GSLGLDM
Citrus	GFLGIGFQP--KWGLKDI PVMPK-----GRYEIMRNYMPKV--GSLGLDM
Gossypium	GFLGIGFQP--KLGLKDI PVMPK-----GRYEIMRNYMPKV--GSLGLDM
Malus	GFLGIGFQP--KWGLKDI PMPK-----GRYDIMRNYMPKV--GTLGLDM
Aquilegia	GFLGIGFQP--KWAIKDI PMPK-----GRYDIMRNYMPQV--GTLGLDM
Vitis	GFLGIGFQP--KWAIKDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Beta	GFLGIGFQP--KWGLKDI PVMPK-----GRYEIMRNYMPKV--GTLGLDM
Allium	GFLGAGFQP--KWGLKDI PMPK-----GRYEIMRNYMPKV--GSMGLDM
Oryza	GFLGIGFQP--KWALSDI PMPK-----GRYEIMRNYMPKV--GSLGLDM
Zea	GFLGLGFQP--KWALSDI PMPK-----GRYEIMRNYMPKV--GTLGLDM
Triticum	GFLGMGFQP--KWALTDI PMPK-----GRYEIMRNYMPKV--GTLGLDM
Physcomitrella1	GFVIGFQP--KWSVADT PMPK-----GRYEIMRNYMPKV--GSFGLDM
Physcomitrella2	GFAGIGFQP--KWSVAET PMPK-----GRYEIMRNYMPKV--GSYGLDM
Physcomitrella3	RFAGIGYEP--KWPLSERPNVPK-----ARYQVIKDYFPKIVI TEVGFET
Volvox	GFLGVGFDP--KWAIQDI PVMPKASRRGGRYSLMKSYPMTV--GSMGLDM
Chlamydomonas	GFLGVGFDP--KWAISDV PMMPK-----GRYKLMKSYPMTV--GSMGLDM
Prototheca	DYMNIGFDP--KWGFEDVPKMPK-----TRYRYMREYMPKV--GTLGHDM
Ostreococcus_lucimarinus	RFLGIGFDP--KWSVAEVPMPK-----GRYKIMRAYMPKK--GTRGHDM
Ostreococcus_tauri	QFMGIGFDP--QWSVAET PMMPK-----GRYKIMRAYMPKK--GTRGLDM
Xanthomonas	GFLGMGFQP--KWRREDEMPWMPK-----GRYQIMKSYMPKV--GSLGLDM
Stenotrophomonas	GFLGMGFQP--KWRREDEMPWMPK-----GRYKIMRAYMPKV--GSLGLDM
Xylella	GFLGMGFHP--KCTRAQMPWMPK-----GRYAIMRAYMPKV--GQLGLDM
Oceanicaulis	GFLGLGFSF--KWSLEET PMMPK-----DRYNIMKAYMPKV--GTLGHQM
Maricaulis	GFLGLGFSF--KWSLEET PMMPK-----ARYGLMKAYMPKV--GTMGHQM
Alkalilimnicola	GMIGLGFHP--TARREDI PWMPK-----GRYAVMRNYMPKV--GTLGLDM
Halorhodospira	GFSGFGFHP--VWRREQI PWMPK-----ARYGVMGRYMPKV--GHLGLDM
Nitrococcus	GLIGLGFQP--DKRREDEMPWMPK-----ARYGIMRNYMPQR--GSLGLDM
Rhodospirillum	GFLGIGFNP--KWRREDI SLMPK-----GRYKLMSEYMPKV--GSLGLDM
Parvibaculum	GFLGLGFTP--NWRREDEI PVMPK-----GRYKIMTEYMKKV--GTMGLDM
Sphingopyxis	GFLGLGMWP--DKRREDEI PMPK-----GRYRIMLDHMPRV--GSLGLDM
Novosphingobium	GYLGLGLWP--DKTREELPMPK-----GRYEIMLRHMPRV--GSMGLDM
Sphingomonas	GFLGLGMWP--DKSRSDLPMPK-----GRYAIMLRHMPRV--GSLGLDM
Erythrobacter	GFLGLGMWP--DKTREELPMPK-----GRYDIMRHRMPRV--GSLGLDM
Zymomonas	GFLGLGLWP--DKKRSDDLMPK-----GRYKIMTEYMPKV--GKLGGLDM
Roseobacter	GFIGLGAAP--IWTHEQMDLMPK-----GRYKLMNDYMTKV--GTMGRVM
Sulfitobacter	GFIGLGAAP--EWSHDQMDLMPK-----GRYKLMNEYMTKV--GTMGRVM
Sagittula	GFIGLGAAP--IWTHDQMP LMPK-----GRYKLMDAYMPKV--GTTGREM
Silicibacter	GFIGLGAAP--IWTHDQMP LMPK-----GRYKLMDAYMQKV--GTMGTTM
Roseovarius	GFIGLGAAP--QWSHEQMP LMPK-----GRYKLMDAYMQKV--GTMGRSM
Jannaschia	DFIGLGAAP--IWRHEDMPLMPK-----GRYKLMGYMQKV--GTMGRTM
Dinoroseobacter	GFIGLGAAP--IWSHEDMPVMPK-----GRYRLMTDYMDRV--GTMGKTM
Oceanicola	GFIGLGAAP--TWSHEDMPLMPK-----GRYKLMDAYMERV--GTAGRVM
Loktanelia	KFIGLGAAP--VWTHDEMPLMPK-----GRYQLMDRYMQSV--GTMGTAM
Paracoccus	GFIGLGAAP--IWTDQMPMPK-----GRYRLMTDYMGRV--GTLGTQM
Rhodobacter	RFLGAGAAP--IWTHDEMVPMPK-----GRYRLMTDYMGRV--GTHGTQM
Parvularcula	GFIGLGAAP--SWSRDDMPKVPK-----GRYTIMRNYMPKV--GSLGLDM
Hyphomonas	GFIGLGASP--IWSMADTPVMPK-----GRYKIMTAYMDKV--GRLGRQM
Caulobacter	GFLGLGFSF--LWKREEVVPMPK-----GRYVIMRNYMPKV--GGLGLDM
Bradyrhizobium	GFLGLGMTP--SWSREDI PVMPK-----GRYKIMRGYMPKV--GHYGLDM
Nitrobacter	GFLGLGMTP--SWSRAQI PMMPK-----GRYRIMSGYMPKV--GGYGLDM
Rhodopseudomonas	GFLGLGMTP--SWSRSEI PVMPK-----GRYKIMTNYMPKV--GSLGLDM
Xanthobacter	GFLGIGMSP--KWTRTETPVMPK-----GRYKIMAGYMPKV--GKLGGLDM
Mesorhizobium	RFLGLGGSP--KWSLAETPKMPK-----SRYEIMTRYMPKV--GKGLDM
Brucella	RFLGVGGSP--KWTLAETPMPK-----SRYKIMTNYMPKV--GHEGLDM
Rhizobium	RFLGIGGSP--KWTYAETPMPK-----SRYEIMTRYMPKV--GKGLDM
Agrobacterium	RFLGIGGSP--LWTSDETPRMPK-----SRYAIMTRYMPKV--GKGLDM
Sinorhizobium	RFLGIGGSP--KWTFAETPRMPK-----SRYAIMTRYMPKV--GTQGLDM
Bartonella	GFLGIGASP--KWTLAETPRMPK-----SRYRIMANYMPKV--GYSGLDM
Aurantimonas	GFLGIGSSP--TWTIDETPVMPK-----SRYEIMRNYMPKV--GTRGLDM
Fulvmarina	GFLGIGSSP--TWTIDETPMPK-----SRYDIMRAYMPEV--GTRGLDM
Stappia	GFLGIGMAP--TWSRADMPRMPK-----SRYDIMTNYMPKV--GSLGLDM
Magnetospirillum	GFLGVGFQP--KWSKDDTPWMPK-----GRYVIMRNYMPEV--GKGLDM
Acidiphilium	GFAPLGFHP--TARRADFDWMPK-----GRYAIMRAYMQKV--GTRGLDM
Granulibacter	GFAPLGFQP--LHSRAAI PWMPK-----GRYEIMRAYMPKV--GTLGLDM
Gluconobacter	GFLPFGFQP--LWPRDAMPWMPK-----SRYAIMRNYMPKV--GSLGLDM
Mariprofundus	GFLGMGFQP--RWGRDDI PWMPK-----ERYAVMRRYMPRV--GNGGLDM
Magnetococcus	AFFGLGVQP--KWRFDI PWMPK-----GRYRIMRDYLPSP--GHLGLDM
Nostoc	-TSLGGSD--RFLRS--PANPY-----HDYIENT-----YGT
Nodularia	-TSLGGSD--RFRSN--PDNPY-----HDYIEHT-----YGT
Anabaena	-TSLGGSD--RFRSD--PANPY-----HDYIEQT-----YGT
Crocospaera	-TSLGDSK--KFHRSD--PINPY-----HSYIENT-----YGT
Thermosynechococcus	-TSLGDTQ--RFYRS--PQNPY-----HTYIEQT-----YGT
Mycobacterium	ELFCAGTHPFARWSAQKLTADPR-----YAEIKRT----QWGR

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	MLRRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Chorispora	MLRRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATAIFANSFFTEGKPNGF
Brassica	MLRRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Pisum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Medicago	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Lotus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Phaseolus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Populus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Lactuca	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Taraxacum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Zinnia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Lycopersicon	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Solanum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Nicotiana	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Citrus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Gossypium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Malus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Aquilegia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Vitis	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Beta	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Allium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Oryza	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Zea	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Triticum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Physcomitrella1	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Physcomitrella2	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Physcomitrella3	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Volvox	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Chlamydomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Prototheca	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Ostreococcus_lucimarinus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Ostreococcus_tauri	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Xanthomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Stenotrophomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Xylella	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Oceanicaulis	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Maricaulis	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Alkalilimnicola	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Halorhodospira	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Nitrococcus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Rhodospirillum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Parvibaculum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Sphingopyxis	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Novosphingobium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Sphingomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Erythrobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Zymomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Roseobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Sulfitobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Sagittula	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Silicibacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Roseovarius	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Jannaschia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Dinoroseobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Oceanicola	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Loktanelia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Paracoccus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Rhodobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Parvularcula	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Hyphomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Caulobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Bradyrhizobium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Nitrobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Rhodopseudomonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Xanthobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Mesorhizobium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Brucella	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Rhizobium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Agrobacterium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Sinorhizobium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Bartonella	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Aurantimonas	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Fulvmarina	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Stappia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Magnetospirillum	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Acidiphilium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Granulibacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Gluconobacter	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Mariprofundus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Magnetococcus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Nostoc	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Nodularia	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Anabaena	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Crocospaera	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Thermosynechococcus	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF
Mycobacterium	MFRTCTVQVNLDFSSSEADMIRKFRAGLALQPIATALFANSFFTEGKPNGF

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	LSMRSHIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFAYRKNK-
Chorispora	LSMRSQIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFAYRKNK-
Brassica	LSMRSHIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFAYRNGK-
Pisum	VSMRSHIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFAYRKKK-
Medicago	VSMRSHIWTDTDKDRTGMLPFVFDSDSFGFEQYVDFALDVPYFVYRKKK-
Lotus	VSMRSHIWTDTDKDRSGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKHK-
Phaseolus	VSMRSHIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKHHR-
Populus	LSKRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKEK-
Lactuca	LSMRSQIWTDTDNNRAGMLPFVFDSDSFGFEQYVEYALDVPYFVYRKKK-
Taraxacum	LSMRSQIWTDTDNNRSGMLPFVFDSDSFGFEQYVEYALDVPYFVYRKNK-
Zinnia	LSMRSQIWTDTDNNRSGMLPFVFDSDSFGFEQYVEYALDVPYFVYRKKK-
Lycopersicon	LSKRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Solanum	LSKRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Nicotiana	LSMRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Citrus	LSMRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Gossypium	LSMRSQIWTDTDKDRTGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Malus	LSMRSQIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRQKK-
Aquilegia	LSMRSHIWSDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Vitis	LSMRSQIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKKK-
Beta	LSMRSHIWSDTDNNRAGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKNK-
Allium	LSKRSHIWTDTDNNRSGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKNK-
Oryza	LSLRSHIWTDTDNNRSGMLPFVFDSDSFGFERVYDVALDVPYFVYRKNK-
Zea	LSLRSHIWTDTDNNRAGMLPFVFDSDSFGFEQYVDYALEVPMYFVYRKNK-
Triticum	LSLRSHIWTDTDNNRSGMLPFVFDSDSFGFEQYVDYALDVPYFVYRKNK-
Physcomitrella1	LSYRSHIWTDDVNNRAGLDLFFVFDGFGFDKYVEYALGVPYFVYRNGR-
Physcomitrella2	LSFRSHIWTDDVDRSGDLFFVFDGFGFEKYVDYALDVPYFVYRNGR-
Physcomitrella3	KTYRSFLWSQFDDDRGTGELFFVFDGDFGFEKYTEYALNVPLMVMHNRDN-
Volvox	VSTRGHVWTDVDSARTGNLFFVFRDMSFERYVDYAMDVPYFVYRNGQ-
Chlamydomonas	LSTRGHVWTDVDSARTGNLFFVFEKDMCFESYVDYAMAVPMYFVYRNGQ-
Prototheca	KSWRLNVWTDVVDNARCGRLFFVFDADFSFARYAEWALDVPYFVYRNGE-
Ostreococcus_lucimarinus	QSMRSDVWRDTRDDRTGTLAWVFDGDFGFEKYCDYVMNVPMYFVYRNGT-
Ostreococcus_tauri	ESMRSDVWRDTRDDRTGTLSWVFDGDFGFEKYCDYVMNVPMYFVYRNGT-
Xanthomonas	LSYRSHIWTDDADRTGMLDFVFDGFGYERYVDYLLDVPYFVYRNGV-
Stenotrophomonas	LSYRSHIWTDDADRTGMLDFVFDGFGYERYVDYLLDVPYFVYRNGV-
Xylella	LSYRSHIWTDDADRTGMLDFVFDGFGYERYVDYLLDVPYFVYRNGV-
Oceanicaulis	LSYRAHVWTDVDDNRTGMLHFAFDEGFGYEQYVDWALDAPYFVYRNSV-
Maricaulis	LSYRAHVWTDVDDNRTGMLHFAFDEGFGYEQYVDWALDAPYFVYRNGE-
Alkalilimnicola	LSYRSHVWTDVDDNRCGMLFFVFDGFGMSFERYVEHVLDVPYFVYRDGR-
Halorhodospira	LSYRSRWEDTDPDRCGMLDFVFDGFGMSFERYVDYVLDVPYFVYRDGQ-
Nitrococcus	LSYRSLIWTDDTDPDRCGMLFFVFEEMGFERYTEHALDVPYFVYRGR-
Rhodospirillum	LSYRSHIWTDDPARTGILFFVFDGGMGFERYVDYLLDVPYFVYRGR-
Parvibaculum	LSYRSHVWTDVDDKARSGMIPFVFDGFGYERYVDYALDVPYFVYRDGK-
Sphingopyxis	LSYRSHIWTDDPARTGMLFFVFDGFGYDRYVDYMLDVPYFVYRDGQ-
Novosphingobium	LSYRSHIWTDDPARTGMLSEVFDGFGYERYVDYMLDVPYFVYRDGH-
Sphingomonas	LSYRSHIWSDTDPARTGMLFFVFDGFGYERYADYALDVPYFVYRDGK-
Erythrobacter	LSYRSHIWSDDPHRTGMLFFVFDGFGYERWTDYMLDVPYFVYRDGK-
Zymomonas	SSYRSHIWTDDPHRTGILFFVFDGFGYERYIDYMLSVPMYFVYRDGR-
Roseobacter	KSWRSRVWRDLDAARTGMLFFVFEFGFGRWVEYALDVPYFVYRDGR-
Sulfitobacter	KSWRSRVWRDLDAARTGMLFFVFEFGFGRWVEYALDVPYFVYRDGK-
Sagittula	KSKRMQIWTDLDDSRGTGMLFFVFEFGFGRWVEYALDVPYFVYRDGK-
Silicibacter	KSWRARVWRDLDAARTGMVFFIFDEGFGFESWVQYALDVPYFVYRDGT-
Roseovarius	KSWRSRVWRDLDAARTGMVFFVFDGFGFEAYAEHALDVPYFVYRDGE-
Jannaschia	KSWRSRVWRDLDPDRGTGTVFFIFDEGFGFEAWVEYALDVPYFVYRDGQ-
Dinoroseobacter	KSWRSRVWRDLDPDRGTGMLFFVFDGFGFDWVYALDVPYFVYRDGQ-
Oceanicola	KSYRNYVWRNLDPARTGMVFFVFDGFGFERWVQYALDVPYFVYRDGE-
Loktanelia	KSWRARVWRDLDAARTGMVFFIFDEGFGFEAWVQVLDVPYFVYRDGK-
Paracoccus	KSWRAHIWQNLDAARTGMLFFAFEDGFGYERWVYVLDVPYFVYRDGK-
Rhodobacter	KSWRSRIWRSLDAARTGMLFFVFEEMGFQRYVDWVLDVPYFVYRDGH-
Parvularcula	RSFRAHIWTDTPDRGTGMLDFVFDGFGYERYTEYMLDVPYFVYRDGD-
Hyphomonas	LSYRSHIWTDLDPDRGTGMLFFVFDGFGYERYVDYALDVPYFVYRGGK-
Caulobacter	LSARANVWTDVDDADRTGLDFVFDGFGDFERYARYALDVPYFVYRGGK-
Bradyrhizobium	LSFRSEIWRDTRDNRSGMIPWAFEDGGMGFERWVYALDVPYFVYRDET-
Nitrobacter	LSFRSEIWRDTRDNRAGMIPWAFEDGGMGFERWVYALDVPYFVYRDET-
Rhodopseudomonas	LSFRSEIWRDTRDNRSGMLPFAFEDGGMGFERWVYALDVPYFVYRSDS-
Xanthobacter	LSFRSEIWRDTRDNRSGMLPFAFEDGGMGFDRYVDYALDVPYFVYRSGES-
Mesorhizobium	QSWRGDIWRDTRDNRSGLEFCFSPDFGFADYVEWALDVPYFVYIRDGH-
Brucella	LSWRSSIWRDTRDNRSGVLPFVFDSENFADYVWALDVPYFVYIRDGH-
Rhizobium	LSWRGDIWRDTRDNRSGLLDFTFRDDFGFRDYAEWALDVPYFVYIRDGR-
Agrobacterium	LSWRGDIWRDTRDNRSGVLPFTFSDDFGFKDYVEWALDVPYFVYIRDGK-
Sinorhizobium	LSWRGDIWRDTRDNRAGLPTAFKPDFGFGDYVEWALDVPYFVYIRDGH-
Bartonella	LSWRSEVWCDDTKQRTGVLFFIFSEHFGFADYVEWALDVPYFVYIRDGH-
Aurantimonas	CSWRSRVWSDVVDNRSGLLFFVFEESFSYRDYAEWALDVPYFVYTRNGR-
Fulvmarina	VSWRSEVWHDVDDNRSGLLFFVFDGFGYERYADYVWALDVPYFVYRGGH-
Stappia	KSFRAQIWTDDHRTGDMFFAFEEGFGYERYVEWALDVPYFVYRSGST-
Magnetospirillum	ISARGDVWTDTRDRTGGLFFAFDEGGMGFERYVDWMLDVPYFVYRDGK-
Acidiphilium	LSNRADAWLTDNARAGIPRMVFDGFGYERYADYMLDIPYFVYRDGR-
Granulibacter	LSYRAHIWTDTPHRTGLGIVFDGFGYERYVEWALDVPYFVYRHPGEG-
Gluconobacter	LSNRARIWTDTRDNRSGQPSVFFEDGFGFEQYVDWALDVPYFVYRSDGK-
Mariprofundus	LSRRGACWLDTPARTGIPACAFEDGFGYQAYTEWALDAPYFVYRDGH-
Magnetococcus	LSYRGEIWRHTDTPDRCGMLFFVFEFGFARYAEYALDVPYFVYRKEGV-
Nostoc	HSTRWGLFPQTPSHVP-----LFASHADHIQWVKDQLVAGTMQNVRLHWT
Nodularia	HSSRWAVFPQTPAHVP-----LFTSHAHHIQWVEAQIAGTMQNVRLHWT
Anabaena	HSTRWGVFPQTPTNVP-----LFTSHAHHIEWVEQQLAIGTMQNVRLHWT
Crocospaera	HSTRWQMFQTPPEKVP-----FFESHNHFIWTEEQQLKTKMNVRLHWT
Thermosynechococcus	HSTRWAIFFKTPPPQVP-----LFTSHAHFIWTEAQLQQTGMQNVRLHWS
Mycobacterium	ASNRAMMFQQLP---TAGLFFHFQRWAEFEGFVYDQKKTGIIIDHMEIR-

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	---YIDCTGMTFRQFLAGKLP-CLPGELPSYNDWENHLTTIFPEVRLKRY
Chorispora	---YVDCTGMTFRQFLAGKLP-CLPGELPTYNDWENHLTTIFPEVRLKRY
Brassica	---YVDCTGMTFRQFLAGKLP-CLPGELPTYNDWENHLTTIFPEVRLKRY
Pisum	---YVDCTGMTFRDFLAGKLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Medicago	---YIDCTGMTFRDFLAGKLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Lotus	---YIDCTGMTFRDFLAGKLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Phaseolus	---YIDCTGKTFRDFLAGRLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Populus	---YIDCTGMSFRDFLAGRLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Lactuca	---YIDCAGLSFRDFMAGKLG-PIPGEYPTLNDWENHLTTIFPEVRLKRY
Taraxacum	---YIDCAGLSFRDFMAGKLA-PIPGEYPTLNDWENHLTTIFPEVRLKRY
Zinnia	---YIDCAGLSFRDFLAGKLP-PIPGEYPTLNDWENHLTTIFPEVRLKRY
Lycopersicon	---YVDCTGLSFRDFMNGKLP-PIPGEYPTLNDWENHLTTIFPEVRLKRY
Solanum	---YVDCSGLSFRDFMNGKLP-PIPGEYPTLNDWENHLTTIFPEVRLKRY
Nicotiana	---YIDCAGMSFRDFMNGKLS-PIPGDYPTLNDWENHLTTIFPEVRLKRY
Citrus	---YIDCAGMSFRDFLAGKLP-CLPGELPTLNDWENHLTTIFPEVRLKRY
Gossypium	---YIDCTGMTFRDFMAGKLP-CIPGELPNLNDWENHLTTIFPEVRLKRY
Malus	---YIDCTGMSFRDFLAGKLP-CIPGELPTLNDWENHLTTIFPEVRLKRY
Aquilegia	---YIDCAGMSFRDFMAGKLP-SAPGELPTLNDWENHLTTIFPEVRLKRY
Vitis	---YIDCTGMSFRDFIAGKLP-SLPGELPNFNDWENHLTTIFPEVRLKRY
Beta	---YVDCSGLSFRDFIDGKLP-VLPGEFPTLNDWENHLTTIFPEVRLKRY
Allium	---YIDCSGLSFRDFMAGKLS-PVPGELPTLNDWENHLTTIFPEVRLKRY
Oryza	---YIDCTGMSFRDFMVGKLP-QAPGELPTLNDWENHLTTIFPEVRLKRY
Zea	---YIDCTGMSFRDFMQGKLP-QAPGELPTLNDWENHLTTIFPEVRLKRY
Triticum	---YIDCTGMSFRDFMAGKLP-QVPGELPTLNDWENHLTTIFPEVRLKRY
Physcomitrella1	---YIDCSGMSFKDFLEGKLS-ALPGEKPTMNDWENHLTTIFPEVRLKRY
Physcomitrella2	---YVDCSGLSFKDFLEGKLS-VLPGERPTLSDWENHLTTIFPEVRLKRY
Physcomitrella3	---WRDVS GASFKNF MAGKLE-AYPGEKPSLNDWMNHLGTMYPPEVRLKRY
Volvox	---YINALGMSWRDFMAGKLP-ALPGEYPTIADWANHLTTIFPEVRLKRY
Chlamydomonas	---YINALGMSWKDFMAGKLP-ALPGEYPTIADWANHLTTIFPEVRLKRF
Prototheca	---YHDVGAQSPFRDFFHGKLA-AFPGLFPTMDDWDVHLTTVFPDVRLLKRF
Ostreococcus_lucimarinus	---YVDVSGESWLD FMEGKLP-QLPGDKPTIDDWEOHLTTVFPPEVRLKRY
Ostreococcus_tauri	---YVDVSGESWLD FMEGKLP-QLPGDTPCLADWEOHLTTVFPPEVRLKRY
Xanthomonas	---YHDASGQSFRDFMQGKLP-ILPGALPTLRDWSDHMTTAFPEVRLKRY
Stenotrophomonas	---YHDASGQSFRDFMQGKLP-ILPGALPTMRDWSDHMTTAFPEVRLKRY
Xylella	---YHDASGQSFRDFLQGRLP-ALPGVLPPTLRDWSDHMTTAFPEVRLKRY
Oceanicaulis	---FQDATGLSFRDFLKGELP-LLPGELPVLSDWEDHLSLTFPEVRLKTF
Maricaulis	---YLDATGKSFRDFLDGRLD-VVPGKEPVLSDWEDHLSLTFPEVRLKTF
Alkalilimnicola	---YIDVAGQSFRDFMCGRLP-GLPGEYPRMSDWEDHLSLTFPEVRLKRF
Halorhodospira	---YIDCAGESFRDFMAGRLP-QLPGERPTLSDWDDHLSLTFPEVRLKQF
Nitrococcus	---YIDASGQSFRDFLAGRLP-ALPGCLPVLSDWEDHLSLTFPEVRLKQF
Rhodospirillum	---YIDATGQSFRDFMAGRLA-ALPGERPTIADWTDHMTTAFPEVRLKRY
Parvibaculum	---YIDATGQSFRDFLNGKLP-ALPGETPTRGDWNNHLTTIFPEVRLKRY
Spingopyxis	---YIDAAGQSFRDFLKGELP-ALPGELPRLSDWTDHLSLTFPEVRLKSF
Novosphingobium	---YIDASGQSFRDFLKGELP-ALPGEKPRQSDWVDHLSLTFPEVRLKSF
Sphingomonas	---YIDAAGLSFRDFLDGKLS-VLPGKEPTEKDWEDHLSLTFPEVRLKSF
Erythrobacter	---YIDAAGHSFRDFLDGKLS-VLPGKEPRPSDWDHLSLTFPEVRLKSF
Zymomonas	---YIDASGQDFRAFLRGELP-ALPNEKPTLSDWVDHLSLTFPEVRLKSY
Roseobacter	---YIDALGLSFRDFLKGELS-ALPGEVATLSDWADHLSLTFPEVRLKRY
Sulfitobacter	---YIDALGQSFRDFLKGKLP-ALPGEIPTLSDWADHLSLTFPEVRLKRY
Sagittula	---YVDALGMSFRDFLEGKLP-ALPGETPTLSDWADHLSLTFPEVRLKRY
Silicibacter	---YINALGQSFRDFLNGRLP-ALPGETPTLSDWADHLSLTFPEVRLKRY
Roseovarius	---YIDALGQSFRDFLRGELP-ALPGEKPTLSDWADHLSLTFPEVRLKRY
Jannaschia	---YIDALGMSFRDFLKGELP-ALPGEKPTLSDWADHLSLTFPEVRLKRY
Dinoroseobacter	---YIDALGQSFRDFLDGKLP-ALPGEVPTLSDWADHLSLTFPEVRLKRY
Oceanicola	---YIDALGQSFRDFLKGELP-ALPGEVPTLSDWADHLSLTFPEVRLKRY
Loktanelia	---YIDALGMSFRDFLRGELP-ALPGEKPRMSDWADHLSLTFPEVRLKRY
Paracoccus	---YIDALGQSFRDFLNGRLP-ALPGEIPTLSDWADHLSLTFPEVRLKRY
Rhodobacter	---YIDALGQSFRDFLKGELP-ALPGEKPTLSDWADHMTTAFPEVRLKRY
Parvularcula	---YVDVAGQSFRD LLAGELP-GAPGQLPTLEDWKDHLSTAFPEVRLKTF
Hyphomonas	---YLDASGLSFRDFMEGKLP-ILPGERPAMDDFVDHLSLTFPEVRLKRF
Caulobacter	---YIDLAGRAFDFIDGKIP-ELPGEYASIKDWADHLSLTFPEVRLKRY
Bradyrhizobium	---YIDVSGTSFRDFFAGKNP-AMAGERPTLSDWANHLSTIFPEVRLKRY
Nitrobacter	---YIDVSGSFRDFFDGRNK-ALPGERPTLSDWANHLSTIFPEVRLKRY
Rhodopseudomonas	---YIDVAGSFRDFFDGKND-KLPGERPTLSDWANHLSTIFPEVRLKRY
Xanthobacter	---YVDVSGTSFRD LLAGRHP-LLPGETATLSDWINHLSTIFPEVRLKRF
Mesorhizobium	---YHDMTHITFRQFMAGAAARNEVPDGLPTMGDWANHLSTLFPDVRLLKRF
Brucella	---YHDC THVTFRQFMNGALKGEVSDPVPNMGDWTNHLSTLFPDVRLLKRF
Rhizobium	---YHDC THVTFRQFMNGALKGEVSDPAPTMGDWTNHLSTLFPDVRLLKRF
Agrobacterium	---YHDC THVTFRQFMNGALKGEIAAWEPTMGDWTNHLSTLFPDVRLLKRF
Sinorhizobium	---YHDC THVTFRQFMNGALKGEIAEWQPTMGDWTNHLSTLFPDVRLLKRF
Bartonella	---YDCTHITFRQFMNGALKGKIANSMPMTMGDWINHLSTLFPDVRLLKRF
Aurantimonas	---YTDVTDVTFRQFMNGALKGRI PDPEPQLGDWTNHLSTLFPDVRLLKTF
Fulvmarina	---YNRATDVTFRQFLDGALNGRFADAEPNMGDWTNHLSTLFPDVRLLKRF
Stappia	---YDVTGTTFRQFMNGALEGKVPDAPNIGDWNHLSTLFPDVRLLKRY
Magnetospirillum	---YIDAAGQSFRDFMAGKLP-AFPGQLPTMGDWADHLSLTFPEVRLKRF
Acidiphilium	---YIDVSGKSFRDFIAGRLEG-LAAEPATIGDFADHTTTTFFPDVRLKRF
Granulibacter	YGGYVDVAGASFRDFMAGRLPG-REGERTPTIGDFADHTTAFPEVRLKRF
Gluconobacter	---NIDVAGCSFRRLAGDVQEP LKGLTPTVGFDFEDHLSLTFPEVRLKQF
Mariprofundus	---YIDCAGNSFRDFMHGRLP-QMAGQYPTMDDWELHISTLFPDVRLLKQY
Magnetococcus	---YSGGGVVFRAFMEGKHP-AKPGVYPTLGDWQTHLSLTFPEVRLKHY
Nostoc	----SVRPNGDRR PYDLNRLLELRICDLVT--DPIALLAITALLEARLLQV
Nodularia	----SVRPNGDRR PHDLNRLLELRICDLVS--DPIALLAISAFLEARLLQL
Anabaena	----SVRPNGDRR PYDLNRLLELRICDLVT--DPIALLAITALLEARLLQL
Crocospaera	----SVRPNGNRPYHLNRLLELRICDLIV--DPIALLAVIALLESRLQL
Thermosynechococcus	----AVRPNGDRR PYDLNRLLELRICDLVT--DPIALLAITALLEARLLQL
Mycobacterium	----WDIRPSP----HLGTLEVRICDGVSNLREL GALVALTHCLIVDLDR

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	LEMRGADGGPWRRRLCALPAFWVGLLYDDDSLQAILDLTADWTPAEREMRLR
Chorispora	LEMRGADGGPWRRRLCALPAFWVGLLYDDDTLQAILDLTADWTAEREMRLR
Brassica	MEMRGADGGPWRRRLCALPAFWVGLLYDEVDLQSVLDLTADWTPAEREMRLR
Pisum	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQRVLDMTADWTLEEREMRLR
Medicago	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQRVLDMTADWTLEEREMRLR
Lotus	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQGVLDLTADWTQQRQMLR
Phaseolus	LEMRGADGGPWRRRLCALPALWVGLLYDEAS LQSLDLTADWTPEERQMLR
Populus	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQSVLDMTADWTSEERQMLR
Lactuca	LEMRGADGGPWRRRLCALPAFWVGLLYDDI SLQNVLDMTADWTAEREMRLR
Taraxacum	LEMRGADGGPWRRRLCALPAFWVGLLYDDAS LQNVLDMTADWTTAEREMRLR
Zinnia	LEMRGADGGPWRRRLCALPAFWVGLYDDI SLQNVLDMTADWTQERQMLR
Lycopersicon	LEMRGADGGPWRRRLCALPAFWVGLLYDEGS LQSVLDMTFDWTAERDMRLR
Solanum	LEMRGADGGPWRRRLCALPAFWVGLLYDEGS LQSVLDMTSDWTAERDMRLR
Nicotiana	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQTVLDMTSDWTAEREMRLR
Nicotiana	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQNVLDMTADWTTGERQMLR
Citrus	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQSVLDMTADWTSEEREMRLR
Gossypium	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQSVLDMTADWTSEEREMRLR
Malus	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQNVLDLTADWTPEERQMLR
Aquilegia	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQSVLDMTADWTPEERQMLR
Vitis	LEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQNVLDIADWTLEERQMLR
Beta	MEMRGADGGPWRRRLCALPAFWVGLLYDEVS LQSVLDMTYDWTVEEREMRLR
Allium	LEMRGADGGPWRRRLCALPAFWVGLLYDDI ALQSVIEITADWTRERDMRLR
Oryza	LEMRGADGGPWRRRLCALPAFWVGLLYDEES LQSVISDMTSDWTNEEREMRLR
Zea	LEMRGADGGPWRRRLCALPAFWVGLLYDEES LQSVISDMTSDWTKEREMRLR
Triticum	MEMRGADGGPWRRRLCALPAFWVGLLYDDI SLQSVISDMTSDWTKEREMRLR
Physcomitrella1	LEMRGADGGPWRRRLCALPAFWTGLLYDEES LQGVLDIINDWTERERAMRLR
Physcomitrella2	LEMRGADGGPWRRRLCALPAFWTGLLYDDI SLQGVLDIINDWTEKERAMRLR
Physcomitrella3	LEMRGADCCPKDFLNLALPAFWVGLLYDEQ SLKNCLEDIRDWSNDRGDFLR
Volvox	LEMRGADGGPWRRRLCALPALWVGLLYDEEA QRQALALVSDWTDSERDYLR
Chlamydomonas	LEMRGADGGPWRRRLCALPALWVGLIYDPEA QRQALALIEDWTPEERDYLR
Prototheca	LEMRGADGGGWEIFITALPALWVGLLYDAGA QAEARLVADWTPPEELDALQ
Ostreococcus_lucimarinus	MEMRGADGGSYEAIMALPALWVGLLYSDKAL DAAEAMVSDWTQVERDALR
Ostreococcus_tauri	LEMRGADGGSFEMALPALWVGLLYSEKAL TAAEKLVSDWSKEERETLR
Xanthomonas	LEMRGADAGPWNRLCALPAFWVGLLYDQTA LDAAWDLVKDFTLAERHALR
Stenotrophomonas	LEMRGADGGPWSRLCALPAFWVGLLYDDT ALDAAWDLVRDFTLAERHALR
Xylella	IEMRGADSGPLPTLALCALPAFWVGLLYDN TALDAAWDLIKDLTLQERHALR
Oceanicaulis	LEQRGADGGPWRRRLCALPAFWVGLLYDQTA LDAAWDLVKDWTAEERDAMR
Maricaulis	LEMRGADSGPWNRLCALPAFWVGLLYDNTAL DAAWDLVKDWTAEERDALR
Alkalilimnicola	IEMRGADGGPWRRRLCALPAFWVGLLYSDR ALDEALQLIADWSVEEISALR
Halorhodospira	LEMRGADGGPWRRRLCALPALWVGLLYD DAAALAAEALIRDWSVEEMRALR
Nitrococcus	IELRGADAGPWSRLCALPAFWVGLLYDDE LALDAASHLVADWTPAEERELR
Rhodospirillum	LEMRGADGGPWRRRLCALPAFWVGLLYDQTA LDAAWDLVKDWTAEERHAQLR
Parvibaculum	MEMRGADGGPWRRRLCALPAFWVGLMYDQ SLDAAAWDLVKDWTAEERQGLR
Spingopyxis	LEMRGADGGPWRRRLCALPALWVGLLYDQD ALDAAWDLVKGWSIAEQQALR
Novosphingobium	LEMRGADGGPWRRRLCALPALWVGLLYDQD ALDAAWDLVKDWDMDGRERLR
Sphingomonas	LEMRGADGGPWRRRLCALPALWVGLLYD S QALDAAWDVVKDWSMEERQALR
Erythrobacter	LEMRGADGGPWRRRLCALPALWVGLLYD S S QALDAAWDLVKDWSMEERQALR
Zymomonas	LEMRGADGASAMMSPALSAFWISILYDSE LLDTASDIKISWSMDDYRNLR
Roseobacter	IEMRGADGGPWRRRLCALPAFWVGLMYDQ T ALDAAWDLVKGFDAETRDALR
Sulfitobacter	IEMRGADGGPWRRRLCALPAFWVGLTYDQ QALDGAWDLVKDWDQAETRQALR
Sagittula	IEMRGADGGPWRRRLCALPAFWVGLMYDQ T ALDAAWDLKRNWTAEQREALR
Silicibacter	IEMRGADGGPWRRRLCALPAFWVGLTYDQ S ALDAAWDLVKGWDAAETRETLR
Roseovarius	MEMRGADGGPWRRRLCALPAFWVGLMYDQ S SLDAAAWDLVKGWDAAETRDALR
Jannaschia	IEMRGADGGPWRRRLCALPAFWVGLTYDQ S ALDAAWDLCKGFDAETREDMR
Dinoroseobacter	IEMRGADGGPWRRRLCALPAFWVGLTYDQ QALDAAWDLVKGWDAAETREALR
Oceanicola	IEMRGADGGPWRRRLCALPALWVGLTYDQ QALDAAWDLVKDWDQAAHALR
Loktanelia	IEMRGADGGPWRRRLCALPAFWTGLLYDQTA LDAAWDLCKNWTAEQRDALR
Paracoccus	IEMRGADGGPWRRRLCALPALWVGLLYDQAA QDAAWDLVKGWDAAETRESWR
Rhodobacter	IEMRGADAGPLSHLVALPAFWVGLMYDGS ALDAAWDLARGWSAETREGLR
Parvularcula	LEMRGADSGSWGRICALPAFWVGLLYDDQ SLNAAWDLVRHWSQETRAAFR
Hyphomonas	LEMRGSDSGPWRDLCAFSAFWTGTFYSQ S SLDAAAWDLVKGWSAEEERAMR
Caulobacter	LEMRGADAGPWSRLCALPALWVGLTYD DAAALATAWDLCKHWTAEADRAGLR
Bradyrhizobium	LEMRGADGVPWGRLPALPAFWVGLLYDDV SLDAAAWDLVKDWTAFERQSLR
Nitrobacter	LEMRGADGVPWGRLPALPAFWVGLLYD TTSLEAAWDAIKRWNAQERQALR
Rhodopseudomonas	LEMRGADGQPWRRRLCALPAFWVGLLYD D TSLDAAWELVKGWSAEEERQALR
Xanthobacter	LEMRGADAGPWRICALPALWVGLLYDQQS LDAAWDLAKDWTAEQRQALR
Mesorhizobium	LEMRGADGGPWRRRLCALPAFWVGLLYD EAALDAAEAL TASWTYKEVLAMR
Brucella	LEMRGADGGPWRRRLCALPAYWVGLLYD EAALAAEELTKDWTYEEVLAALR
Rhizobium	LEMRGADGGPWRRRLCALPAFWVGLLYD EAALAAEDELTKDWSFAEVSALR
Agrobacterium	LEMRGADGGPWRRRLCALPAFWVGLLYN QEALDAADAL TADWSFDEVIALLR
Sinorhizobium	LEMRGADGGPWRRRLCALPAFWVGLLYD DEALSAAEELTRDWSYEEVLAALR
Bartonella	LEMRGADCGSWRRICALSAFWVGLLYD SEALNEAETLTKDWC FEEVLDLRL
Aurantimonas	LEMRGADGGPWRRRLCALPAYWVGLLYD EAALDAAEALTKDWT FEEVSAMR
Fulvmarina	IEMRGADGGPWRRRLCALPAFWVGLLYA PGALDKVEEMTADFTFEEISELR
Stappia	IEMRGADGGPWRRRLCALPALWVGLLYD KGVLDQAWELVKDWTQEBERAAALR
Magnetospirillum	LEMRGADAGPWRRLCALPALWVGLLYD D TALDGAWELVKDWTAEQRREALR
Acidiphilium	IETRGADSGNPAMLLAQPALWVGLTYD DAAALDAASALIRDLSWEQIVHLR
Granulibacter	LEMRGADAGSMAMMLAQSAFWVGLLYD DAALEAATALMNRHGWSLVGLR
Gluconobacter	LEMRGADAGKLEMMVAQSAFWVGLLYD P ATLEAAEKLVREQPWSVYQQLR
Mariprofundus	LEMRGADAGPWPICALPALWVGLLYD AEAEQAAWDMIRDWTHAEVTLAR
Magnetococcus	LELRGADAGNSSL CAMPALWVGLLYD HDE TALLAAWDRVKRWSHEERDRIH
Nostoc	IE--NPNDIPLTQSIFSNELVLTAEANA AAVAADSLDAHLRHWDQD--R
Nodularia	IE--NPDLPLTQSTFSPPELLLTAQNETA AATASLDAQLIHWQDQ--R
Anabaena	IE--NPDLPLTQSKFSPPELLVLT TENEAAAAASLDAQLTHWQDQ--R
Crocospaera	IK--DPTLDPLKSSQLSPDITL DITHSNEMAVAKHSLEAELYHWQDQ--S
Thermosynechococcus	LD--TPDLDPLRWG--DRETLAQLADENE QLAAKRSLEAVLTHWRDR--R
Mycobacterium	RLDAGETLPTMPPHVQENKWRRAARYG-- --LDAVILLDAD--SNERLVTD

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	NKVPVTGLKTPFRD-GLLKHVAEDVLKLAkdGLERRG-----YKEAG
Chorispora	NKVPVTGLKTPFRD-GLLKHVAEDVLKLAkdGLERRG-----YKEAG
Brassica	NKVPVTGLKTPFRD-GLLKHVAEDVLKLAkdGLERRG-----YKEVG
Pisum	NKVTVTGLKTPFRD-GLLKHVAEEVLELAKDGLERRG-----FKESG
Medicago	NKVTVTGLKTPFRD-GLLKHVAEEVLELAKDGLERRG-----FKESG
Lotus	NKVTVSGLKTPFRD-GLLKHVAEDVLKLAkdGLEKRG-----FKESG
Phaseolus	NKVPVTGLKTPFRD-GLLKHVAEDVLQLAkdGLERRG-----FKESG
Populus	NKVPKTLGLKTPFRD-GLLRHVAEEVLELAKDGLERRG-----FKEVG
Lactuca	NKVPVTGLKTPFRD-GLLKHVAQEVVNFADKGLERRG-----YKETG
Taraxacum	NKVPVSGLKTPFRD-GLLKHVAQEVVGFADKGLERRG-----YKETG
Zinnia	NKVPVAGLKTPFRD-GLLKHVAEEVLELAKDGLERRG-----YKETG
Lycopersicon	NKVPKSGLKTPFRD-GLLMHVAQDVVVKLAKEGLERRG-----FKETG
Solanum	NKVPKSGLKTPFRD-GLLMHVAQDVVVKLAKEGLERRG-----FKETG
Nicotiana	NKVPKSGLKTPFRD-GLLKHVAQDVVVKLAKEGLERRG-----FKETG
Citrus	NKVPKTLGLKTPFRD-GLLRHVAQDVVVKLAKDGLERRG-----FKETG
Gossypium	NKVPKTLGLKTPFRD-GLLWHIAEDVLKLAkdGLERRG-----FKESG
Malus	NKVPITGLKTPFRD-GLLKHVAQDVVVKLARDGLERRG-----FKETG
Aquilegia	NKVPKTLGLKTPFRD-GLLRHVAEDVLKLAkdGLERRG-----FKETG
Vitis	NKVPKTLGLKTPFRD-GLLKHVAEDVLKLAkdGLERRG-----FKETG
Beta	NKVPKGLKTPFRD-GYVKHIAEEVLELAKDGLERRG-----FKEIG
Allium	RKVPVTGLKTPFRD-GLLKHVAEDVLKLAQGGLEERRG-----YKEVG
Oryza	RKVPVTGLKTPFRD-GYVRDLAEEILQLSKNGLERRG-----YKEVG
Zea	RKVPSTGLKTPFRD-GYVRDLAEEVLELAKNGLERRG-----YKEVG
Triticum	RKVPVTGLKTPFRD-GYVRDLAEDVLQLAkNGLERRG-----YKEVG
Physcomitrella1	SKVPKLGKTPFRD-GLLKHVAQDVVVKLAkdGLTRRG-----LNETG
Physcomitrella2	NKVPKGLKTPFRD-GLLKHVAQDIKLAkdGLTRRG-----LNETG
Physcomitrella3	KEVNPQGLTTPFRE-GTLQDVAKDVVVKLAkdGLARRG-----LQEGK
Volvox	TEVTRYGLRTRFRG-GSVQDLAKQVVSIAARGGLQRRG-----HDEVN
Chlamydomonas	TEVTRFGLRTPFRA-GTVQDVAKQVVSIAHGGLEERRG-----YDETS
Prototheca	ADVPRQALQATFRS-GTVQDLAKQVLAIAARRGLDARG-----LGEGR
Ostreococcus_lucimarinus	VDVTKDGLSAKFRG-GTANDIAKMMVELSVQGLQERG-----LGEEV
Ostreococcus_tauri	VAVTKDGLSAKFRN-GTARDIARDMVRVLAVDGLKERG-----LGEEV
Xanthomonas	DGVPKALGLPFRNG-VVRDLAEEAVNIAAREGLRRRARLNDRG--QDETG
Stenotrophomonas	DGVPKHAMNLPFRNG-SVRDLAREAVKI SVEGLKRRRAARNADG--QDESK
Xylella	NGVPRHALALPFRNS-TVRTLALQTLDI SRAGLRRAAQNANG--QDETI
Oceanicaulis	AGAAKTALKTPFRNT-TLQETIAQALAI SRSGLKARQRLNAHG--ENEAL
Maricaulis	IGAAGTGLHTPFRD-TLQDIAREALAIARSGLRARARQNHG--ESETL
Alkalilimnicola	EQVPREGLATFRFG-TLREVALEVLAI SRRGLADRAWLDRRG--RDEAH
Halorhodospira	HEVPRTALHTPFRET-TVGALAREVLTIASDGLRNRQRLDQRG--RDERR
Nitrococcus	SAVPRWGLRTPFRGH-LLQEVARETLGIAEQGLRRRARLNEAG--QDETR
Rhodospirillum	AQVPRLLALKTPFRKG-TLQDVALRALEISQAGLAARGRLNSKG--RDETG
Parvibaculum	DGVAKHGLHTPFRFG-TLLDVARRAVEISRAGLDARGNGDGVG--GTEAI
Spingopyxis	DAVPREGLDAPAPGGGTGVLQLAHRVLDIAAAGLAARGEVNSMG--DNEVG
Novosphingobium	AEVPRGLDAALPGGGTLRDLAEEVLAISRSGLSARNRLNEAG--DNETG
Sphingomonas	DSVPKLGDLAPVGGGRKLRDIAREVIDIARSGLAARARLNSG--DNETG
Erythrobacter	NAVPRLLADAPI PGGHRLQDLARVDLKIARQGLTARARLGESE--DNETG
Zymomonas	NEVPKGLKTLIGGRQSLDLGRQLWPLMNDALKRRAILNDKG--QDESR
Roseobacter	VAASVDGLQAKVNG-ISMHDLAREAVAI SQSGLAARARPGAGGMVPDETH
Sulfitobacter	VAASVDGLQAKVGG-VRMHDLAREAVALESEQGLKARARSGAGGMVPDETH
Sagittula	LAATRDALAAEVDG-IRMHDLAREVLEICEAGLKARARPGAGGLVPDETH
Silicibacter	IEASRQGLQARAGN-VRMHDLAREVLAISEAGLKARARPGAGGLVPDETH
Roseovarius	VAASEQALQAKVGG-LNMHDLAREVLDISEAGLKARARPGAGGLVADETH
Jannaschia	VQASVHGLAAEAGG-IKMHDLAREVLAISEVGLAARAMPGAGGLVPDETH
Dinoroseobacter	VAASVDGLQAKVGD-LRMHDLAREVVSIAEAGLAARAMPGAGGMVPDETH
Oceanicola	VAAEGHGLQAKVEG-IDMHDLAREVLAISEAGLKARARPGAGGLVPDETH
Loktanelia	VAASVDGLQAKVEG-IDMHDLAREVVAISEAGLKARALPGAGGLVPDETH
Paracoccus	RGAGLKALDAEVNG-TKMRDLAREVLAISEAGLKARARTGSGGLVPDETH
Rhodobacter	VAASVDGLQGEAGG-VRLDLVAREAVAI AQGGLRARALAGEG--MADEAG
Parvularcula	HDAAKEAMAATVDG-RPVTDLAREVLALSAGLQRRACRGSSG--RDERT
Hyphomonas	QSVRTLGLRTPPI PGGRTMQDLAKDVLAISRAGLKGRVSISSAGD---DETG
Caulobacter	RDVPLGLKAVVAG-RTAQDVAKDFVAIARRGLKRNRAHMNGG--FLDETI
Bradyrhizobium	DDVPRGLGFKARIRN-RYVFEVAKECLTLAHHGLRRRGRIDHLG--RDESR
Nitrobacter	DDVSRMGFKARIK-RYLFETIARECLVLAHAGLRRRRGRVHDHLG--RDESR
Rhodopseudomonas	DEVPRLGFKAKIGN-RFLFEIAKDCVLHAHAGLRRRRGRIDATG--LDSESR
Xanthobacter	DDVPRGLGKAEIAG-RQLRDIARDVLKLEAGLKRRRGRFHDRLG--RDETR
Mesorhizobium	NAVPELGI AAPFRN-ATLREMARVLAISRTGLKNRGKKNRNGD--YDETS
Brucella	NEVPTKALKTDFHG-KPLELIARETLKI SRLGLLNRRKLNDSG--YDETH
Rhizobium	NAVPAEGLQAEFRG-HSLEEMAREVVGISKAGLKSRRKLNAGEG--QDESI
Agrobacterium	NAVPAKGLAAE IAG-KPLLG IARQVLDI SRTGLKNRKRLNAGEG--QDET
Sinorhizobium	NAVPAKALAAEFRS-KSLFDVAREVLAISRNGLKRRNRLNGDG--IDESQ
Bartonella	KRVPKEGLRAPFRQ-TVILELARQAVAI SRKGLKNNRRQYDSGD--FDETN
Aurantimonas	EAVPRDGFRT PFRD-SSVLELARETVRLSRQGLVARNRRNEEG--NDESH
Fulvmarina	RRVPKEGFRT PFRD-GTVLDVARECVRIAREGLIDRANKNDEG--NDESF
Stappia	AGVPTALQTPFRS-GTVLDVAKQVLALESQEGKRRKRLSDGD--LDERV
Magnetospirillum	ATVPARGLAATI HG-RSVREVAADMLSLASAGLKARGRLNDSG--RDETI
Acidiphilium	DEVPRHGLDIALPT-GTLRDLARDVLAIAADGLKSRARTNAA--GEDERI
Granulibacter	SEVARQAVNT PFD-GTVRDLARDVLAIAADGLRAR-----GQGEV
Gluconobacter	AEVPRGLDAAPFG-G-LKPFKRVVLEAEQGLRAR-----VRNEAG
Mariprofundus	QAVPATAMRT PFRD-GDLLPLCEQMVDI SRAGLERLNICNDAG--ENEAR
Magnetococcus	QETPRALQTLTPEGISFRALGLSVLEIARASLHRQGERNANG--CDESI
Nostoc	SILARDWITQMYQDVWAI AKQGFSCFSLPLHKILREG-----NEAQQ
Nodularia	TILARDWIAE IYQDVWAI AKQGHFSCFSLPLQKILRAG-----NEAQQ
Anabaena	SIIARDWVNE IYQEVWAI AKKHGFSCFSLPLQKILREG-----NEAQQ
Crocospaera	KIKATAWIEQ IYQVSP LAKQNGFSCFSLPVNKILRNG-----NLAQQ
Thermosynechococcus	QLTAAAWIAELYEEVWPI AKAQGFSCFSLVPIKILRQG-----NTAQQ
Mycobacterium	DLADVLTRLEPVAKSLNCADELA AVSDI YRDGASYQR-----

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

Appendix

Arabidopsis	FLNAVDEVVRT-GVTPAEKLLLEMYNGEWGQSVDPVFEELLY-----
Chorispora	FLNAVSEVVRT-GVTPAEKLLLELYNGEWGQSVDPVFQELLY-----
Brassica	FLNAVTEVVRT-GVTPAENLLEMYNGEWGQSVDPVFQELLY-----
Pisum	FLNAVAEVVRT-GVTPAERLLELYHGKWEQSVDPVFEELLY-----
Medicago	FLNAVAEVVRT-GVTPAERLLELYHGKWEQSVDPVFEELLY-----
Lotus	FLNEVAEVVRT-GVTPAERLLELYDGKWNQSVDPVFEELLY-----
Phaseolus	FLNEVAEVVRT-GVTPAERLLELYHGKWEQSVDPVFEELLY-----
Populus	FLNAVAEAVST-GITPAEKLLLELYHGKWGQSIDPVFEELLY-----
Lactuca	FLNEVTEVVRT-GLTPAEKLLLELYHGKWGPNVDPVFEELLY-----
Taraxacum	FLNEVTEVVRT-GLTPAEKLLLELYHGKWGQNVDPVFEELLY-----
Zinnia	FLNEVAEVVRT-GLTPAEKLLLELYHGKWGQSVDPVFEELLY-----
Lycopersicon	FLNEVAEVVKT-GVTPAEKLLLELYHGKWGQSVDPVFEELLY-----
Solanum	FLNEVTEVVRT-GVTPAEKLLLELYHGKWGQSVDPVFEELLY-----
Nicotiana	FLNEVTEVVRT-GVTPAEKLLLELYHGKWRQSVDPVFEELLY-----
Citrus	FLNEVAEVVRT-GVTPAEKLLDMYHGKWRQSVDPVFEELLY-----
Gossypium	FLNEVAEVVRT-GVTPAEKLLLELYNGKWGQSVDPVFEELLY-----
Malus	FLNEVAEVVRT-GVTPAEKLLLELYNGKWGQSIDPVFEELLY-----
Aquilegia	FLNALAEVVTT-GVTPAEKLLLELYEGKWRQSVDPVFEELLY-----
Vitis	FLNAVTEVVRT-GVTPAEKLLLEMYHGPWGQCVDPVFEELLY-----
Beta	FLNEVAEVVRT-GVTPAERLLEMYHGKWGQNVDPVFEELLY-----
Allium	FLREVAEVVNT-GVTPAEKLLLELYHGKWCNVDPVFEELLY-----
Oryza	FLREVDVAVISS-GVTPAERLLNLYETKWRQSVDPVFEELLY-----
Zea	FLREVDEVVRT-GVTPAERLLSPYETKWRQNVDPVFEELLY-----
Triticum	FLREVDEVVRT-GVTPAEKLLNLYETKWRQSVDPVFEELLY-----
Physcomitrella1	FLKDIETVRT-GKTPAEHILELYHGKWGQNVDPVFEELLY-----
Physcomitrella2	FLKDIIEIVQT-GKTPAERLLDLYHEKWRQNVDPVFEELLY-----
Physcomitrella3	FLAPLEEVATT-GVTLAERMLKLYEEDWGRVDPVFEELLY-----
Volvox	FLKRLEVVAET-GLTQADHLELYETRWQSVDPVFEELLY-----
Chlamydomonas	FLKRLEVIAET-GLTQADHLELYETRWQSVDPVFEELLY-----
Prototheca	YLDVLDIAIAES-GVTQADRLQLYETE-----
Ostreococcus_lucimarinus	YLRYLQDIVDD-GKSVAAARMSEMNAREWNGDLEKVVYAYATFPDTPKIV---
Ostreococcus_tauri	YLEYLQQVMDG-GATASSRTRALFTNEWKGDLEKVVYAYASYPDVLKPF---
Xanthomonas	FLDVIAEIAET-GVTAERKLLLYHGAWKGDIDPVREFAY-----
Stenotrophomonas	FLDVLQEIIVES-GLTPAERKLLALFHGRWHGDVDPVREFAY-----
Xylella	FLNVLDIAHT-GQTAAQRTLQRYDDTWNHNDINPIFNEYAY-----
Oceanicaulis	FLDDLDEIAKT-GISPAERLLERYHGAWNESVEPIFEEAAY-----
Maricaulis	FLDDLDEIVRT-GRTRAEDLIDRFKGEWDGSIIEPVFTECAY-----
Alkalilimnicola	FLDLLDGIQAS-GRTPAEELLEAWAGRWQDVAVPVREFAY-----
Halorhodospira	HLERLWEIVDA-GRTPAEDLLSAYHGRWGGSLDPIFTEYAY-----
Nitrococcus	FLVELQEIATG-GITPAERLLESWEQRWHHSVEPVFEHAY-----
Rhodospirillum	FLDPLWTIARS-GRTQAEDELLESPLTRWNGEIDPVFTECAY-----
Parvibaculum	HLSAVEAIVAK-GKSPAELLELYHGWRWNGSVDPVFEFAF-----
Sphingopyxis	FLLEPLRRIAKS-GRSPAHDLLDRYEDAWGHDLRSRYDELSF-----
Novosphingobium	YLQPLDEIVAT-GKTPAERLLDLYNGPWGGDLSQIYAEKSF-----
Sphingomonas	YLSFLEDVAAS-GKTNARLLDLYHGGEWQGDLSRVYAGESF-----
Erythrobacter	FLSTLDEIVET-GKVPAAQLLDRYHGGEWGGDIKRVY-KYSF-----
Zymomonas	YLAPIGEIILES-GQSLSDRLRLARYH--QTGNLDFIYQECDDWAQPHILS---
Roseobacter	FLNALHESIET-GKTSADELLDRYHGDWNGDLTRIYDEYSY-----
Sulfitobacter	FLNALKESIET-GKVPADALLELYDGKWKGDLSRIYADYSY-----
Sagittula	FLSALKESVES-GMTPADELLEHYHGDWNGDLSRIYAAYSY-----
Silicibacter	FLNALKDSVES-GKVPADELLDRYQGGDNGDLSRIYAAYSY-----
Roseovarius	FLNALRESVES-GQVPADELLEKYNGEWGDGLDRIYDPYSY-----
Jannaschia	FLNALKDSVET-GQVPADELLEKYSGEWARLDTRIYAEYSY-----
Dinoroseobacter	FLNALKDSIES-GRVPADELLAHYHGDWGGDLSRIYAAYSY-----
Oceanicola	FLNALKESVET-GQVPADELLEAAHYHGDWGGDGLDRIYDPYSY-----
Loktanelia	FLNALKETVET-GKTPADLLDHYHGDWAGDLDRIYTPQYSY-----
Paracoccus	FLNLTLSKESVET-GKVPADELLGKYHGEWAGDLSRIYAEYSY-----
Rhodobacter	HLEVLAEESAAS-GRVVADLLARFHHGAWEGDLRRIYPEFSY-----
Parvularcula	FLDPLVEIAET-GKSFACTLLHDVDAAG-GDFTALFDKYAF-----
Hyphomonas	FLTEMDQIATS-GVTPAERLLQRYGPDWRDVRKVFEEAY-----
Caulobacter	YLGDLLEIADS-GITPAERLLALYHGAWKGDISRVEDFAF-----
Bradyrhizobium	YLEPLDQIIDS-GRSPAEMLEKFNPGWHSVEPAYDEYAF-----
Nitrobacter	HLEPLDQIILDS-GRSPAEMLEKFNAGWGSVEPAYSEYAF-----
Rhodopseudomonas	HLAPLDRIILDN-GHTPAEEMLEKYNAGWGSVEPAYDEFAY-----
Xanthobacter	FLPLEEIVLT-GRSPADKLLALYNGEWDHSVEPAFQALAY-----
Mesorhizobium	FLNLTLDVVAR-GTTSABEEMLSAYHTRWGSIEPVFMEYAY-----
Brucella	FLAPLEEIVAA-ATDAERMLNAYHVSVAWSVDPVIFLEYAY-----
Rhizobium	FLAPLDEVLAK-KATLAEDLLSLYHGRWQGSVEPVFEDYQY-----
Agrobacterium	FLSSLDEVLAK-KTTLAEDLLALYNGRWGGSVVPVFEFYQY-----
Sinorhizobium	FLAPLDEVLAK-KATLAEDMLALYHGRWKSVEPVFADYQY-----
Bartonella	FLTPLEEIVAM-GQTDADKFLSLYHVSVDGTVEPVFLECAAY-----
Aurantimonas	FLAPLEEIVAR-GTTSABEELVRLFEEQWGSMEVFKHLTY-----
Fulvmarina	FLAPLEEIVAR-GTTSABEELVRLYEDQWERSMQQVFKNLAY-----
Stappia	HLAPIEEGLAS-GMCPADVLLQRYNGSWKGDISQVFREYAY-----
Magnetospirillum	YLDPIESVVKT-GKTASEEMLDFAHTRWRGSDIPVIFREYAY-----
Acidiphilium	YLAPLEAIAHG-APTQAEHHLGRYHGAWHGATRIFAEAAAL-----
Granulibacter	YLDPLRDIVGGAPTQAEHHLNRYQSVWAGDVSRIFNEAQVA-----
Gluconobacter	YLDPLHLIADG-GPNQAQYWLDLYNGAWGQSVKPLFTEAAI-----
Mariprofundus	FLTPLMYAVVS-GQTQADRWLAAWHNEWHGDISRVFADAMHP-----
Magnetococcus	FLDPLFITVEN-NQTPAERLLEAYKGRWRGAVEPLFWEEEFESFYAECTKKA-----
Nostoc	WLQLHTVGFDS--QRVITQAILATQEREIELEDKLCSSLSA-----
Nodularia	WLQLHSVGFDS--QRVITQAIIVATAEREVELENKLCSSVIA-----
Anabaena	WLQLHKVGFDT--QCVITQAIISTQERELELQNKLCQTKG-----
Crocospaera	WLKKYQTSPTDI--PLIIAEEIQTVAQKEMLEDKLCDAALLVA-----
Thermosynechococcus	WLAQYAAAGQTI--PEIIMAAQVQEMAANEQEFADQLCPPLATVQG-----
Mycobacterium	-----QLRVAQQHDGDLRAVVDALVAELVI-----

Figure A3 (continued): Alignment of plant and proteobacterial GCL protein sequences.

6.5 Alignment of BjGCL with non-angiosperm plant GCL proteins

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Brassica      MALLSQAGGAYTVPSGHVSSRTGKTIVSGCVNVLRMKETYVSSYSRTLSTKSM LKRSKRGHQLIVAASP
Picea        -----
Physcomitrella 1 -----
Physcomitrella 3 -----
Physcomitrella 2 -----
Ceratopteris -----
Ostreococcus tauri -----
Ostreococcus lucimarinus -----
Chlamydomonas -----
Volvox       -----
Prototheca   -----
Anabaena     -----

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Brassica      PTEEAV--VATEP---LTREDLIAYLASGCKSKEKWRIGTEHEKFGFEV-NTLRPMKYDQIAELLNSIA
Picea        -----
Physcomitrella 1 PSNDAG-SYGGEF---LTRQDLVGYLASGCKPKEKWRIGTEHEKFGFQL-DNLQPMYTPQIKQLLEGLA
Physcomitrella 3 PNNNAGGSRSGEP---LTRQDLVGYLASGCKPKEKWRIGTEHEKFGFQL-DNLQPMYTPQIRQLLEGLA
Physcomitrella 2 PAVEEAAAARAGKDLTKEDLVFLRSGCKPKSEWRIGTEHEKLGFL-DTLKRMTFDQISKLLDGM
Ceratopteris -----
Ostreococcus tauri -----
Ostreococcus lucimarinus TTIRAAATAPARA---VTREECVAFVRRGCKPREAFRIGTEHEKFGYDE-ATKRMDYPVVRHVLSLV
Chlamydomonas ANAPAVAERRTEP---LLQELVDYLSGCRPSAWRIGTEHEKLGFL-ADNSRMNYDQIAQVLRKLE
Volvox       -----
Prototheca   TDKVATPVGAAPH---LTLEDLVENLRKCKPRSSWRIGTEAREVGVRLRLTPCATHQOISDLLTVIH
Anabaena     -----

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Brassica      DRFEWQKVMEGD KI IGLKQKQSI SLEPGGQFELSGAPLETHLCAEVNSHLYQVKAVAEEMGIGFL
Picea        DRFSWQKVMEGD FI IGLKQDKQSI SLEPGGQFELSGAPLETHLCAEVNSHLYQVKAVAEEMGLGFL
Physcomitrella 1 DRFEWQKVMEGD NI IGLTLDGQVSVLEPGGQFELSGAPLETHLCAEVNSHLYQVKAVAEELGLGFV
Physcomitrella 3 DRFEWKKVMEGD NI IGLTFDGGQVSVLEPGGQFELSGAPLETHLCAEVNSHLYQVKAVAEELGLGFA
Physcomitrella 2 SRECYDRVMEGE NI IGLSKDGASVSVLEPGGQFELSGAPLKDVHACRELDTHLEQVNI IIGKELGLRFA
Ceratopteris DRFDWERVTEEG HI IGLKQDKQVSVLEPGGQFELSGAPLETHLCAEVNSHLYQVKAVAEEMGLGFL
Ostreococcus tauri DRYGWAPIYEGE NI IGCTKDKQSVTLEPGGQFELSGAPLENLRQDEEIRWHIETVTALAKEKGHQFM
Ostreococcus lucimarinus DRHGWTPINEAD NI IGCTKDKQSVTLEPGGQFELSGAPLENLRQDEEVRWHIETVNALAREKGHRRFL
Chlamydomonas ARCGWEPIMEEG RI IGVQLDGGQVSVTLEPGGQFELSGAPVETIHKCAEVNSHLYQVKAVAEELQTGFL
Volvox       -----
Prototheca   DSNGWEPYIKEGE AKIGLAHANETVTLEPGGQTELSGAPLKDLHAVAQETKEHLIQVRAACDKVGIDYM
Anabaena     -----

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Brassica      GMGFQPKWRREDIPTMPK----GRYDIRNYMPKV--GSLGLDMFRCTVQVNLDFSEADMIRKFR
Picea        GIGFQPKWAVKDIPVMPK----GRYEIRNYMPKV--GSLGLDMFRCTVQVNLDFSEADMVRKFR
Physcomitrella 1 GIGFQPKWSVADTPMPK----GRYEIRNYMPKV--GSFGLDMFRCTVQVNLDFSEADMVRKFR
Physcomitrella 3 GIGFQPKWSVAETPMPK----GRYEIRNYMPKV--GSYGLDMFRCTVQVNLDFSEADMVRKFR
Physcomitrella 2 GIGYEPKWPLSERPNVPK----ARYQVIKDYFPKIVITEVGFETYQCTAQVNLDFSEQDMINKLR
Ceratopteris GIGFHPKLSIKEIPVMPK----GRYQIIRNYMPKV--GSYGLDMFRCTVQVNLDFSEADMVNKFR
Ostreococcus tauri GIGFDPQWSVAETPMPK----GRYKIIRAYMPKK--GTRGLDMFRCTIQVNLDFEDERDMIRKFR
Ostreococcus lucimarinus GIGFDPKWAISDVPMMPK----GRYKIIRAYMPKK--GTRGHDMFRCTIQVNLDFESEQDMVRKFR
Chlamydomonas GVGFDPKWAIQDIPVMPKASRRGGRYSLKSYMPTV--GSMGLDMFRCTVQVNLDFESEQDMVEKFR
Volvox       GVGFDPKWAIQDIPVMPKASRRGGRYSLKSYMPTV--GSMGLDMFRCTVQVNLDFESERDMVEKFR
Prototheca   NIGFDPKWGFEDVPKMPK----TRRYREYMPKV--GTLGHDMFRCTIQVNLDFESEADMVEKFR
Anabaena     IPGSTLSLGGSDRFRSD----PANPYHDYIEQT----YGTKVVTSVHINVGIDDPEVLMRACR

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Brassica      AGLALQPIATALFANSPFTEGKPNGFLSFSHITDTDKDRTGMLPFVDDSFGFQYVDYALDVPMIF
Picea        AGLALQPIATALFANSPFTEGKPNGFLSFSHITDTDNSRCGMLPFVDDTFGFSYVEYALDVPMIF
Physcomitrella 1 VGLALQPIATALFANSPFTEGKPNGFLSFSHITDVDNNRAGDLPFVEDGFGDKYVEYALDVPMIF
Physcomitrella 3 VGLALQPIATALFANSPFTEGKPNGFLSFSHITDVDKDRSGDLPFVEDGFGFEKYVDYALDVPMIF
Physcomitrella 2 VGLSLQPLATALFANSPFWEGKPAGYKTYSFLSQFDDDRTGELPFVDDDFGFEKYTEYALNVPMIF
Ceratopteris VGLALQPIATALFANSPFTEGKPNGFLSFSHITDVDNNRTGMLPFVEDGFGDKYVEYALDVPMIF
Ostreococcus tauri TSLALQPLATALFANSGIVDGEDTYESMSSDVRDTDDDRTGTLSWVFDDDFGFEKYCDYVMNVPMIF
Ostreococcus lucimarinus TSLALQPLATAMFANSAFVDGADTGYQSMSDVRDTDDDRTGTLAWVFDDDFGFEKYCDYVMNVPMIF
Chlamydomonas IGLALQPIANALFANSPFTEGKPTGYLSTRGHVTDVDASRTGNLPFVFEKDMCFSYVDYAMAVPMIF
Volvox       IGLALQPVANALFANSPFTEGRPTGFVSTRGHVTDVDASRTGNLPFVFRDDMSFERYVDYAMDVPMIF
Prototheca   VSLALQNVAGALFANSPFRDGKPTGYKSWLNVTDVDNARCGRLPFVFDADFSFARYAEWALDVPMIF
Anabaena     LIRVEAPLFLALSASSPFIDGKATGYHSTRWGVFQTPTNVP----LFTSHAHIEWVEQLAIGTMQ

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Appendix

Brassica	AYRNGKYVDC TGM TFRQFLAGKLP C LPGELP TYNDWENHLTTI FPEVRLKRY MEMRGADGGP WRRLCAL
Picea	VYRKKKYVDC SGMSFKDFMKGKLP SLPGELPNLNDWENHLTTI FPEVRLKRY LEMRGADGGP WRRLCAL
Physcomitrella 1	VYRNGRYIDC SGMSFKDFLEGKLSALPGEKPTMNDWENHLTTI FPEVRLKRY LEMRGADGGP WRRLCAL
Physcomitrella 3	VYRNGRYVDC SGLSFKDFLEGKLSVLPGERPTLSDWENHLTTI FPEVRLKRY LEMRGADGGP WRRLCAL
Physcomitrella 2	VHRNDNWRDVS GASFKNF MAGKLEAYPGEKPSLNDWMNHLGTMYP EVRLKRY LEMRGADCCPKDFLNAL
Ceratopteris	AYRNKKYVDC AGMSFRDFMAGKLPNLPDGKASIIDWENHLTTI FPEVRLKRY LEMRGADGGP WKKLCAL
Ostreococcus tauri	VYRNGTYVDVSGESWLD FMEGKLPQLPGDTPCLADWEQHLTTVFP EVRLKRY LEMRGADGGSFEAIMAL
Ostreococcus lucimarinus	VYRNGTYVDVSGESWLD FMEGKLPQLPGDKPTIDDWEQHLTTVFP EVRLKRY MEMRGADGGSYEAIMAL
Chlamydomonas	VYRNGQYINALGMSWKDFMAGKLPALPGEYPTIADWANHLTTI FPEVRLKRY LEMRGADGGP WRMLCAL
Volvox	VYRNGQYINALGMSWRDFMAGKLPALPGEYPTIADWANHLTTI FPEVRLKRY LEMRGADGGP WRMLCAL
Prototheca	LYRDGEYHDVGAQPFRDFHFGKLAAFPGLFPTMDDWDVHLTTVFPDVR LKRY LEMRGADGGGWEFITAL
Anabaena	NVRHLWVSRPNGRRPYDLNRLELRICD-LVTDPI SLLAITALLEARLLQLIENPDLPLTQSKFSPE
Brassica	PAFWVGLLYDEDVLQSVL DLTADWTPAEREMLRNKVPV TGLKTPFRDGLLKHVAEDVLKLAKDGLERRG
Picea	PALWVGLLYDETSLSQSVLDITKDW TLEEMQMLRNKVPKMG LKTPFRDSSLRHVAEDILQLAKEGLQRRG
Physcomitrella 1	PAFWTGLLYDEESLQGALDI INDWTERERAMLRSKVPKLG LNVFRDGLLKHVAQDVFKLAKDGLTRRG
Physcomitrella 3	PAFWTGLLYDDES LQGAFDI INDWTEKERAML RNKVPKEGLNVFRDGLLKHVAQDIFKLAKDGLTRRG
Physcomitrella 2	PAFWVGLLYDEQSLKNCLDI IRDWSNDDRGFLRKEV PNLQGLTVPFRAGTQDVAKQVVSIAHGGLERRG
Ceratopteris	PAFWVGLLYDETS LPRALDI IKDWSLEERSYLRRQVPS FGLKTPFRDGLLR-----
Ostreococcus tauri	PALWVGLLYSEKAL TAAEKLVS DWSKEERETLRVAVTKDGLS AKFRNGTARDIARDMVR LAVDGLKERG
Ostreococcus lucimarinus	PALWVGLLYSDKALDAAEAMVSDWTQVERDALRVDVTKDGLS AKFRGGTANDIAKKMVELSVQGLQERG
Chlamydomonas	PALWVGLLYDPEAQRQALALIEDWTPAERDYLRT EYVTRFGLRTPFRAGTVQDVAKQVVSIAHGGLERRG
Volvox	PALWVGLLYDEEAQRQALALVSDWTD SERDYLRTEVTRYGLRTRFRGGSVQDLAKQVVS IARGGLQRRG
Prototheca	PALWVGLLYDAGAQAEAAARLVADWTP EELDALQADVPRQALQATFRSGTVQDLAKQVLA IARRGLDARG
Anabaena	ELVTLTTENEAAAASASLDAQLTHWQDGRS I IARDWVNEIYQEVWAI AKKHGFS CFLSPLQKILREGNE
Brassica	YKEVGF LNAVTEVVRTGVT PAENLLEMYNGEWGQSVDPVFQELLY-----
Picea	CKESGFLNEVAEVVRTGITPAERLLDLYHGK WGNVDPVFEEELLY-----
Physcomitrella 1	LNETGFLKDIEETVRTGKTPAEHILELYHGK WGENVDPVFEKLLY-----
Physcomitrella 3	LNEAGFLKDIEEIVQGTGKTPAEERLLDLYHEK WNRNVDTVFEEELLY-----
Physcomitrella 2	LQEGKFLAPLEEVATTGVT LAERMLKLYEEEW DGRVDPIFNELRL-----
Ceratopteris	-----
Ostreococcus tauri	LGEEVYLEYLQQMVDGGATASSRTRALFTNEWKGD LAKVYEYASYPDVLPKF-
Ostreococcus lucimarinus	LGEEVYLRYLQDIVDDGKSVAARMSEM NAREWNGDLEKVEYATFPDTPKIV
Chlamydomonas	YDETSFLKRLEVI AETGLTQADHLL ELYETKQWRSVDPLYKEFMY-----
Volvox	HDEVNFKRLEVVAETGLTQADHLL ELYETRWQRSVDPLYTELMY-----
Prototheca	LGEGRYLDVLD AIAESGVTQADRLLQLYETE-----
Anabaena	AQHWLQLHKVGFDTQC VITQAISTTQERELELQNK LCTQLKG-----

Figure A4: Alignment of GCL protein sequences from *Brassica juncea*, the gymnosperm *Picea glauca* (partial, translated from DV974075 and DR584455), the moss *Physcomitrella patens*, the fern *Ceratopteris richardii* (CV735771, partial sequence) and the green algae *Ostreococcus lucimarinus* and *O. tauri*, *Chlamydomonas reinhardtii*, *Volvox carteri* (partial sequence), and *Prototheca wickerhamii*, and the cyanobacterium *Anabaena variabilis* (YP_323696). Residues involved in substrate binding and catalysis in *Brassica* are marked in red, residues involved in the formation of the dimer interface are highlighted in blue, and cysteine residues forming the two disulfide bridges in *Brassica* are marked in yellow. Predicted transit peptides are depicted in blue letters.

6.6 Predicted localization of plant GCL proteins

Plant species	Predicted localization of GCL
<i>Allium cepa</i>	Plastidic (3)
<i>Arabidopsis thaliana</i>	Plastidic (4), confirmed (Wachter et al., 2005)
<i>Aquilegia sp.</i>	Cytosolic (4)
<i>Beta vulgaris</i>	Mitochondrial (5), confirmed plastidic (Müller, 2006)
<i>Brassica juncea</i>	Plastidic (2), confirmed (Wachter et al., 2005)
<i>Citrus sinensis</i>	Plastidic (3)
<i>Gossypium raimondii</i>	Plastidic (5)
<i>Lactuca perennis</i>	Plastidic (1)
<i>Lotus corniculatus</i>	Plastidic (4)
<i>Lycopersicon esculentum</i>	Plastidic (4)
<i>Malus domestica</i>	Plastidic (5)
<i>Medicago trunculata</i>	Plastidic (1)
<i>Nicotiana tabacum</i>	Plastidic (3), confirmed (this work)
<i>Oryza sativa</i>	Plastidic (5)
<i>Phaseolus vulgaris</i>	Mitochondrial (3)
<i>Physcomitrella patens</i> #1	Cytosolic (2)
<i>Physcomitrella patens</i> #2	Mitochondrial (4)
<i>Physcomitrella patens</i> #3	Mitochondrial (5)
<i>Pisum sativum</i>	Plastidic (3)
<i>Populus trichocarpa</i>	Plastidic (3)
<i>Solanum tuberosum</i>	Cytosolic (5)
<i>Triticum aestivum</i>	Mitochondrial (4)
<i>Taraxacum officinale</i>	Plastidic (3)
<i>Vitis vinifera</i>	Plastidic (4)
<i>Zinnia elegans</i>	Plastidic (3)

Table A5: Predicted localization of plant GCL proteins: Predictions were conducted using the targetP program (Emanuelsson et al., 2000). The value in parenthesis behind the prediction is the reliability class, with lower numbers indicating a higher reliability. Accession numbers for all sequences are found in the Appendix, Table A1

9 Publication record

Gromes, R., Hothorn, M., Scheffzek, K., and Rausch, T. (2007). Activation of γ -glutamylcysteine ligase by redox-regulated monomer-dimer transition is a mechanism unique to plants. **In preparation**

Rausch, T., Gromes, R., Liedschulte, V., Müller, I., Bogs, J., Galovic, V., and Wachter, A. (2007). Novel insight into the regulation of GSH biosynthesis in higher plants. **Plant Biol** **9**, 565-572

Hothorn, M., Wachter, A., Gromes, R., Stuwe, T., Rausch, T., and Scheffzek, K. (2006). Structural Basis for the Redox Control of Plant Glutamate Cysteine Ligase. **J. Biol. Chem.** **281**, 27557-27565.

Zhigang, A., Cuijie, L., Yuangang, Z., Yejie, D., Wachter, A., Gromes, R., and Rausch, T. (2006). Expression of BjMT2, a metallothionein 2 from *Brassica juncea*, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. **J. Exp. Bot.** **57**, 3575-3582.

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