Functional Analysis of Norcoclaurine Synthase in Coptis japonica

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(S)-Norcoclaurine is the entry compound in benzylisoquinoline alkaloid biosynthesis and is produced by the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) by norcoclaurine synthase (NCS) (EC 4.2.1.78). Although cDNA of the pathogenesis-related (PR) 10 family, the translation product of which 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 iso-

Higher plants produce divergent chemicals such as alkaloids, terpenoids, and phenolic compounds in secondary metabolism. Among these chemicals, alkaloids are very important in medi-
cine because of their high biological activities. Alkaloids are low molecular weight, nitrogen-containing compounds that are found in ~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. Although 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 secorogina, 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 ~2,500 defined structures. Nor-
coclaurine produced from tyrosine is a key entry compound from which various benzylisoquinoline alkaloids such as anal-
gesic morphine, colchicines, antibacterial berberine, palmatine, and sanguinarine are produced through a multistep process. (S)-Norcoclaurine is produced by norcoclaurine synthase (NCS)3 (EC 4.2.1.78), which condenses dopamine and 4-hy-
droxyphenylacetaldehyde (4-HPAA) (Fig. 1) (3, 4). Because 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 pro-
ducing large amounts of benzylisoquinoline alkaloid. Using this system, many enzymes in berberine biosynthesis, including norcoclaurine 6-0-methyltransferase, cocaurine N-methyltransferase, and N-methylcoclaurine 3'-hydroxylase, have been iso-
lated and characterized (7–9). In this report, we describe the characterization of NCS in C. japonica cells. Because selected cultured C. japonica 156-1 cells expressed the genes for enzymes in berberine biosynthesis in greater amounts than
non-selected cells\(^4\) (9–11), we screened expressed sequence tag (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 Escherichia 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 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

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors for Recombinant CjNCS1 and CjPR10A**—Expression vectors were constructed for full-length cDNAs of CjNCS1, CjPR10A, and AtSRG1 were introduced into E. coli cells containing each plasmid were grown at 37 °C in Luria Bertani medium. After induction with 1 mM isopropylthiogalactoside, 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 Luria Bertani medium with the extraction Tris buffer (0.1 mM Tris-HCl, pH 8.0, containing 10% glycerol, 5 mM EDTA, and 5 mM 2-mercaptoethanol) and then purified to homogeneity. Crude extract was centrifuged at 10,000 \( \times g \) for 10 min, and the supernatant was subjected to fractionation with (NH\(_4\))\(_2\)SO\(_4\). The desired recombinant protein was precipitated between 30 and 60% (NH\(_4\))\(_2\)SO\(_4\) saturation and made soluble in the extraction buffer again. The protein solution was dialyzed in the same buffer and applied to a DEAE-Sepharose CL-4B column (Amersham Biosciences) (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 mM NaCl solution. The recombinant protein fractions were detected with SDS-PAGE, and these fractions were pooled and then applied to a hydroxyapatite column (Amersham Biosciences) (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 mM) of KPB solution (100 ml). The active fractions were pooled and then dialyzed 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

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4 E. Dubouzet, J. G. Dubouzet, K. Yazaki, and F. Sato, unpublished data.
conversion of tyramine with monoamine oxidase. 4-HPAA was prepared from the deamination of tyramine by purified tyramine oxidase (1 unit/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 h, 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. 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. Because we did not have an (S)-norcoclaurine standard, norcoclaurine was converted to coclaurine by recombinant 6-O-methyltransferase from *C. japonica* (7) and compared with an (S)-coclaurine standard.

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

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 NH₄OAc (0.05% trifluoroacetic acid, D₂O)/CH₃CN (0.05% trifluoroacetic acid) (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 stereo-specific 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 stereo-specific, 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, because (S)-norlaudanosoline, which is produced by the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde, was once thought to be a common pathway intermediate in benzylisoquinoline alkaloid biosynthesis (22, 23), whereas (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-dihydroxyphenylacetaldehyde, 4-HPAA, benzaldehyde, and pyruvic acid) were reacted with dopamine as an amine substrate, phenylacetaldehyde, 3,4-dihydroxyphenylacetaldehyde, 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**—Because 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 the advantage that transcripts of berberine biosynthetic enzyme have been highly enriched. When the sequences of our EST library were determined, we noticed that it contained many oxygenase-like proteins that might be related to berberine biosynthesis. Additional RNA gel blot analysis showed that such oxygenase-like protein cDNA could be related to berberine biosynthesis, because 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). Because 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*. Although no enzyme activity was detected for tyrosine, 3,4-dihydrophenylalanine (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, although 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 report. 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 was predicted to be cytosolic protein.
**FIGURE 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 Data base 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); G20OX, 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).
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 with 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 immunoprecipitated with antiserum against recombinant CjNCS1. The activity of native NCS after immunoprecipitation with anti-CjNCS1 antibodies was markedly reduced, whereas antibodies against Cj3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase, another biosynthetic enzyme in the berberine pathway, did not affect the NCS activity (Fig. 5A). Because 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.

Because 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-Phenanthro-
line 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, because 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-dihydroxyphenylacetaldehyde, and 4-HPAA), whereas 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, whereas 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 has structural characteristics similar to those of CjNCS1: requirement for ferrous ion, but not 2-oxoglutarate (25). Because isopenicillin N synthase 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 that catalyzed the cyclase reaction without oxygen and was different from isopenicillin N synthase.

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 (His-228 and Asp-230) that 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. Isopenicillin N synthase 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. Because AtSRG1 retained a binding domain for 2-oxoglutarate, we examined the NCS activity of AtSRG1 expressed in *E. coli*. Although 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).

**FIGURE 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 Fig. 3.

**DISCUSSION**

Norcoclaureine 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. Although 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 stereospecifically, it has not been investigated empirically. In this study, we established an LC-MS system with chiral column to determine that NCS from *C. japonica* produced (S)-norcoclaurine stereospecifically (Fig. 2). Because 6-O-methyltransferase and coclaurine N-methyltransferase in late steps of benzyliso-
quinoiline alkaloid biosynthesis are not stereo-specific (7, 8, 30). NCS plays an important role in producing optical isomers in the biosynthesis. Although 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, whereas 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.5 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 norlaudanosine 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 N-terminal (6) and were predicted to be localized in a vesicular compartment. On the other hand, major biosynthetic enzymes such as tyrosine/DOPA decarboxylase, 6-O-methyltransferase, coclaurine N-methyltransferase, and 3’-hydroxy-N-methylcoclaurine 4’-O-methyltransferase in the early steps in the isoquinoline alkaloid biosynthesis are cytosolic (32). Although 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 Papaver somniferum and Papaver 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 RNA interference 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.

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 P. somniferum and E. californica (21). Extreme instability has also been reported for 1-aminocyclop propane-1-carboxylate oxidases and anthocyanidine synthase, which are 2-oxoglutamate-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. (38) 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. RNA interference of codeninone reductase was also suggested to interrupt the metabolic channel from reticuline to codeine in P. somniferum (39). Although 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.

Although 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 insight into the reaction mechanism of NCS, because 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 is that CjNCS1 lacks the 2-oxoglutamate-binding sequence, whereas it is highly homologous to plant 2-oxogluturate-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 isopenicillin N synthase, another cyclase in the dioxygenase family. Isopenicillin N synthase 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, isopenicillin N synthase also has no 2-oxoglutarate binding region. Crystallographic characterization of the three-dimensional structure of CjNCS1 would provide important cues for the understanding of the 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. Although there have been previous reports on hyoscyamine.

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5 H. Minami and F. Sato, unpublished data.
6 E. Fukusaki, C-I. An, A. Kobayashi, and F. Sato, data not shown.
Functional Analysis of NCS in C. japonica

6β-hydroxylation in scopolamine biosynthesis (43) and desacetoxylvindoline 4-hydroxylase 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 isooquinoline alkaloid biosynthesis is universal in the plant kingdom. During evolution, the catalytic properties of the key enzymes were created by amino acid substitution, and as 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. (29) reported the monophyletic origin of benzylisoquinoline alkaloid biosynthesis prior to the emergence of the eudicots. The molecular characterization of NCS as key entry gene in comparison with cytochrome P450, such as SRG1 expression in Arabidopsis NCS activity. SRG1 protein in detail. As reported above, a CjNCS1-like protein, although we could not characterize the function of NCS. CjNCS1 could offer a new perspective for studies on the diversity and evolution of alkaloid biosynthesis.

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