

Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom

Sylvain Jeandroz,^{1,2} Daniel Wipf,^{2,3} Dennis J. Stuehr,⁴ Lorenzo Lamattina,⁵ Michael Melkonian,⁶ Zhijian Tian,⁷ Ying Zhu,⁷ Eric J. Carpenter,⁸ Gane Ka-Shu Wong,^{7,8,9} David Wendehenne^{2,3*}

Nitric oxide (NO) signaling regulates various physiological processes in both animals and plants. In animals, NO synthesis is mainly catalyzed by NO synthase (NOS) enzymes. Although NOS-like activities that are sensitive to mammalian NOS inhibitors have been detected in plant extracts, few bona fide plant NOS enzymes have been identified. We searched the data set produced by the 1000 Plants (1KP) international consortium for the presence of transcripts encoding NOS-like proteins in over 1000 species of land plants and algae. We also searched for genes encoding NOS-like enzymes in 24 publicly available algal genomes. We identified no typical NOS sequences in 1087 sequenced transcriptomes of land plants. In contrast, we identified NOS-like sequences in 15 of the 265 algal species analyzed. Even if the presence of NOS enzymes assembled from multipolypeptides in plants cannot be conclusively discarded, the emerging data suggest that, instead of generating NO with evolutionarily conserved NOS enzymes, land plants have evolved finely regulated nitrate assimilation and reduction processes to synthesize NO through a mechanism different than that in animals.

Introduction

Nitric oxide (NO) is a ubiquitous intra- and intercellular messenger that functions in many physiological processes in most, if not all, kingdoms of life. In animals, NO is produced by a family of enzymes called NO synthases (NOSes), which exist in three major isoforms plus some splicing variants. In humans, the neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) isoforms are produced constitutively, and their activity is strictly dependent on the intracellular Ca^{2+} concentration, whereas the inducible NOS (iNOS or NOS2) isoform is Ca^{2+} -independent (1). NOSes are active as homodimers and produce NO and L-citrulline from L-arginine (L-Arg) through two successive steps of oxidation in the presence of O_2 and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (2). NOSes have an N-terminal oxygenase (NOSoxy) domain and a C-terminal reductase (NOSred) domain that are connected by a calmodulin (CaM)-binding motif (1, 3). Two conserved cysteine (Cys) residues from the NOSoxy domain of each monomer form a zinc coordination site that facilitates NOS dimerization (4). The NOSoxy domain binds a heme prosthetic group and the redox factor tetrahydrobiopterin (H_4B) (4, 5). The NOSred domain displays similarities with NADPH-cytochrome P450 reductase and transfers NADPH-derived electrons to the heme through flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) during catalysis (5–8). In addition, human NOS1 has an N-terminal PDZ domain that mediates protein-protein interactions (9). Recently, Campbell *et al.* (10)

determined structures of the three mammalian NOS isoforms. This study revealed that all isoforms have a similar overall structure and range of conformations. More precisely, the NOSoxy domains mediate homodimerization, and each NOSoxy domain contacts CaM. Each NOSoxy domain of the homodimer is flanked by two separate and flexible NOSred domains of varying conformations. This structural analysis also showed that only a single NOSred at a time contributes to electron transfer. Regarding the evolution of this multidomain protein, it has been proposed that NOSoxy was acquired by horizontal gene transfer from bacteria and that NOSred may have evolved from a bacterial sulfite reductase acquired from the proto-mitochondrial endosymbiont (11).

During the past 15 years, NO has been recognized as a key player in major plant physiological processes, including development and adaptive responses to biotic and abiotic stresses (12). NO is produced by nitrate reductase (NR), from hydroxylamines and polyamines, and through non-enzymatic routes (13–17). NOS-like activities sensitive to mammalian NOS inhibitors have been detected in plant extracts (18). For example, an L-Arg-dependent NOS activity requiring the same cofactors as animal NOS activity was measured in pea leaf peroxisomes (19, 20). Western blot analysis of the peroxisomal fractions using a polyclonal antibody that recognizes murine iNOS revealed an immunoreactive polypeptide of about 130 kD. Furthermore, putative NOS-like enzymes were identified in *Nicotiana tabacum* and *Arabidopsis thaliana*, but the ability of these enzymes to catalyze NOS activities has been questioned (21, 22). More generally, no gene or protein with sequence similarity to animal or bacterial NOSes has been reported in *A. thaliana* or in the higher plant genomes sequenced to date. Therefore, the hypothesis that plants have NOSes is a subject of intense discussion (23, 24). A gene encoding one functional NOS that resembles human NOSes has recently been discovered in the green alga *Ostreococcus tauri* (Mamiellophyceae, Chlorophyta), an early-diverging class within the green plant lineage (25). More recently, Kumar *et al.* (26) identified from public data banks NOS-like sequences from two additional members of this class, *Bathycoccus prasinos* and *Ostreococcus lucimarinus*.

Here, we used data from the 1000 Plants (1KP) international multidisciplinary consortium's transcriptome database (www.onekp.com/) and

¹AgroSup Dijon, UMR 1347 Agroécologie, BP 86510, 21000 Dijon, France. ²Equipe de Recherche Labellisée CNRS 6300, BP 86510, 21000 Dijon, France. ³Université Bourgogne Franche-Comté, UMR 1347 Agroécologie, BP 86510, 21000 Dijon, France. ⁴Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA. ⁵Instituto de Investigaciones Biológicas, UNMdP-CONICET, CC 1245, 7600 Mar del Plata, Argentina. ⁶Botanical Institute, Department of Biological Sciences, Universität zu Köln, Köln D-50674, Germany. ⁷BGI-Shenzhen, Beishan Industrial Zone, Yantian District, Shenzhen 518083, China. ⁸Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada. ⁹Department of Medicine, University of Alberta, Edmonton, Alberta T6G 2E1, Canada.

*Corresponding author: E-mail: wendehen@dijon.inra.fr

publicly available algal genome sequences to investigate the presence of NOS-like enzymes in over 1000 species of land plants and algae. This work addresses whether the presence of NOS in the three species of Mamiellophyceae is common among the algae or an exception, and whether NOS sequences are present in embryophyte land plants, including in the poorly studied genomes and transcriptomes of seedless plants.

In Silico Search for NOS Homologs in Plants

On the basis of *O. tauri* NOS and human NOS1, 26 sequences from 20 different species putatively homologous to NOS were identified in silico from the 1KP project database, which includes transcriptomes from 1328 species (Table 1 and Fig. 1). These candidates are distributed in seven different classes of green algae and in the transcriptomes of other photosynthetic organisms. No NOS homologs were found in the 1087 sequenced transcriptomes of land plants. We only identified in land plants shorter sequences that could not be classified as NOS because of their limited sequence similarity with *O. tauri* NOS and human NOS1 and the absence of structural elements characteristic of NOS complexes. Furthermore, we did not find any NOS sequences in the available genomes of plants in which NOS-like activities have been measured, including *A. thaliana*, *N. tabacum*, and *Pisum sativum* (www.arabidopsis.org, https://solgenomics.net, and www.coolseasonfoodlegume.org).

Animal NOSes have a bidomain structure containing NOSoxy and NOSred domains. Nevertheless, we cannot exclude the possibility that NOS activities measured in land plants could be catalyzed by a multimeric complex composed of independent NOSoxy and NOSred partners. To test

this hypothesis, we performed separate sequence similarity searches using the *O. tauri* NOSoxy (residues 1 to 557) and NOSred (residues 558 to 1081) domains. With NOSoxy as the query for searching the land plants transcriptome database, no results were returned. Using NOSred as the query, we found sequences showing a low degree of similarity with a minimum expect value (*E* value) of 3×10^{-30} . However, according to nucleotide and protein sequence comparisons using BLAST (Basic Local Alignment Search Tool) and InterPro (protein sequence analysis and classification, www.ebi.ac.uk/interpro/) analyses, these sequences most likely belong to the cytochrome P450 reductase family. Thus, these data suggest that land plants do not have an enzyme homologous or structurally related to animal NOSes. Further supporting this conclusion and in agreement with our results, Butt *et al.* (27), using a proteomic approach, previously showed that antibodies that recognize the NOSred domain of mammalian NOSes did not recognize NOS-like proteins in extracts from *Zea mays* embryos. If land plants have no NOSes, then what accounts for the NOS-like activities detected in land plants? As previously discussed by Corpas *et al.* (18), we cannot exclude the possibility that one or several proteins structurally unrelated to NOSes may account for the observed NOS-like activities in land plants. Identification of these proteins remains a major challenge.

Structure of Algal NOSes

Closer sequence analysis indicated that 13 algal sequences contain both the NOSoxy and NOSred domains typical of mammalian NOSes, connected by a sequence showing similarities to the CaM-binding domain found in the *O. tauri* NOS (Table 1 and Fig. 1) (25). Other sequences displaying similarities

NADPH-binding domain; -ZBS, -NOSoxy = missing zinc-binding site and oxygenase domain. *E* values were derived from BLASTp analysis using the 1KP database option (www.bioinfodata.org/Blast4OneKP/) with human NOS1 and *O. tauri* NOS (CAL57731) as query sequences.

Table 1. List of plant protein sequences showing highest similarities with described NOSes. Complete = complete NOS; ZBS, NOSoxy = zinc-binding site and NOSoxy domain; FAD, NADPH = FAD- and NADPH-binding domains; NOSred = FMN-, FAD-, and NADPH-binding domains; NADPH partial = NOS with incomplete

Sequence code	Domains	Species name	Clade	Order	Class	<i>E</i> value
DRGY-7431	Complete	<i>Chaetosphaeridium globosum</i>	Green algae	Coleochaetales	Coleochaetophyceae	1×10^{-129}
WDGV-56501	Complete	<i>Cosmarium subtumidum</i>	Green algae	Desmidiales	Zygnematophyceae	1×10^{-120}
LETF-25172	Complete	<i>Planophila terrestris</i>	Green algae	Ultrichales	Ulvophyceae	1×10^{-133}
LSHT-41223	Complete	<i>Bolbocoleon piliferum</i>	Green algae	Ulvales	Ulvophyceae	6×10^{-96}
AJAU-4151	Complete	<i>Helicodictyon planctonicum</i>	Green algae	Ultrichales	Ulvophyceae	1×10^{-120}
BZSH-7369	Complete	<i>Golenkinia longispicula</i>	Green algae	Sphaeropleales	Chlorophyceae	1×10^{-127}
PRIQ-7883	Complete	<i>Pleurastrum insigne</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-120}
RYJX-11655	Complete	<i>Pandorina morum</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-120}
ZIVZ-8856	Complete	<i>Phacotus lenticularis</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-128}
VIAU-5304	ZBS, NOSoxy	<i>Carteria crucifera</i>	Green algae	Volvocales	Chlorophyceae	5×10^{-60}
VIAU-7897	FAD, NADPH	<i>Carteria crucifera</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-39}
OFUE-44553	ZBS, NOSoxy	<i>Lobochlamys segnis</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-28}
OFUE-10388	NOSred	<i>Lobochlamys segnis</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-87}
ACRY-42445	ZBS, NOSoxy	<i>Pteromonas</i> sp.	Green algae	Volvocales	Chlorophyceae	4×10^{-53}
LNIL-1516	NOSred	<i>Pteromonas angulosa</i>	Green algae	Volvocales	Chlorophyceae	2×10^{-96}
BCYF-46343	ZBS, NOSoxy	<i>Chlamydomonas cribrum</i>	Green algae	Volvocales	Chlorophyceae	3×10^{-59}
TSBQ-5508	NOSred	<i>Chlamydomonas</i> spM2762	Green algae	Volvocales	Chlorophyceae	2×10^{-44}
JWGT-5959	ZBS, NOSoxy	<i>Volvox aureus M1028</i>	Green algae	Volvocales	Chlorophyceae	1×10^{-41}
JWGT-5952	NOSred	<i>Volvox aureus M1028</i>	Green algae	Volvocales	Chlorophyceae	8×10^{-53}
ZNUM-4613	Complete	<i>Leptosira obovata</i>	Green algae	<i>Ordo incertae sedis</i>	Trebouxophyceae	1×10^{-129}
FMVB-38388	Complete	<i>Scherffelia dubia</i>	Green algae	Chlorodendrales	Chlorodendrophyceae	1×10^{-106}
ISIM-37076	NADPH partial	<i>Nephroselmis pyriformis</i>	Green algae	Nephroselmidales	Nephroselmidophyceae	1×10^{-100}
JJZR-6120	Complete	<i>Rhodochaete parvula</i>	Red algae	Rhodochaetales	Compsopogonophyceae	1×10^{-103}
TZJQ-124063	-ZBS, -NOSoxy	<i>Prorocentrum micans</i>	Alveolata	Prorocentrales	Dinophyceae	8×10^{-66}
JGGD-14376	ZBS, NOSoxy	<i>Sargassum muticum</i>	Chromista	Fucales	Phaeophyceae	6×10^{-82}
JGGD-80461	NOSred	<i>Sargassum muticum</i>	Chromista	Fucales	Phaeophyceae	5×10^{-22}

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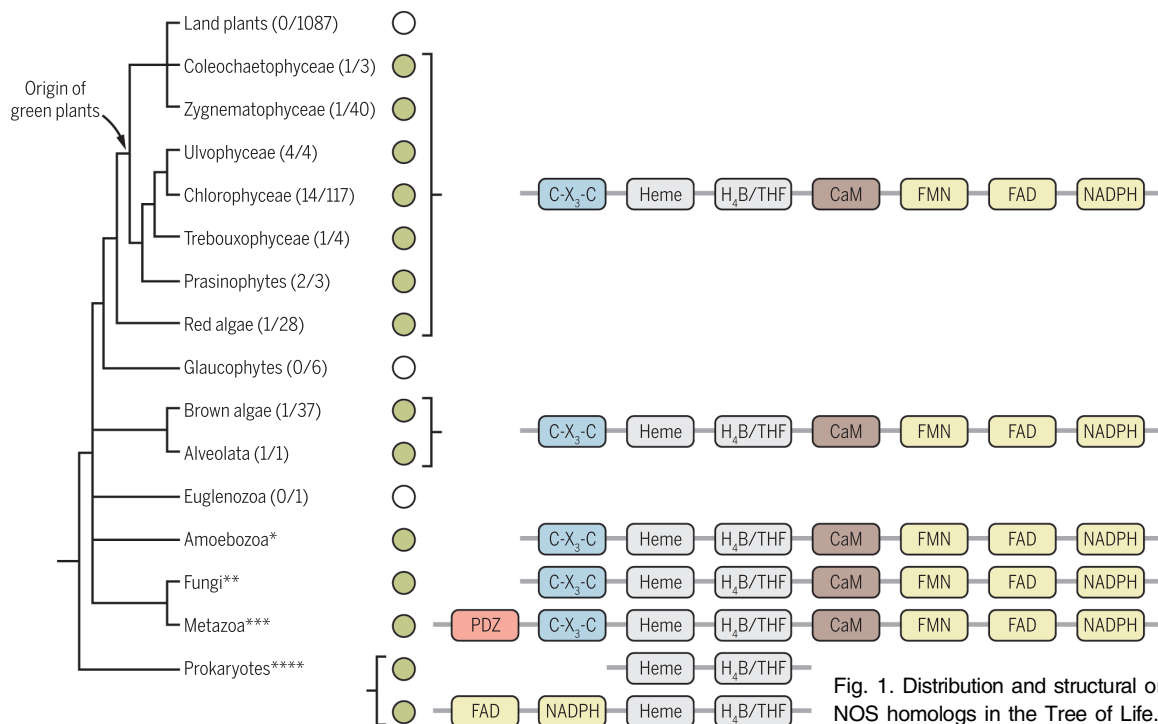


Fig. 1. Distribution and structural organization of NOS homologs in the Tree of Life. The number of identified complete or partial NOSes in each

taxon/number of taxa present in the 1KP database is indicated in parenthesis after the name of each group. Green circles, NOS is present; open circles, NOS is absent. The schematic structures of each type of NOS are represented according to (25), (28), and (58). The established binding motifs of mammalian NOSes for FMN, FAD, NADPH, CaM, heme, H₄B, and zinc (C-X₃-C and C-X₄-C) were conserved in all eukaryotic NOSes. * indicates NOS has been reported in *Physarum polycephalum* (59); **, deduced from *Aspergillus oryzae* sequence (XP_001825673); ***, the N-terminal PDZ domain involved in protein-protein interactions in human NOS1 is not conserved in all metazoans (28); ****, bacterial NOSes were initially identified as only containing the oxidase domain (NOSoxy) with the exception of the *Sorangium cellulosum* NOS, in which the relative position of the two domains is inverted, with the reductase domain (NOSred) at the N terminus and the NOSoxy domain at the C terminus (31).

to NOS lack either the NOSoxy or NOSred domain or contain only a partial NOSoxy domain. Protein sequence similarity between overlapping segments of algal NOS orthologs and human NOS1 is near 30%. Average pairwise similarity calculated between sequences of the monophyletic group of green and red algae is low (37%) compared to the similarity between metazoan NOS sequences, which is nearly 60% between humans and cnidarians (28). In addition to the investigation of the 1KP transcriptome data set, we also searched for NOS-like sequences in the 24 algal genomes that are available in public databases. These species were not included in the 1KP database. We performed searches using the protein sequence of *O. tauri* NOS as a query against these complete genomes, and the results are summarized in Table 2. Two proteins showing homology to the *O. tauri* NOS were found in *Klebsormidium flaccidum* (ID: Kf1000760350; 1130 amino acids) and in *Thalassiosira oceanica* (ID: EJK55330; 1245 amino acids). For both proteins, the InterPro analysis allowed the identification of complete NOSoxy and NOSred domains, indicating that these belong to the NOS family. The sequences identified in 10 other genomes showed *E* values ranging from 2×10^{-16} to 1×10^{-37} and likely correspond to cytochrome P450 reductase, because only the C-terminal portions of the sequences display some homology to the *O. tauri* NOS. Finally, no sequences with significant similarity were found in the 12 other algal genomes.

The NOSoxy domain

Comparison of the sequences of the NOS homologs from the algae *Cosmarium subtumidum* and *O. tauri* to human and mouse NOSes and an NOSoxy

sequence from *Bacillus subtilis* reveals interesting similarities and differences (Fig. 2). The NOSoxy domain of mammalian NOSes has an extended N-terminal portion that consists of a predicted β -hairpin hook, a zinc-binding site, and residues that contribute to forming the dimer interface and to binding the dihydroxypropyl side chain of H₄B (29, 30). The hook contains five conserved residues in which mutations alter the native properties of the protein, including homodimer formation, H₄B binding, and activity (29). With the exception of a NOS homolog in *Sorangium cellulosum* that contains both NOSoxy and NOSred domains (31), bacterial NOS-like proteins consist of only an NOSoxy domain and lack the extended N-terminal sequence found in mammals (30, 32). According to Pant *et al.* (33), the absence of the N-terminal hook and zinc- and pterin-binding regions in the *B. subtilis* NOSoxy had little effect on this protein's conformation or its ability to form a homodimer. The algal NOSes contain an N-terminal extension (Fig. 2). However, compared to mammalian NOSes, algal NOSes lack the key conserved residues of the N-terminal hook as well as those involved in binding the dihydroxypropyl side chain of H₄B (Fig. 2). On the other hand, a putative zinc-binding site (Cys-X₃-Cys instead of the Cys-X₄-Cys in mammalian NOSes) is present. A more general structural analysis of the NOSoxy domain shows that algal NOSes conserve the key structural features of mammalian and bacterial NOSoxy, including the proximal heme ligand Cys, the L-Arg- and H₄B-binding residues, and the so-called helical lariat and helical T region that are involved in binding the pterin cofactor and in the interactions between monomers that help stabilize the homodimer (Fig. 2).

Table 2. Search for NOS-like proteins encoded in complete algal genomes. Two complete NOS sequences were identified (bold). Protein sequences have been retrieved from complete algal genomes available in the NCBI (National Center for Biotechnology Information) Genome database and in three species-specific genome portals (The *Cyanidioschyzon merolae* Genome Project:

<http://merolae.biol.s.u-tokyo.ac.jp/>; the Kazusa DNA Research Institute: <http://genome.microbedb.jp/klebsormidium/nies-2285/>; the Joint Genome Institute Genome Portal: <http://genome.jgi.doe.gov/MicpuC2/MicpuC2.home.html>). Similarity to *O. tauri* NOS was estimated using BLASTp (percentage of coverage and *E* values) and InterPro domain analysis.

Species name	Database	BLASTp results (<i>E</i> value)
<i>Bigelowiella natans</i>	NCBI	2×10^{-16}
<i>Chlorella variabilis</i>	NCBI	2×10^{-25}
<i>Chlorella vulgaris</i>	NCBI	No hits
<i>Chondrus crispus</i>	NCBI	4×10^{-25}
<i>Coccomyxa subellipsoidea C-169</i>	NCBI	2×10^{-32}
<i>Cymbomonas tetramitiformis</i>	NCBI	No hits
<i>Cyanidioschyzon merolae</i>	<i>Cyanidioschyzon merolae</i> Genome Project	No hits
<i>Ectocarpus siliculosus</i>	NCBI	6×10^{-24}
<i>Emiliania huxleyi</i>	NCBI	2×10^{-29}
<i>Gracilaria chilensis</i>	NCBI	No hits
<i>Guillardia theta</i>	NCBI	1×10^{-37}
<i>Helicosporidium</i>	NCBI	1×10^{-34}
<i>Heterococcus</i>	NCBI	No hits
<i>Klebsormidium flaccidum</i>	Kazusa DNA Research Institute	9×10^{-177}
<i>Micromonas pusilla</i>	Joint Genome Institute	No hits
<i>Monoraphidium neglectum</i>	NCBI	6×10^{-30}
<i>Nannochloropsis gaditana</i>	NCBI	3×10^{-31}
<i>Picochlorum</i>	NCBI	No hits
<i>Polytomella magna</i>	NCBI	No hits
<i>Saccharina japonica</i>	NCBI	No hits
<i>Thalassiosira oceanica</i>	NCBI	2×10^{-130}
<i>Thalassiosira pseudonana</i>	NCBI	4×10^{-24}
<i>Trebouxia gelatinosa</i>	NCBI	No hits

Tetrahydrofolate as a redox factor

The pterin cofactor H₄B plays a crucial role in NOS catalysis by acting as an electron donor (4). We did not find in the IKP database, even in algae carrying NOS genes, genes encoding the major enzymes for H₄B biosynthesis, specifically 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase. This suggests that the algal NOS may instead bind an H₄B analog to support NO synthesis. It has been demonstrated that the closely related pterin, tetrahydrofolate (H₄F), can act as an electron donor in *O. tauri* NOS (25, 34), and bacterial NOS-like proteins, such as that from *Deinococcus radiodurans*, synthesize NO efficiently from H₄F (35). Furthermore, plants produce H₄F (36). At the structural level, as highlighted above, the extended N-terminal portion of mammalian NOS participates in H₄B binding. According to Tejero and Stuehr (4), the absence of the N-terminal extension in the bacterial NOS-like proteins allows them to bind larger pterins such as H₄F. It is conceivable that this may also hold true for the algal NOSes, which do not contain the conserved residues for binding H₄B in their N-terminal extensions.

The NOSred domain

The NOSred domains of algal NOSes display structural characteristics typical of mammalian NOSes, including the FMN-, FAD-, and NADPH-binding sites (Fig. 2). As noted for the *O. tauri* NOS by Foresi *et al.* (25), algal NOSes do not contain the autoinhibitory loop that is present in the FMN-binding site of constitutive mammalian NOSes. This sequence of 40 to 50 residues, which is also absent in mammalian iNOS, confers Ca²⁺ sensitivity to mammalian constitutive NOSes by destabilizing CaM binding at low Ca²⁺ concentrations and influences the rate of electron transfer from

FMN to heme (1, 37). However, a putative CaM-binding site was predicted in the *O. tauri* NOSred domain (25). CaM-binding sites usually are non-homologous in sequence but form a basic, amphipathic α helix. In the case of mammalian NOSes, CaM-binding domains display the classical Ca²⁺-dependent “1-5-8-14” motif, in which the numbers indicate the position of key hydrophobic residues in the domain that are essential for CaM binding (38). This motif was found in the *C. subterminum* NOSred domain, and, with the exception of a serine residue at position 5 instead of a more hydrophobic residue, it was also found in the *O. tauri* NOSred domain (Fig. 2). Nevertheless, it should be noted that in contrast to mammalian NOS CaM-binding sites, analysis of the secondary structure of the putative CaM-binding site of *O. tauri* and *C. subterminum* NOSes did not predict an α helix, bringing into question the ability of this domain to interact with CaM. Accordingly, purified recombinant *O. tauri* NOS retained up to 70% of its activity in the absence of CaM (25).

Evolution of NO Synthesis in Plants

Two conclusions arise from our analysis. First, among the 241 transcriptomes and 24 available genomes of algal species that we screened for the presence of NOSes, only 15 species had orthologs displaying the entire multidomain structure of mammalian NOSes. These NOSes are unequally distributed and did not correspond to phylogeny, indicating a partial loss of NOS-encoding genes in some algal groups. Second, we observed an absence of NOS transcripts in the monophyletic group of land plants. Land plants that were screened include species in which NO production that is sensitive to mammalian NOS inhibitors, or NOS-like activities, or

C. subterminum -----VAVTRCP-LM-----HGSVAGRLASCSPGLQVEICPEG-C-----KPEPKLVSGREGEGPMETLVREALEFQDVFHADKGSSEKAKET
O. tauri -----ATDDDDGVDVPSRCP-FA-----HGTVTVDPPYPGYVHGKNRVRVCP-PRG-C-----VPRP-----PSKPTESAESALRREAEYIIRLFGKEHGWDDEVRVM
Baa223061 -----VAVTRCP-LM-----HGSVAGRLASCSPGLQVEICPEG-C-----KPEPKLVSGREGEGPMETLVREALEFQDVFHADKGSSEKAKET
humanNOS1 PPTSQKSPKTKNGSPKSPKPRFLKVKNNWETEVLVDTLHLKSTLETGCTEYICMGSIMHPSQHARRPE-DVVRTKQGLFPLAKEFIDQYSSIKRFGSKAHM
humanNOS2 -----SLVKLDATPLSS-----PRHVRIKNNWGSMTFDTLHLKAKGIITCRSKSCLGSIMTPKSLTRGPRDKPTPDELLPQALFVFNQYSSYGSFKEAKIEEHL
humanNOS3 -----PSSPLTQPPEG-----PKFPRIKNNWEGSITTYDTLHSAQAQDQPCPTFRRLCLGSLVTPRKLQGRPSGPPAPELLELSQARDFINQYSSIKRSGSQAEH
mouseiNOS -----SLDKLHVTSTR-----EQYVRIKNNWGSGLILHDTLHKKATSDFITCKSKSCLGSIMNPKSLTRGPRDKPTPLEELPHATIEFINQYSSYGSFKEAKIEEHL

N-terminal hook Zinc loop Pterin-binding

C. subterminum RVRQ-VMREIQATGTYSHTEWELQHGARLAWRNAPKCSSRKFWEELNVIDARHAFTGAQMLEACLEMLEKATLSCVSSANIVVFRPQTPTGEDGPRVWNN
O. tauri RVNE-VLTSIRETGTAYHTLDEIRHGARVARNAPKICINRKYWSTLDVIDARGATNEDMFEAIKEHLHRGVEADHVPVLMTVFRPQTPTGNDGPRVWNS
Baa223061 DRLADIKSEIDLGLSGYVHTKEELEHGAKMAWRNAPKICIRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPTITIFPEEKEGKQ-VEIWNH
humanNOS1 ERLEEVENKEIDTTSTYQLKDTLEIYGAKHAWRNASRCVGRIQWSLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSATITFPQRTDQKHD-FRVWNS
humanNOS2 ARVEAVTKEIETTGTQYQLTGDELIFATKQAWRNAPKICIGRIQWSNLQVFDARSCSTAREMFEHICRHVRYSTNNGNRSATITVFPQRSDDGKHD-FRVWNA
humanNOS3 QRLQVEVAEVAATGTQYQLRESELVFGAKQAWRNAPKICVGRIQWSLQVFDARCSAQEMFPTICNHIKYATNNGNLRSATITVFPQRSDDGKHD-FRIWNS
mouseiNOS ARLEAVTKEIETTGTQYQLTLELIFATKMAWRNAPKICIGRIQWSNLQVFDARNCSAQEMFQICRHILYATNNGNRSATITVFPQRSDDGKHD-FRLWNS

C. subterminum QIIRFCGFRQPDGSLVLDGPAEVDFTELCIKRFGWKPPAPTPTQTSANGKFRWEVLPLLLQDPPEEAPLFEIPEQYVVRVPIRHPEYEAISALGLQWYAV
O. tauri QLMRYAGHRDASGGVVDPAEVDFTDVLKXKHFVWAKETG-----MFDALPIVQINPETPAMPVLPDQCFLEVPVIRHPTIRGISQLGLKWKYGI
Baa223061 QLRIRYAGYE-SDGERIGDPAESCILTAACEE-LGWRGERTD-----FDLLPLIFRMKGDEQPVVYELPRSLVIEVPIHPDIEAFSDLELKWYGV
humanNOS1 QLRIRYAGYKQPDGSLVLDGPAEVDFTDVLKXKHFVWAKETG-----MFDALPIVQINPETPAMPVLPDQCFLEVPVIRHPTIRGISQLGLKWKYGI
humanNOS2 QLRIRYAGYQMPDGSIRGDPANVEFTQLCID-LGWKPKYGR-----FDVPLVLLQANGND-PELFIQIPPELVEVIRHPKFEWFKDLGLKWKYGL
humanNOS3 QLRIRYAGYQMPDGSIRGDPANVEFTQLCID-LGWKPKYGR-----FDVPLVLLQANGND-PELFIQIPPELVEVIRHPKFEWFKDLGLKWKYGL
mouseiNOS QLRIRYAGYQMPDGTIRGDAATLEFTQLCID-LGWKPKYGR-----FDVPLVLLQANGND-PEVFEIIPDVLVLEVPTMEHPKYEWFGLKWKYGL

C. subterminum PAFSSLELSVGGVLYTACPFNGWYADTEIV-RNLTDEGRNLNLCVAVRSGLDVRSAWDRDAVFEIGVAVMHSFREAAGMGMVDQHTLAANFCRWYQR
O. tauri PAVSNITMDLGGHYTAAPFNGWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE
Baa223061 PIIISDMKLEVGGIHYNAAPFNGWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE
humanNOS1 PAVSNMMLLEIGGLEFSACPFNGWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE
humanNOS2 PAVANMLLEVGLEFPFGCPFNWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE
humanNOS3 PAVSNMMLLEIGGLEFPFAAPFNGWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE
mouseiNOS PAVANMLLEVGLEFPACPFNGWYMTETIATRNFGDESRYNLLPQIAEAMGLDSTHDTLWRDHAAALNAYVDFSKFRDRVSIADHHTCAEAFADWYVE

C. subterminum ERKARGFTPPGDWASVCPAAAHSDVSLQEHQFMEYTIKPGYWAAGRWYLERLLERLLENPRALPFGATTPAQAGCTATAGAALGGQAGSAAADGEAANDT
O. tauri ELKTRGYAP-GNWKWIVPPTASSSTSSYIYGLGNKMEYTLKLPALVGGMLRSLRRAKADAG-----FLTSDAFSTVSIIRVAIAAAKFRR
Baa223061 EEEAGRKLTD-GDWTLVLPISPAATHIFH--RSYDINSIVKPNYFQDKP-----PDKKRAIGPKKLAEAVKFSAKLMGQ
humanNOS1 EYRCRGGCP-ADWVWIVPMSGSIPTVFH--QEMLNRYLTPSFYYQDPWNTHVWKGNTGT-----PKRR-EIPLKLVKAVLFCMLMRK
humanNOS2 EYRSRGGCP-ADWIVLVPMSGSIPTVFH--QEMLNRYLTPSFYYQDPWNTHVWKGNTGT-----PKRR-EIPLKLVKAVLFCMLMRK
humanNOS3 EQKARGGCP-ADWAWIVPISGSLTPVFH--QEMVNYFLSPAFRYQDPWKGSAAKGTGIT-----RKKT--FKEVANAVKISASLMGT
mouseiNOS EYRARGGCP-ADWIVLVPVSGSITPTVFH--QEMLNRYLTPSFYYQIEPWKTHIWQNEKLR-----PRRR-EIRFRVLVKKVFFASLMRK

Helical lariat

Helical T

CaM-binding site

C. subterminum ASQRQARVLLIYASVSGTTTYQAQVAAVVGKGAAVRVLNMEDFERDKWAMEIQSDVVVLMSTYGPAGPSSAAAKFVHWIQGSHQEAEAEAFK-----
O. tauri NKLKVGKGIIVLYASDGGRSQSRATWLSFLFQRLPMVRVNVANPDVPLTDLLSNVEFLLFLASTTSGSQVPTGSDSFIETWK--TEHGRALR-----
Baa223061 AMAKRKATILYATETGKSQAYAKTLCEIFKHAFDAKVMSEYD----IVHLEHETLVLVVSTFTFNGDPPENGEKFGCALMEMRHPNSVQ---EERKS
humanNOS1 TMSRVRVTLFATETGKSEALAWDLGALFCAFNPKVVCMDKYD----LSCLEERLLVSTFTFNGDPCPNGEKLLKKSFLMR---EERKS
humanNOS2 VMKRKATILYGSETGRAQSYAQQLGRLFRKAFDPRVLCMEYD----VVSLEHETLVLVVSTFTFNGDPPENGEFSAALMEMSGPYNSSPRPEQHS
humanNOS3 VMASRVATVLFATETGKSEALARDLATLFSYAFNTKVVCMQDYK----ASTLEEEQLLVVSTFTFNGDPCPNQTLKKSFLMLR-----
mouseiNOS

C. subterminum -----GKSIYVVGFGNSCYPRFCAAADLLESLLSIGAVLPLVPAVKADALAGEEAVWGWAKELVGCVA
O. tauri -----GKYFAVCAFGRSAYPKFCAGGEQFAAALAAAGAVAMPITRCDQLNGEDASVHSYTMRLFDWLLD
Baa223061 YKVRFNSSVSSYSDSQKSSGDGPDLRDNFESAGPLANVRFVFLGSGRAYPHFCFAHVDTLLEELGGERIILKMRGDELQCGEEAFRTWAKVFKAACD
humanNOS1 -----ELN-----NKFRYAVFGLGSSMYPRFCFAHVDTLLEELGGERIILKMRGDELQCGEEAFRTWAKVFKAACD
humanNOS2 YKIRFNISCSDDLVSWRKRKESSTDSAGALGTLRFVFLGSGRAYPHFCFAHVDTLLEELGGERIILKMRGDELQCGEEAFRTWAKVFKAACD
humanNOS3 -----ELN-----HTFRYAVFGLGSSMYPRFCFAHVDTLLEELGGERIILKMRGDELQCGEEAFRTWAKVFKAACD
mouseiNOS

Autoinhibitory loop

FMN-binding domain

C. subterminum KDGCMSDQOQESIMAKLPLTDANQVQ-AFQPTHAVIRLSGDAVRPAREKCHGWTEVLETEELLAPAGERTSTRLIHIDIDNLPCTGTVHAGDEVSWAEND
O. tauri ADRIPIPSLR--DLMVDQINGGVAIEP-AFTLKVRRQDVNGRIHLGPTVQHGTAABELIDRAITNGARMNTVWLKFRILIHAREEVQYKPKGDHVAVWPQTS
Baa223061 VFCVGDVNIKANNSLISNDRSWKRNKFRITFAEAPELTQGLSNVHKRVSAARLLSRQNLQSPKSSRSTIFVRLHTNGSQELOYKPGDHLGVFPGNH
humanNOS1 TFDVVRGKQ--HIQIPKLYTSNVWDPHHYRLVQDSQPLDLSKALSSMHAKNVFTMLRKSQNLQSPKSSRSTIFVRLHTNGSQELOYKPGDHLGVFPGNH
humanNOS2 TFCVGEDA--KAAARDIFSPPKRSWKRQYRLSAQAEGQLPLGLIHVHRRKMFQATIRSVENLQSSKSTRATILVRLDTGGQEGLOYKPGDHLGVFPGNH
humanNOS3 TFDVRSKH--HIQIPKRFTSNATWEPQYRLIQSPEPLDLNRLSIAHAKNVFTMLRKSQNLQSEKSSRSTIFVRLHTNGSQELOYKPGDHLGVFPGNH
mouseiNOS

FAD-binding site

C. subterminum PALVEEYAAHLG-VTAAHLDDMFLQNTKEDDSSLPLGGLDHLPLNPTFRITLARHIGLSEKLPFAALSVLATRAPEDQLQKLGDRYD-EYTAWSAET
O. tauri EARARVFAAHFG-LTFRDVLLEVPKSDALLVKKSID----PAIPNVVSVEHLFRVLDINGEASAAALSALAHYAFNEDARQDWAEMTG-ARIFSIHF
Baa223061 EDLVNALIERLEDAPPVQMVKVELLEERNALGVISNWTDELRLPCTIFQAFKYLLDITTPPTPLQQLQFASLATSEKEKQRLVLSKGLVSEYEEWK
humanNOS1 PALVQGIILERVVDGPTPHQTVRLEALDESGY-----WVSDKRLPPCSLSQALTYFLDITTPPTQLLQKLAQVATEEPERQRLEALCQ-PSEYKWK
humanNOS2 PGLVEALLSRVEDPAPTEPVAVEQLB-KGSPGPGPPGWVRDPRLPCTLRQALTFFLDITSPSPQLRLSLTAEPEPQOELEALSQDPRRYEYEWK
humanNOS3 TALVQGIILERVVDCTPHQTVCLVLEDESGY-----WVSDKRLPPCSLSQALTYFLDITTPPTQLLQKLAQVATEEPERQRLEALCQ-PSEYNDWK
mouseiNOS

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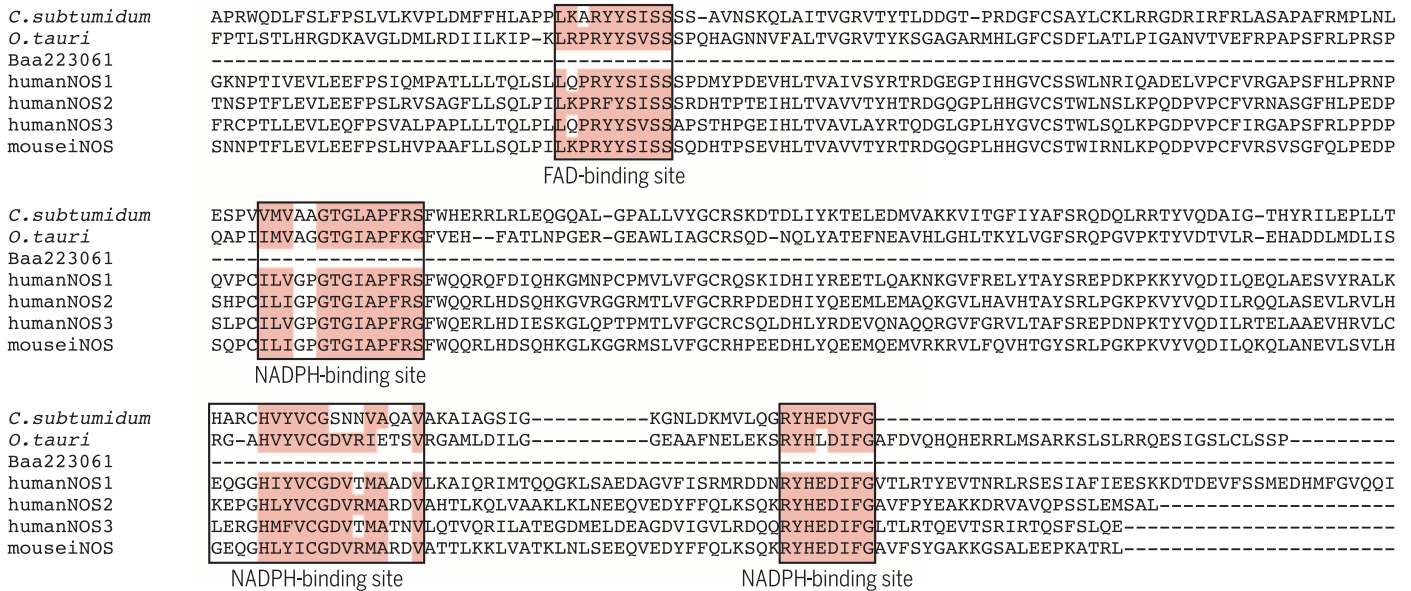


Fig. 2. Sequence alignments of a sample of NOS proteins. Sequences of algal (*C. subtumidum* and *O. tauri*) NOSes, *B. subtilis* NOSoxy (Baa223061), human NOS1 (starting at residue 285), human NOS2 (starting at residue 70), human NOS3, and mouse iNOS (starting at residue 65) are shown. The dashed blue arrow delimits the NOSoxy and NOSred domains. Features of the NOSoxy domain: The extended N-terminal portion of mammalian NOSes consists of the N-terminal hook, a zinc loop, and a pterin-binding site. Residues that make up these structural elements are boxed, with conserved residues indicated by orange letters or, in the case of the Cys ligands for binding zinc, with yellow highlighting. The five conserved residues of the

N-terminal hook where mutation alters the native properties of mouse iNOS (29) are underlined. The Cys ligand for heme iron and H₄B-binding residues are shown with blue and gray highlighting, respectively. The residues involved in L-Arg binding are indicated by blue letters. Features of the NOSred domain: The NOSred is absent from bacteria NOS. The residues that make up the CaM-binding site and the binding sites for FMN, FAD, and NADPH are boxed. Within the CaM-binding domain, the key hydrophobic residues for CaM binding are shown with pink highlighting. Within the binding sites for FMN, FAD, and NADPH, conserved residues are shown with pink highlighting.

both, has been reported. Two non-exclusive scenarios might account for this phylogenetic distribution of NOS in plants: (i) vertical gene transmission from a eukaryotic ancestor before the separation of the eukaryotic supergroups, followed by gene loss in some lineages, including all land plants; (ii) acquisition by horizontal gene transfer in algal lineages. Both scenarios implicate numerous and repeated evolutionary events (losses or gains of NOS-encoding genes), and it is difficult to rigorously test these two hypotheses. Nevertheless, a phylogenetic analysis showed that a phylogenetic tree of NOS proteins is globally congruent with the organismal tree (Fig. 3), given the low resolution of a single gene phylogeny. Therefore, multiple horizontal gene transfer events (for example, from bacteria or other sources) seem unlikely. Moreover, bidomain NOS sequences typical of metazoans are not present in prokaryotes, implying vertical transmission of an ancestral NOS that was lost in most plant lineages, including all of the land plants.

The basic mechanisms of NO signaling in plants are similar to those in animals. NO acts through complex networks of second messengers [such as Ca²⁺, cyclic GMP (guanosine 5'-monophosphate), cyclic ADP (adenosine 5'-diphosphate)-ribose, and lipids], modulates the activity of signaling-related proteins (including receptors, protein kinases, and transcription factors), and contributes to the regulation of the expression of numerous genes. Furthermore, the actions of NO-derived chemical species are, in part, mediated by posttranslational protein modifications, including tyrosine nitration, Cys-nitrosation, and metal-nitrosylation. Also, plant and animal cells share key enzymes involved in the regulation of NO effects such as nitrosogluthathione reductase, thioredoxins, thioredoxin reductases, and hemoglobin (39–43). In animals, constitutive NOSes are part of protein

complexes that play a fundamental role in transducing NO signaling activities. Given all this, it is remarkable that NOS was not conserved in land plants during evolution.

Nitrate metabolism is considered to be the first NO-generating system in living organisms (44), and plants have efficient systems for nitrate uptake, reduction, compartmentalization, long distance transport, and remobilization. Accordingly, nitrate reduction mediated by NR has emerged as the main enzymatic source of NO in plants. Nitrite also generates NO nonenzymatically under acidic conditions (13), can be reduced by chloroplasts to NO in a light-driven reaction (45), and can be reduced to NO in mitochondria under conditions of anoxia or hypoxia, probably through the action of cytochrome c oxidase (17, 46). Besides nitrite, hydroxylamines and polyamines can also act as substrates for NO synthesis (17, 47), and several enzymes, including catalase, xanthine oxidoreductase, and horseradish peroxidase, are suspected to have the ability to produce NO in addition to their primary enzymatic activities [recently reviewed in (21)]. Therefore, plants have many routes for synthesizing NO, suggesting that a NOS enzyme may be unnecessary in plants. The existence of many mechanisms for NO synthesis also raises questions about the respective contributions and regulation of these distinct NO sources in physiological processes.

The observation that, in photosynthetic organisms, only a few algal species contain NOS orthologs introduces the question of why this enzyme is absent in the other algal species. Here, too, nitrogen metabolism could offer some clues. Indeed, NR catalyzes NO synthesis in several algal species, such as the unicellular green algae *Chlamydomonas reinhardtii* (48) and *Scenedesmus obliquus* (49), and, similarly to terrestrial plants,

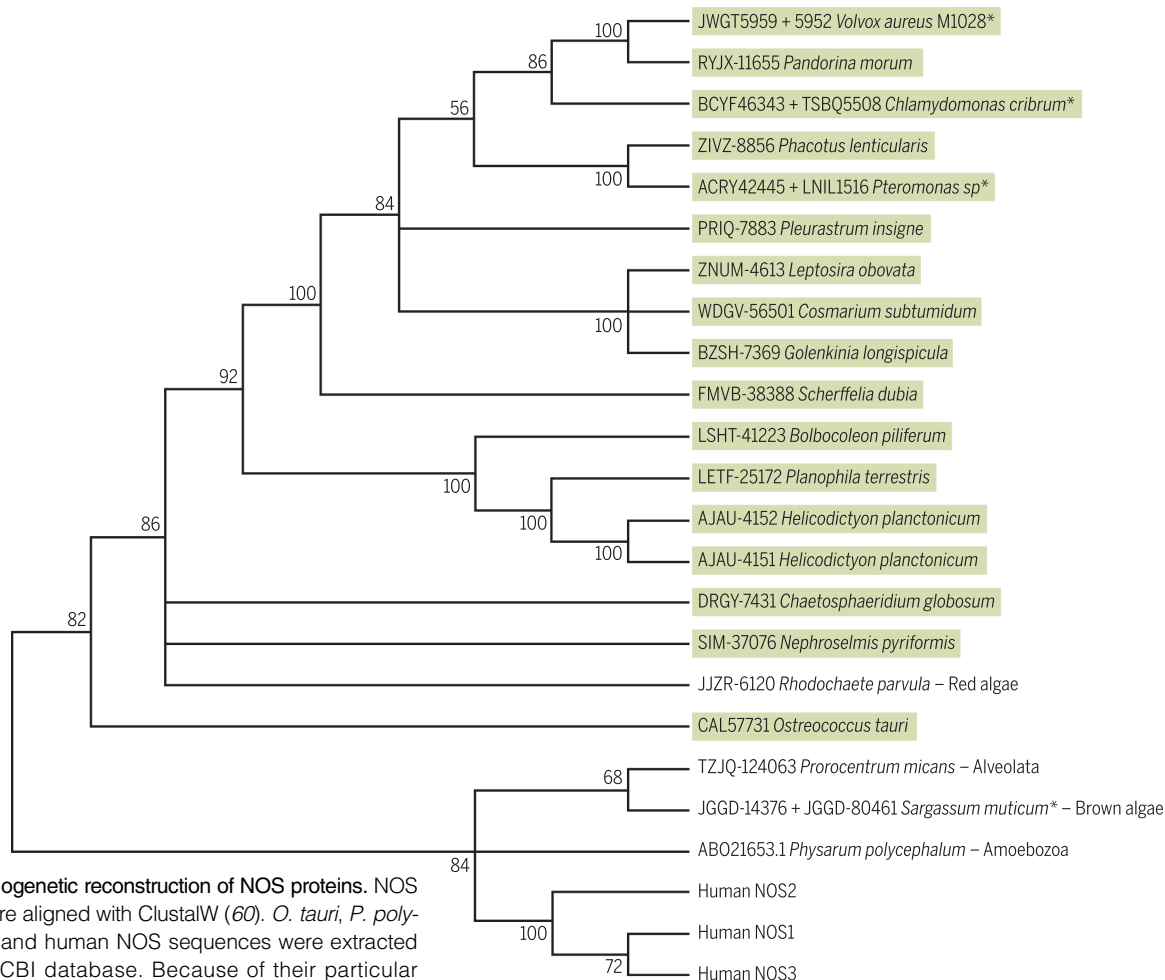


Fig. 3. Phylogenetic reconstruction of NOS proteins. NOS proteins were aligned with ClustalW (60). *O. tauri*, *P. polycephalum*, and human NOS sequences were extracted from the NCBI database. Because of their particular structures, prokaryotic NOSes were not included. The phylogenetic tree was constructed by maximum likelihood methods using MEGA (55), and bootstrap analysis was carried out using 100 replicates. Sequences indicated by an asterisk correspond to NOS reconstituted from two partial overlapping sequences. Green algae are indicated with green highlighting.

could constitute a major enzymatic NO source. On the other hand, the presence of both NOS and NR in some algal species is intriguing. Does this peculiarity reflect the pressures of specific environmental conditions? Foresi *et al.* (25) reported that *O. tauri* cells have a high rate of NOS-dependent NO production under light irradiances that cause photoinhibition. According to the authors, NOS-derived NO may reduce oxidative damage caused by light-induced stress. The occurrence of both nitrite- and NOS-dependent pathways could be extended to mammals and fish. Indeed, in addition to NOSes, mammals and fish also have a nitrite-dependent pathway for NO synthesis (50, 51). This biosynthetic pathway operates under normoxic conditions but becomes more active when oxygen availability is reduced. Accordingly, several reports highlight a role for nitrite-derived NO in cytoprotection during hypoxia in mammals (52). The underlying mechanisms are still under investigation, but several enzymes involved in nitrate-to-nitrite reduction and nitrite-to-NO reduction have been identified, such as xanthine oxidoreductase (53), deoxyhemoglobin (51), and sulfite oxidase (54). Similar to fish, algae and land plants frequently face hypoxic or anaerobic conditions in their natural habitats. It is therefore tempting to speculate that the nitrite-dependent NO pathways constitute an evolutionary process that helps plants and algae to adapt to such environments.

In conclusion, it seems plausible that NOS has participated in the past, and still participates, in NO production together with NR in some non-embryophyte photosynthetic organisms, but not in land plants. More generally, we can assume that the ability of land plants to assimilate and reduce nitrate, together with the frequent hypoxic conditions encountered by plants (mainly during their earlier evolution in water) and the later diversification to new and drastically different environmental conditions of terrestrial habitats, has led these sessile organisms toward developing the efficient production of NO from NR as the main strategy of survival.

Appendix

Protein sequences with similarity to *Ostreococcus tauri* NOS and human NOS1 were identified from the IKP database (<http://onekp.com/project.html>) using BLASTp and tBLASTn searches. The sequences were aligned using Muscle. Phylogenetic analyses, conducted using MEGA version 5 (55), allowed identification of the most divergent sequences and were drawn according to the Tree of Life project (<http://tolweb.org/tree/>). Domain organization was analyzed using InterProScan (56, 57). Conserved domains were further identified in protein sequence alignments using the human NOS1 and the *O. tauri* NOS as references.

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