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CB5C affects the glucosinolate profile in *Arabidopsis thaliana*

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ABSTRACT

Cytochrome b_5 (CB5) proteins are small heme-binding proteins, that influence cytochrome P450 activity. While only one CB5 isoform is found in mammals, higher plants have several isoforms of these proteins. The roles of the many CB5 isoforms in plants remain unknown. We hypothesized that CB5 proteins support the cytochrome P450 enzymes of plant specialized metabolism and found *CB5C* from *Arabidopsis thaliana* to co-express with glucosinolate biosynthetic genes. We characterized the glucosinolate profiles of 2 T-DNA insertion mutants of *CB5C*, and found that long-chained aliphatic glucosinolates were reduced in one of the mutant lines – a phenotype that was exaggerated upon methyl-jasmonate treatment. These results support the hypothesis, that *CB5C* influences glucosinolate biosynthesis, however, the mode of action remains unknown. Furthermore, the mutants differed in their biomass response to methyl jasmonate treatment. Thereby, our results highlight the varying effects of T-DNA insertion sites, as the 2 analyzed alleles show different phenotypes.

Abbreviations: CB5, cytochrome b5; ER, endoplasmic reticulum; GLS, glucosinolates; LC, long-chained aliphatic; MeJA, methyl-jasmonate; P450, cytochrome P450; SC, short-chained aliphatic; 4MOI3M, 4-methoxyindole-3-methyl glucosinolate

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Introduction

Cytochrome b_5 (CB5) proteins are small (~15 kD) heme-binding proteins, anchored to the endoplasmic reticulum (ER). These proteins have been studied extensively in animal systems due to their role in cellular detoxification and drug metabolism.[1,2,3,4] CB5 proteins interact with ER-anchored cytochrome P450 (P450) enzymes; a large family of versatile enzymes able to catalyze the transformation of otherwise inert molecules.[5] CB5 proteins influence P450 enzymes either by supplying electrons – similar to the NADPH-dependent P450 reductases - or by modulating their activity via physical interaction independent of electron donation.[6,7] The majority of studies have focused on mammalian CB5 proteins, whereas CB5 proteins from other organisms have been examined to a lesser extent. In contrast to mammals, which have a single copy of the *CB5* gene,[8] the genomes of higher plants encode multiple CB5 isoforms,[9] e.g. 6 in the model plant *Arabidopsis thaliana*[10] and 11 in *Glycine max* (soybean).[11] The purpose of the increased number of CB5 isoforms remains elusive.

As sessile organisms, plants excel in their capability to synthesize diverse specialized chemicals - e.g., toxins, fragrances and pigments – which enable them to interact with, and adapt to, their ever-changing environment.[12] Essential for this specialized metabolism are P450 enzymes that carry out central catalytic steps in several of these specialized biosynthetic pathways.[13,14] The number of these enzymes are widely expanded in higher plants, with *A. thaliana* having more than 240.[15] Plants may therefore have evolved a larger number of CB5 isoforms to keep up with the increased number of P450s.[16,17] A connection between CB5 proteins and specialized plant metabolism was found in petunia, where discoloration of the flower petals was observed in a *CB5* knock-out mutant.[18] The change in flower color coincided with a decrease in anthocyanins and a decrease in the activity of the biosynthetic P450, flavonoid 3′, 5′-hydroxylase, indicating that this enzyme requires CB5 for full enzymatic activity. Further evidence for a role of CB5 proteins in specialized metabolism comes from the biosynthesis of artemisinic acid – the precursor for the anti-malarial drug, artemisinin. Upon heterologous expression of the artemisinic acid biosynthetic pathway (including a P450) from *Artimisia annua*, co-expression of a CB5 resulted in higher levels of artemisinic acid.[19] These findings suggest that CB5 proteins can be critical for metabolic efficiency, possibly through interactions with the P450 enzymes.

A. thaliana produces glucosinolates (GLS) as their major class of specialized metabolites involved in plant defense.[20,21] P450s carry out essential enzymatic steps in the GLS biosynthetic pathway. Based on the idea that CB5 proteins might be involved in plant specialized metabolism through interactions with P450s, we hypothesized that a CB5 protein plays a role for the efficiency of this pathway.[22] In this study, we investigated the role of the *CB5* isoform C, which co-expresses with the GLS pathway in *A. thaliana*. We characterized GLS profiles of

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2 T-DNA insertional mutants of *CB5C* in the presence and absence of the GLS-inducing hormone methyl-jasmonate (MeJA). Our results show that *CB5C* affects GLS profiles by influencing especially long-chained aliphatic (LC) GLS.

Results

Co-expression analysis revealed that only one of the 6 *A. thaliana CB5* genes – *CB5C* (also referred to as *CYB5-1*) [23] – co-expresses with the GLS biosynthesis genes (Table 1). We obtained 2 T-DNA insertion lines, *cb5c-1* and *cb5c-2* (Fig. 1) and analyzed the expression levels of *CB5C* (Fig. 2). As previously reported, *cb5c-2* is a *knock-down* mutant,[23] and also *cb5c-1* shows reduced transcript levels compared to wildtype (WT).

Analysis by mass-spectrometry enabled quantification of 15 different GLS in individual seedlings. Total levels of GLS were similar in mutants and WT plants (Table 2, Table S1). However, the mutants showed differences in the levels of individual GLS. *cb5c-1* showed significantly reduced levels of the LC GLS 7-methylsulfinylheptyl and 8-methylsulfinyloctyl GLS. By contrast, *cb5c-2* showed reduced levels of the seed-derived 3-benzoyloxypropyl GLS. Common for both mutants was an increased accumulation of the short-chained aliphatic (SC) 4-methylthiobutyl GLS ($p = 0.072$ and 0.012 for *cb5c-1* and *cb5c-2*, respectively).

As GLS levels are inducible upon application of exogenous jasmonate,[22,24] we treated the plants with methyl-jasmonate (MeJA) to assess whether this would exaggerate the subtle chemotypes of *cb5c-1* and *cb5c-2*, thus revealing a potential condition-specific role of *CB5C*. MeJA treatment led to an increase in almost all measured GLS, and indeed resulted in more pronounced differences in GLS between genotypes (Table 2). Interestingly, MeJA-treated WT plants showed an increase in 3-benzoyloxypropyl and 4-benzoyloxybutyl GLS. These GLS originate from the seed, but are not *de novo* synthesized in the seedlings.[25] A similar – but less pronounced – increase upon MeJA, was observed for the *cb5c-2* mutant, but no significant increase was found in *cb5c-1*.

In the MeJA-treated *cb5c-1* mutant LC GLS continued to be lower compared to WT, but the decrease now involved all LC GLS. In addition, one SC GLS (4-benzoyloxybutyl GLS) was significantly lower in *cb5c-1* upon MeJA treatment compared to the WT. Similarly, in *cb5c-2*, we found the levels of 3-benzoyloxypropyl GLS to be lower than WT when treated with MeJA. In this mutant line, the 4-methoxyindol-3-ylmethyl GLS (4MOI3M) also showed a decrease (Table 2).

By visual inspection of the plates, we noticed a difference in growth between the genotypes in response to MeJA treatment. Quantification of this phenotype revealed a difference in growth between genotypes upon hormone treatment, whereas

Table 1. Genes co-expressing with *A. thaliana CB5C*

Locus	Alias (Short description)	Role in glucosinolate biosynthesis	MR
At2g46650	CB5C		0
At4g39940	APK2		1
At1g18590	**SOT17**	**aliphatic and indolyl**	**2**
At2g20610	**SUR1**	**aliphatic and indolyl**	**3.5**
At5g44720	sulfurase		4.1
At2g14750	AKN1		4.2
At5g63980	SUPO1		4.7
At5g07460	PMSR2		6.3
At4g30530	**GGP1**	**aliphatic and indolyl**	**6.5**
At3g58990	**IPMI1**	**aliphatic**	**6.6**
At3g03190	**GSTF11**	**aliphatic**	**7.1**
At1g24100	**UGT74B1**	**indolyl**	**7.8**
At3g23570	alpha/beta-Hydrolases		9.5
At4g39950	**CYP79B2**	**indolyl**	**9.5**
At4g12030	**BAT5**	**aliphatic**	**9.5**
At1g74090	**SOT18**	**aliphatic**	**10**
At4g13770	REF2		10.2
At2g43100	**IPMI2**	**aliphatic**	**10.4**
At2g22330	**CYP79B3**	**indolyl**	**11.4**
At1g24625	ZFP7		12.1
At3g19710	**BCAT4**	**aliphatic**	**13**
At2g30860	**GSTF9**	**indolyl**	**13.2**
At1g62560	**FMO GS-OX3**	**aliphatic**	**14**
At1g65860	**FMO GS-OX1**	**aliphatic**	**14.4**
At1g74100	**SOT16**	**indolyl**	**14.8**
At4g14680	APS3		15
At2g31790	transferase		16.9
At5g23010	**MAM1**	**aliphatic**	**17.1**
At5g01500	TAAC		17.2
At1g78370	**GSTU20**	**aliphatic**	**17.3**
At4g31500	**SUR2**	**indolyl**	**17.7**
At5g60890	**MYB34**	**Indolyl**	**17.7**

The top 31 genes co-expressing with *CB5C*. MR is a co-expression value used by the ATTED-II database. Enzymes involved in glucosinolate biosynthesis are highlighted in bold.

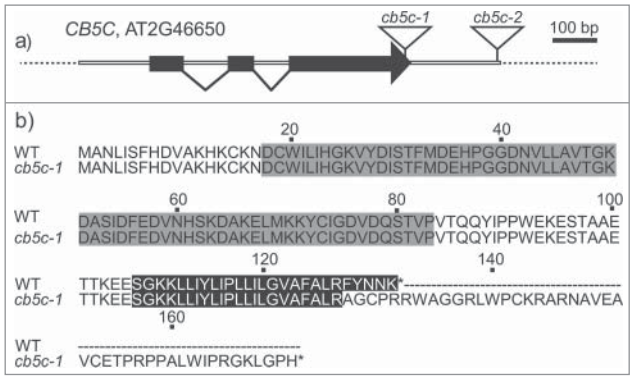

Figure 1. Sequence structure of *CB5C* and mutants. a) Gene structure of *A. thaliana CB5C*, showing the T-DNA insertion sites for the 2 mutants (*cb5c-1* and *cb5c-2*). Black boxes depict exons; white boxes indicate introns and untranslated regions. b) Sequence comparison between the protein products of WT and *cb5c-1*. The N-terminal heme-binding domain (gray), the C-terminal transmembrane helix (black) and the additional domain of the *cb5c-1* mutant.

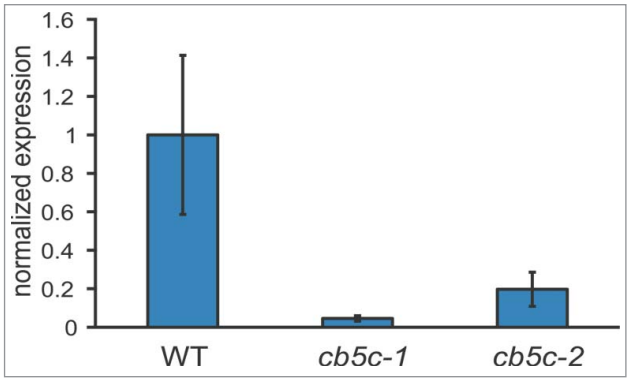

Figure 2. Expression levels of *CB5C*. The three genotypes (WT, *cb5c-1* and *cb5c-2*) were analyzed for *CB5C* expression. Bar graphs display the mean expression (normalized to WT levels) and error-bars display the standard deviation of 3 independent biological replicates for each genotype.

Table 2. Glucosinolate levels in seedlings of the *cb5c* mutants (nmol/g FW).

Genotype Treatment	WT ctrl			*cb5c-1* ctrl			*cb5c-2* ctrl			WT MeJA			*cb5c-1* MeJA			*cb5c-2* MeJA		
Total GLS	1673	±	142	1307	±	139	1438	±	143	4162	±	364	3615	±	295	3210	±	196°
Short-chained aliphatic GLS	267	±	20	258	±	27	276	±	30	390	±	33	356	±	38	385	±	37
3-methylsulfinylpropyl	11	±	1	12	±	2	11	±	1	22	±	3	21	±	5	24	±	5
3-hydroxypropyl	4	±	1	4	±	1	3	±	1	3	±	1	3	±	1	2	±	1
3-benzoyloxypropyl	28	±	3	31	±	3	**19**	±	**2***	37	±	3	38	±	3	**25**	±	**2****
4-methylthiobutyl	30	±	4	79	±	12°	**79**	±	**14***	73	±	11	88	±	15	93	±	14
4-methylsulfinylbutyl	111	±	12	103	±	13	103	±	11	154	±	20	139	±	28	155	±	26
4-hydroxybutyl	37	±	3	33	±	5	32	±	4	37	±	3	33	±	4	32	±	3
4-benzoyloxybutyl	53	±	3	38	±	6°	45	±	5	71	±	4	**48**	±	**6****	58	±	4°
5-methylsulfinylpentyl	21	±	2	18	±	1	18	±	1	33	±	3	28	±	4	29	±	4
Long-chained aliphatic GLS	258	±	34	174	±	28°	194	±	27	767	±	52	**554**	±	**56***	731	±	41
7-methylthioheptyl	34	±	5	30	±	3	34	±	4	98	±	8	**63**	±	**7****	87	±	5
7-methylsulfinylheptyl	51	±	6	**30**	±	**4****	36	±	4°	82	±	5	**60**	±	**6****	80	±	7
8-methylthiooctyl	53	±	9	65	±	6	48	±	7	276	±	20	**195**	±	**25***	250	±	15
8-methylsulfinyloctyl	135	±	17	**98**	±	**15***	109	±	14	312	±	22	**242**	±	**22***	314	±	22
Indolyl GLS	1127	±	103	879	±	94	952	±	97	2973	±	328	2699	±	250	2066	±	192°
Indol-3-ylmethyl	195	±	13	186	±	17	159	±	15	627	±	78	566	±	108	394	±	68°
4-methoxyindol-3-ylmethyl	131	±	12	103	±	10	108	±	11	648	±	41	610	±	53	**478**	±	**38***
N-methoxyindol-3-ylmethyl	801	±	83	655	±	73	684	±	76	1698	±	355	1523	±	289	1193	±	218

Values are given as mean ± s.e.m.
$^{**}p < 0.01$ $^{*}p < 0.05$ $^{\circ}p < 0.1$ different from WT via pairwise t-test, n = 30. Control treatment (ctrl). Methyl-jasmonate (MeJA). Glucosinolates (GLS).
See Table S1 for the ANOVA results.

no significant difference between untreated plants was observed. WT and *cb5c-1* plants showed decreased weight upon treatment, whereas the weight of *cb5c-2* was unaffected (Fig. 3).

Discussion

We analyzed GLS levels of 2 T-DNA mutants of the *CB5C* gene. The two mutants have different T-DNA insertion sites. The *cb5c-1 knock-down* mutant has an insertion within the coding sequence of the *CB5C* gene, causing a disruption of the reading frame that is likely to result in a recombinant protein with 39 new C-terminal residues (Fig. 1). As the residues C-terminal to the transmembrane helix determine the localization of CB5 proteins in *A. thaliana*,[10] an altered amino acid sequence at the C-terminus could result in mislocalization of the CB5C protein. The *cb5c-2* mutant carries a T-DNA insertion in the 3′-untranslated region of the gene, classifying it as a "*knock-about*" mutant (Fig. 1).[26] Although the 2 mutants show comparable decreases in transcript levels (Fig. 2), the corresponding proteins might differ fundamentally in their C-terminal amino acid sequence. The recombinant CB5C-1 mutant protein likely mislocalizes, whereas the *cb5c-2* mutant encodes the native CB5C protein, with full functionality. This may render the phenotypic effects of the T-DNA insertions very different, although *CB5C* transcript levels are comparable in the 2 mutant lines.

Both mutants show subtle but distinct alterations in the levels of individual GLS. Treatment with MeJA exaggerates the differences between WT and mutants. Prolonged treatment of *A. thaliana* seedlings with exogenous MeJA – as done in these experiments - does likely not reflect physiological conditions, but allows us to induce GLS levels, [27] and provoke a stronger phenotype. These findings support our hypothesis that *CB5C* influences GLS biosynthesis, in a condition-specific manner. The function of *CB5C* does not seem to be essential for GLS biosynthesis, but it affects accumulation of particularly LC GLS, especially when induced by MeJA treatment. The decrease in almost exclusively LC GLS seen for the *cb5c-1* mutant suggests specificity in the function of *CB5C* and potentially links the gene to the CYP79F enzymes that catalyze a key step in the biosynthetic step of specifically LC GLS.[28,29] We observed no differences in LC GLS in the *cb5c-2* mutant, where we anticipate that CB5C is properly localized. Here, the residual expression might be able to sustain its role in supporting GLS biosynthesis, whereas the *cb5c-1* mutant protein may be mislocalized and therefore non-functional. Another discrepancy between the 2 mutants is their relatively low levels of the seed-specific 3-benzoyloxypropyl and 4-benzoyloxybutyl GLS. Upon MeJA treatment WT plants accumulate more of these GLS, indicating less GLS turnover. The *cb5c-1* did not show a

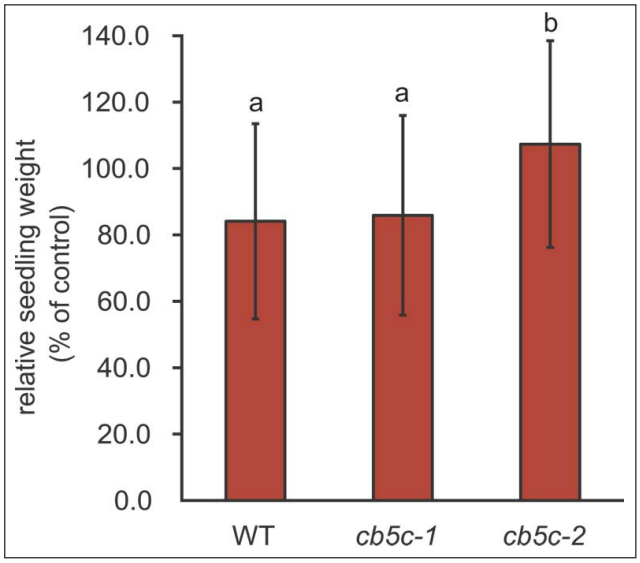

Figure 3. Biomass response to MeJA. Biomass responses of 10-day-old seedlings of the 3 genotypes (WT, *cb5c-1* and *cb5c-2*). The weight of seedlings treated with methyl-jasmonate (MeJA) was normalized to the weight of untreated seedlings (ctrl). Different letters refer to statistical groupings (pairwise *t*-test as post-hoc, $p < 0.05$, $n = 30$). See Table S1 for the ANOVA table.

significant change in these 2 GLS upon MeJA treatment, whereas *cb5c-2* showed an increase comparable to - but less pronounced than - WT plants. This indicates that the effect of *CB5C* on GLS profiles results from increase in turnover, as is seen for these seed-specific glucosinolates.

The different phenotypic consequences of the 2 T-DNA insertions are further illustrated by the differences in biomass response to MeJA treatment (Fig. 3). Exogenously applied MeJA reduces seedling biomass,[30-32] but strikingly, seedlings of the *cb5c-2* mutants did not show this response. In comparison, WT and *cb5c-1* mutants showed reduced growth upon MeJA treatment. Noticeably, *A. thaliana* CB5 proteins interact with a series of other proteins than P450s such as 1) a plasma membrane-localized sucrose transporter,[23] 2) several enzymes involved in fatty acid metabolism[11,33] and 3) the ethylene response regulator RTE1.[34] This – together with our findings – suggests that *CB5C* could play a broader physiological role, in addition to directly influencing GLS metabolism.

Methods

Co-expression analysis

Genes co-expressing with *CB5C* were identified using the ATTED-II database (http://atted.jp/).[35]

Plant lines and growth conditions

Two T-DNA insertion lines targeting *CB5C* (AT2G46650) – *cb5c-1* (SALK_027748C) and *cb5c-2* (*cyb5-1*; SALK_065187) - were acquired from the Nottingham Arabidopsis Stock Center (NASC). The *cb5c-1* line was homozygous. The *cb5c-2* line was heterozygous; a homozygous line and a segregating wildtype (referred to as WT) were obtained from this heterozygote. Plants were germinated and grown on MS (Murashige and Skoog medium including vitamins; M0222, Duchefa) agar plates with 1% sucrose. For hormone treatment, the medium was supplemented with 10 μM MeJA (392707–5ML,Sigma-Aldrich). Seeds were sterilized by incubating in 70% (v/v) ethanol, 0.05 % (v/v) Triton X-100 for 5 min and washing in 70% (v/v) ethanol. The air-dried seeds were plated and cold-stratified for 24 hours at 4°C in the dark. Seedlings were grown for 10 d at 20°C under 16 hours of light with an intensity of 110–140 μE.

Quantitative RT-PCR

RNA was extracted using TRIzol® reagent (Invitrogen 15596–018). For each biological repetition, 3 seedlings were pooled and placed in a tube with 0.5 mL TRIzol and a chrome ball. Samples were homogenized in a Retch Mixer Mill 303 at 30 Hz for 30 sec. Samples were then incubated 5 min at room temperature. 0.2 mL chloroform was added and the samples mixed before being centrifuged for 15 min at 12.000x *g* at 4°C. The upper phase was collected and mixed with an equal volume of cold isopropanol. The samples were kept on ice for 30 min before being centrifuged for 10 min at 15.000x *g* at 4°C. The resulting pellet was washed with 75 % ethanol and centrifuged for 5 min at 7.500x *g* at 4°C. Supernatant was removed and the resulting pellet was air-dried before resuspension in 20 μL nuclease free water. The extracted RNA was DNase treated using DNaseI (AMPDK1-1KT, Sigma-Aldrich) according to manufactures instructions. 1 μg of RNA was reverse transcribed into cDNA with the iScript cDNA synthesis kit (#1708891, Bio-Rad) following the manufactures instructions.

qRT-PCR was performed using DyNAmo Flash SYBR Green qPCR Kit (F-415L, Thermo Scientific) and the following primers to quantify *CB5C* expression: 5′-AGCACTTTCATGGACGAACA-‘3 and 5′-TTGGCATCTTTGCTATGGTTC-‘3. PEX4/UBC21 (AT5G25760)[36] was used as reference gene using the following primers: 5′- CTGAGCCGGACAGTCCTCTTAACTG-‘3 and 5′-CGGCGAGGCGTGTATACATTTGTG-‘3. Two rounds of technical replicates were run, each including all 3 biological replicates. The C_T values for *CB5C* were normalized to PEX4/UBC21 – taking primer efficiencies into account (close to 1 for both primer sets). Finally, the *CB5C* expression values of each mutant were further normalized to WT.

GLS and biomass analysis

GLS were extracted from single 10-day-old seedlings. The extraction was performed according to Andersen et al.[37] using 1 nmol *p*-hydroxybenzyl GLS as internal standard. Quantification was performed via liquid chromatography coupled to mass-spectrometry according to Jensen et al.[38] Two independent rounds of experiments were performed with 15 seedlings for each combination of genotype and treatment per round – seedlings of these experiments were weighed individually on a scale with sub-milligram sensitivity. Prior to any statistical analysis, the weight of MeJA-treated seedlings were normalized to the mean weight of the corresponding untreated seedlings – yielding the biomass response values. Statistical analysis of biomass response and GLS levels was performed across experimental rounds via ANOVA, using the following linear models: GLS = TREATMENT + GENOTYPE + EXPERIMENT + EXPERIMENT:GENOTYPE + TREATMENT:GENOTYPE + EXPERIMENT:TREATMENT and Biomass response = genotype + experiment + genotype:experiment. *Holm*-corrected pairwise t-tests were used for *post hoc* testing.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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