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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 b$_5$; 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

**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. 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. 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. 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, the genomes of higher plants encode multiple CB5 isoforms, e.g. 6 in the model plant *Arabidopsis thaliana* and 11 in *Glycine max* (soybean). 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. Essential for this specialized metabolism are P450 enzymes that carry out central catalytic steps in several of these specialized biosynthetic pathways. The number of these enzymes is widely expanded in higher plants, with *A. thaliana* having more than 240. Plants may therefore have evolved a larger number of CB5 isoforms to keep up with the increased number of P450s. 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. 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 artemisinin – the precursor for the anti-malarial drug, artemisinin. Upon heterologous expression of the artemisinic acid biosynthetic pathway (including a P450) from *Artemisia annua*, co-expression of a CB5 resulted in higher levels of artemisinic acid. 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. 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. 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
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-benzoxoxypropyl 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 and 0.012 for cb5c-2 (Fig. 2). As previously reported, cb5c-2 and cb5c-1 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.23 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 | sulfatase | 4.1 |
| At2g14750 | AKR1 | 4.2 |
| At5g63980 | SUP01 | 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 |
| At3g22570 | 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 | BCA4 | aliphatic | 13 |
| At2g30860 | GST9 | 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 |
| At4g31600 | 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.
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*, 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). 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, 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. 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 decrease in these GLS.

**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-hydroxycarbonyl |       | 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-benzoxycarbonyl |       | 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-methylthiohexyl |       | 34 ± 5 | 30 ± 3 | 34 ± 4 | 98 ± 8 | 63 | 7 |
| 7-methylsulfinylhexyl |       | 51 ± 6 | 30 ± 4 | 36 ± 4 | 82 ± 5 | 60 ± 6 | 80 ± 7 |
| 8-methylthiooctyl |       | 53 ± 9 | 65 ± 6 | 48 ± 7 | 278 ± 20 | 195 ± 25 | 250 ± 15 |
| 8-methylsulfinyloctyl |       | 135 ± 17 | 98 ± 15 | 109 ± 14 | 312 ± 22 | 242 ± 22 | 314 ± 22 |
| Indol GLS |       | 1127 ± 103 | 879 ± 94 | 952 ± 97 | 2973 ± 328 | 2699 ± 250 | 2066 ± 192 |
| Indol-3-yethyl |       | 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, ***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.

**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 (Sigma-Aldrich). Seeds were sterilized by incubating in 70% (v/v) ethanol for 24 hours at 4°C under 16 hours of light with an intensity of 110 μmol photon/m²/s.

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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