

# Discovery and characterization of small molecule inhibitors of cystathionine gamma-synthase with *in planta* activity

Itai Bloch<sup>1</sup>, Hadar Haviv<sup>1</sup>, Irena Rapoport<sup>1</sup>, Elad Cohen<sup>1</sup>, Rotem Shelly Ben Shushan<sup>1</sup>, Nesly Dotan<sup>1</sup>, Inbal Sher<sup>3</sup>, Yael Hacham<sup>1,2</sup>, Rachel Amir<sup>1,2,\*</sup>  and Maayan Gal<sup>3,\*</sup> 

<sup>1</sup>Migal – Galilee Technology Center, Kiryat Shmona, Israel

<sup>2</sup>Tel-Hai College, Upper Galilee, Israel

<sup>3</sup>Department of Oral Biology, The Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

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\*Correspondence (Tel +972 507 987 058 (MG) and +972 507 395 696 (RA); emails mayyanga@tauex.tau.ac.il (MG) and Rachel@migal.org.il (RA))

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## Summary

The synthesis of essential amino acids in plants is pivotal for their viability and growth, and these cellular pathways are therefore targeted for the discovery of new molecules for weed control. Herein, we describe the discovery and design of small molecule inhibitors of cystathionine gamma-synthase, a key enzyme in the biosynthesis of methionine. Based on *in silico* screening and filtering of a large molecular database followed by the *in vitro* selection of molecules, we identified small molecules capable of binding the target enzyme. Molecular modelling of the interaction and direct biophysical binding enabled us to explore a focussed chemical expansion set of molecules characterized by an active phenyl-benzamide chemical group. These molecules are bio-active and efficiently inhibit the viability of BY-2 tobacco cells and seedlings growth of *Arabidopsis thaliana* on agar plates.

## Introduction

Weeds cause significant losses in agricultural produce and yield as they compete with crops for the most vital resources such as light, water and fertilizers. Thus, major efforts have been invested in trying to develop substances capable of inhibiting vital plant processes that could act as herbicides (Gianessi, 2013; Harker, 2001; Soltani *et al.*, 2016; Swanton *et al.*, 1993). This need comes together with the observation that herbicidal resistance is constantly on the rise (Zimdahl, 2018), and that climate change is enabling weeds to attack and compete more effectively with the crop plants (Arnell *et al.*, 2002). However, gaps in research and development have hampered the discovery of new herbicides (Duke, 2012). Researchers searched for various plants' specific targets leading to herbicides that inhibit photosynthesis and mitosis through direct interaction with tubulin, those that lead to the overproduction of superoxide radicals, and the synthesis of cell walls, lipids, carotenoid, folate, phyto-hormones, as well as other plant-specific processes (Dayan, 2019; Duke, 1990). Regardless of the mechanism, new herbicides should be chemicals with a low toxicity that are safe to both user and environment (Corsi, 2015). As of today, the most common way to manage weeds is by using herbicides in the form of synthetic molecules (Hicks, 2012; Kaschani, 2007). However, although extensive research has been invested, no herbicide acting in a new mode of action has been commercially developed in recent decades, and some of the existing herbicides are being removed from the market by regulators due to environmental and toxicological concerns (Chuprov-Netochin *et al.*, 2016; Duke, 2012; McCourt, 2010; Meesters *et al.*, 2014; Peng *et al.*, 2013).

Moreover, heavy use of herbicides is applying constant selection pressure on weeds, accelerating the evolution of herbicidal resistance (Heap, 2014; Heap, 2018; Powles, 2008; Service, 2013), and cases where multiple herbicide resistance developed were documented (Bell *et al.*, 2013). Within the realm of specificity, interference with the synthesis of essential amino acids that are produced by plants and not by animals is a promising target (Kishore, 1988; Muhitch *et al.*, 1987; Duke and Dayan, 2011; Wendler, 1990). Indeed, small molecules targeting such enzymes have the potential of disrupting plant growth and development. For instance, imidazolinones and sulphonylureas that inhibit enzyme acetolactate synthase, which is involved in the biosynthesis of branched-chain amino acids, and glyphosate, which inhibits 5-enolpyruvylshikimate-3-phosphate synthase that is involved in the biosynthesis of aromatic amino acids, have proven to be very successful herbicides (Dor *et al.*, 2017; Hall *et al.*, 2020). More recently, aspterric acid was discovered as a potent inhibitor of the enzyme dihydroxy acid dehydratase catalysing the last synthesis step of Val, Leu and Ile (Yan *et al.*, 2018). Efforts have also been invested in the inhibition of histidine synthesis by interfering with the activity of imidazoleglycerol phosphate dehydratase (Gohda *et al.*, 1998).

Among the essential amino acids, the biosynthesis pathway of methionine has been utilized less for developing new inhibitors compared to other amino acid synthesis pathways (Ravanel *et al.*, 1998b). Methionine is a fundamental metabolite in plant cells, since, in addition to its role as a protein constituent and its central role in the initiation of mRNA translation, it controls the levels of several essential metabolites through its important derivative, S-adenosyl-methionine (SAM). In plants, methionine regulates a

variety of cellular processes such as synthesis of the plant hormone ethylene, vitamins such as biotin, and the polyamines spermidine and spermine, playing crucial roles in many aspects of plant growth, including cell proliferation and differentiation, apoptosis, homeostasis and gene expression (Roje, 2006). In addition, SAM is the precursor for the methyl group required for DNA and RNA methylation and many secondary metabolites. Therefore, methionine is considered a key player in plant metabolism.

Cystathionine gamma-synthase (CgS) is the key regulatory enzyme of the methionine biosynthesis pathway in plants. The importance of CgS activity for normal plant metabolism, together with the lack of CgS in animals and humans, marks it as an interesting herbicide target (Amir, 2010; Hirase, 2003). CgS binds O-phospho-homoserine, which is initially formed from aspartate, and cysteine, the first sulphur-containing organic compound, in order to form cystathionine, the first unique metabolite in the methionine pathway (Ravanel *et al.*, 1998b). The key role of CgS in methionine synthesis was revealed by the findings that overexpression of the *A. thaliana* CgS (*AtCgS*) gene in the leaves and seeds of transgenic plants led to significantly higher levels of methionine (Hacham *et al.*, 2008; Heap, 2014). However, overexpression of the two other enzymes in the methionine pathway, cystathionine  $\beta$ -lyase and methionine synthase, exhibited a weaker role in regulating methionine levels (Maimann *et al.*, 2001; Nikiforova *et al.*, 2002). Furthermore, reduced activity of CgS is correlated with abnormal phenotypes such as the inhibition of plant growth, non-regular morphology and the inability to produce flowers (Cohen *et al.*, 2017, 2014; Gakiere, 2000; Kim, 2000). As part of the pyridoxal-5 phosphate (PLP)-dependent enzymes, CgS catalyses the  $\gamma$ -replacement reaction of L-cysteine and L-homoserine derivatives (Alexander *et al.*, 1994; Eliot, 2004). Figure 1a illustrates the chemical reaction and the different homoserine derivatives in plants and microorganisms. On the structural level, CgS is organized as a homotetramer with a molecular mass ranging from 130 kDa to 220 kDa in different plants (Clausen *et al.*, 1998; Sagong, 2017; Steegborn *et al.*, 1999b), where each homodimer produces two active sites. The binding site is highly conserved and requires the co-factor of PLP for its catalytic activity (Clausen *et al.*, 1999). In the core of its binding site, CgS has a positively charged arginine residue interacting with the carboxyl group of different substrates and a tyrosine interacting with the co-factor of PLP (Steegborn *et al.*, 2001; Steegborn *et al.*, 1999b). The structural features of the enzyme's catalytic site bound to the PLP are illustrated in Figure 1b. The notion of CgS as a potential target for the development of inhibitors led to the identification of the putative binding site for small molecules and the characterization of potential herbicides (Hirase and Molin, 2003; Steegborn *et al.*, 2001).

Herein, we aim at discovering novel small organic molecules that inhibit CgS and thus inhibit plant cells and *in planta* activity. Unlike studies that employed phenotypic screening, we applied a structure-based approach whereby a large virtual small molecule library was screened *in silico* and we selected a set of compounds for further study via biochemical, cellular and *in planta* assays.

## Results and discussion

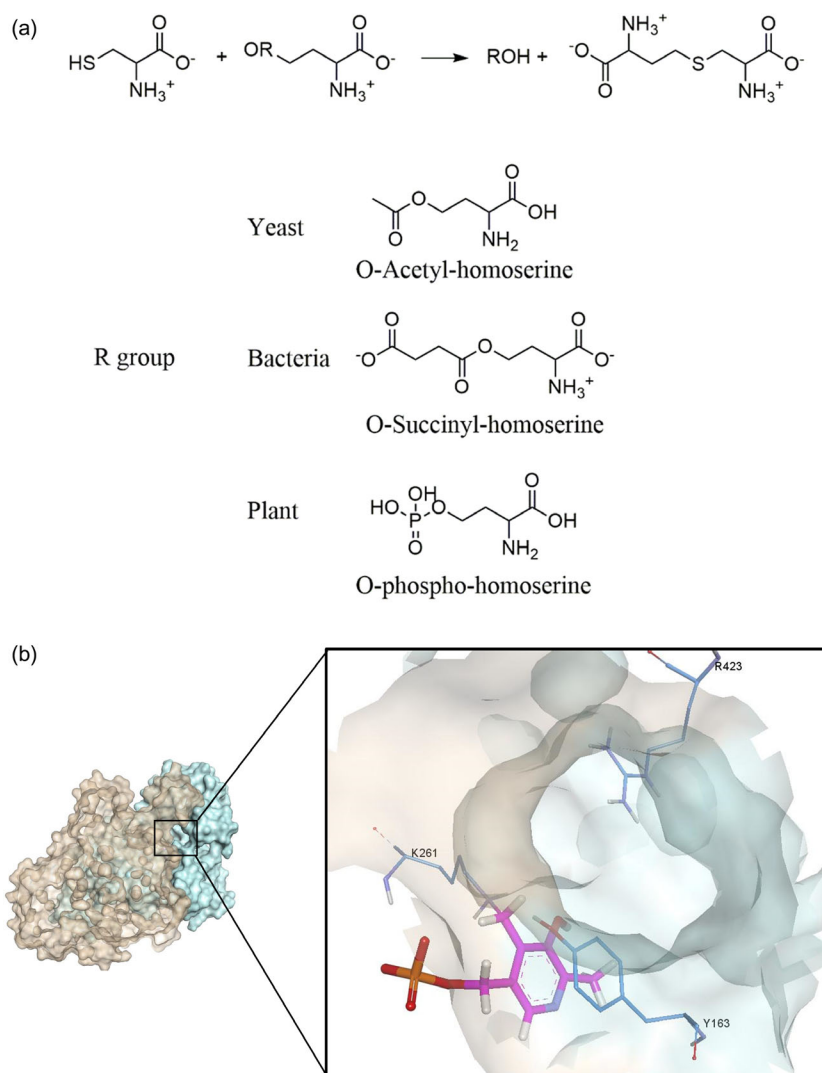
### *In silico* library construction and screening

The current study focussed on the discovery and design of new small molecules targeting the methionine pathway. In the past, pesticides in general and herbicides in particular had been identified based on direct screening on the plants. Successful compounds were selected according to their ability to impart the

desired output in what is considered phenotypic screening. In phenotypic screening, successful compounds supposedly contain all the requirements for an active herbicide. However, the method in general reached a dead-end in terms of delivering a new mechanism of action, coping with current environmental regulations, possessing the ability to develop selective herbicides and improving affinity binding that is often based on the physico-chemical binding properties of the molecule to the target protein. The result is that most of the current existing and approved commercial herbicides act in one of the following modes of action, acetolactate synthase, photosystem II, 5-enolpyruvylshikimic acid-3-phosphate synthase, Acetyl CoA Carboxylase (ACCase) or protoporphyrinogen oxidase (Dayan, 2019). Thus, the search for new chemical entities capable of inhibiting weeds has shifted in recent years to what is known as target-based discovery (Lein *et al.*, 2004; Ward, 1999). This approach required computer *in silico* approaches together with biophysical and biochemical validation. An example of such a computational approach is the discovery and optimization of molecules targeting the pyruvate dehydrogenase complex (Peng *et al.*, 2007).

Herein, we used a multidisciplinary approach in which the integration of computational and biophysical/biochemical tools enabled us to screen a large library of small molecule candidates and validate the activity of the selected virtual hits towards CgS. In the target-based computational approach, the target protein and putative binding site were selected, and a large virtual library of compounds was filtered to select for preliminary hits, that is, compounds showing initial binding affinity to CgS. This differs from the phenotypic-based screening approach in which lower numbers of chemical compounds are applied onto the target plant and candidates are selected according to their ability to kill the target plant (Alfred *et al.*, 2012; Jianyi *et al.*, 2002). An additional advantage of computational approaches is the ease of filtering according to custom-defined criteria. For instance, in the current protocol, we utilized the profile of known herbicides and applied a filtration accordingly. This resembles the cheminformatics analysis (Quareshy *et al.*, 2018) and docking application used for identifying promising inhibitors of ACCase (Ye *et al.*, 2018).

In order to find new small molecule inhibitors targeting the CgS enzyme, we virtually screened a database of approximately 30 million commercially available small molecules. This process included the following steps: (i) carrying out a structural analysis of all available structures of CgS in order to characterize the potential binding site and generate 3D models for docking; (ii) carrying out an analysis of known CgS structures and binders from previously published studies, co-crystals and natural ligands for the extraction of required pharmacophore-like constraints; (iii) generating a profiled screening library based on the analysis of all known herbicides in order to fit our library to the chemical and physical properties of herbicides and bio-active compounds in plants; (iv) filtering of the profiled screening virtual library according to the required pharmacophore-like features and known herbicides properties; (v) docking and clustering of the virtual compounds passing the first pharmacophore filter; and (vi) ranking and manually selecting top-ranked compounds for the next *in vitro* validation. Figure 2a illustrates the workflow of the virtual screening process designed to search and filter potential CgS inhibitors. To filter the molecular library based on physico-chemical properties of known herbicides, we used the interactive herbicide database that comprises 334 known herbicides (Gandy *et al.*, 2015). Chemical properties including



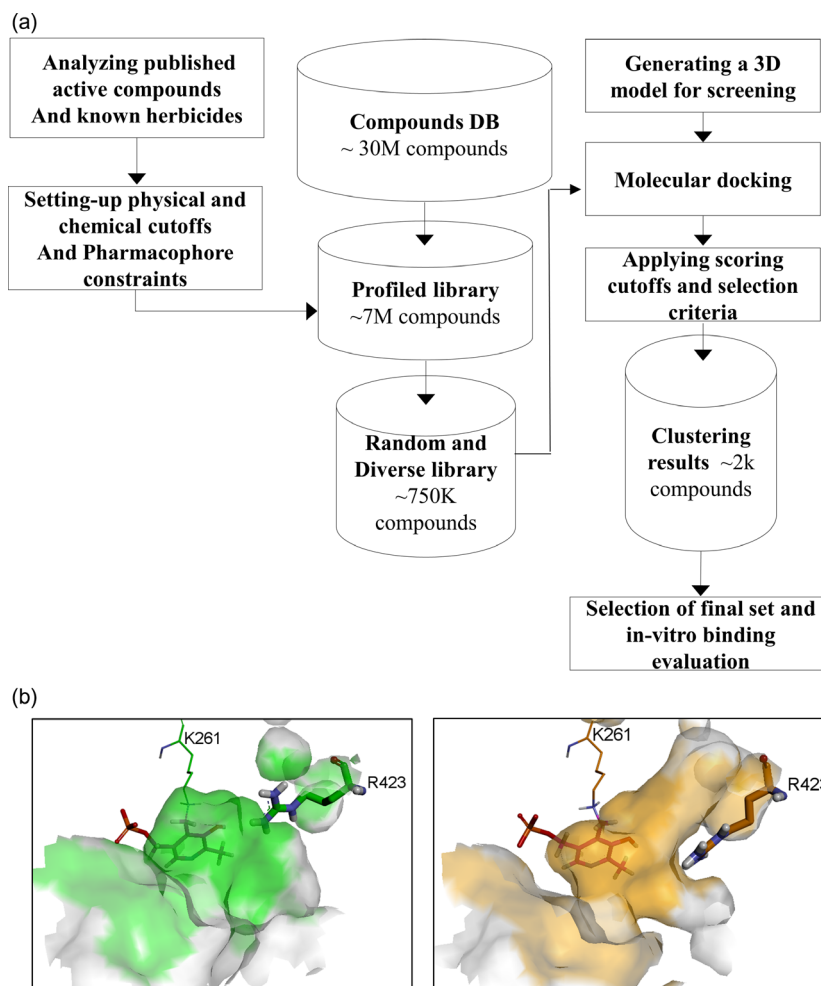
**Figure 1** CgS structure and function. (a) Chemical reaction catalysed by CgS in yeast bacteria and plants, and the different substrates in each organism. (b) CgS active site is composed of homodimer and requires the co-factor PLP (purple) that binds Lys261 of CgS to the substrate. Y163 is essential for PLP binding to the substrate and acts as an acid–base catalyst (Steegborn *et al.*, 1999b).

AlogP, molecular weight, molecular volume, number of atoms, number of H acceptors/donors, number of positive/negative atoms, number of rings and number of aromatic rings of these herbicides were extracted. Minimal and maximal boundaries were set, and the full molecular database was filtered based on these properties.

Crystal structure analysis of CgS (Steegborn *et al.*, 2001; Steegborn *et al.*, 1999b) revealed two distinct conformations of the active site, globular or extended, depending on the side chain-rotamer state of Arg423 and Asn211 fluctuation. Based on the different conformations, a structural ensemble was prepared and used as the basis for the molecular docking of the profiled *in silico* library. Figure 2b shows the globular and extended enzyme model that was used to screen the small molecule library. Following the *in silico* screening, we purchased a subset of ~200 compounds for the *in vitro* screening. This set of compounds covers the largest possible chemical space while still addressing the desired physio-chemical criteria to ensure optimal binding.

### Setup of the *in vitro* screening system

A crucial step of *in silico* screening is the follow-up of robust *in vitro* validation. Therefore, in our next step, we embarked on determining whether the small molecule compounds directly influence the methionine pathway. To this end, we set up a screening system including an engineered MetB knockout *E. coli* that is an auxotroph to methionine (Hacham *et al.*, 2002; Hacham *et al.*, 2006). The bacterial screening assay (Figures 2 and 3) was designed specifically to differentiate between small molecules targeting the methionine biosynthesis pathway and molecules affecting other pathways. Having a bacterial assay as a secondary screening step enables cost-effective and rapid filtering of a large subset of molecules. Moreover, this assay can also be used as a stand-alone for the screening of a larger *in vitro* library. Indeed, a previous study described a high-throughput screening assay for the discovery of the inhibitors of human 4-hydroxyphenylpyruvate dioxygenase using bacteria (Neuckermans *et al.*, 2019).



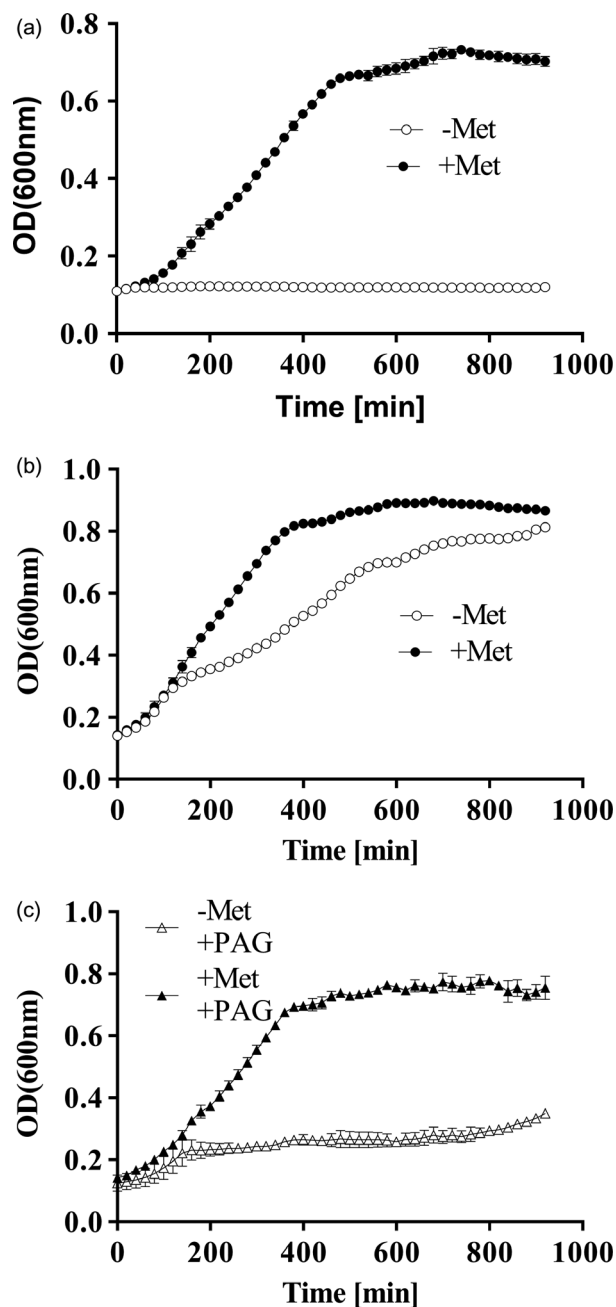
**Figure 2** *In silico* screening of a small molecule library against CgS structure. (a) Illustration of the virtual screening procedure used to filter a final set of potential inhibitors from the initial ~30 million compounds. Pharmacophore constraints were used for the initial crude filtering of the molecular library. Molecules in the final filtered libraries were docked onto an extended averaged structure of CgS. Scoring function was applied to rank the best predicted inhibitors. (b) Fluctuations of R423 change the active site geometry from a globular (left panel) to an extended mode (right panel). Both structures were used in the molecular docking of the small molecule library.

Figure 3a shows the methionine auxotroph (MetB knockout) *E. coli* growth curves on M9 minimal media containing glucose and ammonium chloride as the sole carbon and nitrogen source, respectively, with and without the addition of external methionine. The methionine auxotroph cells require the addition of methionine to grow (black circles, Figure 3a) and cannot grow without it (white circles, Figure 3a). To set up a screening system for the selected small molecules, we complemented the MetB knockout bacteria with a gene of *AtCgS* that lacks its transit peptide (Hacham *et al.*, 2002). This was shown to complement the MetB knockout, and so we transformed the PQE-30 plasmid containing the *AtCgS* gene to the bacterial cells. Figure 3b shows the bacterial growth curves with and without methionine (Kim *et al.*, 2002; Ravelle *et al.*, 1998a). Though at a lower growth rate, cells with the *AtCgS* gene grew without any external methionine validating the activity of the complemented *AtCgS* gene. In order to validate that the assay is suitable for identifying small molecule inhibitors of CgS, we further tested the effect of a known CgS inhibitor, propargyl-glycine (PAG) (Thompson *et al.*, 1982). Figure 3c shows the same experimental setup described

in Figure 3b but with the addition of 50  $\mu\text{M}$  PAG in the M9. When no external methionine is supplemented, the PAG inhibits the *AtCgS* enzyme and therefore significantly inhibits cell growth. However, with the addition of methionine, the cells grew in the presence of PAG. The bacterial assay is therefore suitable for the selection of small molecule modulators of CgS and the methionine pathway.

#### *In vitro* selection of active small molecules

Having an *in vitro* system for selecting CgS small molecule inhibitors, we tested the effect of the molecules with and without the addition of external methionine on bacterial growth following transformation with *AtCgS*. Each molecule was dissolved in a well within the bacterial minimal media culture to a final concentration of 50  $\mu\text{M}$  in a 96 well-plate format. The growth curves in M9 were monitored under the same duration and conditions shown in Figure 3, and the slope and maximal OD value of each growth curve were evaluated. Figure 4a shows the effect of the screened molecules on the *AtCgS* transformed bacteria with (bottom) and without (top) methionine. Given that the molecules' stock



**Figure 3** Setup of the bacterial assay for the screening of CgS small molecule inhibitors. (a) Growth curves of MetB knockout bacteria auxotroph to methionine with and without the supplementation of 40 mg/L external methionine. (b) Growth curves of bacteria following the transformation of *AtCgS* with and without the supplementation of methionine. (c) Growth curves of the previous bacteria on M9 with the supplementation of external methionine with and without the addition of the known CgS inhibitor PAG.

solution is in DMSO, each bar in Figure 4a represents the ratio of growth slope of bacteria treated with a single molecule to bacteria treated with an equal concentration of DMSO as a control. Potential small molecule inhibitors in the aforementioned screening are molecules that inhibit bacterial growth in the absence of methionine (top panel), but have less or no effect on bacterial growth in the presence of methionine (bottom panel).

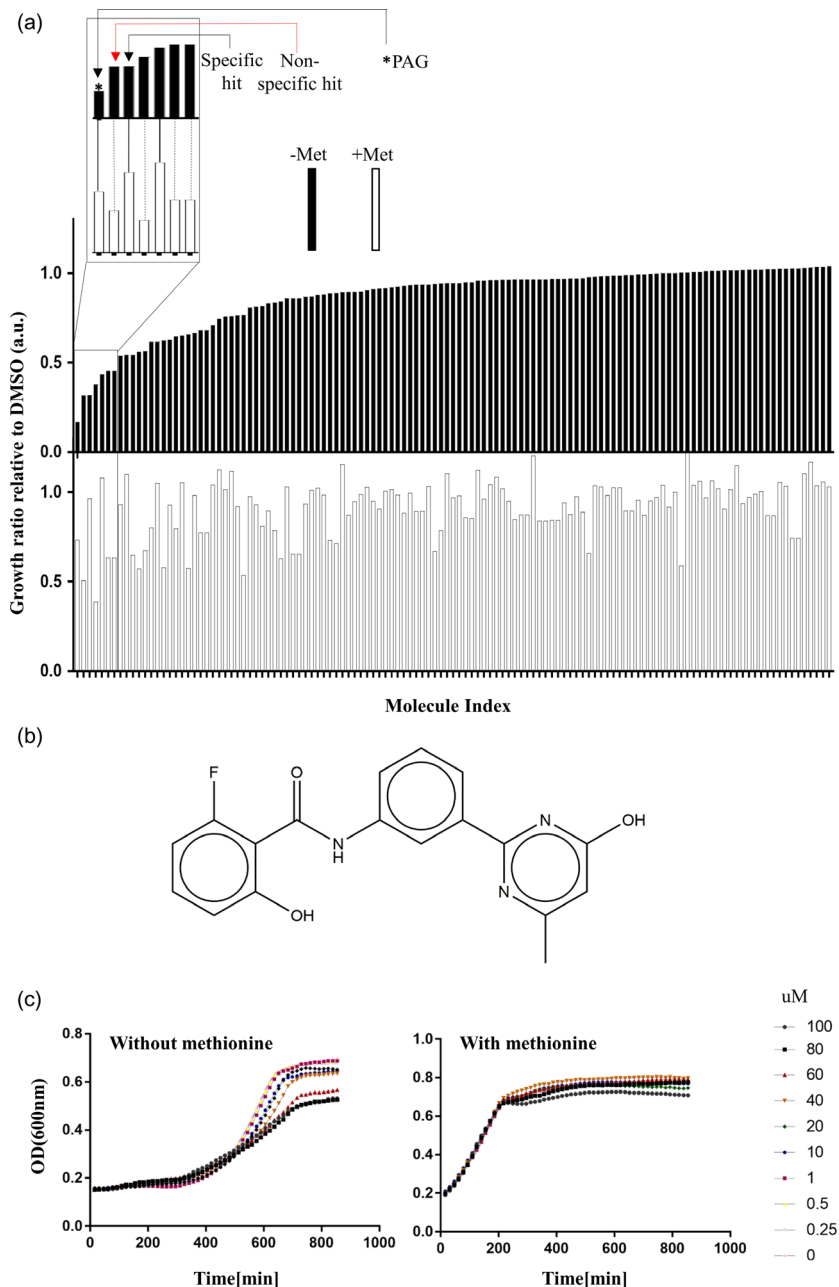
Figure 4a is sorted in ascending order in the top panel, and thus potential CgS inhibitors are represented by bars located on the left side of the top panel where the growth rate is lowest without methionine. However, specific methionine modulators should have no effect on the growth rate with methionine (bottom panel). Molecules that are considered positive *in vitro* hits or non-specific modulators are shown in the inset. Specific hits are represented by bars linked by filled lines within the inset. Indeed, the bar on the left side (marked by \*) corresponds to bacteria treated with the known positive control molecule, PAG. Bars linked by dashed lines represent cells that were affected by the molecule also in the presence of external methionine (upper bar in bottom panel, Figure 4a). Such bars are considered a 'non-specific hit' as marked by the red arrow. This means that the molecule was active with and without methionine; thus, growth inhibition is not directly related to modulation of the methionine pathway but to an alternative anti-bacterial mechanism.

A 'specific hit' is represented by the third bar from the left (marked by a red arrow as a 'specific hit') modulator. This molecule is active in the absence of methionine and not active in its presence. Figure 4b shows the chemical structure of the molecule 2-fluoro-6-hydroxy-N-(3-(4-hydroxy-6-methylpyrimidin-2-yl)phenyl)benzamide (CGSI-1). It has a central phenyl-acetamide with a 3-fluorophenol on the amide side and a substituted pyrimidine on the other. We then tested the activity of CGSI-1 in a dose-response manner. Figure 4c shows bacteria growth curves in M9 containing variable concentrations of CGSI-1 with (right panel) and without (left panel) external methionine. In the presence of methionine, there is no effect of CGSI-1. However, a clear dose-response behaviour is observed when no methionine is supplemented to the M9 medium. This result indicates that the molecule interferes with the methionine pathway within the bacterial cell.

### Chemical expansion and direct binding validation of a chemical analogous to CgS

The next step was to explore the direct binding of CGSI-1 and its chemical analogs to CgS. Following structural analysis of the binding pose of CGSI-1 to the CgS enzyme, we designed and synthesized a set of chemical analogs that were tested for the desired activity. Figure 5 shows the chemical structures of a selected analogous chemicals containing the central phenyl-acetamide and alternative functional groups at the pyrimidine and benzene rings on the two sides of the molecule. This step served two purposes: the first is to find chemical analogs with improved affinity towards the target protein; the second is to ensure that the molecular activity is indeed related to the chemical scaffold and not a singularity within the molecular chemical space.

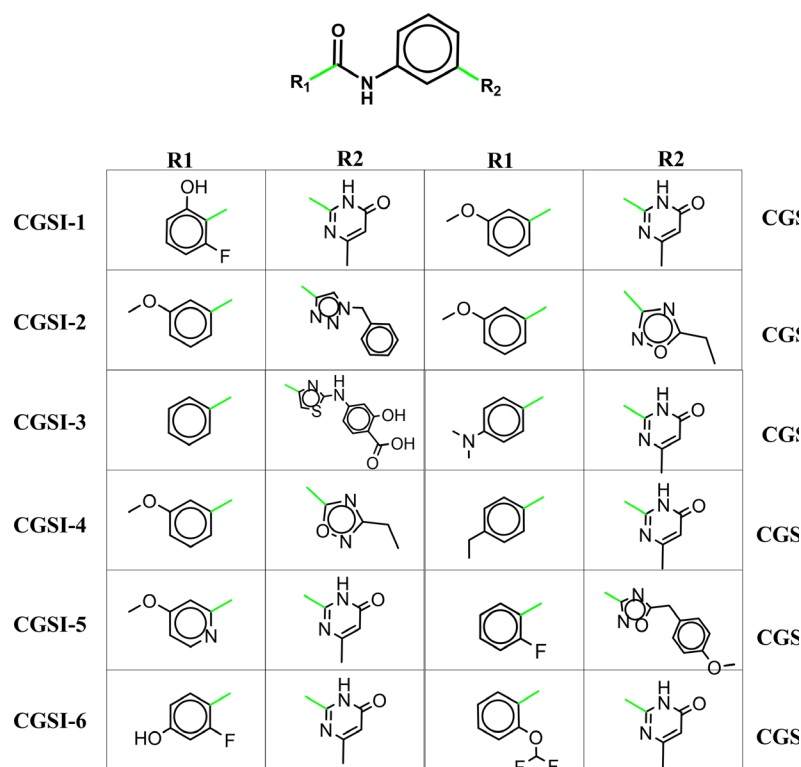
To validate that the selected molecules interact directly with CgS, we conducted a Micro Scale Thermophoresis (MST) experiment as an orthogonal secondary assay to evaluate the binding of the preliminary hit to the target protein (Jerabek-Willemsen *et al.*, 2011; Magnez *et al.*, 2017; Wienken *et al.*, 2010), as illustrated in Figure 6. Among the limited number of biophysical approaches capable of monitoring direct binding between a protein to a small molecule, MST requires relatively low amounts of purified protein and at least in theory can read the binding of low molecular weight molecules (<500 Da) to the much larger target protein (Jerabek-Willemsen *et al.*, 2011). To this end, recombinant CgS was labelled with an amine reactive fluorescent molecule and MST signal intensity was measured in the presence



**Figure 4** *In vitro* screening of selected small molecules in methionine auxotroph bacteria transformed with *AtCgS*. (a) Bars showing the ratio of growth slopes relative to the DMSO of cells in duplicate treated with 50  $\mu\text{M}$  of each selected molecule. Top and bottom panels correspond to with and without the addition of external methionine, respectively. The bar marked with \* in the inset represents bacteria treated with PAG. (b) Chemical structure of CGSI-1. (c) Growth curves in the presence of different CGSI-1 concentrations with (right) and without (left) the supplementation of external methionine.

of variable concentrations of the active molecule. Table 1 summarizes the values of the dissociation constant ( $K_d$ ) of each molecule to CgS. A low  $K_d$  value refers to higher affinity to the enzyme. The MST binding curves of molecules CGSI-1 to 4 are shown in Figure 6a. As expected, a range of affinities is observed, corresponding to the effect that different chemical groups have on the enzyme binding efficiency. It is important to note the different binding curves' shape and axis values. While the baseline of the curve reflects the free protein ( $x = 0$ ), the top region of the

curves corresponds to the relative fluorescence intensity of the labelled CgS when bound to each of the molecules and is thus different in each of the curves. These differences are related to the rate of protein migration in the MST capillary and depend on several factors including molecular weight, surface charge and the hydration state of the protein. Whereas the differences in molecular weight are negligible, the other parameters are compound-dependent and thus give rise to different migration rates and fluorescence intensity readout. Additional difference



**Figure 5** Chemical expansion of CGSI-1. Alternative R1 and R2 groups are shown for each of the analogous chemicals.

that should be considered is the solubility of the compounds, which limits the highest possible concentration of the compounds in the solution.

#### Inhibition of recombinant CgS enzymatic activity

Following the evaluation of direct binding of the small molecules to CgS, we further monitored the effect of the molecules on the enzyme's enzymatic activity. To this end, a recombinant CgS enzyme was expressed in BL21 cells and purified to homogeneity. A reaction containing the purified CgS enzyme incubated with 2 mM cysteine and 3 mM O-phospho-homoserine with 50 μM of the different compounds was monitored for 3 h. The level of free phosphorous in the solution was then correlated with the enzymatic reaction. To determine Pi concentration, aliquots from each reaction sample were mixed with ascorbic acid solution to yield coloured product at 882 nm. Figure 6b shows the inhibition percentage of compound relative to equal concentrations of DMSO with CGSI-2 and CGSI-3 leading to the highest inhibitory effect.

#### Inhibition of BY-2 cellular viability

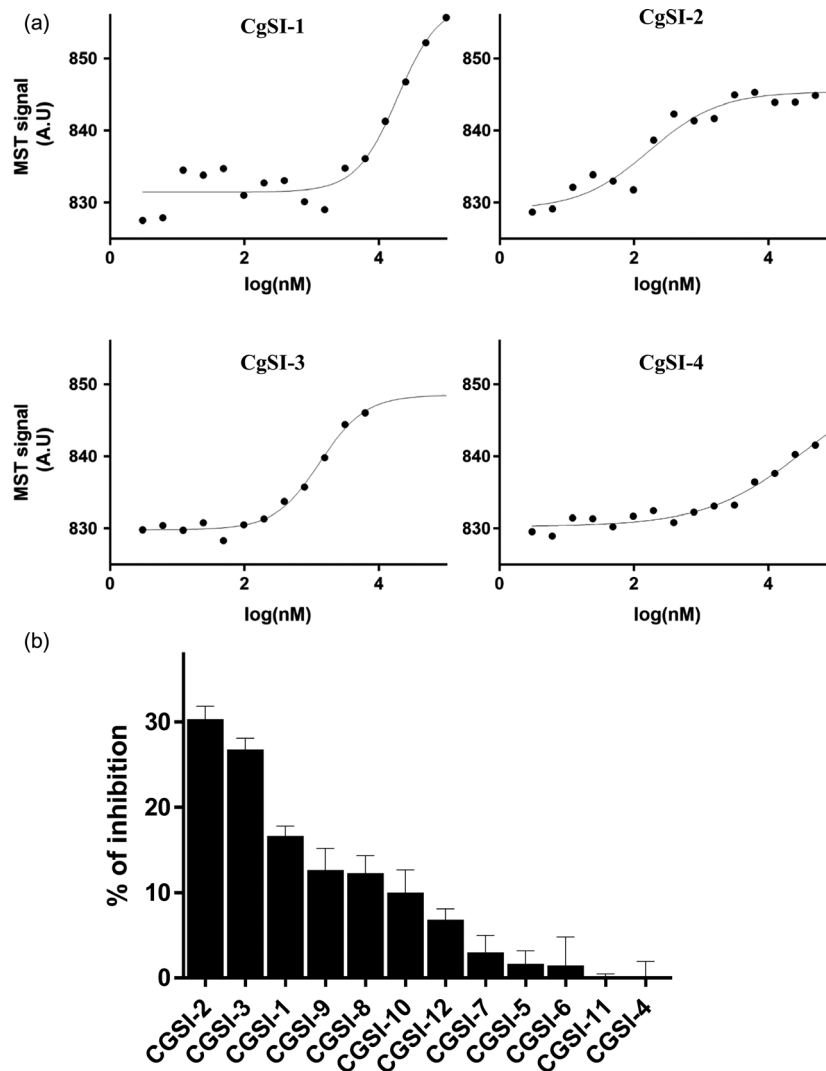
Taken together, the bacterial assay, enzymatic activity and MST validate that the preliminary hits, as well as the focussed structure-similar molecular set, are active in the desired mechanism of action. Indeed, the focussed and cost-effective structure-activity relation study showed activity towards the CgS. Based on the relatively high conservation and structural similarity of the CgS active site (Clausen *et al.*, 1998; Steegborn *et al.*, 1999b) and the putative site of interaction with the small molecules, it is expected that the molecules will act as a general inhibitor and be active in a broad range of plants. For this reason, following the mechanism validation, we tested cellular and *in planta* activity in BY-2 tobacco cells (Dauphinee *et al.*, 2019), and on the

germination and development of *Arabidopsis thaliana* seedlings (Figures 7 and 8). The latter is a cost-effective and rapid way to monitor the effect of various plant inhibitors (Crişan *et al.*, 2009; Dyer, 1995; Moschetto *et al.*, 2019; Travlos *et al.*, 2020). Even though does not always correlate with a successful weed control, it gives a fast and effective evaluation for the *in planta* activity of the non-formulated compound. Moreover, various phenotypes can be observed directly such as the albino effect on cotyledons of several compounds.

To this end, BY-2 tobacco cells were cultured, and cell viability with and without the presence of the CgS inhibitors was monitored. Considering that the sequence and structure of the CgS catalytic centre are highly conserved in plants, it is thus expected that inhibitors targeting this epitope will also be active in tobacco BY-2 cells. Figure 7a presents bar plots of BY-2 viability, monitoring the number of live cells following treatment with each compound relative to cells treated with an equal amount of DMSO. Molecules CGSI-2, 4 and 8 are bio-active and inhibit cell growth. It can be observed that molecules with a higher affinity do not necessarily exhibit the best cellular activity. The CGSI-2 molecule shows a consistent activity profile, whereas the CGSI-4 shows high activity in the BY-2 cells and only modest activity in the seedlings developmental assay. These differences between direct binding and cellular activity often arise due to poor cell penetration, variable solubility or chemical alteration of the molecules within the cell.

#### The effect on *A. thaliana* seedlings

In our last step, we evaluated *in planta* activity quantitatively in *A. thaliana* seeds germination and in seedlings by monitoring germination rate and root length and root mass on agar plates. These parameters are commonly used for determining the toxicity of compounds to the target plant (Bhattacharya, 2010). To this



**Figure 6** MST binding curves and enzymatic inhibition. (a) Fluorescently labelled recombinant CgS at a concentration of 100 nM in each MST capillary was incubated with variable concentrations of each compound. MST signal was fitted to a single site-binding model yielding the dissociation constants (Kd). (b) Purified CgS was incubated with the different small molecules, and the inhibition level relative to the DMSO of each compound in the chemical reaction is shown.

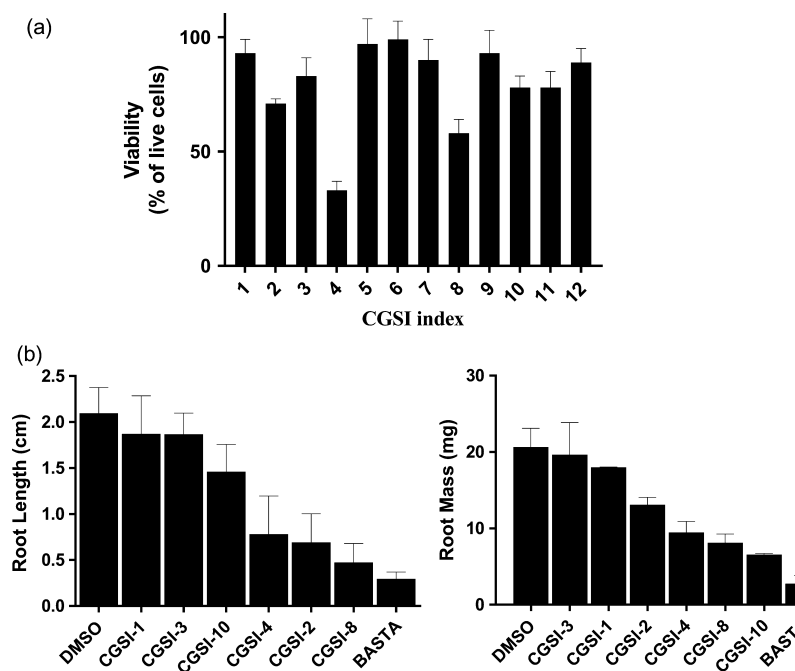
**Table 1** Dissociation constants (Kd) of chemicals analogous to CgS that were measured by MST

CgSI	Kd (nM)
1	29 355
2	182.4
3	1783
4	5040
5	6698
6	9278
7	13 968
8	17 795
9	39 563
10	NA
11	NA
12	NA

end, 50  $\mu$ M of the small molecule inhibitors was added to the medium plates containing *A. thaliana* seeds. The different molecules tested did not inhibit germination of the seeds. However, these compounds affected the root length of the seedlings when compared to the roots of seedlings growing on media containing DMSO. As a control, we also used plates having glufosinate as a known herbicide. No significant differences were observed in germination rate. However, high inhibition of root growth occurred when the plates had CgSI-2, 4 and 8, which is congruent with the BY-2 viability assay (Figures 7b, 8a). In the final step, we characterized the concentration-dependent effect of CgSI-2 on the root length of *A. thaliana* seedlings. Figure 8b shows a clear dose-response effect with an *in planta* IC50 of 14  $\mu$ M for CgSI-2 and IC50 of 19  $\mu$ M for CgSI-8.

Taken together, this study succeeded in defining a small molecule that binds CgS, enabling the inhibition of the enzyme activity, the proliferation of BY-2 cells and the development of seedling roots. Together with these achievements, some open





**Figure 7** BY-2 viability assay and qualitative seed germination assay. (a) Bars showing the % of live cells for the different CGSI compounds relative to the DMSO-treated cells. (b) Average root length (left) and mass (right) of *Arabidopsis thaliana* seeds on an agar plate containing 50  $\mu\text{M}$  of the indicated CGSI compounds.

questions still remain and should be clarified in the future. For instance, what is the off-target activity and how it will affect mammals. The latter is an important issue, since the human enzyme cystathionine  $\gamma$ -lyase shares structural similarity features with CgS (Kraus *et al.*, 2009; Messerschmidt *et al.*, 2003; Steegborn *et al.*, 1999a). In addition, other questions remain related to the application of the molecules in the field. These include the effect of spraying on leaves, large-scale synthesis cost of the molecule, as well as stability and environmental effects. Although all these parameters will have to be tested and tuned, we did establish a preliminary proof-of-concept (POC) for the applicability of CGSI-2 and CGSI-4 in spraying applications. Figure S1 shows the effect of glyphosate and glufosinate as well as CGSI-2, -4 and -8 on Arugula (*Eruca sativa*) leaves after five days. Although not yet as efficient as a herbicide should be, a clear effect is observed for CGSI-2 and -4. Considering that the molecules originated based on the computational filtering of known herbicidal properties makes it a promising candidate for future study and optimization of the latter parameters including the chemical formulation of the vehicle. In addition to the discovery of a long-sought-for new herbicide, an additional important aspect of the target-based approach is the ability to develop engineered plant strains that are resistant to the prospective herbicide. This route could be applied using protein-engineering tools such as protein-directed evolution, which would enable us to evolve a CgS variant that does not interact with the small molecule inhibitor and that retains its enzymatic activity. The latter approach is more challenging if the target protein is not predefined. Another possibility is to screen EMS- or fast-neutron-generated mutants of crop plants for tolerance to these compounds.

## Materials and methods

### Molecular docking and *in silico* compound selection

Two crystal structures of *Nicotiana tabacum* CGS – without and with the inhibitor CTCPO – were used in this study, PDB code:

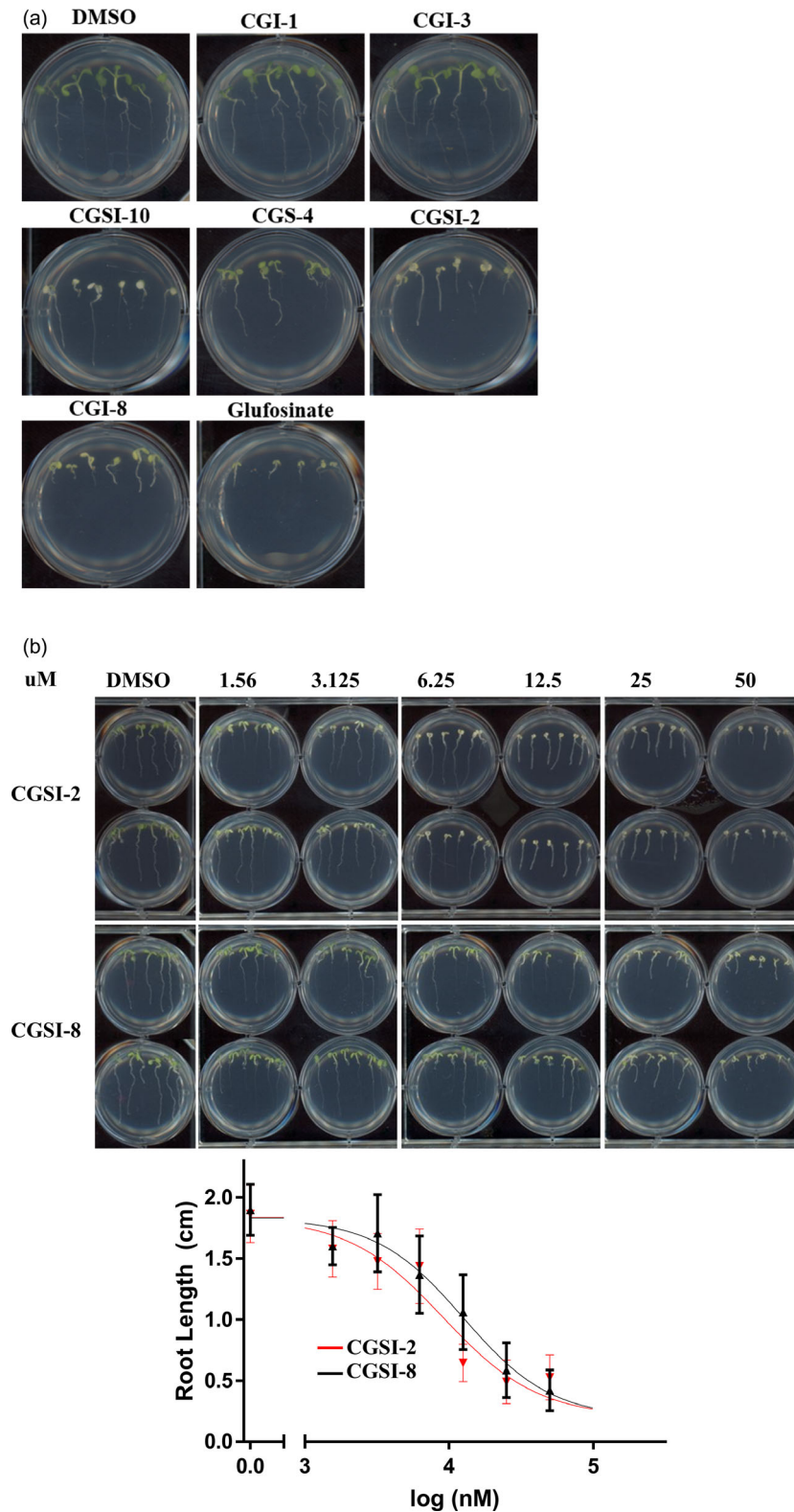
1QGN and 1I48, respectively (Steegborn *et al.*, 2001; Steegborn *et al.*, 1999b). Structure preparation and forcefield assignment were carried out using CHARMM, and ensemble molecular docking of the profiled library was done using the GOLD program (Jones *et al.*, 1997; Verdonk *et al.*, 2005) and Glide, Schrödinger with preset genetic algorithm settings optimized for virtual screening (Friesner *et al.*, 2006; Halgren *et al.*, 2004). A post-docking scoring function was applied to rank each docking pose. A cut-off value was used to filter out compounds that did not satisfy specific location constraints and to account for putative ligand interacting groups in addition to the structure-based complementary ones.

### *In vitro* screening with methionine auxotroph bacteria

In this assay, methionine auxotroph *E. coli* mutant LE392, as was used elsewhere (Hacham *et al.*, 2003), was transformed with PQE-30 vector containing the *AtCgS* gene, as previously described (Hacham *et al.*, 2002; Hacham *et al.*, 2006). In order to test the effect of the small molecule inhibitors on the CgS enzyme and the methionine pathway, growth curves of the *E. coli* in M9 minimal media + 100  $\mu\text{M}$  IPTG were measured with and without the addition of external methionine (40 mg/L) in the presence of different compounds. *E. coli* growth curves were measured by following the OD (600 nm) of bacteria in 96-well plates placed within a Tecan M200 plate reader in an orbital shaking mode at 37 °C. OD readings (600 nm) were made every 20 min for about 1000 min. The slope of each curve was estimated using the 'linest' function in Excel. Final 50  $\mu\text{M}$  molecule concentration was reached by  $\times 1000$  dilution of 50 mM stock in 100% DMSO. Negative control experiments containing DMSO only were thus executed in the presence of 0.1% DMSO.

### BY-2 viability assay

In this assay, the viability of the BY-2 tobacco cell line was tested following treatment with the small molecule compounds. To this end, cells were cultured in Murashige and Skoog basal medium (Sigma-Aldrich, cat#: M0404), supplemented with 2,4-



**Figure 8** Pictures showing the germination of *Arabidopsis thaliana* seeds growing on agar plates treated with selected inhibitors. (a) Effect of 50  $\mu$ M of each molecule on seed length and mass. (b) Concentration-dependent effect of CGSI-2 and CGSI-8 on root length.

dichlorophenoxyacetic acid (0.2 mg/L) and sucrose (3%), incubated in the dark at 25 °C and subcultured at a 1:15 dilution in fresh media every seven days. Before treatment, the cell

suspension was adjusted to OD (600 nm) = 0.4 and seeded in six-well plates in a volume of 3 ml per well. Compounds were then added to the wells at a final concentration of 25  $\mu$ M (0.05%

DMSO) and incubated for 48 h. Cellular viability was measured using the commercially available PrestoBlue assay (Thermo Fisher Scientific, PrestoBlue™ Cell Viability reagent, cat#:A13261). Ten  $\mu\text{L}$  of the PrestoBlue reagent was added to 90  $\mu\text{L}$  of the treated cells, and fluorescence measurement was done in Exc/Ems of 560/590 nm in a Tecan M200 plate reader. The results show cell viability relative to cells treated with 0.1% of DMSO only measured in triplicate.

### CGS expression and purification

Recombinant AtCgS protein was expressed in *E. coli* BL21 cells and purified to homogeneity. To this end, pET-11 vector containing the DNA sequence of AtCgS 6x-HIS tag followed by a TEV-cleavable GB1 solubility tag was cloned and transformed into the bacteria cells. Cells were cultured to OD = 0.8, and protein expression was induced with the addition of 1 mM IPTG. The cells were cultured for an additional 16 h at 25 °C. Following cellular lysis, the soluble fraction was passed through a nickel column and the bound HIS-tagged protein was eluted by 300 mM Imidazole. The 6x-HIS and GB1 tags were cleaved in dialysis and the mixtures passed again through a nickel column to separate the CGS from the TEV and HIS-GB1 constructs. As a final step, the CgS was run on S75 size exclusion chromatography.

### MST assay

To measure the binding of the compounds to the CgS protein, the latter was labelled with commercially available amine reactive fluorophore (NanoTemper, cat# MO-L001) according to manufacturer's instructions. MST binding curves were then measured using the Monolith NT.115 apparatus by incubating the labelled protein with the indicated compounds in a dose–response manner. Baseline corresponding to fluorescence intensity of the free protein was normalized to a relative value based on the average of the three initial low concentrations data points.

### Enzymatic inhibition assay

Purified CgS enzyme at a dose of 0.75  $\mu\text{M}$  was incubated with 50  $\mu\text{M}$  of different compounds in a total volume of 100  $\mu\text{L}$  MOPS buffer (50 mM, pH 7) with 150 mM NaCl for two hours at room temperature. Following incubation, 20  $\mu\text{L}$  of the 15 mM phospho-homo-serine solution (Sigma 04668) and 4  $\mu\text{L}$  of the 50 mM cysteine solution (Sigma W326305) were added for a final concentration of 3 mM and 2 mM, respectively, and reactions were incubated for three hours at room temperature. Following  $\times 6.8$  dilution in H<sub>2</sub>O of 102.5  $\mu\text{L}$  of ascorbic acid assay solution, 12.5  $\mu\text{L}$  from each enzymatic reaction sample was transferred into it. After an additional 30 min, equilibration at room temperature absorbance was measured at 882 nm.

### Ascorbic acid assay solution

H<sub>2</sub>SO<sub>4</sub> 1.25 M, ammonium molybdate 4.9 mM, ascorbic acid 30 mM and antimony potassium tartrate 0.22 mM.

### Inhibition of *A. thaliana* seeds

Seeds were first washed with 70% ethanol for 1 min and then with sterile water. Sterilization was done with 50% bleaching solution (3%) and 0.2% Triton x-100 for 10 min followed by a five-time wash with sterile water. The seeds were placed in six-well plates with half Murashige and Skoog medium (MS; Duchefa, Haarlem, Netherlands) containing 1% sucrose (w/v), pH 5.8. Plates with the *A. thaliana* seeds were placed at 4 °C for two days and then transferred to a growth chamber for seven

days (22  $\pm$  2°C) at day/light cycles of 16/8 h. The plates were placed vertically. In each plate, a control including 0.1% DMSO in the medium was placed and the small molecule compounds were tested by diluting 1000 $\times$  fold of 50 mM stock in DMSO leading to a final concentration of 50  $\mu\text{M}$  with 0.1% DMSO. For the dose–response assay, molecules were diluted to the desired concentration and transferred to the medium.

### Curve analysis

Curve fitting was done by Graphpad prism 7 using a non-linear regression analysis tool set.

### Small molecule chemistry

All molecules were purchased or synthesized by Akos GmbH or Enamine Ltd.

### Conflict of interest

This research was supported by a grant from the Israeli Innovation Authority (IIA) #58634 and by Adama Ltd. Itai Bloch and Maayan Gal are the scientific founders of Projini Ltd. and serve as the company's CSO and scientific advisor, respectively. The chemical scaffolds herein were applied for patent application WO2019142192A1. Elad Cohen, Rotem Shelly, Ben Shushan and Nesly Dotan are currently employees of Projini Ltd.

### Author contributions

I.B. and E.C. designed the model, performed the computational screening and docking, and analysed the computational data. In addition, they selected the virtual hits. H.H. and I.R. set up the *in vitro* methods and executed all experiments and protein purification assays. R.S., B.S, N.D. and Y.H. executed the *in planta* experiments and analysed the data. I.S read the manuscript and gave critical comments. Y.H., R.A. and M.G wrote the manuscript, supervised the project and together with I.B., conceived the original idea and planned the experiments.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Application of spraying of the molecules.