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FUNCTIONAL ANALYSIS OF NORCOCLAURINE SYNTHASE IN *COPTIS JAPONICA*

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(*S*)-Norcoclaurine is the entry compound in benzylisoquinoline alkaloids biosynthesis and is produced by the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) by norcoclaurine synthase (NCS; EC 4.2.1.78). Whereas cDNA of the pathogenesis-related (PR) 10 family, which translation product catalyzes NCS reaction, has been isolated from *Thalictrum flavum*, its detailed enzymological properties have not yet been characterized. We report here that a distinct cDNA isolated from *Coptis japonica* (CjNCS1) also catalyzed NCS reaction as well as a PR10 homologue of *Coptis japonica* (CjPR10A). Both recombinant proteins stereo-specifically produced (*S*)-norcoclaurine by the condensation of dopamine and 4-HPAA. Since a CjNCS1 cDNA that encoded 352 amino acids showed sequence similarity to 2-oxoglutarate-dependent dioxygenases of plant origin, we characterized the properties of the native enzyme. Sequence analysis indicated that CjNCS1 only contained a Fe^{2+}-binding site and lacked the 2-oxoglutarate-binding domain. In fact, NCS reaction of native NCS isolated from cultured *Coptis japonica* cells did not depend on 2-oxoglutarate, nor oxygen, but did require ferrous ion. On the other hand, CjPR10A showed no specific motif. The addition of *o*-phenanthroline inhibited NCS reaction of both native NCS and recombinant CjNCS1, but not that of CjPR10A. In addition, native NCS and recombinant CjNCS1 accepted phenylacetaldehyde and 3,4-dihydroxyphenylacetaldehyde, as well as 4-HPAA, for condensation with dopamine, whereas recombinant CjPR10A could use 4-hydroxyphenylpyruvate and pyruvic acid in addition to the above aldehydes. These results suggested that CjNCS1 is the major NCS in *Coptis japonica*, whereas native NCS extracted from cultured *Coptis japonica* cells was more active and formed a larger complex compared to recombinant CjNCS1.

Higher plants produce divergent chemicals, such as alkaloids, terpenoids, and phenolic compounds, in secondary metabolism. Among these chemicals, alkaloids are very important in medicine due to their high biological activities. Alkaloids are low-molecular-weight, nitrogen-containing compounds that are found in about 20% of plant species. Most alkaloids are derived from amines produced by the decarboxylation of amino acids, such as histidine, lysine, ornithine, tryptophan, and tyrosine. Whereas the coupling of amines to other products is the first important step in producing diverse alkaloids, this entry reaction has been poorly characterized. (*S*)-Strictosidine is a central intermediate for indole alkaloids. Strictosidine synthase, which catalyzes the formation of (*S*)-strictosidine from tryptamine and secologanin, is a rare exception in that its cDNA has been cloned from *Catharanthus roseus* and *Rauvolfia serpentine* (1,2).

Benzylisoquinoline alkaloids are a large and diverse group of pharmaceutical alkaloids with about 2,500 defined structures. Norcoclaurine produced from tyrosine is a key entry compound, from which various benzylisoquinoline alkaloids, such as analgesic morphine, colchicines, antibacterial berberine,

palmatine, and sanguinarine, are produced through a multistep process. (*S*)-Norcoclaurine is produced by norcoclaurine synthase (NCS; EC 4.2.1.78)[1], which condenses dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA)(Fig. 1)(3,4). Since this condensation reaction can occur relatively easily by a chemical reaction, it has been difficult to characterize the biochemical and molecular biological properties of NCS. Only recently has NCS been isolated and characterized from cell suspension cultures of meadow rue (*Thalictrum flavum* ssp. *glaucum*)(5,6), but the mechanism of the NCS reaction, including the stereo-specificity of product, has not yet been determined.

Cultured *Coptis japonica* cells are a unique system for producing large amounts of benzylisoquinoline alkaloid. Using this system, many enzymes in berberine biosynthesis, including norcoclaurine 6-*O*-methyltransferase (6OMT), coclaurine *N*-methyltransferase (CNMT), *N*-methylcoclaurine 3'-hydroxylase (CYP80B2), 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT), have been isolated and characterized (7-9). In this report, we describe the characterization of NCS in *C. japonica* cells. Since selected cultured *C. japonica* 156-1 cells expressed the genes for enzymes in berberine biosynthesis in greater amounts than non-selected cells (Dubouzet E. unpublished data)(9-11), we screened EST clones with higher expression in a selected line and examined its NCS activity. Our isolated candidate cDNA (CjNCS1) had a dioxygenase-like protein family gene domain, and we examined its roles in NCS activity using both recombinant CjNCS1 produced in *E. coli* and native NCS isolated from cultured *C. japonica* cells, in comparison with recombinant Cj pathogenesis-related (PR) 10-like protein (PR10A) isolated based on sequence homology with *Thalictrum* NCS. All of our data suggested that the CjNCS1 gene product corresponds to native NCS in *C. japonica* cells. We discuss the significance of the co-existence of both CjNCS1 and CjPR10A proteins with NCS activity in *C. japonica* and the molecular evolution of NCS.

EXPERIMENTAL PROCEDURES

Cultured cells and RNA preparation - Four *C. japonica* cell lines, CjY, Cj8, 156-1, and 156-1S, were maintained in suspension culture as described previously (12). CjY was an unselected cell line, whereas 156-1 and Cj8 were selected cell lines for high alkaloid production (12), but Cj8 had lost its high productivity after inadequate subculture. The cell line 156-1S was transgenic 156-1 cells that had been transformed with SMT cDNA expression vector driven by cauliflower mosaic virus 35S promoter (13). Total RNA was extracted from 1-, 2-, and 3-week-old cells with TriZOL and used for RNA blot analysis (10, 14).

Construction and sequencing of a cDNA library of C. japonica - Poly $(A)^+$ RNA was isolated and a cDNA library was constructed as described elsewhere (8). The cDNA fragments were ligated into pDR196 vector (15). Sequencing of the cDNA library was performed using a MegaBACE 1000 DNA Sequencing System (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions.

RNA gel blot analysis - Ten µg of total RNA isolated from each cultured cell line was electrophoresed on formaldehyde gels and then blotted onto BioDyne A™(Pall) membranes. The EST clone sequences were amplified by PCR with primers (5'-GAA AGA AAA AAA ATA TAC CCC AGC-3' and 5'-TTT CGT AAA TTT CTG GCA AGG TAG AC-3'), separated on agarose gel and recovered using GenElute™ (Sigma-Aldrich) to prepare probes. PCR product was labelled with ^{32}P-radioisotope and RNA hybridization and signal detection were carried out according to standard protocols as reported previously (16).

Construction of expression vectors for recombinant CjNCS1 and CjPR10A - Expression vectors were constructed for full-length CjNCS1 and CjPR10A cDNAs without the fused peptide derived from the vector sequence in pET-41a vector (Novagen, Madison, WI). CjNCS1 and CjPR10A cDNAs were amplified by PCR with 5'-GGG AAT TCC ATA TGA GCA AGA ATC TTA CTG GTG-3' (*Nde*I restriction site is underlined) and 5'-CCG CTC GAG TTA TAG TTT CAT CTG ATC CAA TAA GCT CTT ACC-3' (*Xho*I adaptor is underlined) primers for CjNCS1 and 5'- GGC ATA TGA GGA TGG AAG TTG TTC TAG-3'

(*Nde*I restriction site is underlined) and 5'-GGC TCG AGT TAC TCT GAA CTC TTG TGT TTG-3' (*Xho*I adaptor is underlined) primers for CjPR10A. The PCR products were digested with *Nde*I and *Xho*I, and ligated into the pET-41a vector digested with *Nde*I and *Xho*I using a DNA Ligation Kit (Takara Shuzo Co.). The resulting plasmid/vector was sequenced to confirm the correct construction.

Isolation of Arabidopsis homologue (AtSRG1) to CjNCS1 and construction of expression vector - Total RNA was extracted from 5-week-old callus cultures of *A. thaliana* maintained on Linsmaier and Skoog medium supplemented with 3% sucrose, 10 μM 1-naphthaleneacetic acid (NAA), and 1 μM benzyladenine (BA) using an RNEasy kit (Qiagen). Total RNA (2.5 μg) was converted to cDNA using Superscript III according to the protocol of the manufacturer, and the AtSRG1 (GenBank™ accession number S44261), which is highly homologous to CjNCS1, was amplified by PCR using 5'-GGG AAT TCC ATA TGG AAG CAA AAG GGG CAG CA-3' (*Nde*I restriction site is underlined) and 5'-CCG CTC GAG TTA GAT TCT CAA AGC ATC TAG-3' (*Xho*I adaptor is underlined) as primers. PCR product was digested with *Nde*I and *Xho*I and ligated into pET-41a as described above.

Heterologous expression of recombinant CjNCS1, CjPR10A, and AtSRG1 in E. coli - The expression vectors for full-length cDNAs of CjNCS1, CjPR10A, and AtSRG1 were introduced into *E. coli* BL21 (DE3). *E. coli* cells containing each plasmid were grown at 37°C in LB medium. After induction with 1 mM isopropylthiogalactoside (IPTG), *E. coli* cells containing CjNCS1 plasmid were incubated at 16°C for 48 h, and *E. coli* cells containing CjPR10A and AtSRG1 plasmids were incubated at 37°C for 4 h.

The resulting recombinant CjNCS1 protein was extracted from a 1-liter culture of *E. coli* grown in LB medium with the extraction Tris buffer (0.1 M Tris-HCl, pH 8.0, containing 10% glycerol, 5 mM EDTA and 5 mM 2-mercaptoethanol), then purified to homogeneity. Crude extract was centrifuged at 10,000×g for 10 min, and the supernatant was then subjected to fractionation with $(NH_4)_2SO_4$. The desired recombinant protein was precipitated between 30 and 60% $(NH_4)_2SO_4$ saturation and made soluble in the extraction buffer again. The protein solution was dialysed in the same buffer and applied to a DEAE Sepharose CL-4B column (Pharmacia, 2.5 × 16 cm) that had been equilibrated with the extraction buffer. After the column was washed with 2 volumes of the same buffer, recombinant protein was eluted with 250 ml of a linear gradient of extraction buffer with 0-0.5 M NaCl solution. The recombinant protein fractions were detected with SDS-PAGE and these fractions were pooled and then applied to a hydroxyapatite column (Pharmacia, 2.5 × 9 cm) that had been pre-equilibrated with 10 mM potassium phosphate buffer (KPB)(pH 8.0). After the column was washed with 10 mM KPB, CjNCS1 was eluted with a linear gradient (0.01-0.5 M) of KPB solution (100 ml). The active fractions were pooled, and then dialysed in 50 mM KPB (pH 8.0).

All operations were performed at 4°C, and all of the buffers contained 5 mM 2-mercaptoethanol and 10% glycerol unless stated otherwise.

Measurement of NCS activity - Enzyme activity was determined in reaction buffer (50 mM Tris-HCl (pH 7.4), 10% glycerol, and 5 mM 2-mercaptoethanol) containing 1 mM dopamine and 1 mM 4-HPAA. 4-HPAA was produced by the enzymological conversion of tyramine with monoamine oxidase. 4-HPAA was prepared from the deamination of tyramine by purified tyramine oxidase (1 U/ml) from *Arthrobacter* sp. (SIGMA) in reaction buffer (50 mM Tris-HCl (pH 7.4) containing 2 mM tyramine). After incubation at 30°C for 2 hr, the reaction was stopped by boiling for 5 min. The sample was centrifuged, the supernatant was used as 2 mM 4-HPAA solution, and the reaction was considered to be complete. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 1/10 volume of 20% trichloroacetic acid (TCA). After protein precipitation, the formation of norcoclaurine was determined by liquid chromatography-mass spectroscopy (LC-MS; LCMA-2010A, Shimadzu, Kyoto, Japan) with an LC-10A system: column,

Inertsil ODS-3 (4.6 × 250 mm; GL Sciences Inc.); solvent system, acetonitrile/methanol/300 mM acetic acid (2:2:5); flow rate, 0.5 ml/min at 40°C.

To distinguish (*R*) and (*S*) forms of norcoclaurine, we used a chiral column (SUMICHIRAL-CBH, Sumika Chemical Analysis Service) with LC-MS analysis. The solvent system was acetonitrile/0.3% acetic acid (5:95), pH 5.2; flow rate, 0.4 ml/min at 25°C. Since we did not have a (*S*)-norcoclaurine standard, norcoclaurine was converted to coclaurine by recombinant 6OMT from *C. japonica* (7) and compared it with a (*S*)-coclaurine standard.

To examine the effect of ferrous ion in the NCS reaction, the enzyme solution was pre-incubated with 5 mM EDTA or 0.5 mM *o*-phenanthroline for 5 min at 4°C. After pre-incubation with chelators, the substrates were added to the reaction mixture, and the NCS activity was then assayed as described above.

Immunoprecipitation of NCS activity with antiserum against CjNCS1 - Mouse polyclonal antibodies for *C. japonica* NCS were prepared against purified recombinant CjNCS1 at Japan Clinical Laboratories, Inc. (Kyoto, Japan). These antibodies were specific to CjNCS1 and no signal was detected by SDS-PAGE immunoblotting except for CjNCS1 in crude *C. japonica* cell extract. For immunoprecipitation experiments, the same volume of polyclonal antiserum for CjNCS1 was added to cell lysates. After 2 hr of incubation at 4°C, 20 µl Protein G Plus/Protein A Agarose Suspension (CALBIOCHEM) was added to an antiserum/enzyme mixture, and the suspension was then mixed gently for another 12 h at 4°C. The supernatant was recovered by centrifugation and NCS activity was determined as described above.

Alignment analysis - The protein sequences were aligned using ClustalW (17, 18) and Boxshade (www.ch.embnet.org/software/BOX-form.html). The phylogenetic tree was calculated by the neighbor-joining method using ClustalW (19).

LC/NMR method - LC was performed on an Inertsil ODS-3 reversed-phase column; solvent system, 0.1 M NH_4OAc (0.05% TFA, D_2O)/CH_3CN (0.05% TFA) (4:1); flow rate, 0.5 ml/min at 40°C. NMR analysis was performed as described elsewhere (20).

RESULTS

NCS activity in C. japonica cells - (*S*)-Norcoclaurine, the central precursor of pharmaceutically important benzylisoquinoline alkaloids, is produced by NCS, which condenses dopamine and 4-HPAA (Fig. 1). NCS activity is usually detected by the formation of total norcoclaurine (21), whereas racemic norcoclaurine is also formed by a chemical reaction during the enzyme reaction. Therefore, we established a system to detect the stereospecific formation of (*S*)-norcoclaurine to estimate the native NCS reaction. Crude extract from cultured *C. japonica* cells clearly showed that the native enzymic reaction was stereospecific, that is, it gave the (*S*) form, whereas the product of the chemical reaction was a mixture of (*R*) and (*S*) forms (Fig. 2).

Next, we examined the substrate-specificity of NCS, since (*S*)-norlaudanosoline, which is produced by the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA), was once thought to be a common pathway intermediate in benzylisoquinoline alkaloid biosynthesis (22, 23), while (*S*)-norcoclaurine is now thought to be a natural precursor in benzylisoquinoline alkaloid biosynthesis (4, 24). The substrate-specificity of NCS was examined with several amines and aldehydes. When the condensation activity of NCS from *C. japonica* was examined for dopamine, tyramine, tryptamine, noradrenaline, 2-phenylethylamine, and 3-hydroxy-4-methoxyphenethylamine with 4-HPAA as an aldehyde donor, only dopamine produced a condensation product, norcoclaurine (data not shown). On the other hand, when several aldehydes and carboxylic acids (phenylacetaldehyde, 3,4-DHPAA, 4-HPAA, benzaldehyde, and pyruvic acid) were reacted with dopamine as an amine substrate, phenylacetaldehyde, 3,4-DHPAA, and 4-HPAA produced 1-benzyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, norlaudanosoline, and norcoclaurine, respectively (data not shown). Thus, NCS from *C. japonica* could condense dopamine and a

phenylacetaldehyde group.

Molecular cloning of a C. japonica NCS cDNA candidate - Since it was rather difficult to assay NCS due to the background chemical reaction until the recent development of chiral-column chromatographic analysis, we tried to isolate NCS cDNA using an EST library prepared from high berberine-producing cultured *C. japonica* cells. In fact, our EST library of *C. japonica* 156-1 cells has an advantage that transcripts of berberine biosynthetic enzyme have been highly enriched (E. Dubouzet in preparation). When the sequences of our EST library were determined, we noticed that it contained many oxygenase-like proteins, which might be related to berberine biosynthesis. Additional RNA gel blot analysis showed that such oxygenase-like protein cDNA could be related to berberine biosynthesis, since the expression pattern of oxygenase-like protein was highly correlated with the expression of other biosynthetic genes in several *Coptis* cell lines with different berberine productivities (Supplemental Data). Since cDNAs for early steps in norcoclaurine biosynthesis from tyrosine have not yet been characterized, we examined the enzyme activity of this oxygenase-like protein.

To examine the enzyme activity of oxygenase-like protein, we produced recombinant protein in *E. coli*. Whereas no enzyme activity was detected for tyrosine, DOPA, or tyramine, this protein produced a product with dopamine and 4-HPAA. The reaction product was identified to be (*S*)-norcoclaurine by LC-MS, whereas the enzyme activity was considerably lower than that of native enzyme. The other recombinant protein (putative 2-nitropropane dioxygenase) and pET 41-a vector, as the control, did not show NCS activity or any other enzyme activity for the early reaction from tyrosine to norcoclaurine. Thus, this clone was named CjNCS1 in this manuscript. We determined the full-length sequence of CjNCS1 and found that this EST had 1172 nucleotides with an open reading frame that encoded 352 amino acids (Supplemental Data). The deduced amino acid sequence of CjNCS1 was similar to 2-oxoglutarate-dependent dioxygenases of plant origin (Fig. 3). CjNCS1 had no putative signal peptide and predicted to be cytosolic protein.

CjNCS1 was purified to electrophoretic homogeneity by ammonium sulfate fractionation, with DEAE Sepharose CL-4B and hydroxyapatite column chromatography. The molecular mass of the enzyme was determined to be 40 kDa by Superdex 200 HR 10/30 gel filtration (data not shown). SDS-PAGE of CjNCS1 gave a single band corresponding to a molecular mass of 40 kDa, indicating that the recombinant CjNCS1 was a monomer (data not shown).

During our characterization of CjNCS1, NCS was isolated from a *T. flavum* cell culture and the amino acid sequences predicted from its cDNA showed 28-38% identity with the Betv1 allergen and belonged to the PR10 protein family, whereas no homology with CjNCS1 was found (6). Thus, a gene homologous to *T. flavum* NCS (*C. japonica* PR10A) was isolated from a *C. japonica* EST library and compared to native NCS in *C. japonica* cells. Recombinant CjPR10A was produced in *E. coli*, and the protein was used as crude extract without further purification. Both CjNCS1 and CjPR10A catalyzed the condensation of dopamine and 4-HPAA, and produced (*S*)-norcoclaurine, although the elution time of the products differed between CjNCS1 and CjPR10A in LC-MS analysis (Fig. 4). Confirmation of the structure of the product of the NCS reaction with CjNCS1 or CjPR10A by LC-NMR also showed that the products of both CjNCS1 and CjPR10A were norcoclaurine (data not shown).

Characterization of C. japonica NCS based on the sequence information of CjNCS1 - To characterize which gene product corresponds to native NCS in *C. japonica*, native NCS extracted from cultured *C. japonica* cells was immuno-precipitated with antiserum against recombinant CjNCS1. The activity of native NCS after immunoprecipitation with anti-CjNCS1 antibodies was markedly reduced, while antibodies against Cj4'OMT, another biosynthetic enzyme in the berberine pathway, did not affect the NCS activity (Fig. 5A). Since a measurable amount of NCS activity remained even after immunoprecipitation with anti-CjNCS1, we examined whether CjPR10A might be involved in the NCS reaction in *C. japonica* cells as shown below.

Since the sequence of CjNCS1 suggested that ferrous ion may be required (Fig. 3),

we examined the effect of chelators on the NCS activity of native enzymes and both recombinant proteins. *o*-Phenanthroline, a chelator for ferrous ion, inhibited NCS activity in *C. japonica* cells and recombinant CjNCS1 (Fig. 5B), whereas EDTA did not (data not shown). *o*-Phenanthroline as well as EDTA did not inhibit the NCS activity of CjPR10A. This result again showed CjNCS1 was involved in native NCS in *C. japonica*. However, it was possible that CjPR10A was still involved in native NCS, since residual NCS activity was visible.

When we examined the substrate specificity of the NCS reaction, we found that the substrate specificity of CjPR10A was different from that of native NCS in *C. japonica* and CjNCS1. CjNCS1 and the crude extract from cultured *C. japonica* cells only accept dopamine and a phenylacetaldehyde group (phenylacetaldehyde, 3,4-DHPAA, and 4-HPAA), while CjPR10A used pyruvic acid and 4-hydroxyphenylpyruvate for the condensation with dopamine (data not shown). This result clearly indicated that the native NCS reaction in *C. japonica* involved CjNCS1, not CjPR10A.

The activity of recombinant CjNCS1 was lower than that of the native enzyme extracted from cultured *C. japonica* cells on a protein basis. Therefore, the compositions of recombinant CjNCS1 and native NCS were compared. The fractions by gel filtration for purified recombinant CjNCS1 and crude extract from *C. japonica* cells were analyzed by western blotting. As a result, NCS from *C. japonica* was a complex, while recombinant CjNCS1 was a monomer (data not shown).

2-Oxoglutarate was not needed in the NCS reaction, as expected from the sequence of CjNCS1. Isopenicillin N synthase (IPNS) has structural characteristics similar to those of CjNCS1: requirement for ferrous ion, but not 2-oxoglutarate (25). Since IPNS requires oxygen for its enzyme activity, we examined the requirement for oxygen in the NCS reaction. The depletion of dissolved oxygen in the reaction mixture by the glucose oxidase-catalase system (26) did not reduce NCS activity. Thus, we concluded that NCS of *C. japonica* was a novel 2-oxoglutarate-independent dioxygenase-like protein which catalyzed the cyclase reaction without oxygen and was different from IPNS.

Sequence comparison of CjNCS1 and other plant dioxygenases - The sequence of CjNCS1 was compared with those of other representative 2-oxoglutarate-dependent dioxygenases in plants to estimate the molecular basis of reaction (Fig. 3). The sequence of CjNCS1 showed a typical 2-oxoglutarate-dependent dioxygenase-like protein sequence as in other plant genes. CjNCS1 had two completely conserved histidine and aspartate residues (His228 and Asp230) which would act as ferrous ligands, as reported for other 2-oxoglutarate-dependent dioxygenases (27), and the motif His X Asp (53-57 amino acids) X His (28)(indicated by asterisks). However, CjNCS1 did not have the highly conserved consensus sequence for binding of the co-substrate, 2-oxoglutarate,
Asn-Tyr-Tyr-Pro-Pro-Cys-Pro-Gln-Pro (Fig. 3). This corresponded to the result that 2-oxoglutarate was not required in the reaction of NCS. IPNS also does not have this sequence and does not require 2-oxoglutarate (25).

A homology search revealed that *A. thaliana* has AtSRG1, which is highly homologous to CjNCS1 (Fig. 6). AtSRG1 is a 2-oxoglutarate-dependent dioxygenase-like protein but has a conserved 2-oxoglutarate-binding domain. Since AtSRG1 retained a binding domain for 2-oxoglutarate, we examined the NCS activity of AtSRG1 expressed in *E. coli*. Whereas recombinant AtSRG1 was successfully expressed in *E. coli* in soluble form, crude extract did not show any NCS activity with dopamine and 4-HPAA (data not shown).

DISCUSSION

Norcoclaurine formation is the key reaction in benzylisoquinoline alkaloid biosynthesis. NCS activity has been detected in most plant species that have been shown to produce benzylisoquinoline alkaloids (29). However, it has been difficult to characterize NCS due to the background activity of a chemical reaction. While a gene in the PR10 family has been isolated from *T. flavum* and opium poppy (6,29) during our characterization of CjNCS1, their physiological role was not yet been identified.

Although NCS produce (*S*)-norcoclaurine stereo-specifically, it has not been investigated empirically. In this study, we established a LC-MS system with chiral column to determine that NCS from *C. japonica* produced (*S*)-norcoclaurine stereo-specifically (Fig. 2). Since 6OMT and CNMT in late steps of benzylisoquinoline alkaloid biosynthesis were not stereo-specific (7,8,30), NCS plays an important role to produce optical isomers in the biosynthesis. While we also found that two gene products (CjNCS1 and CjPR10A) isolated from *C. japonica* cells could catalyze the stereo-specific condensation of dopamine and 4-HPAA, further studies showed that these enzymes had different enzymological properties and substrate specificities.

CjNCS1 had a dioxygenase-family domain and a ferrous ion-binding site, but CjPR10A did not. Furthermore, while CjPR10A accepted dopamine and 4-hydroxyphenylpyruvate as substrates for norcoclaurine formation, native NCS and CjNCS1 did not. Native NCS enzymes from *Eschscholzia californica*, *Nandina domestica*, and *Corydalis pallida* var. tenuis cells also did not accept phenylpyruvate as a substrate as did *Coptis* enzyme (unpublished data). Whereas the formation of norcoclaurine by the condensation of dopamine and 4-HPAA is now clearly established as the central pathway in isoquinoline alkaloid biosynthesis (4,24), the formation of norcoclaurine via the condensation of dopamine and 4-HPAA or via the condensation of dopamine and 4-hydroxyphenylpyruvate was also intensively investigated. The formation of norlaudanosoline through norlaudanosoline 1-carboxylic acid by the condensation of dopamine and 3,4-dihydroxyphenylpyruvate was once seriously considered to be a possible pathway using in vitro and in vivo tracer experiments (23,31). The formation of norcoclaurine from dopamine and 4-hydroxyphenylpyruvate by recombinant CjPR10A suggested that a PR10-like protein might catalyze such an artificial reaction. Our current result provides the biochemical basis for solving the long-standing mystery of artificial activity through norlaudanosoline 1-carboxylic acid in isoquinoline alkaloid biosynthesis.

CjPR10A and NCS from *T. flavum* had the signal peptide at N-terminal (6) and predicted to be localized in a vesicular compartment. On the other hand, major biosynthetic enzymes such as tyrosine/DOPA decarboxylase, 6OMT, CNMT, and 4'OMT in the early steps in the isoquinoline alkaloid biosynthesis are cytosolic (32). While dopamine synthesized in cytosol from L-DOPA by DOPA decarboxylase would be transported and accumulated within a vacuole at the concentration of 1 mg/ml in the latex of *P. somniferum* and *P. bracteatum* (33,34), dopamine produced in cytosol would be more efficient substrate for CjNCS1 and the following biosynthetic reactions. Immunoprecipitation with antiserum against CjNCS1 clearly indicated that native NCS in *C. japonica* cells consisted of CjNCS1 and not CjPR10A. Our preliminary characterization of the function of CjNCS1 using the transient RNAi in *C. japonica* protoplasts (35) showed that accumulation of berberine clearly decreased with double stranded RNA of CjNCS1, supporting the involvement of CjNCS1 in berberine biosynthesis (Fukusaki et al., data not shown).

Although recombinant CjNCS1 expressed in *E. coli* showed NCS activity, this activity was lower than that of native enzyme on a protein basis as estimated by immunoblot analysis. This low activity might be due to the instability of NCS, as reported in crude extracts of *Papaver somniferum* and *E. californica* (21). Extreme instability has also been reported for 1-aminocyclopropane-1-carboxylate oxidases (ACC) and anthocyanidine synthase, which are 2-oxoglutarate-dependent oxygenases (36,37). Partially distorted folding or modification in *E. coli* also might decrease the activity. Our chromatographic analysis suggested that the native NCS extracted from cultured *C. japonica* cells would form a complex, whereas recombinant CjNCS1 was a monomer. Thus, the low activity of recombinant CjNCS1 might be caused by the difference in complex formation. Recently, Kristensen *et al.* reported that the coordinated expression of two cytochrome P450 genes with a glucosyltransferase from Sorghum enhanced the production of dhurrin in the heterologous host plant *Arabidopsis* through metabolon formation (38). RNAi of codeninone reductase was also suggested to interrupt the metabolic channel from

reticuline to codeine in *P. somniferum* (39). While the NCS reaction in isoquinoline alkaloid biosynthesis may also form a similar complex to produce norcoclaurine from tyrosine, we need a more detailed characterization of the enzyme complex in isoquinoline alkaloid biosynthesis.

Whereas we determined that CjNCS1 and CjPR10A produced (*S*)-norcoclaurine in LC-MS analysis, the different elution time of the products may suggest the different mechanism of NCS reaction between CjNCS1 and CjPR10A. The sequence similarity of CjNCS1 with the plant dioxygenase family would provide the insight of reaction mechanism of NCS, since genes in the dioxygenase family have been reported to be involved in divergent biosynthetic pathways as hydroxylase, hydroxylase/epoxidase, cyclase, desaturase, and ring expandase (40,41). One unique feature of CjNCS1 was that CjNCS1 lacks the 2-oxoglutarate-binding sequence, while it is highly homologous to plant 2-oxoglutarate-dependent dioxygenase. While the significance of this lack of a 2-oxoglutarate domain should be clarified in future studies, we expect that this modification would be an important step in the development of NCS activity. The alignment between NCS and homologous genes indicated that CjNCS1 was similar to IPNS, another cyclase in the dioxygenase family. IPNS has a degree of homology with deacetoxycephalosporin C synthetase and deacetylcephalosporin C synthase, two 2-oxoglutarate-dependent dioxygenases involved in the later steps of cephalosporin biosynthesis (28). Interestingly, IPNS also has no 2-oxoglutarate-binding region. Crystallographic characterization of 3D structure of CjNCS1 would provide important cues for the understanding of molecular mechanism of NCS reaction.

The molecular evolution of new enzymological activity has been discussed for 4,5-extradiol dioxygenase in betalain biosynthesis (42). Alignment of this gene family revealed a conserved motif that is present in all organisms except plants that synthesize betalain. In the alignment of CjNCS1 and other plant 2-oxoglutarate-dependent dioxygenases, the N-terminal domains are poorly conserved. The poorly conserved N-terminal domain may also represent binding sites for specific alkaloid substrates. 2-Oxoglutarate-dependent dioxygenases have also been reported to be involved in alkaloid biosynthesis in plants. While there have been previous reports on hyoscyamine 6β-hydroxylase (H6H) in scopolamine biosynthesis (43) and desacetoxyvindoline 4-hydroxylase (D4H) in vindoline biosynthesis (44), this is the first report of the cloning of cyclase-type dioxygenase-like protein in alkaloid biosynthesis in plants. We expect that more dioxygenase-like proteins may play a role in alkaloid biosynthesis.

It has been suggested that an early pathway in isoquinoline alkaloid biosynthesis is universal in the plant kingdom. During evolution, the catalytic properties of the key enzymes were created by amino-acid substitution, and such the enzymes became part of a biosynthetic pathway. In *C. japonica*, the universal pathway involving NCS plays a role in berberine biosynthesis. Recently Liscombe *et al.* reported the monophyletic origin of benzylisoquinoline alkaloid biosynthesis prior to the emergence of the eudicots (29). The molecular characterization of NCS as key entry gene in comparison with cytochrome P450, such as CYP80 and CYP719, which are specific for isoquinoline alkaloid biosynthesis, would be useful for better understanding the evolutionary pathway of this unique secondary metabolism.

It is interesting that *A. thaliana* and rice (e.g., AK072706) have a CjNCS1-like protein, although we could not characterize the rice CjNCS-like protein in detail. As reported above, *Arabidopsis* SRG1 protein had a 2-oxoglutarate-binding domain and no NCS activity. SRG1 expression in *Arabidopsis* has been reported to be regulated by ethylene-induced-senescence. While the true function of SRG1 has not yet been clarified, it might be involved in the detoxification of amines or aldehydes as senescence proceeds, since both amine and aldehyde are highly toxic to physiological functions.

While the origin of alkaloid biosynthesis is not clear, the key entry enzyme genes are gradually being isolated. For example, strictosidine synthase couples tryptamine and

secologanin as the first committed step in monoterpenoid indole alkaloid biosynthesis (2). Another example is homospermidine synthase (HSS), which catalyzes the first specific step in pyrrolizidine alkaloids from putrescine and spermidine (45,46). HSS has been shown to be recruited from deoxyhypusine synthase by independent gene duplication in several different angiosperm lineages during evolution (47). The amino acid sequences of both genes are considerably different from that of NCS. CjNCS1 could offer a new perspective for studies on the diversity and evolution of alkaloid biosynthesis.

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FOOTNOTES

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[1]Abbreviations: NCS, norcoclaurine synthase; 4-HPAA, 4-hydroxyphenylacetaldehyde; PR10, pathogenesis-related 10; 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; 4'OMT, 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase; LC-MS, liquid chromatography-mass spectroscopy; 3,4-DHPAA, 3,4-dihydroxyphenylacetaldehyde; IPNS, isopenicillin N synthase; ACC, 1-aminocyclopropane-1-carboxylate oxidases.

FIGURE LEGENDS

Fig. 1. Coupling of dopamine and the phenylacetaldehyde group. (*S*)-Norcoclaurine is produced by the condensation of dopamine and 4-HPAA by NCS.

Fig. 2. Analysis of the optical isomers of norcoclaurine. In the analysis of optical isomers, a SUMICHIRAL CBH column (4.0 × 100 mm; Sumika Chemical Analysis Service) was used. The solvent system was acetonitrile/0.3% acetic acid (5:95), pH 5.2; flow rate, 0.4 ml/min at 25°C. Norcoclaurine was converted to coclaurine by recombinant 6OMT of *C. japonica* (7), and was compared to a (*S*)-coclaurine standard.

Fig. 3. Alignment of CjNCS1 and 2-oxoglutarate-dependent dioxygenases of plant species. The dioxygenase amino acid sequences were obtained from GenBank™. Solid highlighting with white characters indicates identical amino acid residues in all proteins. Gray highlighting with white characters indicates similarities. EFE, tomato ethylene-forming enzyme (GenBank/EMBL Database accession number X58885); ACCO, tomato 1-aminocyclopropane-1-carboxylate oxidase (P05116); AtSRG1, senescence-regulated gene 1 from *A. thaliana* (S44261); AOH, apple tree anthocyanidin synthase (X71360); FLS, petunia flavonol synthase (Z22543); G_{20}OX, gibberellin 20-oxidase from *A. thaliana* (X83379); D4H, desacetoxyvindoline 4-hydroxylase from *C. roseus* (AF008597); H6H, hyoscyamine 6β-hydroxylase from *H. niger* (M62719); IPNS, isopenicillin *N*-synthase from *A. nidulans* (M18111).

Fig. 4. Comparison of the products of CjNCS1 and CjPR10A. A SUMICHIRAL CBH column was used. The solvent system was 10% 2-propanol; flow rate, 0.4 ml/min at 25°C.

Fig. 5. (A) Immunoprecipitation for *C. japonica* NCS. NCS activities remaining in supernatants after immunoprecipitation with anti-CjNCS1 antibodies (○; the crude extract from cultured *C. japonica* cells, □; 50 mM Tris-HCl (pH 7.4) buffer) or with anti-4'OMT antibodies (◇) were assayed. Data represent means of two experiments. (B) The effect of chelator on NCS activity. *o*-Phenanthroline was used to examine the effect of a chelator on NCS activity. Error bars represent the standard deviation of three independent measurements.

Fig. 6. Phylogenetic tree of CjNCS1 and 2-oxoglutarate-dependent dioxygenases of plant species. The length of the horizontal lines indicates the evolutionary distance between neighbors. The abbreviations are the same as in the legend to Figure 3.

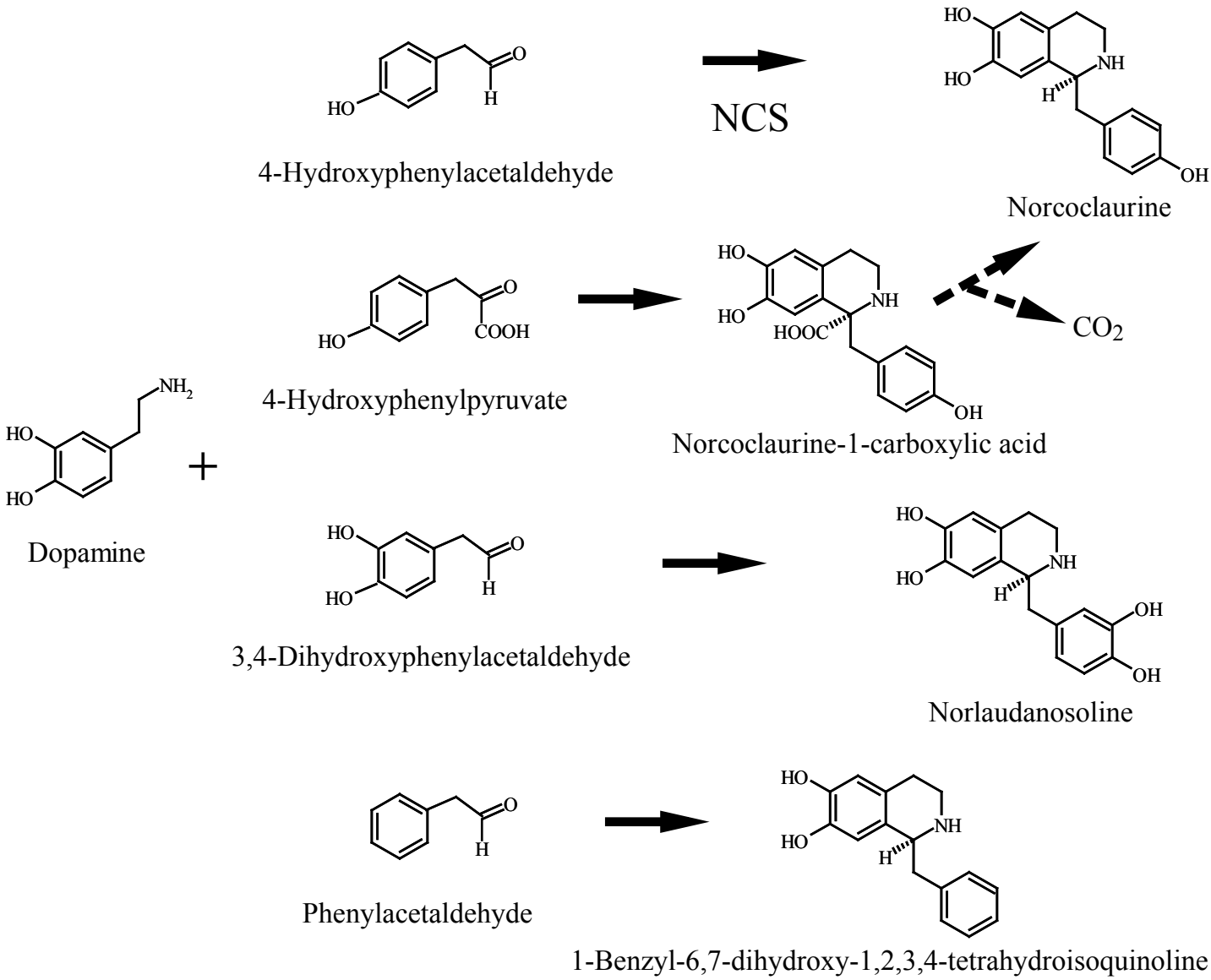


Figure 1

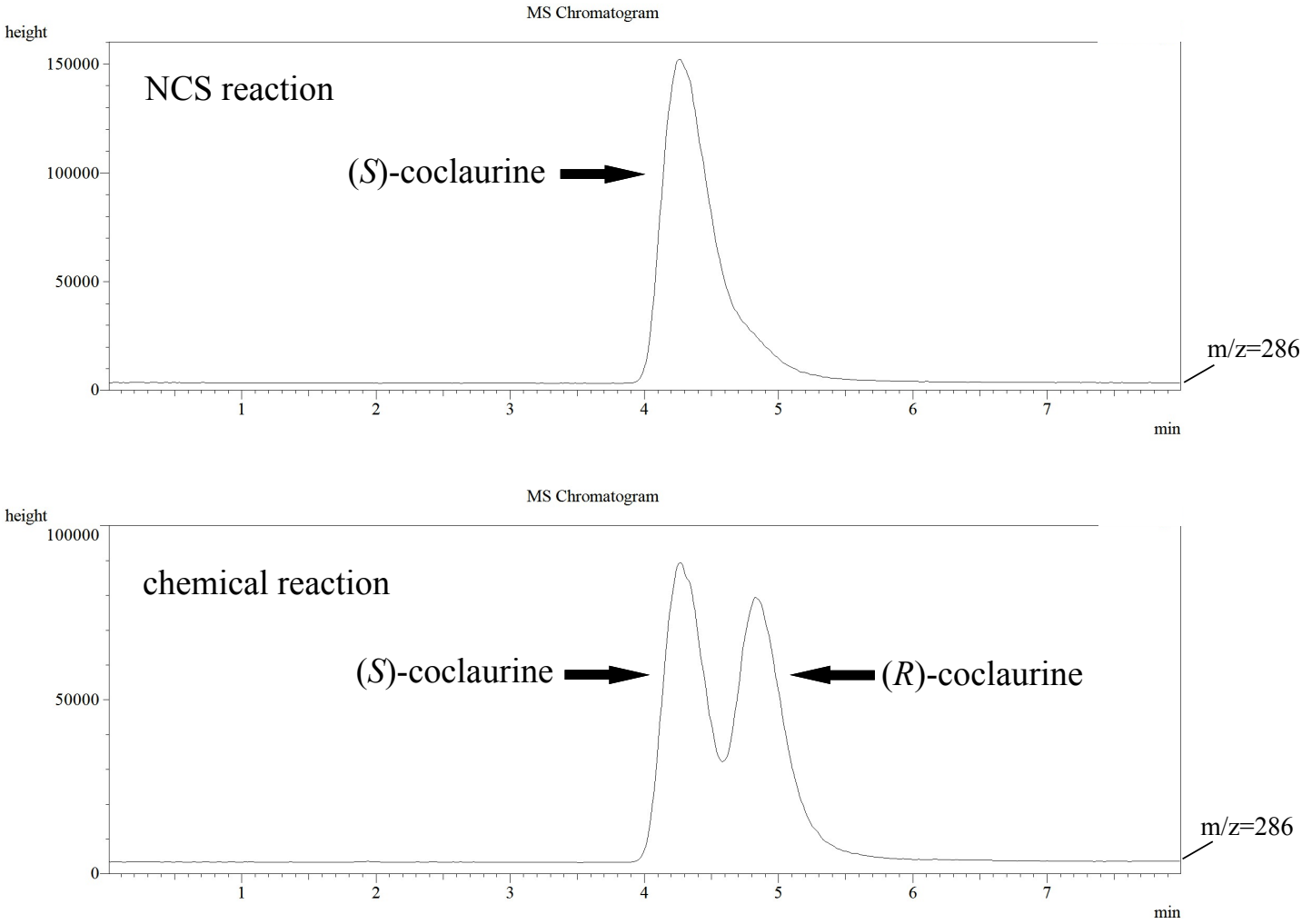


Figure 2

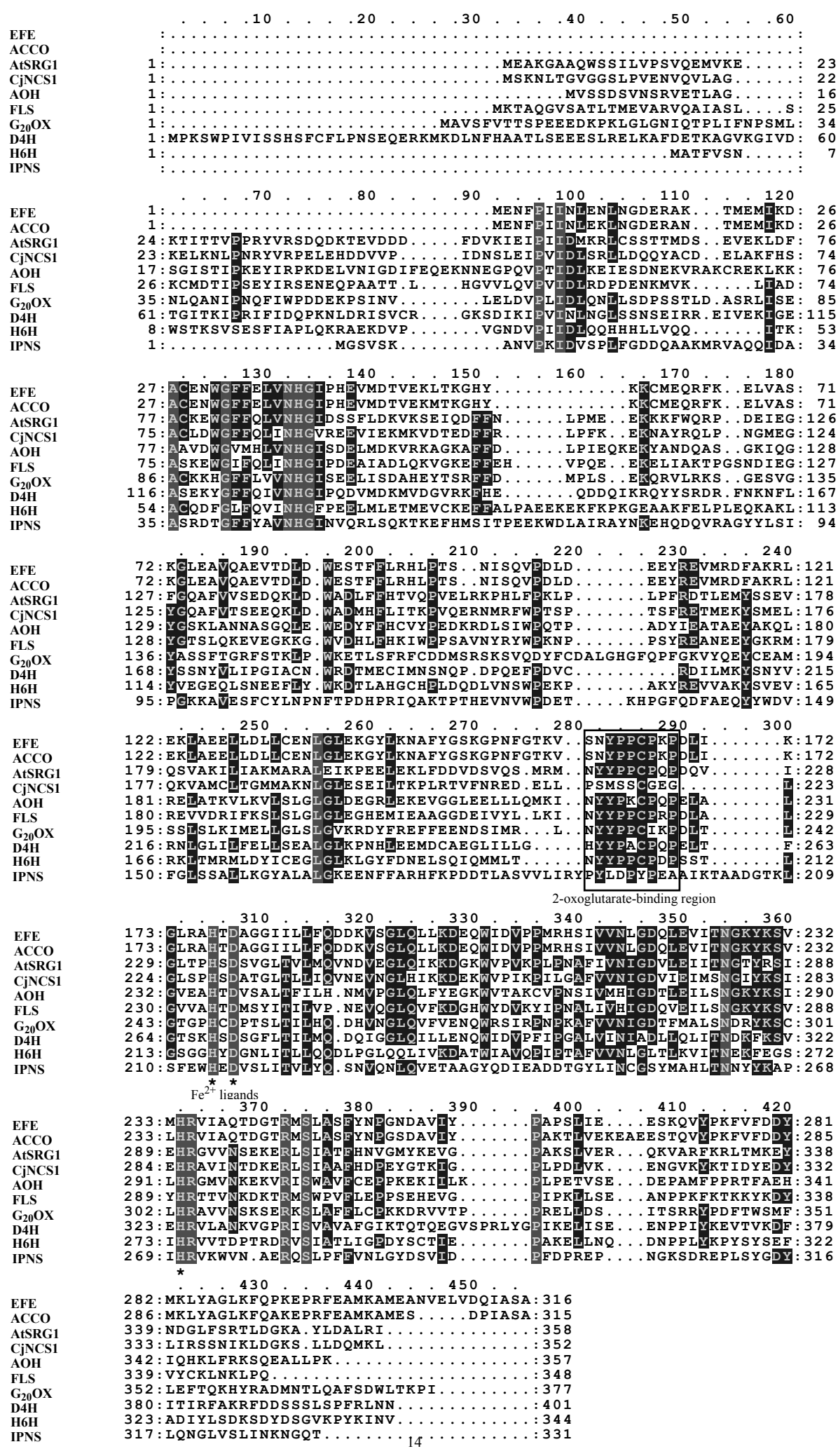


Figure 3

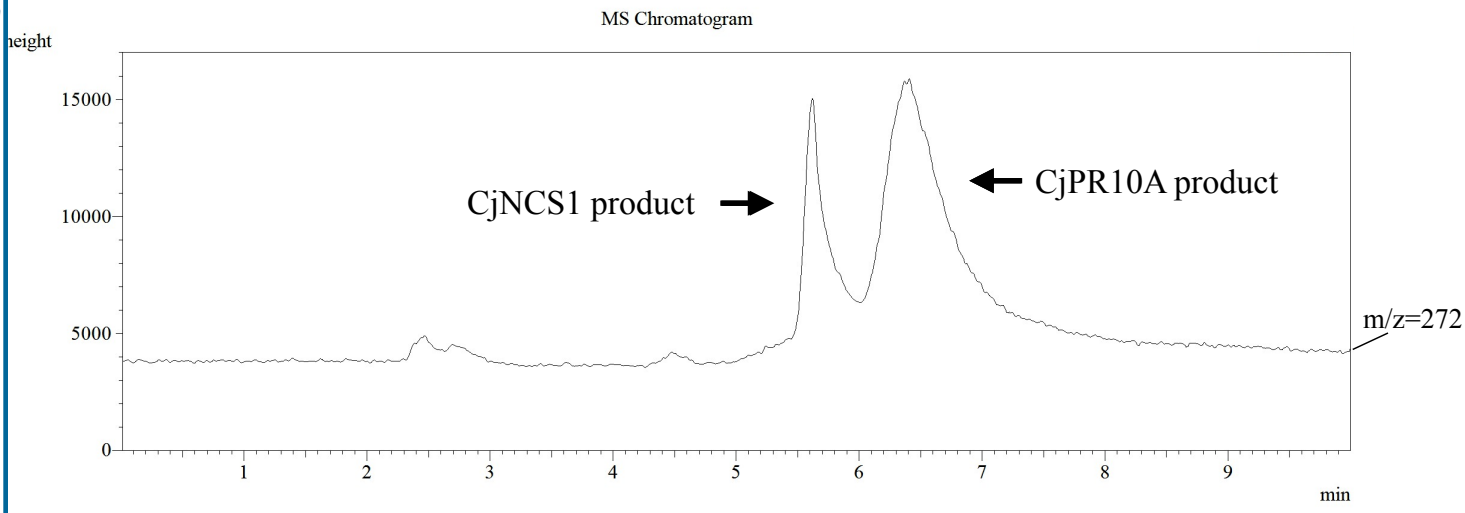
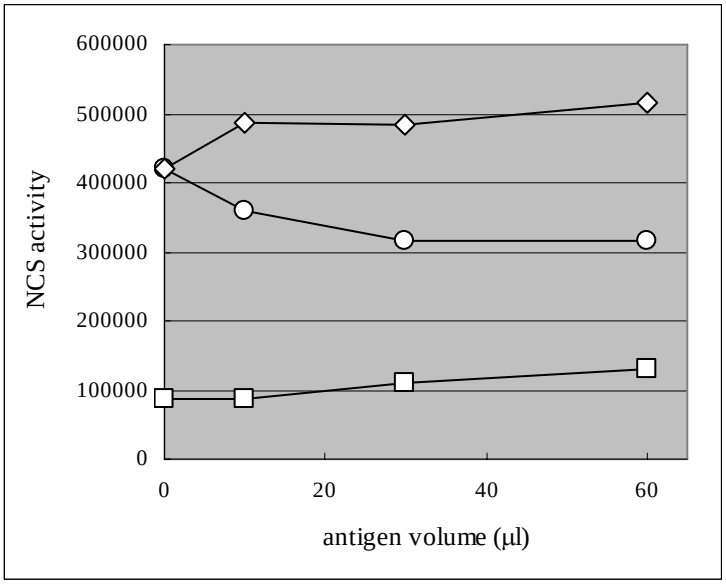


Figure 4

(A)



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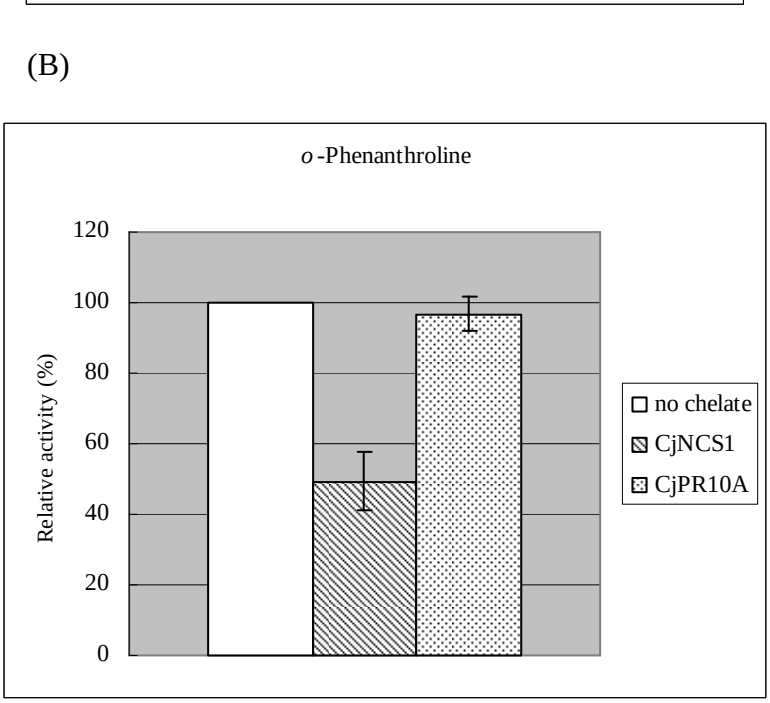

Figure 5

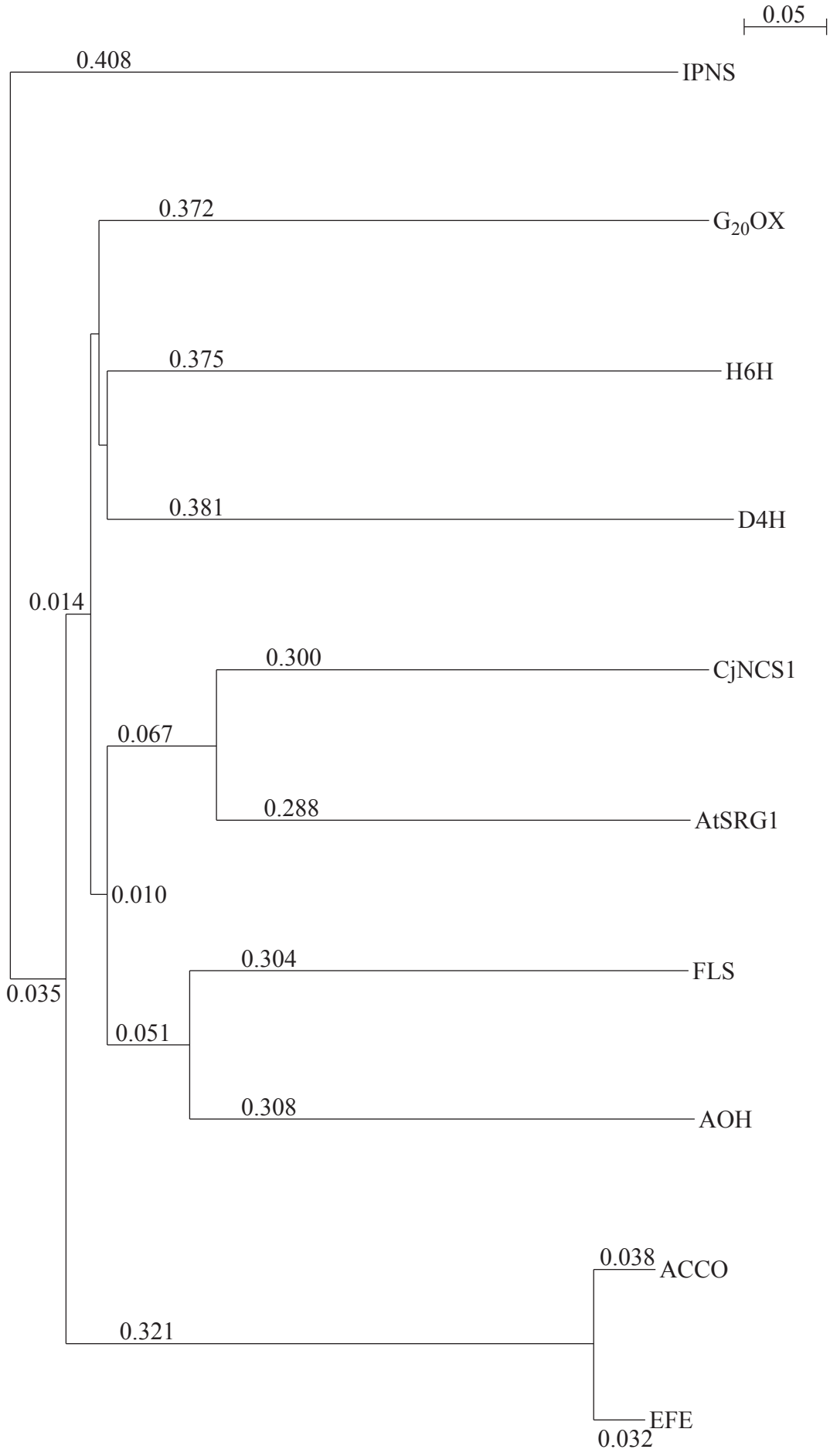

Figure 6