Overexpression of gamma-Glutamyl Cyclotransferases 2;1 (CsGGCT2;1) Reduces Arsenic Toxicity and Accumulation in Camelina sativa (L.)

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Abstract

Environmental arsenic contamination adversely affects human health, and its accumulation diminishes food quality. Glutathione (GSH) is known to bind and detoxify arsenic and other toxic metals. GSH homeostasis in living cells is maintained via γ-glutamyl cycle. Previously, γ-glutamyl cyclotransferases (*GGCT*s) have been shown to be involved in GSH degradation in plants and increasing tolerance to toxic metals. Here we characterized the *GGCT2;1* homolog from *Camelina sativa* for its role in arsenic tolerance and accumulation. Overexpressing *CsGGCT2;1* in Camelina resulted in strong tolerance to arsenite (AsIII). Compared to wild-type, overexpression (OE) lines had significantly higher shoot (2.6-3.5-fold) and root (7-10-fold) biomass on AsIII containing media and accumulated 40-60% less arsenic in root and shoot tissues. Further, OE lines had higher chlorophyll content, lower lipid peroxidation and oxidative stress. There was a small but nonsignificant increase in 5-oxoproline (5-OP) in OE lines and the upregulation of *Oxoprolinase 1* (*OXP1*) suggested accelerated conversion of 5-OP to glutamate, which is then utilized for GSH resynthesis for maintaining homeostasis. Overall, overexpression of GGCT2;1 and it homologs in Camelina and other crops will enable their cultivation on contaminated marginal lands to reduce As accumulation, addressing food safety issues as well as future food and biofuel needs.

Introduction

With the growing world population and changing climate conditions, feeding the world in the future will pose a severe challenge (Dhankher and Foyer 2018; Ray *et al* . 2013). Global food, feed, and fuel requirements are putting tremendous stress on our limited land resources. To achieve food safety and security goals, utilizing degraded and contaminated land for agricultural production safely and sustainably will assist in feeding the future population. Heavy metals and metalloids are major contributors to soil contamination (Li *et al* . 2019). Metal(loid)s such as arsenic (As) can be present naturally in agricultural soil or due to anthropogenic activities like fertilizer and pesticide application, sewage waste, coal burning, and industrial pollution (Li *et al* . 2019). The presence of metal(loid)s in soil impacts the growth and development of food crops as these toxic elements compete with nutrient uptake, negatively impact photosynthesis, create reactive oxygen species (ROS), and damage crucial biochemical processes in plant cells (Farooq *et al* . 2016; Li et al. 2019). Besides severely impacting food production, toxic metals also affect food quality and safety. Food contaminated with toxic metals poses serious threats to the health of humans and livestock. Chronic exposure to As can cause skin, liver, and kidney cancer (Farooq *et al* . 2016; Jomova *et al* . 2011), mercury (Hg) exposure leads to nervous system damage (Yang *et al* . 2020), cadmium (Cd) can cause kidney diseases and cancers (Genchi *et al* . 2020), and lead (Pb) causes reduced cognitive skills and mental development issues (Mason, Harp, and Han 2014). In order to utilize degraded land resources, we need to develop strategies for

the safe use of contaminated sites without compromising the quality and quantity of the produce grown on them. One such approach is utilizing genetic engineering to create plants that can withstand phytotoxicity while limiting the uptake and accumulation of toxic metals.

One of the significant toxic metalloids of global concern is As. Arsenic is a highly toxic, group I carcinogen, and unlike some heavy metals like iron, nickel, and cobalt, it is not required by plants (Farooq *et al* . 2016). Globally, As contamination is a major concern in ˜108 countries, impacting more than 230 million people (Shaji *et al* . 2021). Arsenic toxicity also reduces plant growth and development by damaging the plant's cellular and molecular functions (Meselhy *et al* . 2021; Verma *et al* . 2021). Plants can take up As in two primary forms – inorganic forms such as arsenite (AsIII) and arsenate (AsV), and organic forms such as methylated arsenic –monomethylarsenic acid (MMA) and dimethylarsenic acid (DMA) (Bali and Sidhu 2021; Farooq *et al* . 2016). Plants take up inorganic AsV through phosphate transporters and AsIII, MMA, and DMA via membrane-bound aquaporin transporters, like nodulin 26-like proteins (NIPs) and plasma membrane intrinsic proteins (PIPs) (Ali 2022; Farooq*et al* . 2016; Mosa *et al* . 2012). After entering plants, As impacts the plants' physiology and biochemistry by hindering photosynthetic, respiratory, and other growth and development processes. To protect themselves from the damaging effects of As and other toxic metals, plants utilize their innate capability to detoxify toxic metal(loid)s efficiently via binding with glutathione (GSH) and GSH derivatives, phytochelatins (PCs). Plant cells maintain GSH homeostasis via the γ-glutamyl cycle (Paulose *et al.* , 2013; Noctor *et al.* , 2012; Emamverdian *et al.* , 2015; Hasanuzzaman *et al.* , 2017). Glutathione and its oligomer PCs can help sequester heavy metals in the vacuole and extracellular spaces and limit their uptake and transport, thereby preventing the plant's cellular machinery from damaging toxic effects (Hasanuzzaman et al. 2017). Exogenous GSH application has improved plant tolerance to Hg, cesium (Cs), silver nanoparticles (AgNPs), and salinity in Arabidopsis, soybean, and poplar (Adams *et al* . 2020; Akram *et al* . 2017; Kim *et al* . 2017; Ma *et al* . 2020). Moreover, improving GSH synthesis via overexpression of the *GSH1* gene showed increased tolerance to Cd and As in Arabidopsis (Guo *et al* . 2008) and enhanced tolerance to Pb in poplar (Samuilov *et al* . 2016) and better overall growth of poplar grown in soil contaminated with heavy metals (Ivanova *et al* . 2011). Overexpression of bacterial *γ-E˜Σ, a ΓΣH1*homolog*,* also leads to increased tolerance to As in Arabidopsis (Dhankher *et al.* 2002; Li *et al.* 2005). Similarly, overexpressing GSH recycling genes, *GGCT2;1* (*gamma-glutamyl cyclotransferases 2;1*), improved tolerance to As in Arabidopsis which led to higher biomass, lower As accumulation, and better nutrient utilization as compared to wild-type plants (Paulose *et al.*2013). Here, we translate those Arabidopsis research findings of Paulose*et al.* (2013) into *Camelina sativa* , a biofuel crop, to potentially develop plants that can provide economic returns while cultivated on soils contaminated with toxic metals.

The selection of Camelina as the crop to work with was based on its unique properties. *Camillina sativa* , commonly known as the gold of pleasure or false flax, is an oilseed crop of the Brassicaceae family and is considered a dedicated biofuel crop (Chhikara *et al* . 2018). Camelina has various agronomic advantages for production, including early maturity, low requirement for water and nutrients, adaptability to adverse environmental conditions, and resistance to common cruciferous pests (Abdullah *et al* . 2018; Kagale *et al* . 2014; Stamenković *et al* . 2021). Camelina seeds contain more than 30% oil which can be utilized to produce biodiesel. Based on its shorter life cycle (85-100 days), it can be included in double and relay cropping systems. Life cycle assessment studies of Camelina have shown its economic viability and sustainability for use as a biofuel (Berti*et al* . 2017; Keske *et al* . 2013). However, Camelina is still an underutilized resource, and its potential as a biofuel crop could be increased by translating and applying beneficial research findings from other model plants (Sainger *et al* . 2017; Stamenković *et al* . 2021).

In congruence with this aim to improve Camelina, as a translational approach, we overexpressed the *CsGGCT2;1* gene in Camelina and analyzed its effect on the plant's ability to grow on arsenite-contaminated media. Our results showed that overexpression of*CsGGCT2;1* enabled Camelina plants to grow better than wild-type (WT) plants on media supplemented with AsIII. The *CsGGCT2;1*transgenic lines also had significantly higher shoot and root biomass, improved chlorophyll content, lower lipid peroxidation, and lower arsenic accumulation than WT plants.

Material and Methods

Cloning of CsGGCT2;1 and generation of the gene construct

A Complementary DNA (cDNA) library generated from Camelina wild-type leaves was used to perform PCR with sense (5' TACGTCGTCGACGTTTAAACATGGTTTTGTGGGTA 3') and antisense primers (5' AGATTCGAATCATGATACAAAGACTCTTTGC 3') designed from Camelina*GGCT2;1* (Csa08g024050.1, www.Camelinadb.ca) gene coding sequences. The PCR amplification conditions were 94°C for 2 min (1 cycle); 94°C for 45 sec, 55°C for 1 min, 72°C for 45 sec (40 cycles); final extension at 72°C for 10 min (1 cycle). The resulting 654 nucleotide PCR product was gel purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) and ligated into pGem-T easy cloning vector (Promega Corporation, Madison, WI). The insert was verified by sequencing and then subcloned into the binary pCambia Redseed vector using *Sal* 1 and *Xba* 1 sites for overexpression in plants. The expression of the *CsGGCT2;1* gene is driven under an enhanced 35S promoter. The 35S promoter was amplified from the pEarlygate 103 plasmid via PCR with primers (5' TAGCTGGGGCCCGGCGCGCCGAGATCTCC 3'), (5' CGTCGACACTAGTTCCTCT-CCAAATGAAA 3') and after sequence verification, it was subsequently sub-cloned at *Apa* 1 and *Sal* 1 sites of pCambia Redseed vector. The pCambia RedSeed vector contains a DsRed reporter gene and a hygromycin resistance gene for transgenic seed selection using a DsRed filter or hygromycin antibiotics. The resulting plasmid *pCambiaRedSeed/35Sp::CsGGCT2;1* (Figure 1) was transformed into plants for driving constitutive expression of the *CsGGCT2;1*gene.

Plant Materials and generation of CSGGCT2;1 overexpression lines

Camelina sativa (L.) Crantz cultivar 'Sunseon' was used as the wildtype plant. *CsGGCT2;1* overexpression lines were generated by inoculating wild-type plants with *Agrobacterium* via vacuum infiltration (Lu and Kang 2008). *Agrobacterium tumefaciens* strain EHA105A with modified pCAMBIA1300 plasmid containing*CaMV35Sp::CsGGCT2;1::Nos-t* construct was used for transformation (Figure 1a). After transformation, homozygous lines were generated using DsRED as a selection marker (Figure 1b). The gene expression level was confirmed by using isolated RNA from 3-week-old plants, synthesizing cDNA, and using quantitative real-time PCR (qRT- PCR) (Figure 1c). T_3 homozygous lines were used for all analyses.

Analysis of differential regulation of *CsGGCT2;1* transcript

For differential gene expression analysis, WT plants were germinated on $\frac{1}{2}$ x MS media, and 2-week-old plants were transferred to $\frac{1}{2}$ x Hoagland's solution. After acclimatization for 4-5 days, the hydroponic solution was replaced with $\frac{1}{2}$ x Hoagland's solution containing 25 µM sodium arsenite (AsIII). Plants were harvested at 0 (Control), 6, 12, 24, and 48 h of AsIII exposure. Finally, plants were flash-frozen in liquid nitrogen and stored at -80°C until further analysis of gene expression.

Total RNA was isolated from the roots and shoots of Camelina seedlings using a Plant RNA isolation kit (Sigma-Aldrich Inc., St. Louis, MO) following the manufacturer's protocol. The cDNA was synthesized using the Verso-cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA).*CsGGCT2;1* forward (5'CTACAGCTACTGGACCATGTG 3') and reverse (5' TCACTTCCTCTGGCAAATCG 3') and housekeeping gene *CsEF1* forward (5' CTGCTAACTTCACCTCCCAG 3') and reverse (5' GCTCCTTCT-CAATCTCCTTACC 3') primers were used to perform qRT-PCR. The qRT-PCR amplification program was 95°C for 5 min (1 cycle); 95°C for 15 seconds, 60°C for 45 seconds, 72°C for 1 min (32 cycles), and final extension at 72°C for 10 min (1 cycle). The relative expression level was analyzed by using the delta-delta Ct ($2^{-[?][?]}$Ct) method (Livak and Schmittgen 2001).

Experimental set-up for AsIII treatment

For AsIII treatments, plants were grown on 1/2 x MS media (Phytotechnology Laboratories, LLC, Lenexa, KS) containing 1.5% Sucrose and 0.8% Phytoblend (Caisson Labs, Smithfield, UT) with pH adjusted to 5.7 in growth chamber. Growth conditions were 24°/22°C day/night temperature and 16h/8h light/dark cycle, with a light intensity of 100-120 µmol/m^2/s. For AsIII treatments, 20 µM sodium arsenite was added to the

½ x MS media and plants were grown for 21 days. Fresh shoot and root biomass were recorded at harvesting, and the samples were flash-frozen in liquid nitrogen and stored at -80°C until further analysis.

Chlorophyll content

Chlorophyll was measured using the protocol found in (Ma *et al* . 2016) with adjustments for lower weight and volume. To extract chlorophyll, approximately 10 mg of crushed leaves were added to 1.5 ml of 95% (v/v) ethanol. Samples were incubated in the dark for two days at room temperature (RT) and absorbance of the supernatant was measured at 664.2 nm and 648.6 nm by UV-Vis spectrophotometer (Biotek Epock microplate reader, Aligent Technologies Inc., Santa Clara, CA). Total chlorophyll was determined by Chla = 13.36x(A664.2) - 5.19x(A648.6); Chlb = 27.43x(A648.6) - 8.12x(A664.2), and total chlorophyll = Chla + Chlb.

Lipid peroxidation analysis

Lipid peroxide was measured using the protocol mentioned in Chen and Zhang (2016). Briefly, 50 mg of crushed plant tissues were placed in a microcentrifuge tube and 1 ml phosphate buffer (PBS) (100mM, pH 7.8) was added. After vortexing briefly, tubes were centrifuged for 20 mins at 10,000 rpm. Then 100 µl of supernatant was taken in a fresh tube and 1ml of 0.25% Thiobarbituric acid (TBA) in Trichloroacetic acid (TCA) was added to it. PBS was used as a control. The samples were heated on a heating block for 15 mins at 90°C. Holes were poked in the centrifuge tube lids to avoid popping. The samples were then cooled on ice for 5 mins and the absorbance readings were taken using a UV-Vis spectrophotometer (Biotech Epoch microplate reader, Aligent Technologies Inc., Santa Clara, CA) at 532nm and 600nm. The final Malondialdehyde (MDA) concentration was calculated based on Lambert-Beer's equation (extinction coefficient of MDA is 155 mM/cm).

Analysis of Reactive Oxygen Species (ROS) production

Total ROS were measured in control and AsIII-treated WT and *CsGGCT2;1* overexpressing (OE) seedlings by using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA). Roots were soaked in a solution of 1 mM DCFDA for 15 min in the dark, washed, and imaged. A Nikon Eclipse Ni microscope equipped with a DS-Ri2 digital camera (Nikon Instruments Inc., Melville, NY) was used for imaging. The fluorescence from H_2DCFDA was observed under eGFP epifluorescence (Ex470-440, DM4951p, BA525/550) with exposure time set at 150 milliseconds at 4x magnification. ImageJ software was used to quantify the average signal intensity in regions of interest (1cm of root tip).

Arsenic accumulation analysis

Shoot and root samples from plants grown on media containing 20 µM AsIII for 3-weeks were harvested separately. The samples were washed with 10 mM sodium-EDTA to remove excess As adsorbed on surface and then rinsed twice with DI water. The samples were then dried at 70°C for 48 hours. Dried samples were weighed, roughly crushed, and digested using concentrated nitric acid (15.8N) and 30% hydrogen peroxide (H_2O_2) on a heat block digester at 115°C for 45 minutes. Digested samples were then diluted and analyzed for arsenic using ICP-MS (ICPMS-2030, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). Translocation factor from root to shoot was calculated using this formula: TF = As concentration in shoot/ As concentration in root

Analysis of thiol compounds and amino acids

For thiol compounds analysis, approximately 100 mg of fresh seedling tissues (a homogenous mixture of root and shoot) was collected in a 1.5 ml microcentrifuge tube containing 1 ml of extraction buffer (6.3 mM diethylenetriamine pentacetic acid mixed with 0.1% trifluoroacetic acid). The extracts were used for derivatization and analysis of thiol compounds – Cysteine, γ-EC, GSH, and PCs as described in Minocha*et al* . 2008. For amino acid analysis, a homogeneous mixture (shoot and root) of 100 mg fresh tissue was collected in a 1.5 ml microcentrifuge tube containing 1ml of 5% (v/v) perchloric acid and stored at -20° C. The samples

were prepared and analyzed via HPLC as per Minocha and Long (2004) with minor modifications described in (Majumdar *et al* . 2018).

Analysis of 5-Oxoproline (5-OP) For 5-OP analysis, samples were homogenized in 80% chilled ethanol. After vortexing briefly, the samples were centrifuged at 13000 rpm for 15 mins. Supernatants were transferred to new tubes and evaporated under vacuum at RT for 16 to 24 hours. Samples were then lyophilized and re-dissolved in 10 mM ammonium acetate. Samples were processed with LC-MS-MS (UPLC/Xevo TQD QQQ-MS, Waters Corporation, Milford, MA) using the protocol from (Eckstein *et al* . 2008) with minor modifications. Briefly, Synregi 4µ Hydro-RP 80R analytical column (Phenomenex, Torrance, CA) was used at a flow rate of 0.3ml/min, with a total run time of 7 minutes per sample.

Analysis of *OXP1* transcript level in *CsGGCT2;1* overexpression lines

WT and *CsGGCT2;1* OE lines were grown for 21 days on $\frac{1}{2}$ x MS and $\frac{1}{2}$ x MS media supplemented with AsIII (20 µM). After RNA isolation and cDNA synthesis, qRT-PCR was performed using *CsOXP1* forward (5' GTCTTCTGCGTTGACACCCA 3') and reverse primers (5' GGTGCCAAGCCTCCATCAGA 3'). *CsEF1* (housekeeping) primers and qRT-PCR conditions were the same as mentioned earlier.

Statistical analysis

Statistical analysis was done using the Student's and Dunnett's T-test performed using SAS software (version 9.4) with Camelina wild-type as the control. Bars represent the average and error bars represent the standard error of the mean. Statistical significance is represented as "*", "**" and "***" for p [?] 0.05, p [?] 0.01, and p [?] 0.001, respectively.

Results

Camelina *GGCT2;1* share strong homology with its relative Arabidopsis

Camelina and Arabidopsis have three homologs of *GGCTs –GGCT1* , *GGCT2;1* , and *GGCT2;2* . *Camelina sativa* , being a hexaploid, has three copies of *GGCT2;1* – Csa20g051600, Csa08g024050, Csa13g034230 (www.camelina.db, Kaegel, *et al* . 2014; Figure S1 a). Based on multiple sequence alignment analysis, three copies of *CsGGCT2;1* homeolog sequences had 91 to 97% identity at the amino acid levels. The CsGGCT2;1 (Csa08g024050) and AtGGCT2;1 (AT5G26220) exhibited approximately 92.92% identity (Figure S1 a&c). The phylogenetic analysis showed *CsGGCT2;1* has a close relationship with *AtGGCT2;1* as compared to other GGCTs (Figure S1 b).

Generation of Camelina lines overexpressing *CsGGCT2;1*

To overexpress *CsGGCT2;1* in Camelina, the gene construct *CaMV35Sp::CsGGCT2;1::Nos-t* (Figure 1a) was introduced into the Camelina nuclear genome under the control of the constitutive *35S* promoter via flower dip method. Resulting seeds from the transgenic lines were identified using DS Red fluorescence (Figure 1b) and grown up to T_3 generation to obtain homozygous lines. Two weeks old seedlings of T_3 homozygous lines were analyzed for the confirmation of the overexpression of *CsGGCT2;1* transcripts using qRT- PCR. The PCR assays of three elite lines indicated that the relative expression of *CsGGCT2;1* transcripts was 6 to 17-fold higher in the Camelina transgenic lines compared to WT seedlings (Figure 1c). The qRT-PCR findings confirmed that the *CsGGCT2;1* construct resulted in *GGCT2;1* transcriptional overexpression in Camelina.

Differential expression of *CsGGCT2;1* gene

To gain insight into the potential function of *CsGGCT2;1* for arsenic tolerance or sensitivity in Camelina, its differential regulation was analyzed in root and shoot tissues exposed to AsIII. Camelina wild-type plants grown in Hoagland's solution and exposed to AsIII for 0, 6, 12, 24, and 48 hours were used for the differential regulation of *CsGGCT2;1* transcripts using qRT-PCR. The results show that the *CsGGCT2;1* gene was induced after 6 h of exposure to AsIII in both root and shoot (Figure 2). In shoots, the expression level at 6 h showed a 3-fold increase and then decreased to control levels after 48 h exposure (Figure 2a). In

roots, there was a 17-fold increase in the expression of *CsGGCT2;1* after 6 h of AsIII exposure, that then waned to a 6-fold difference at 12 h, and then again increased to 15-fold difference at 48 h (Figure 2b).

Analysis of Camelina wild-type and *CsGGCT2;1* OE lines on AsIII containing media

Camelina wild-type and *CsGGCT2;1* OE lines were grown on $\frac{1}{2}$ x MS media (control) and $\frac{1}{2}$ x MS media supplemented with 20 µM sodium arsenite for 21 days and then harvested (Figure 3a). There were no significant differences in the root and shoot biomass of wild-type and OE lines under the non-stress $\frac{1}{2}$x MS control conditions (Figure 3). However, under AsIII treatment, *CsGGCT2;1* OE lines showed strong tolerance and attained significantly higher root and shoot biomass than WT plants. The *CsGGCT2;1* OE lines had approximately 2.6-3-fold higher shoot biomass and approximately 7-fold higher root biomass than WT plants (Figure 3 b&c).

Analysis of chlorophyll content and lipid peroxidation

Chlorophyll content was measured in Camelina WT and *CsGGCT2;1* OE lines exposed to control and AsIII treatments. There were no significant differences between WT and OE lines under control conditions. However, under the AsIII treatment, all the *CsGGCT2;1* OE plants had leaves that were darker green in color and had higher chlorophyll content than the WT plants (Figure 4a). *CsGGCT2;1* OE lines #9, #102, and #103 had 70%, 123%, and 115%, respectively, higher chlorophyll than the WT plants.

MDA levels as an indication of membrane damage due to lipid peroxidation were measured under normal and AsIII stress conditions. Like biomass and chlorophyll content, WT and *CsGGCT2;1* OE lines had no significant difference in lipid peroxidation under control conditions. However, WT plants had significantly higher MDA content than *CsGGCT2;1* OE lines under AsIII treatment (Figure 4b). In response to AsIII stress, the *CsGGCT2;1* OE lines had approximately 30% lesser MDA than WT, indicating that *CsGGCT2;1* prevents membrane damage due to AsIII-induced oxidative stress.

ROS analysis in Camelina seedlings

ROS was analyzed in Camelina WT and *CsGGCT2;1* OE lines grown on $\frac{1}{2}$ x MS and AsIII treatments using H_2DCF-DA staining. The seedlings were grown for two weeks on the media, and the root tips were stained with H_2DCF-DA, washed with DI water, and then observed under confocal fluorescent microscopy. As depicted by fluorescence, WT roots had significantly higher ROS under AsIII treatments as compared to the *CsGGCT2;1* OE lines (Figure 4c). Based on quantification of fluorescence, there was a significant (˜ 40%) reduction in fluorescence in *CsGGCT2;1* OE lines than in the WT plants (Figure 4d).

Arsenic accumulation in roots and shoots of *CsGGCT2;1* OE and WT plants

Arsenic concentration was determined in the shoot and root tissue of both *CsGGCT2;1* OE and WT seedlings. *CsGGCT2;1* OE lines accumulated ˜ 40-60% lesser arsenic in the roots (Figure 5a) and ˜ 60% less in the shoots than the WT plants (Figure 5b). The translocation factor (TF) from root to shoot was also 23 to 36% lower for *CsGGCT2;1* OE lines relative to WT seedlings (Figure 5c).

Analysis of 5-Oxoproline level and upregulation of 5-Oxoprolinase (*OXP1*) gene

GGCT2;1 is involved in GSH degradation and the recycling of its constituent amino acids (Paulose *et al* ., 2013). The immediate product of GGCTs activity is 5-oxoproline (5-OP) resulting from the degradation of GSH or its precursor γ-glutamyl cysteine (γ-EC). We measured the 5-OP levels in WT and two *CsGGCT2;1* OE lines #102 and #103 under normal and AsIII treatments using triple-quadrupole mass spectroscopy (UPLC/Xevo TQD QQQ-MS, Waters Corporation, Milford, MA). No significant differences were observed between WT and OE lines under normal or AsIII treatment. However, the OE lines had approximately 21-24% more 5-oxoproline levels under MS control conditions than the WT (Figure 6a). Additionally, the 5-OP level increased in both WT and OE lines by 19-33% under AsIII treatment relative to non-stress control conditions. Still, the OE lines maintained 10-20% higher 5-OP levels than WT under AsIII treatment.

Since the immediate product of GGCTs activity is 5-oxoproline (5-OP), significantly higher levels of 5-OP

were expected in the lines overexpressing *CsGGCT2;1* . 5-OP is further converted to glutamate (Glu) by the endogenous activity of Oxoprolinase1 (OXP1). Our findings prompted us to study the upregulation of the endogenous *OXP1* gene in *CsGGCT2;1* OE lines using qRT-PCR under normal and AsIII stress conditions. Our results showed that under control conditions the expression of CsOXP1 was upregulated 2 to 3.5-fold in the OE lines compared to WT plants and 1.5 to 3.5-fold under AsIII stress conditions (Figure 6b).

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The levels of GSH and γ-EC were analyzed via HPLC in WT and two *CsGGCT2;1* OE lines (#102 and #103) exposed to MS control and AsIII treatments. There were no significant differences in GSH levels between WT and OE lines under MS control or in response to AsIII treatment (Figure 7a). However, under AsIII treatment, overall GSH levels increased relative to MS control (Figure 7a). For γ-EC analysis, *CsGGCT2;1* OE lines had significantly (50-70%) lower γ-EC levels than WT under MS control, but under AsIII treatment, there were no significant differences between WT and OE lines (Figure 7b). As with GSH, overall γ-EC levels increased in the plants grown on AsIII stress relative to MS control (Figure 7b).

Analysis of Amino acid levels

Amino acid (AAs) levels were analyzed via HPLC in WT and two *CsGGCT2;1* OE lines #102 and #103 under MS control and AsIII treatment. Glycine (Gly), cysteine (Cys), and glutamate (Glu) are three constituent amino acids of GSH. Therefore, we analyzed the level of all amino acids in WT and OE lines under normal and AsIII stress conditions. No significant differences in the levels of Cys and Glu were observed in WT and OE lines under normal and AsIII treatment (Figure 7 c&d). However, under AsIII treatment, their levels increased relative to normal non-stress conditions. Still, within treatments, there were no significant difference in the levels of Cys and Glu between WT and OE lines (Figure 7c&d). For Gly, the *CsGGCT2;1* OE lines had 15-30% less than the WT under normal conditions. Though Gly levels increased by 45-140% in all plants in response to AsIII, relative to MS control, again there were no significant differences in Gly content between WT and OE lines (Figure 7e).

For glutamine (Gln), there was no significant difference between WT and OE lines under MS control. Under AsIII treatment, Gln content was 18-21% lower in OE lines than WT plants (Figure 7f). Further, no significant differences were found in serine, arginine, threonine, alanine, prolines, tryptophane, ornithine, and lysine (Figure S2). For Asp and GABA, other than line #103 on MS control, *CsGGCT2;1* OE lines had lower levels in both MS and AsIII treatments. No difference was observed for isoleucine (Ile) between WT and OE lines growing on MS control, but in response to AsIII treatment, OE lines had lower levels than WT. For phenylalanine (Phe) and histidine (His), higher levels were observed in OE lines relative to WT under MS control, but under AsIII, there was no significant differences in either AA (Figure S2).

Discussion

In this study, we characterized *CsGGCT2;1* gene in Camelina for its role in providing arsenite tolerance by maintaining GSH homeostasis. Under AsIII stress, *CsGGCT2;1* OE lines had significantly higher shoot and root biomass, improved chlorophyll content, lower lipid peroxidation, and accumulated less arsenic than the WT. Further analysis showed that relative to non-stress MS control conditions, resources in the plants treated with AsIII shifted towards the γ-glutamyl cycle as Glu, Cys, Gly, GSH, and 5-OP levels increased; some slightly to some multiple fold differences.

Paulose *et al* . (2013) investigated the role of *AtGGCT2;1* and its involvement in GSH degradation and the recycling of glutamate in Arabidopsis plants under AsIII stress. The authors observed that *AtGGCT2;1* gene was strongly upregulated in roots exposed to AsIII treatment but not in the shoots. Overexpression of *AtGGCT2;1* in Arabidopsis led to increased tolerance to AsIII as evidenced by significantly higher shoot biomass in the OE lines growing on media containing AsIII, and a significant decrease in arsenic accumulation in shoots with an increased accumulation in roots of OE plants relative to WT. Overall levels of GSH were similar in WT and *AtGGCT2;1* OE lines. However, *AtGGCT2;1* OE lines also had a significantly higher accumulation of 5-OP than WT plants in both root and shoots, but the level was up to 30-fold higher in

shoots. Based on an N^{15} Glu assay, Paulose *et al* . (2013) concluded that the *AtGGCT2;1* OE lines had better tolerance and less accumulation of arsenic in shoot tissue due to an enhanced γ-glutamyl cycle and more efficient glutamate recycling.

Building on the previous work from Paulose *et al* . (2013), we translated the research from the model plant *Arabidopsis thaliana*to the oilseed crop *Camelina sativa* . Both Camelina and Arabidopsis belong to the Brassicaceae family, and each have three homologs of *GGCT* s – *GGCT1* (or *GGCT2;3*),*GGCT2;1* , and *GGCT2;2* (Figure S1). CsGGCT2;1 and AtGGCT2;1 have high amino acids sequence similarity (˜92%) (Figure S1a). This high protein sequence similarity with Arabidopsis may be one reasons why we observed comparable results in Camelina. We used 20 µM AsIII concentration to analyze Camelina plants, as the concentration used for Arabidopsis (35 µM), by Paulose *et al* . (2013), was too toxic for the WT Camelina plants to grow. We found that AsIII treatment upregulated *CsGGCT2;1* gene in Camelina WT plants. However, contrary to *AtGGCT2;1* differential regulation in Arabidopsis, we observed the upregulation of *CsGGCT2;1*transcripts in both roots and shoots (Figure 2). Camelina, being a hexaploid, have three homeologs of *GGCT2;1* and could have a different expression profile compared to Arabidopsis. Relative to WT, the overexpression of *CsGGCT2;1* also provided stronger tolerance to AsIII in Camelina (Figure 3). We observed a 40-60% less arsenic in both roots and shoots in the Camelina *GGCT2;1* OE lines compared to the WT (Figure 5). We also found no difference in GSH levels between the WT and *CsGGCT2;1* OE lines (Figure 7a). Contrary to the high levels of 5-OP in shoots reported for *AtGGCT2;1* OE lines of Arabidopsis (30-fold relative to WT) (Paulose *et al* ., 2013), we detected only a 20% increase in 5-OP in combined root and shoot tissues from 21 old-day seedlings of *CsGGCT2;1* OE lines relative to CsWT (Figure 6a). This combination of results indicates that the observed tolerance to As was more robust in the Camelina lines overexpressing*CsGGCT2;1* than that reported in Arabidopsis by Paulose *et al.* (2013).

Arsenic is not a required element in plants, and once taken up by plants leads to various adverse effects on plants' growth and development (Bali and Sidhu 2021; Farooq *et al* . 2016). Arsenic impacts the plants' morphological, physiological, and metabolic attributes, e.g., lowering shoot and root biomass, reducing plant height, impacting root growth, decreasing chlorophyll content and photosynthetic rate, etc. (Ahmad*et al* . 2020a; Majumder *et al* . 2020; Singh *et al* . 2019). Arsenic also disrupts plants' cellular oxidative state by making reactive oxygen species (ROS) and causing lipid peroxidation (Ahmad*et al* . 2020a; Kofroňová, Mašková, and Lipavská 2018; Majumder*et al* . 2020; Singh *et al* . 2019). We observed a significant reduction in the growth of WT Camelina plants exposed to AsIII, whereas there was minimal impact on the growth of the *CsGGCT2;1* OE lines; empirically, the OE lines had growth almost similar to unchallenged plants. Arsenic is known to induce oxidative stress in plants, therefore, to understand the mechanism of CsGGCT2;1 role in protecting plants from its toxicity, we explored the effects of AsIII treatment by analyzing chlorophyll content, MDA, and ROS levels. Chlorophyll content is a predictor of plants' photosynthetic efficacy. Researchers have found chlorophyll decreased due to arsenic exposure, thereby limiting the growth and development of the plant (Ahmad *et al* . 2020b; Anjum *et al* . 2017; Meselhy *et al* . 2021). We observed that AsIII treatment decreased the chlorophyll content in the WT plants, while there was no impact on chlorophyll in the *CsGGCT2;1* OE lines (Figure 4a).

Malondialdehyde (MDA) indicates lipid peroxidation and membrane damage in living cells and many researchers have reported increased MDA levels in plants exposed to AsIII (Anjum *et al* . 2017; Khare *et al* . 2017; Pandey *et al* . 2016; Yadu *et al* . 2019). We observed an increase in MDA levels in WT Camelina seedlings exposed to AsIII treatment. However, MDA in *CsGGCT2;1* OE lines was similar under both MS control and AsIII treatment (Figure 4b). One reason for this increase in the level of MDA is an increase in ROS due to oxidative stress induced by the AsIII treatment. Arsenic exposure increased ROS levels in Indian mustard (Pandey *et al* . 2016), Arabidopsis (Khare*et al* . 2017), and pigeon peas (Yadu *et al* . 2019). We also visualized ROS levels using a fluorescent probe (H_2DCF-DA staining) in the plant's root tips (˜1cm) and quantified them using ImageJ software. As expected, we found increased ROS in the root tips of AsIII treated WT Camelina relative to WT under control (MS) conditions. Also somewhat expected, root tips of the *CsGGCT2;1* OE lines had lower levels of ROS as compared to WT, under both MS control and AsIII treatments, however the differences were only significant for the AsIII treatment (Figure 4c&d). Higher chlo-

rophyll, lower lipid peroxidation, and lower ROS levels under AsIII treatment could be why there was better growth of *CsGGCT2;1* OE lines relative to WT.

After arsenic uptake, plants limit its damaging effects on cellular machinery by sequestering it in vacuoles. One crucial redox molecule involved in AsIII detoxification via conjugation is GSH. Glutathione levels are maintained in the plants through the γ-glutamyl cycle, a continuous cycle of synthesis of GSH from glutamate, cysteine, and glycine and its subsequent breakdown into component amino acids (Hasanuzzaman *et al* . 2017; Noctor *et al* . 2012; Paulose*et al* . 2013). Glutathione and its oligomer, PCs, complex with AsIII in the cytosol and the complex is then transported to the vacuole via protein transporters (ABCC transporters) (Bali and Sidhu 2021; Noctor *et al.* 2012; Tang *et al* . 2019). Overexpression of genes related to the γ-glutamyl and PCs synthesis pathway has resulted in increased tolerance in Arabidopsis (Dhankher *et al* . 2002; Guo*et al* . 2008; Li *et al* . 2005; Paulose *et al* . 2013; Song *et al* . 2010), Brassica (Gasic and Korban 2007; Reisinger*et al* . 2008) and Cottonwood (LeBlanc *et al* . 2011). Similarly, we observed that overexpressing *CsGGCT2;1* , a gene involved in the γ-glutamyl cycle, enhanced AsIII tolerance in Camelina. On analyzing levels of thiols i.e., GSH, γ-EC, and cysteine, we observed no significant differences between WT and *CsGGCT2;1* OE lines, except for γ-EC under MS control conditions, which was significantly lower than WT plants (Figure 7b). The decrease in γ-EC may have been due to the direct breakdown of γ-EC by GGCTs. Kumar *et al* . (2015) also reported the breakdown of γ-EC by Arabidopsis GGCTs in*in-vivo* functional assays in yeast and the authors found very low activity of *GGCT2;1* directed towards γ-EC breakdown, the*GGCT2;1* homeolog in Camelina could have more effectively increased activity towards γ-EC breakdown than that in Arabidopsis (*AtGGCT2;1*) (Kumar *et al* . 2015). It could also be that the heightened activity of *CsGGCT2;1* leads to the breakdown of GSH, triggering the upregulation of the glutathione synthase (GS) gene. As a result, the synthesis of additional GSH from γ-EC and Gly is induced. Further, no difference in γ-EC levels was observed under AsIII-treated conditions because the overall γ-glutamyl pathway was induced to counteract AsIII toxicity. The lack of difference in the levels of GSH and Cys between the WT and OE lines could be due to the efficient recycling of these by γ-glutamyl cycle, as reported by Paulose *et al.* (2013). In response to AsIII treatment, we observed an overall 1.5 to 3-fold increase in the level of these thiols for both CsWT and*CsGGCT2;1* OE lines, indicating a shift of resources towards the γ-glutamyl cycle (Figure 7). The same trend was observed for Glu and Gly as well, the remaining amino acid components of GSH, also indicative of a resource shift towards the γ-glutamyl cycle for enhanced survival under AsIII stress.

Further, we found a 19-33% increase in 5-OP levels in OE lines relative to WT under MS control and AsIII treatments. *GGCT2;1* is shown to directly acts on GSH and γ-Glu-AA and converts both into 5-OP (Ohkama-Ohtsu *et al* . 2008; Paulose *et al* . 2013). Though Paulose *et al.* (2013) reported a substantial build-up of 5-OP levels in the *AtGGCT2;1* OE lines, there was not a considerable accumulation in Camelina (Figure 6a). Based on our qRT-PCR analysis,*CsOXP1* expression was upregulated in the OE lines (Figure 6b), which indicates that the overexpression of *CsGGCT2;1* has upregulated the downstream gene *OXP1* , which in turn increased the recycling of 5-OP to maintain steady state levels of GSH in the seedlings.

Better survival of OE lines (relative to WT) under AsIII treatment also seems to be attributable to lower arsenic uptake and accumulation within the transformed plants. *CsGGCT2;1* OE lines had ˜ 40 to 60% lower arsenic accumulation relative to WT while maintaining a similar level of GSH (Figures 5 & 7). This could be because of efficient γ-glutamyl cycle via faster degradation of γ-EC and GSH in the*CsGGCT2;1* OE lines, thus faster recycling of Cys, Glu, and Gly for subsequent GSH synthesis. This efficient degradation and synthesis of γ-EC and GSH might prevent the opportunity of binding of AsIII to these thiols for sequestration and accumulation of As in vacuole. When calculated as GSH per unit of arsenic, the *CsGGCT2;1* OE lines had more GSH available Therefore, the damaging effects of As at the cellular level were more efficiently limited in the OE lines. Low accumulation of arsenic could also be due to the role of *CsGGCT2;1* in GSH distribution in the root and root architecture. Joshi *et al.*(2019) noted differences in the growth and GSH distribution in the roots of Arabidopsis WT and *ggct2;1* knockdown lines (Joshi *et al* . 2019). Less accumulation could also due to induction of some aquaporin channels involved in both the uptake and efflux of As back into the environment (Kumar *et al* . 2018; Lindsay and Maathuis 2016). Further investigation is

needed to investigate the relationship between *GGCTs* and metal transporters/channel genes.

Conclusion

This study concluded that overexpression of *CsGGCT2;1* in Camelina increased AsIII tolerance. Compared to WT, *CsGGTC2;1* OE lines had significantly higher biomass, lower arsenic accumulation, better chlorophyll content, and lower MDA and ROS levels. The *CsGGCT2;1*OE lines had slightly higher levels of 5-OP and increased levels of the*CsOXP1* gene, indicating a role of *GGCT2;1* in increasing the rate of GSH recycling and enhancing survival under AsIII stress via improved GSH homeostasis. This research demonstrates the successful translation and applications of findings from the model plant Arabidopsis to Camelina, a dedicated biofuel crop. These results highlight the potential of modified plants to thrive under AsIII stress with reduced accumulation, thus enabling the cultivation of crops on contaminated marginal lands to ensure food safety and future food security.

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Author contributions

OPD, GS, and KA conceived and designed the study. GS, HL, and KA conducted experiments and collected and analyzed the data. SL and RM helped with HPLC analysis of thiols and amino acids. GS and OPD wrote and revised the manuscript. All authors helped revise the manuscript and provided valuable critical comments.

Conflict of Interest Statement

The Authors declare that they have no competing or conflict of interests.

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