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Engineering *Nicotiana benthamiana* for production of active cannabinoid synthases via secretory pathway optimization

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ABSTRACT

The production of cannabinoid compounds such as Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabichromene (CBC) with potential pharmaceutical applications is growing sharply. However, challenges such as the low yield of minor cannabinoids, legal restrictions on cultivation, and the complexity and cost of purification from the Cannabis sativa plant necessitate a biotechnological approach. Since the biosynthetic pathway is disclosed, cannabinoids have been produced in yeast, insect cells and plants mainly by the heterologous expression of tetrahydrocannabinol acid synthase (THCAS). THCAS and cannabidiolic acid synthase (CBDAS) use cannabigerolic acid (CBGA) as a substrate. In this study, we transiently expressed recombinant forms of THCAS and CBDAS in leaves of Nicotiana benthamiana. Our results demonstrate that efficient expression in the secretory pathway relies on replacing the endogenous signal peptide with a heterologous one. Both proteins were successfully secreted to the apoplast. MS-based analysis of the purified proteins revealed that they are heavily glycosylated with mainly Golgi-processed complex type N-glycans. In planta enzymatic removal of N-glycans indicated that glycosylation plays a role for CBDAS protein folding or stability. Finally, in vitro assays with CBGA showed that the plant-made recombinant CBDAS and THCAS are enzymatically active.

1. Introduction

Cannabis sativa is a valuable herb which naturally produces organic compounds called cannabinoids. The most studied cannabinoids include cannabidiol (CBD), tetrahydrocannabinol (THC), cannabichromene (CBC), and their respective acid forms (cannabidiolic acid (CBDA), tetrahydrocannabinol acid (THCA) and cannabichromenic acid (CBCA) [1]. Due to their physiological and psychotropic properties, cannabinoids have been subjected to extensive biological research. Their effects have been assessed for treating symptoms of epilepsy, multiple sclerosis, and chronic pain as well as appetite stimulation in cancer or AIDS patients [2,3,4,5]. The endogenous production of cannabinoids in *C. sativa* involves the intervention of four enzymes, where unsaturated fatty acid compounds represent the initial point of the biosynthetic pathway. These enzymes comprise tetraketide synthase (CsTKS), olivetolic acid cyclase (CsOAC), prenyltransferase-4 (CsPT4), tetrahydrocannabinolic acid synthase (CsTHCAS), cannabidiolic acid synthase (CsCBDAS) or cannabichromenic acid synthase (*Cs*CBCAS) [6]. THCAS and CBDAS exhibit a protein identity of >80 %, including the conserved berberine bridge type (BBE) domain which is required for the binding of the flavin adenine dinucleotide (FAD) moiety. The THCAS crystal structure (PDB: 3VTE) indicates that the FAD binding region (77–251aa) consists of two distinct α-β domains with the molecule covalently bound via His114 and Cys176, an additional disulfide bond located between residues Cys37 and Cys99. Tyr484 serves as the base that withdraws the proton from the C4 hydroxyl group of cannabigerolic acid (CBGA), initiating the catalytic reaction [7]. In the case of CBDAS this residue is Tyr483, and the proton is extracted from the terminal methyl group [8]. These differences cause the production of distinct cannabinoid compounds. In vitro functional studies indicate that these oxidocyclase enzymes may be capable of producing multiple products, potentially reflecting the promiscuity of the ancestral enzyme [9]. The *C. sativa* THCAS possesses

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eight putative *N*-glycosylation sites (N65, N89, N168, N297, N305, N329, N467 and N499) [7], whereas CBDAS contains seven (N45, N65, N168, N296, N304, N328, and N498). However, no comprehensive characterization of the glycosylation and *N*-glycans composition of THCAS and CBDAS enzymes has been reported, and the role of the glycans is unclear.

In the past two decades, plants have emerged as commercially relevant green biofactories for pharmaceutical products, including vaccines, therapeutic proteins, polymers, and industrial enzymes. For instance, the production of insulin in transgenic safflowers was accomplished by SemBioSys Genetics Inc. in Canada. In the case of vaccines, a plant-based COVID-19 vaccine consisting of coronavirus VLPs was manufactured by Medicago Inc. and approved by Health Canada in 2022 [10]. Protalix Biotherapeutics developed the production of the enzyme taliglucerase alfa in carrot cells. This became the first plant-produced recombinant protein biopharmaceutical to be commercialized for human use [11].

In the field of chemical production, there is a growing interest in developing sustainable solutions for the assembly of biosynthetic pathways in engineered host organisms. Heterologous expression of THCAS in yeast, insect cells, and plants has shown the successful production of cannabinoids such as THCA, CBDA, THCVA, and CBDVA [12,13,14,15,16]. The heterologous expression of CBDAS using insect cells demonstrated enzymatic activity through the measurement of molecular oxygen consumption and hydrogen peroxide production [17]. In industrial hemp, the overexpression of CBDAS and silencing of THCAS genes in female flowers via RNA interference using vacuum *Agrobacterium* infiltration led to a substantial increase of CBD and the reduction of THC content compared with mock plants. The use of this approach showed the high effectiveness for metabolic engineering of cannabinoids in hemp [18].

The use of engineered plants represents a sustainable and potentially cost-effective alternative to chemical synthesis, due to the lower energy and resource inputs required. Compared with the low-yield and expensive extraction process from the host plant, *C. sativa,* metabolic engineering of heterologous plants or chemoenzymatic synthesis of cannabinoids with plant-produced recombinant enzymes emerges as a promising alternative [19].

In the present study, the signal peptide-dependent transient expression of THCAS and CBDAS in the apoplast of infiltrated *Nicotiana benthamiana* leaves is shown. The *N*-glycosylation pattern of the expressed proteins was determined, and enzymatic activity was confirmed using an in vitro assay.

2. Materials and methods

2.1. Chemicals

Cannabigerolic acid (CBGA), cannabidiolic acid (CBDA), and Δ^9-tetrahydrocannabinolic acid A (THCA-A) were purchased from Cayman Chemical (Michigan, USA).

2.2. Gene design and construction of the pEAQ-HT based vectors

The full length of the *C. sativa* cannabidiolic acid synthase (*Cs*CBDAS) and tetrahydrocannabinolic acid synthase (*Cs*THCAS) (GenBank accession no NP_001384865.1 and no Q8GTB6.1) genes were designed following codon optimization and supplied in pBluescript II SK (+) and pUC57-BsaI-Free cloning vectors, respectively. The genes were synthesized by Biomatik (Ontario, Canada). The primers CsCBDAS_1F, CsCBDAS_2R, CsCBDAS_3R, CsTHCAS_1F, CsTHCAS_2R and CsTHCAS_3R were designed to introduce the sequence for the attachment of a 6x His-Tag and 5′*Age*I and 3′*Xho*I restriction sites for subsequent subcloning into the pEAQ-*HT* expression vector [20]. Overlap extension PCR (OE-PCR) [21], was used to insert the coding region for the barley α-amylase signal peptide in order to replace the native signal peptide in CsCBDAS and CsTHCAS and generate the αSP-CsCBDAS and αSP-CsTHCAS sequences that were subsequently cloned into pEAQ-*HT* expression vector. For OE-PCR the primers SP-fwd, SP-rev1-CsCBDAS, SP-rev2-CsTHCAS, CsCBDAS_2F, CsTHCAS_2F, CsCBDAS_3F, CsCBDAS_4F, CsCBDAS_3R were designed (Supplementary Table S1). *Escherichia coli* DH5α carrying positive clones were detected by colony-PCR and Sanger DNA sequencing (Mycrosynth AG, Switzerland). The expression vectors were electroporated into the *Agrobacterium tumefaciens* UIA143 strain and the recombinant clones were selected by kanamycin resistance [22].

2.3. Transient expression in N. benthamiana plants

The transient expression of the *Cs*CBDAS and *Cs*THCAS was carried out in leaves of *N. benthamiana* WT or glycoengineered ΔXT/FT (ΔXF) plants which almost completely lack *N*-glycans with β1,2-xylose and core α1,3-fucose residues [23]. The plants were grown and maintained at 23–25 °C and infiltrated 4–5 weeks after seedling pickout. The first three mature leaves of each plant were selected for infiltration. Agrobacterium strains were cultured and grown overnight, pelleted, and resuspended at OD_{600} 0.2 in the infiltration buffer (10 mM MES, 10 mM $MgSO_4$, 100 μM acetosyringone), and the leaves were infiltrated using a blunt-ended syringe as described previously [23].

2.4. Protein expression analysis and purification

Total soluble protein (TSP) or apoplastic fluid (AF) was obtained at 4 days post infiltration (dpi) to analyze the expression of the CBDAS and THCAS proteins. For TSP extraction, 100 mg of leaves were frozen in liquid nitrogen, pulverized using metallic beads in a mixer mill (Retsch MM 400), and subsequently homogenized in 400 μl of 1 x PBS. The extracts were clarified by centrifugation at 16,000 *g* for 15 min at 4 °C. The extracts were denatured by boiling in the presence of 1 x Laemmli buffer for 5 min at 95 °C. The AF from agroinfiltrated leaves was obtained by 1 x PBS infiltration followed by low-speed centrifugation as described previously [24]. For small-scale purification, NaCl and imidazole were added to the collected apoplastic fluid to adjust the buffer to 20 mM Na_2HPO_4, 500 mM NaCl, and 10 mM imidazole (pH 7.4). His-tagged αSP-CsCBDAS and αSP-CsTHCAS were purified using 50 μl of His Mag Sepharose™ Ni magnetic beads (Cytiva) according to the manufacturer's instructions. The proteins were eluted with 60 μl 20 mM Na_2HPO_4, 500 mM NaCl, and 500 mM imidazole (pH 7.4), and the concentration was measured using a nanodrop spectrophotometer (Implen, NanoPhotometer®) [25]. The eluted proteins were denatured by boiling, and the SDS-PAGE separated proteins were either visualized by Coomassie Brilliant Blue staining or subjected to immunoblotting.

2.5. SDS-PAGE and immunoblot analysis

The proteins were resolved by electrophoresis in 4–12 % polyacrylamide gels under denaturing conditions, and then were blotted onto nitrocellulose membranes (Cytiva). Blots were blocked with 3 % BSA dissolved in 1 x PBS + 1 % Tween (PBS-T) for 2 h at 25 °C and incubated overnight with the 6x-His tag monoclonal antibody (Thermo Fisher Scientific). Horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) was added, and the membranes were incubated for 2 h at 25 °C. Antibody binding was detected by incubation with Pierce® ECL Western Blotting Substrate solutions (Bio-Rad, USA). Molecular weight marker PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) was used to determine the size of the expressed proteins.

2.6. In vitro and in planta deglycosylation

The deglycosylation reaction of the αSP-CsCBDAS and αSP-CsTHCAS proteins using Endo H (NEB) and PNGase F (NEB) was carried out according to the manufacturer's instructions. Briefly, 20 μl of apoplastic

fluid containing denaturing buffer was incubated with one unit of Endo H or PNGase F for 1 h at 37 °C, after heat inactivation, the reactions were analyzed by immunoblotting. For *in planta* deglycosylation, *Nicotiana benthamiana* WT plants were co-infiltrated with pEAQ-αSP-CsCBDAS or pEAQ-αSP-CsTHCAS and p20-ST-EndoH vector [26]. The TSP and AF were obtained at 4 dpi and analyzed by immunoblotting.

2.7. N-glycan analysis

The *N*-glycan composition of *N. benthamiana*-produced CBDAS and THCAS was analyzed using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The purified proteins were separated in reducing SDS-PAGE and Coomassie stained. The 80 kDa bands were excised, S-alkylated with iodoacetamide, and in-gel digested with trypsin or in-solution with chymotrypsin. The digested samples were loaded on a nanoEase C18 column (nanoEase M/Z HSS T3 Column, 100 Å, 1.8 µm, 300 µm X 150 mm, Waters) using 0.1 % formic acid as the aqueous solvent. A gradient from 1 % B (B: 80 % Acetonitrile, 0.1 % FA) to 40 % B in 30 min was applied, followed by a 5 min gradient from 40 % B to 95 % B that facilitates elution of large peptides, at a flow rate of 6 µL/min. Detection was performed with an Orbitap MS (Exploris 480, Thermo Fisher Scientific) equipped with the standard H-ESI source in positive ion, DDA mode (= switching to MSMS mode for eluting peaks). MS-scans were recorded (range: 350–3200 m/z), and the 8 highest peaks were selected for fragmentation. Instrument calibration was performed using Pierce FlexMix Calibration Solution (Thermo Fisher Scientific).

The possible glycopeptides were identified as sets of peaks consisting of the peptide moiety and the attached N-glycan varying in the number of HexNAc, hexose, deoxyhexose, and pentose residues. The theoretical masses of these glycopeptides were determined with a spread sheet using the monoisotopic masses for amino acids and monosaccharides. Manual glycopeptide searches were made using FreeStyle (Thermo Fisher Scientific). For the relative quantification of the different glycoforms the peak intensities of the deconvoluted spectra were compared (using an in-house software tool (Freestyle_parser_v0.3.R); https://glycotoolkit.com/Tools/GlycoGlyph/ accessed on 1 May 2023)).

2.8. CBDAS and THCAS activity assay

To determine the enzymatic activity of CBDAS and THCAS, 500 ng or 100 ng of the purified proteins were mixed with 100 µM CBGA and incubated in the reaction buffer (100 mM trisodium citrate, pH 5.5) at 37 °C for 2 h After termination, the reactions were stored at -20 °C until the analysis.

2.9. LC ESI-MS/MS analysis of cannabinoids

The CBDAS and THCAS enzymatic assays were analyzed by LC-ESI-MS/MS using an Orbitrap Tribrid mass spectrometer (LC-OTMS^n, Orbitrap IQ-X Tribid, Thermo Fisher Scientific) equipped with a H-ESI source in positive ion mode. A tSIM method was developed for the ions with m/z values 359.2217 and 361.2373 and a gradient from 21.1 % B (B: 0.1 % FA in 95 % ACN) to 94.7 % B in 7 min at a flow rate of 300 µL/min was applied (13 min total run time). For separation, a Waters BioResolve column (2.1 × 5 mm) was used at a column oven temperature of 60 °C. Orbitrap Resolution was set to 60.000, RF Lens (%) to 60 with a Maximum Injection Time (ms) of 118. Data were analyzed using FreeStyle (Thermo Fisher Scientific) software and TraceFinder (Thermo Fisher Scientific). For confirmation of the expected products the retention times of CBDA and THCA-A as authentic standards were compared with the peaks observed in the samples. MS2 fragmentation information was utilized to confirm the identity of the molecules with greater confidence.

3. Results

3.1. CBDAS and THCAS are successfully expressed in N. benthamiana and secreted to the apoplast

Both native and heterologously expressed THCAS and CBDAS are secreted to the extracellular space [27,14,17]. Consequently, two distinct expression constructs were generated for each gene, differing solely in the coding sequence for the N-terminal signal peptide, which is responsible for targeting the protein to the secretory pathway. The expressed proteins consisted of either the native signal peptide (nSP) or the barley α-amylase signal peptide (α-SP), which is frequently used to target recombinant proteins to the secretory pathway in *N. benthamiana* (Fig. 1A). The CBDAS and THCAS proteins were transiently expressed in *N. benthamiana* leaves by *Agrobacterium*-mediated infiltration. Four days post-infiltration, the expression of CBDAS and THCAS could only be confirmed in the leaves infiltrated with the expression vectors encoding the proteins with the α-SP. The CBDAS and THCAS proteins were detected in the total soluble protein (TSP) extracts and the apoplastic fluid (AF) (Fig. 1B). CBDAS and THCAS in the AF were purified by binding to magnetic Ni-NTA sepharose beads. After purification, the molecular weight and integrity of the proteins were analyzed using SDS-PAGE and immunoblotting. Both proteins displayed single bands of approximately 80 kDa (Fig. 1C). The expression level in the AF was 2.5 µg/ml of fresh AF for CBDAS and 20 µg/ml of fresh AF for THCAS (Supplementary Fig. S1). The final yields after purification were up to 28.8 µg/ml of AF for CBDAS and 107.7 µg/ml of AF for THCAS (Fig. 1D). Taken together, the data indicate that the proteins can be expressed, but the expression levels and final yields differ (4–8 fold) between the two proteins. The expression of THCAS was higher than CBDAS.

3.2. CBDAS and THCAS produced in N. benthamiana are N-glycosylated

The molecular weight of CBDAS and THCAS expressed in *N. benthamiana* appeared to be higher than the calculated molecular weight based on the amino acid sequence. This discrepancy could be attributed to posttranslational modifications, such as glycosylation. To check if both proteins are *N*-glycosylated, the AF extracts from infiltrated WT or ΔXF were subjected to either Endo H or PNGase F treatment to remove the *N*-glycans. The two proteins expressed in WT were predominantly Endo H-insensitive and displayed only a faint band at approximately 65 kDa that corresponds to deglycosylated forms of the proteins. While CBDAS and THCAS expressed in WT plants were largely resistant to the PNGase F treatment, the proteins expressed in the glycoengineered ΔXF line were sensitive to PNGase F digestion and exhibited a clear shift in the molecular weight. This finding indicates that secreted CBDAS and THCAS are extensively *N*-glycosylated with core α1,3-fucose containing complex *N*-glycans. The presence of core α1,3-fucose prevents the cleavage by PNGase F of complex *N*-glycans from glycoproteins derived from WT plants but not from ΔXF plants (Fig. 2A). The presence of Golgi processed *N*-glycans is consistent with the detection of the CBDAS and THCAS in the AF.

3.3. In planta deglycosylation has a negative impact on CBDAS expression

To determine the role of the *N*-glycans for the expression and secretion to the AF, we transiently co-expressed an Endo H (ST-EndoH) variant that was targeted to the secretory pathway in plants [26]. ST-EndoH cleaves oligomannosidic *N*-glycans in the ER and Golgi apparatus, and the co-expressed glycoproteins will mostly carry *N*-glycans consisting of a single GlcNAc residue attached to the *N*-glycosylation site. Immunoblot analysis of the TSP and AF demonstrated that the expression levels of CBDAS were substantially reduced in the presence of ST-EndoH (Fig. 2B). In contrast to that, a THCAS variant that migrated faster in the presence of ST-EndoH was observed in both, the TSP and

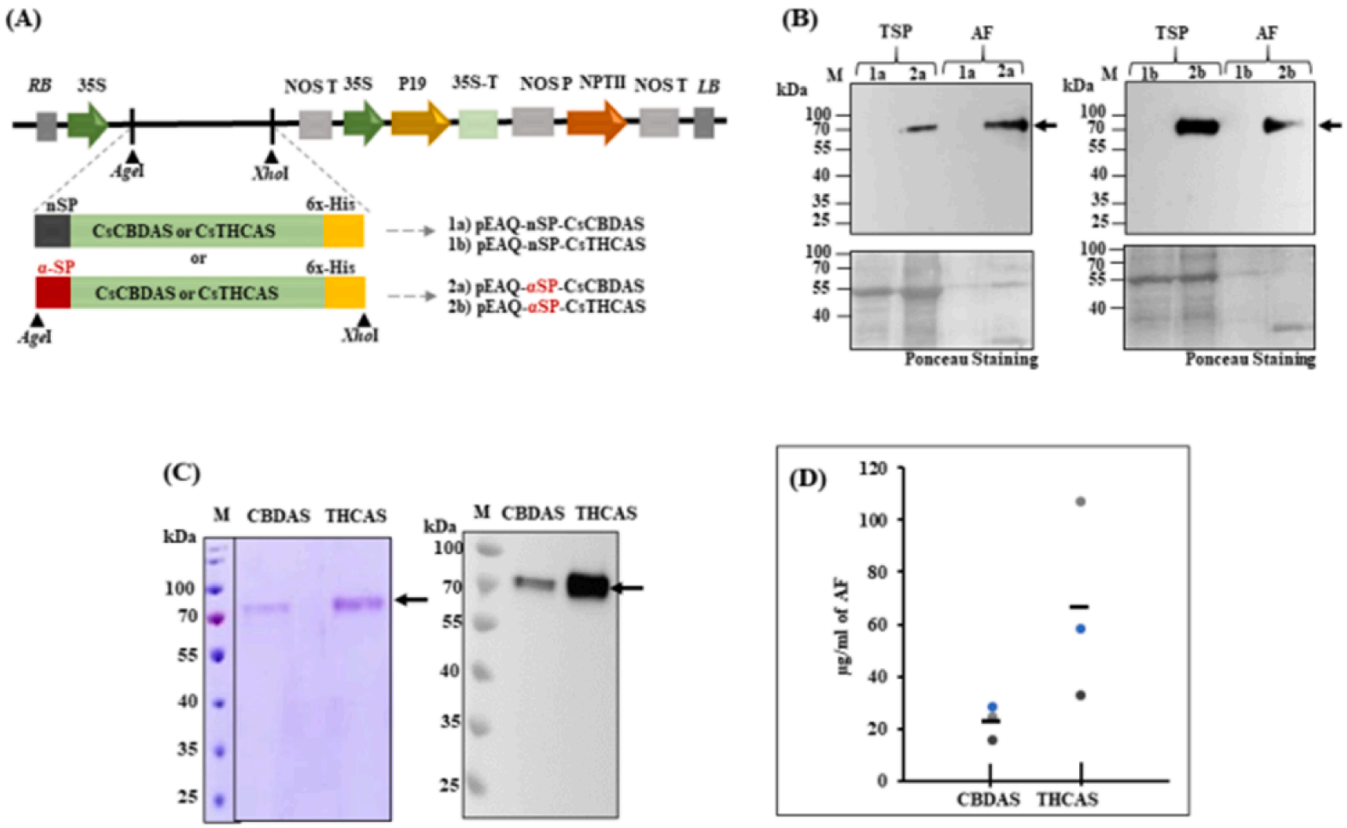


Fig. 1. CBDAS and THCAS are secreted to the apoplast. (A) Schematic representation of the expression cassettes. The expression vectors 1a) pEAQ-nSP-CsCBDAS and 1b) pEAQ-nSP-CsTHCAS contained the *Cannabis sativa* cbdas and thcas native signal peptide sequences, respectively, and both 2a) pEAQ-αSP-CsCBDAS and 2b) pEAQ-αSP-CsTHCAS the barley α-amylase signal peptide. *RB*: right border; 35S: cauliflower mosaic virus 35S constitutive promoter; nSP: native signal peptide; αSP: barley α-amylase signal peptide; 6x-His: 6x-His tag; NOS T: nopaline synthase terminator; P19: suppressor of gene silencing; 35S-T: cauliflower mosaic virus terminator; NOS P: nopaline synthase promoter; NPTII: kanamycin resistance gene; *LB*: left border. (B) The CBDAS and THCAS expression constructs were transiently expressed in *N. benthamiana* WT. Four days post-infiltration, the total soluble protein (TSP) and apoplastic fluid (AF) were extracted, and the presence of the proteins was analyzed by immunoblotting with anti-His antibodies. (C) SDS-PAGE under reducing conditions and immunoblotting of proteins purified from the AF. The CBDAS and THCAS bands are indicated by arrows. (D) For quantification of yield after purification from the AF, three independent purifications are shown by colored dots. The mean value is indicated (black bar).

AF, indicating the *in planta N*-glycan removal. A smaller amount of fully glycosylated THCAS was also present, indicating that some proteins escaped the in vivo cleavage by ST-EndoH (Fig. 2C).

3.4. The N-glycosylation sites of CBDAS and THCAS contain complex and truncated N-glycans

The two proteins purified from the apoplastic fluid of *N. benthamiana* WT plants were proteolytically digested and the glycopeptides were analyzed by LC-ESI-MS. Six of the seven *N*-glycosylation sites from CBDAS and seven from the eight sites from THCAS were properly analyzed. The glycosylation of site NKT in both proteins could not be examined, as neither trypsin nor chymotrypsin produced detectable glycopeptides (Fig. 3A). For CBDAS, 51.61 % of all *N*-glycans belong to complex *N*-glycans with GnGnXF, GnMXF, and GnMX as the frequent structures. In addition, large amounts of truncated *N*-glycans (32.26 %) containing MMXF, MMX, MUX, and MUXF glycoforms and minor amounts of Lewis A type structures carrying additional fucose and/or galactose residues (AMX, AMXF, AGnXF and AGnXF2) are present. Overall, similar results were obtained for THCAS, which mainly comprise complex *N*-glycans (40.43 %), truncated glycoforms (38.30 %; mainly MMXF, MUXF, and MUX), and Lewis A type structures (12.77 %) (Fig. 3B).

3.5. Site-specific differences in N-glycan processing appear conserved between CBDAS and THCAS

While minor site-specific differences in the *N*-glycan profile were detected at individual *N*-glycosylation sites of CBDAS and THCAS (Fig. 3C), common characteristics between shared sites can be observed. Of the total sites analyzed, five of them possess the same *N*-glycosylation sequence and position in the proteins (i.e., NST, NLS, NIT, NKS, and NYT). The NST site is mostly glycosylated with two complex *N*-glycans in a similar abundance between the two proteins: GnMXF (35.25 % in CBDAS and 38.53 % in THCAS) and GnMX (32.41 % in CBDAS and 32.98 % in THCAS). With respect to the NLS site, the complex N-glycan with two terminal GlcNAc residues (GnGnXF) was the most predominant structure with a high abundance in both proteins (75.74 % for CBDAS and 63.66 % for THCAS). Interestingly, in both proteins, the NIT site was less processed, and the complex *N*-glycans completely lacked core fucose. This suggests that the *N*-glycans at this site are not as accessible due to local protein conformations, and this structural constraint is conserved in both proteins. Similarly, the NKS site is only partially glycosylated in both proteins.

Three differential sites were analyzed. While the NAT site is only present in CBDAS, the sites NNS and NFT are found in THCAS. With regard to the content of *N*-complex glycans, the profile for NAT is very similar to the NLS sites: however, more truncated glycoforms were detected, and Lewis A type structures were also found at low levels. In the case of NNS and NFT sites, the majority of *N*-glycan structures were

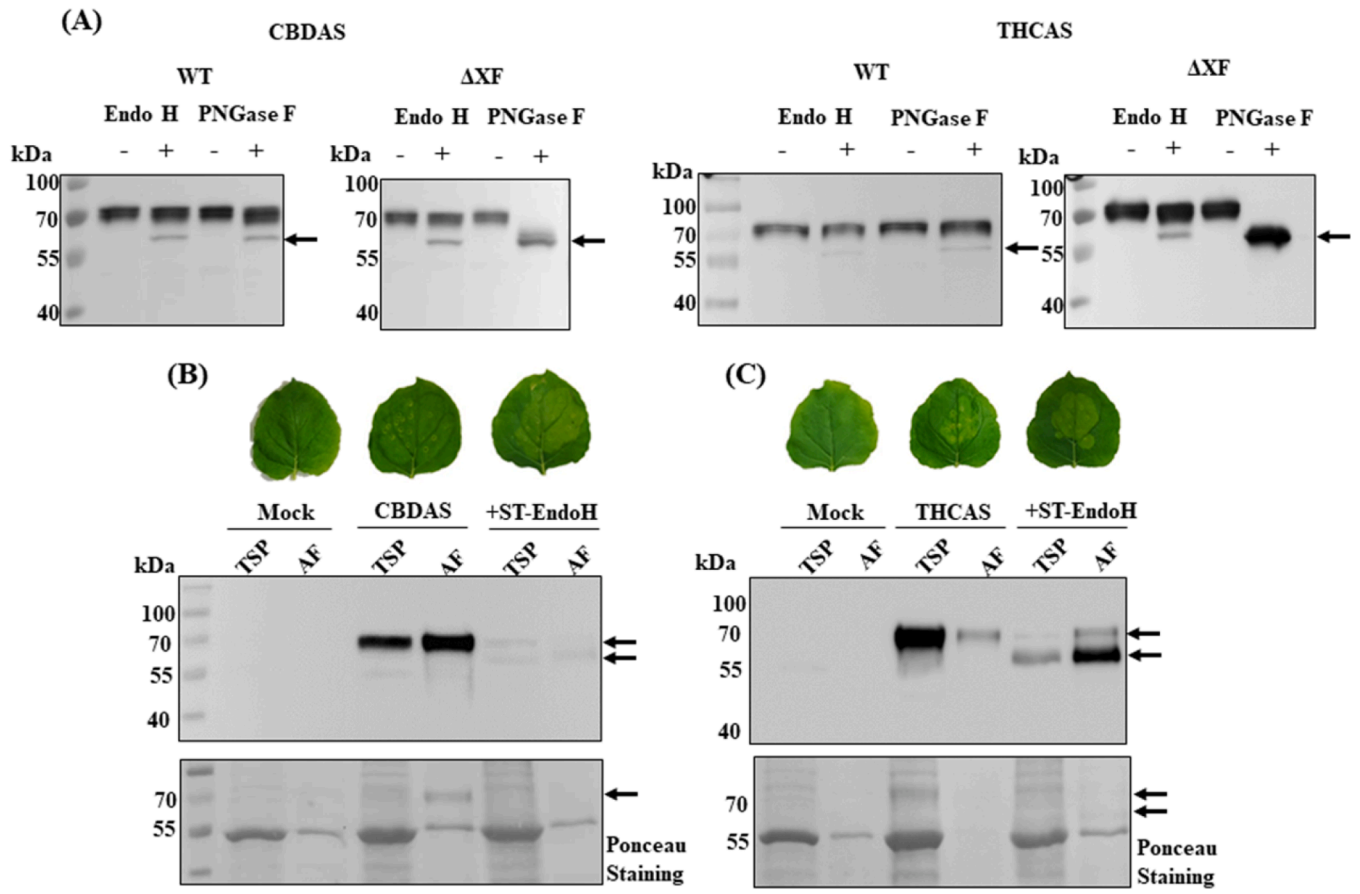


Fig. 2. CBDAS and THCAS are extensively glycosylated. (A) Immunoblot analysis of *in vitro* deglycosylated CBDAS and THCAS. CBDAS and THCAS were transiently expressed in WT or ΔXF *N. benthamiana* plants, the apoplastic fluid extract was digested with Endo H or PNGase F, and immunoblot detection was carried out with anti-His antibodies. (B) and (C) *In planta* deglycosylation by co-expression of CBDAS or THCAS with ST-EndoH in WT *N. benthamiana* plants. Mock: apoplastic fluid from uninfiltrated leaf; TSP: total soluble protein extract; AF: apoplastic fluid extract. Glycosylated and deglycosylated variants are marked with arrows.

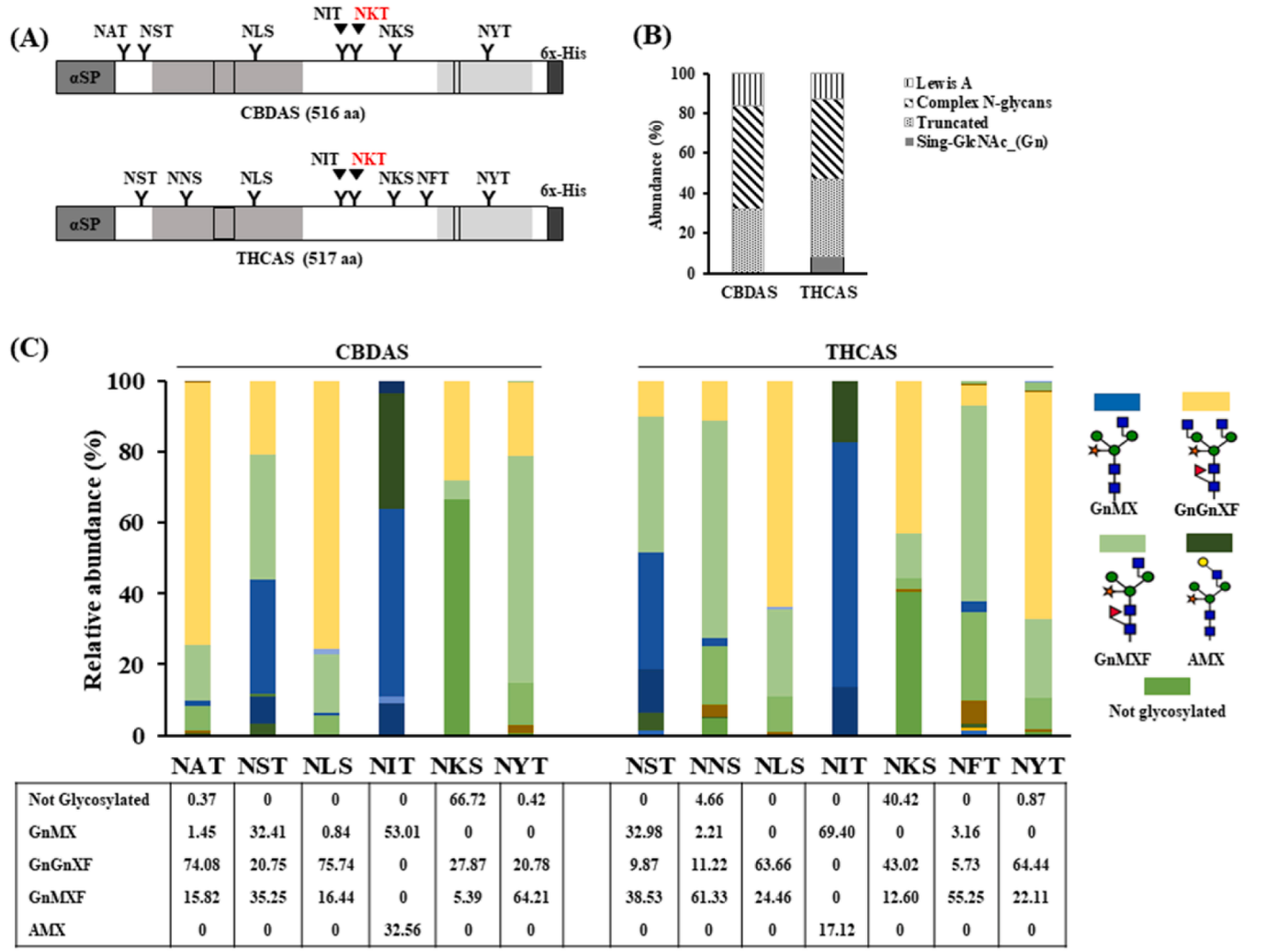
found to comprise complex glycans with similar abundance between them (Supplementary Table S2 and S3).

3.6. Recombinant CBDAS and THCAS are active and produce CBDA and THCA

To determine the enzymatic activity of the plant-made CBDAS and THCAS, in *vitro* enzymatic assays were carried out with the purified CBDAS and THCAS proteins. Two different amounts of each protein (100 and 500 ng) and 100 μM of CBGA as substrate were used, and the products of the assays were analyzed by LC-ESI-MS/MS. In the control reaction which contained only the CBGA substrate, no peaks of the expected products were detected. The reactions containing 500 ng of CBDAS or THCAS showed the production of CBDA and THCA. Additionally, different isomers were detected (Fig. 4A and B). The mixture containing 500 ng of CBDAS produced 1.75 μM of CBDA and 0.005 μM of THCA, while the reaction with 500 ng of THCAS generated 12.2 μM of THCA and no CBDA was detected (Fig. 4C). The MS spectra of the THCA and CBDA produced in *N. benthamiana* showed masses and fragments that were identical to the standards (Supplementary Fig. S2 and Fig. S3). Taken together, this demonstrates that both plant-derived enzymes are active and capable of producing the desired product.

4. Discussion

The enzymes that catalyze the formation of the bioactive cannabinoids CBDA and THCA in *C. sativa* are CBDAS and THCAS, respectively. Their heterologous expression in different hosts, such as yeast, bacteria, and plants has been addressed in attempts to obtain these valuable compounds in a rapid and efficient way by chemoenzymatic synthesis. This allows precise and high-yield production without the use of growing the narcotic hemp plant. In addition, three non-well-known enzymes belonging to the BBE superfamily from the organisms *Brassica rapa, Nicotiana attenuata,* and *Papaver somniferum,* which have shown to accept CBGA as a substrate have been heterologous expressed in yeast and produced cannabielsoin (CBE) [28]. In the present study, we tested the expression of CBDAS and THCAS in *N. benthamiana* plants using either the native or the barley α-amylase signal peptide. The expression was only achieved when the native signal peptide was substituted. This is consistent with a previous study where the expression of THCAS in *N. benthamiana* plants was only achieved when the protein was targeted to the ER using a heterologous signal peptide [14]. In tobacco hairy roots expressing the full-length enzyme with the native signal peptide, the enzyme was not found to be secreted to the medium [16]. By contrast, using the same sequence in insect cells, the native THCAS accumulated in the medium, suggesting the cleavage of the signal peptide and secretion of the mature enzyme [16]. Similarly, recombinant CBDAS produced in insect cells showed activity only when the measurement was performed with the culture medium. This indicates that the signal peptide was correctly cleaved and the majority of the enzyme was secreted from the cells [17]. In yeast, the THCAS expression was accomplished when codon optimization was applied, and the native signal peptide was removed [29]. A limited number of studies have been conducted on the recombinant expression of CBDAS. However, our data indicate that CBDAS behaves similarly to THCAS. Together, these studies highlight the critical role of the signal peptide in



	NAT	NST	NLS	NIT	NKS	NYT		NST	NNS	NLS	NIT	NKS	NFT	NYT
Not Glycosylated	0.37	0	0	0	66.72	0.42		0	4.66	0	0	40.42	0	0.87
GnMX	1.45	32.41	0.84	53.01	0	0		32.98	2.21	0	69.40	0	3.16	0
GnGnXF	74.08	20.75	75.74	0	27.87	20.78		9.87	11.22	63.66	0	43.02	5.73	64.44
GnMXF	15.82	35.25	16.44	0	5.39	64.21		38.53	61.33	24.46	0	12.60	55.25	22.11
AMX	0	0	0	32.56	0	0		0	0	0	17.12	0	0	0

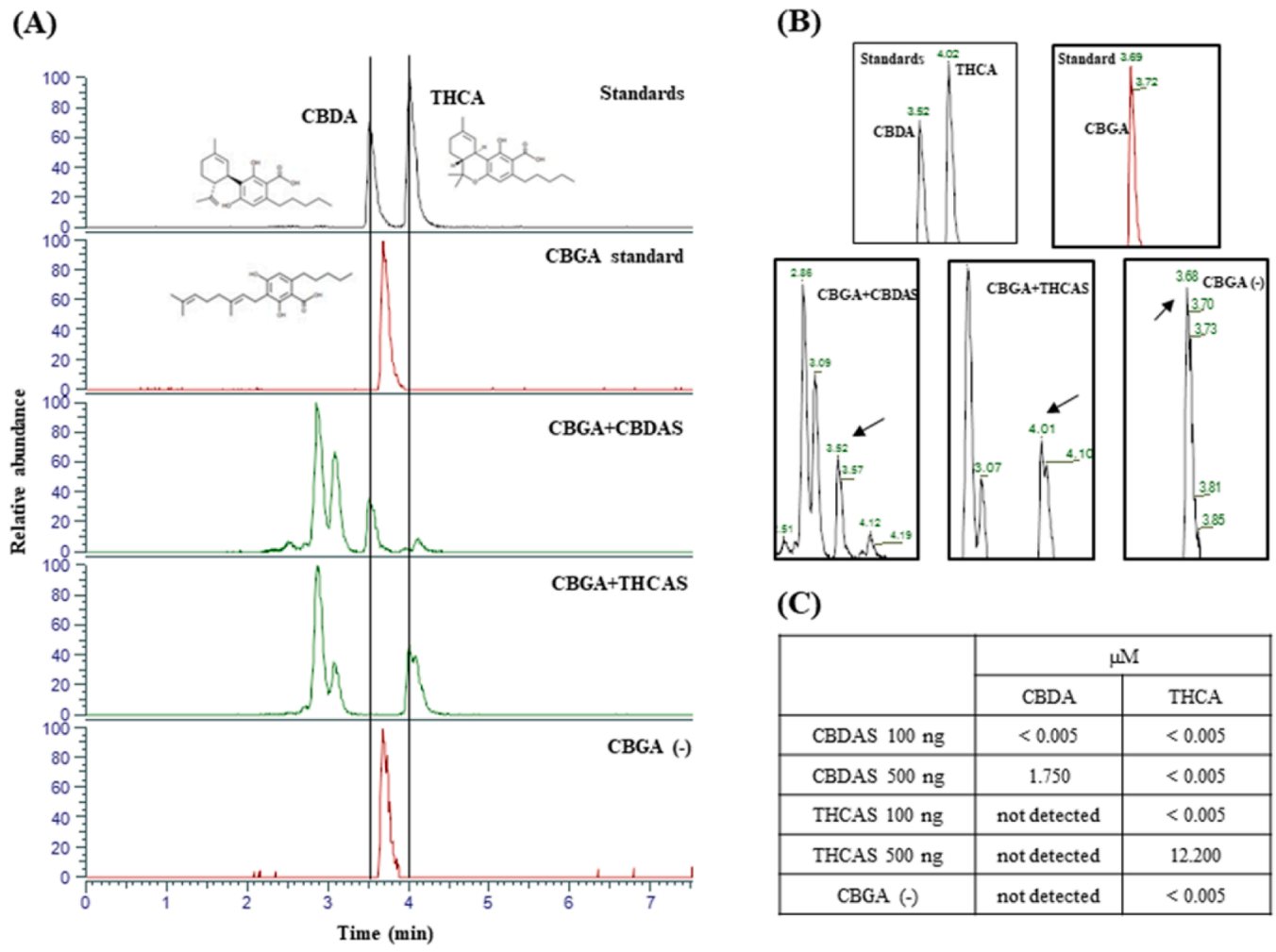
Fig. 3. N-glycan analysis of purified CBDAS and THCAS. (A) Illustration of the plant-expressed CBDAS (amino acid region 29–544) and THCAS (amino acid region 29–545), the N-glycosylation sites are shown, the site NKT in either CBDAS or THCAS was not analyzed. Dark gray rectangle: FAD binding domain; outlined dark gray rectangle: FAD binding site; light gray rectangle: berberine bridge type domain (BBD); outlined light gray rectangle: active site: αSP: barley α-amylase signal peptide; 6x-His: 6x-His tag. (B) Total N-glycan composition of the plant-made CBDAS and THCAS. Truncated: processed N-glycans lacking GlcNAc residues at the non-reducing end (i.e. MMXF, MMX, MUX, MUXF); complex N-glycans: structures containing at least one GlcNAc residues at the non-reducing end (i.e. GnGnXF, GnMXF, GnMX); Lewis A: complex N-glycans that contain Lewis A-type terminal modifications (AMX, AMXF, AGnXF, AGnXF2). For a detailed explanation of the nomenclature see Altmann et al., 2024 [34]. (C) Site-specific analysis of the most prominent *N*-glycans detected on CBDAS and THCAS proteins. The visual representation highlights the abundant glycan structures for each N-glycosylation site which are presented in order from N- to C-terminus. The table below shows the relative abundance of the not-glycosylated and the most predominant glycoforms for each *N*-glycosylation site. For a list of all detected N-glycans see Table S2 and S3.

the expression of the recombinant enzymes. One possible explanation for this phenomenon is that the signal peptide from *C. sativa* might be inefficiently recognized by the *N. benthamiana* translocation machinery. This could lead to targeting of the proteins to the cytosol and accumulation as insoluble proteins reported for expression in bacteria [16], or the presence of the uncleaved signal peptide in the ER leads to misfolding and rapid protein degradation. Further studies are required to determine the characteristics of the *C. sativa* THCAS and CBDAS signal peptides.

While THCAS and CBDAS share quite a high sequence similarity, a clear difference in expression yields between THCAS and CBDAS was observed. Following purification, a 3.7-fold higher yield was obtained for THCAS with an estimated 8-fold higher expression in the AF. Both proteins are extensively glycosylated, and differences in N-glycosylation could have an impact on their expression or catalytic activity [17]. THCAS harbors one additional N-glycosylation site, and after in planta deglycosylation the CBDAS expression was almost undetectable, indicating that CBDAS glycosylation is important for protein folding and stability. Similar findings have been previously reported for recombinant human enzymes expressed in different animal cells [30,31].

MS-based analysis of glycopeptides showed that all detected peptides with N-glycosylation sites carry N-glycans that are of the complex or truncated types. Site-specific differences were detected, and some complex N-glycans were further modified with Lewis A type structures. The function of Lewis A type structures on plant glycoproteins remains unclear [32]. A previous study showed that in vitro deglycosylated yeast-derived THCAS displayed increased catalytic activity [8]. Further studies are necessary to determine whether distinct N-glycan modifications affect the activity of recombinant CBDAS and THCAS.

Previously, the enzymatic activity of recombinant THCAS produced in yeast, insect cells, and plants has been reported. Furthermore, the cannabinoid precursors were successfully expressed in *N. benthamiana* [33]. This study demonstrates the production of active recombinant THCAS and, for the first time, the production of an active recombinant



	µM	
	CBDA	THCA
CBDAS 100 ng	< 0.005	< 0.005
CBDAS 500 ng	1.750	< 0.005
THCAS 100 ng	not detected	< 0.005
THCAS 500 ng	not detected	12.200
CBGA (-)	not detected	< 0.005

Fig. 4. CBDAS and THCAS purified from the apoplastic fluid extract are active with CBGA as a substrate. (A) CBDAS and THCAS were incubated with CBGA and the production of CBDA and THCA was analyzed by LC-ESI-MS/MS. Peaks corresponding to references are marked with a vertical black line. Additionally, isomers of the products with varying intensities were detected (peaks eluting at earlier time points). (B) Retention times for standards and enzymatic assays. (C) Quantification of the products (CBDA and THCA) after the in vitro enzymatic assays using two different amounts of plant-made CBDAS and THCAS (100 and 500 ng).

CBDAS heterologously expressed in plants. These indicate the potential use of *N. benthamiana* as a biofactory for cannabinoid production. In terms of product yield, THCAS demonstrated greater efficiency in the generation of the specific reaction product than CBDAS. This was evident by the final concentration of THCA after the in vitro enzymatic assay, which was 12.20 µM, in comparison to the 1.75 µM of CBDA produced by CBDAS. However, the yield was still low relative to the amount of substrate used. The MS analysis revealed the presence of substantial amounts of isomers with the same precursor mass and fragment masses as the expected products, which exhibited a higher polarity and structural similarity to CBDA and THCA. Further efforts are required to optimize the assay conditions, including temperature, time of the reaction, and enzyme concentration, in order to improve the concentration of the final products.

5. Conclusions

The CBDAS and THCAS enzymes from *Cannabis sativa* can be produced in *N. benthamiana* plants by targeting the enzymes to the secretory pathway using a heterologous signal peptide. The plant-made enzymes are heavily glycosylated and the characterization of the *N*-glycans at individual *N*-glycosylation sites showed the presence of processed complex *N*-glycans. The impact of *in planta N*-glycan removal on expression was demonstrated. Both recombinant proteins were found to be active enzymes and produced the cannabinoid compounds: CBDA and THCA. There are, however, some fundamental issues that require further investigation: 1) the inefficient use of the native signal peptide from *C. sativa* CBDAS and THCAS in *N. benthamiana*; 2) the great differences between CBDAS and THCAS expression, before and after the *in planta* production of the deglycosylated variants; 3) the differences in catalytic efficiency between the two plant-made enzymes; and 4) the conditions for the enzymatic assays in order to generate greater amounts of the specific products. Consequently, efforts directed towards the study of these cannabinoid synthesising enzymes will result in the accumulation of knowledge that is necessary to achieve the maximum biotechnological potential of these enzymes in the production of bioactive compounds.

CRediT authorship contribution statement

Omayra C. Bolaños-Martínez: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – original draft. **Anna Urbanetz:** Methodology, Formal analysis, Data curation. **Daniel Maresch:** Formal analysis, Data curation. **Richard Strasser:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft. **Sornkanok Vimolmangkang:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2024.e00865.

Data availability

The datasets used during the current study are available from the corresponding author on the reasonable request.

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