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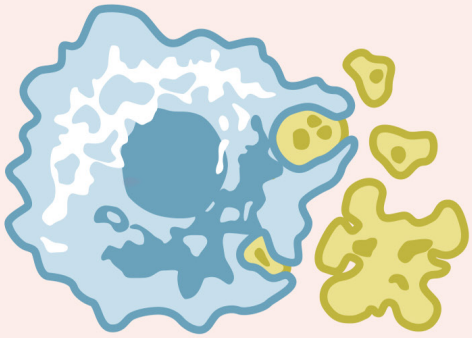

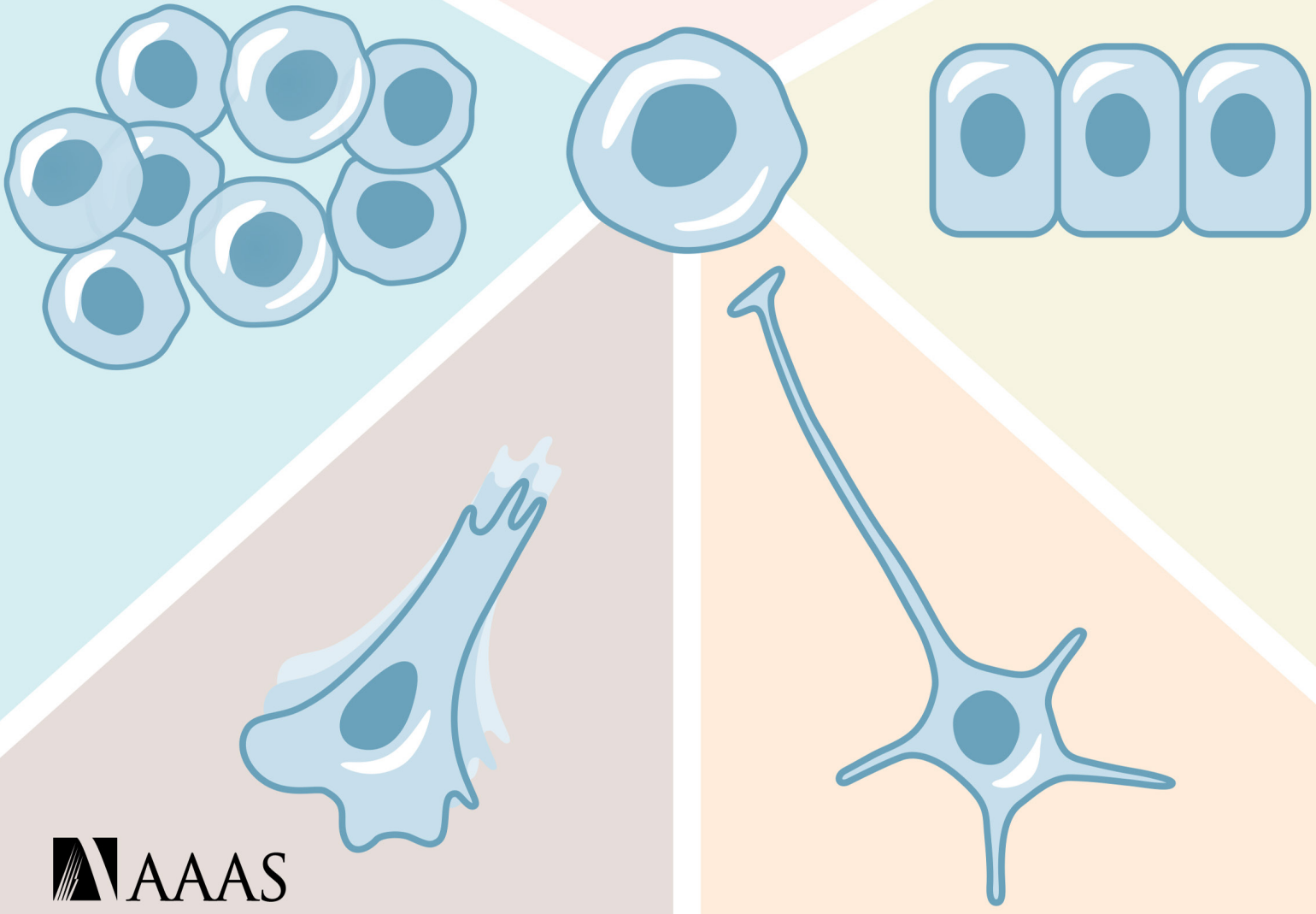

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PLANT BIOLOGY

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

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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

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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

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 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.

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

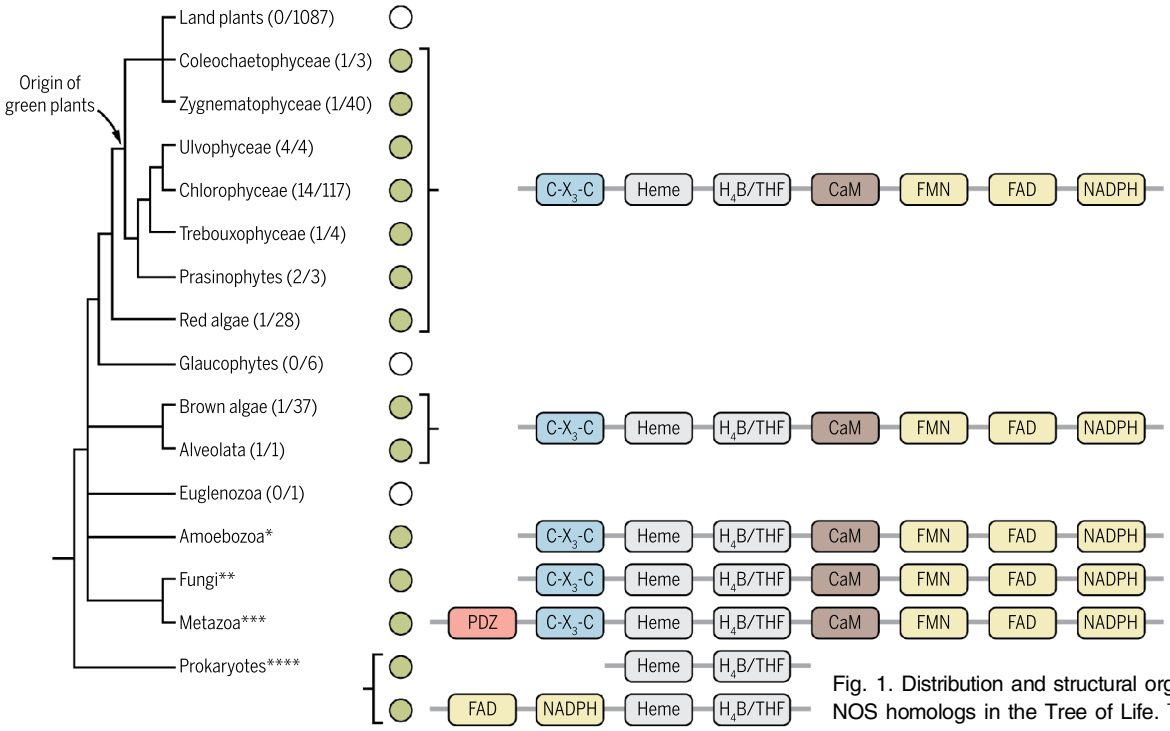


Fig. 1. Distribution and structural organization of NOS homologs in the Tree of Life. The number of identified complete or partial NOSes in each taxon/number of taxa present in the 1KP database is indicated in parenthesis after the name of each group. Green circles, NOS is present; open circles, NOS 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, H_4B, and zinc (C-X_3-C and C-X_4-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 H_4B (*29*, *30*). The hook contains five conserved residues in which mutations alter the native properties of the protein, including homodimer formation, H_4B 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 H_4B (Fig. 2). On the other hand, a putative zinc-binding site (Cys-X_3-Cys instead of the Cys-X_4-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 H_4B-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 (*E* value)
Bigelowiella natans	NCBI	2×10^{-16}
Chlorella variabilis	NCBI	2×10^{-25}
Chlorella vulgaris	NCBI	No hits
Chondrus crispus	NCBI	4×10^{-25}
Coccomyxa subellipsoidea C-169	NCBI	2×10^{-32}
Cymbomonas tetramitiformis	NCBI	No hits
Cyanidioschyzon merolae	*Cyanidioschyzon merolae* Genome Project	No hits
Ectocarpus siliculosus	NCBI	6×10^{-24}
Emiliania huxleyi	NCBI	2×10^{-29}
Gracilaria chilensis	NCBI	No hits
Guillardia theta	NCBI	1×10^{-37}
Helicosporidium	NCBI	1×10^{-34}
Heterococcus	NCBI	No hits
Klebsormidium flaccidum	**Kazusa DNA Research Institute**	$\mathbf{9 \times 10^{-177}}$
Micromonas pusilla	Joint Genome Institute	No hits
Monoraphidium neglectum	NCBI	6×10^{-30}
Nannochloropsis gaditana	NCBI	3×10^{-31}
Picochlorum	NCBI	No hits
Polytomella magna	NCBI	No hits
Saccharina japonica	NCBI	No hits
Thalassiosira oceanica	**NCBI**	$\mathbf{2 \times 10^{-130}}$
Thalassiosira pseudonana	NCBI	4×10^{-24}
Trebouxia gelatinosa	NCBI	No hits

Tetrahydrofolate as a redox factor

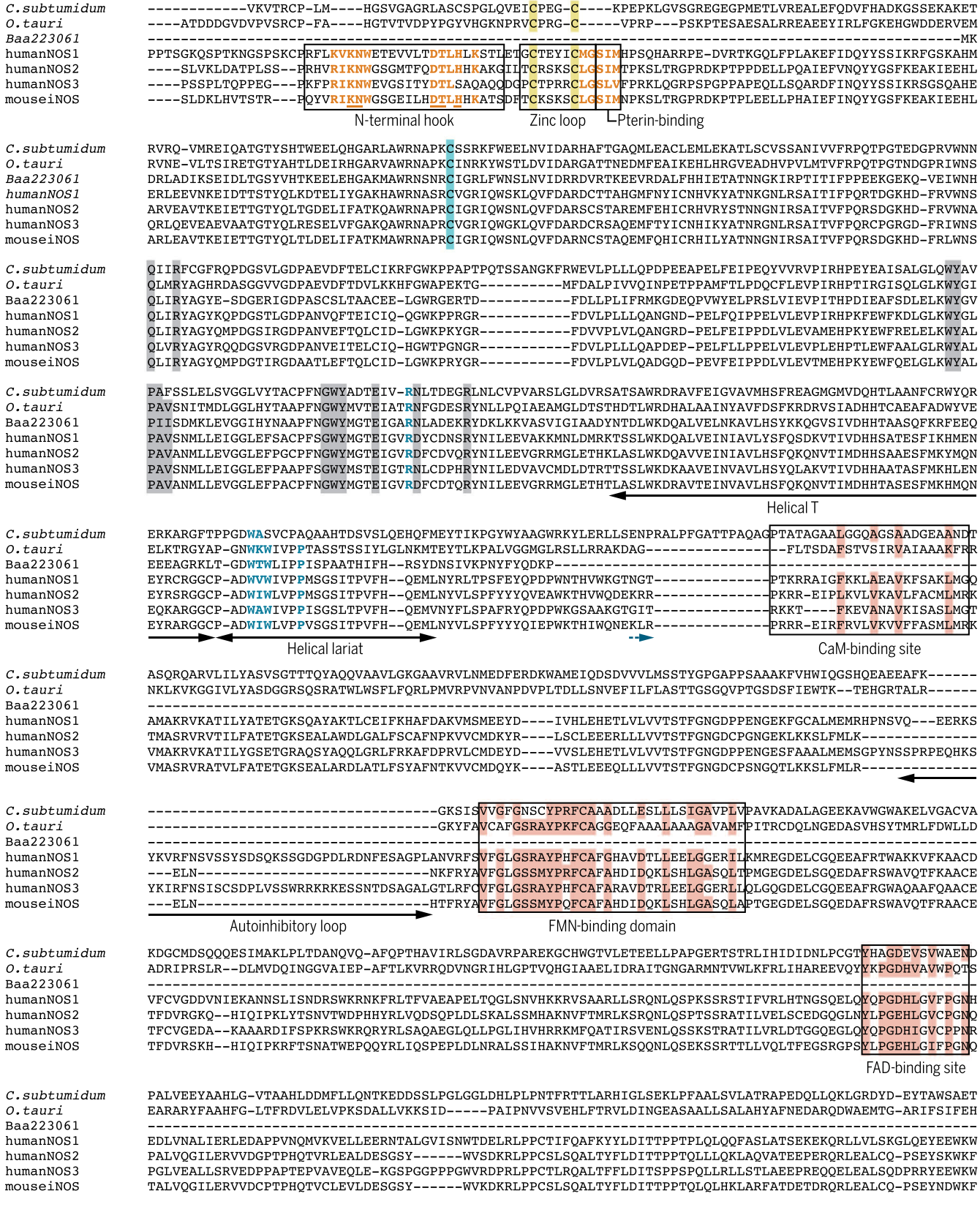
The pterin cofactor H_4B plays a crucial role in NOS catalysis by acting as an electron donor (*4*). We did not find in the 1KP database, even in algae carrying NOS genes, genes encoding the major enzymes for H_4B biosynthesis, specifically 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase. This suggests that the algal NOS may instead bind an H_4B analog to support NO synthesis. It has been demonstrated that the closely related pterin, tetrahydrofolate (H_4F), 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 H_4F (*35*). Furthermore, plants produce H_4F (*36*). At the structural level, as highlighted above, the extended N-terminal portion of mammalian NOS participates in H_4B 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 H_4F. It is conceivable that this may also hold true for the algal NOSes, which do not contain the conserved residues for binding H_4B 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 Ca^{2+} sensitivity to mammalian constitutive NOSes by destabilizing CaM binding at low Ca^{2+} 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 Ca^{2+}-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. subtumidum* 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. subtumidum* 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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O.tauri
Baa223061
humanNOS1
humanNOS2
humanNOS3
mouseiNOS
N-terminal hook
Zinc loop
Pterin-binding
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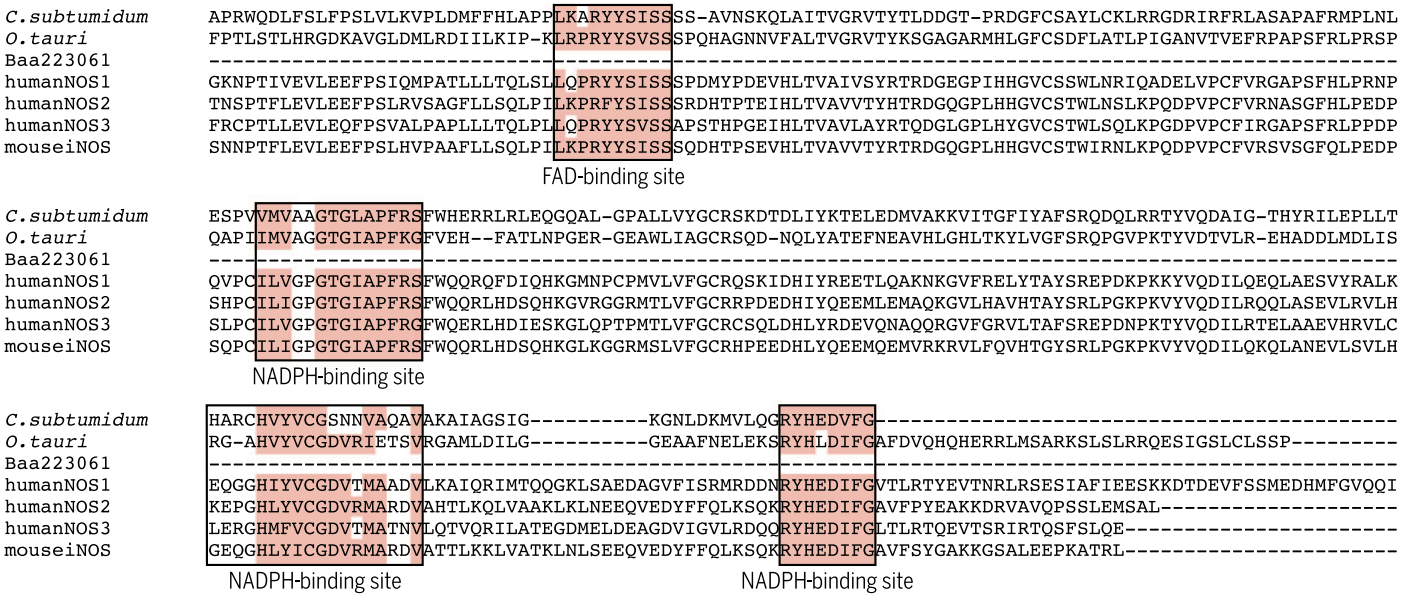

Fig. 2. Sequence alignments of a sample of NOS proteins. Sequences of algal (*C. subtumidum* and *O. tauri*) NOSes, *B. subtilis* NOSoxy (Baa223061), human NOS1 (starting at residue 285), human NOS2 (starting at residue 70), human NOS3, and mouse iNOS (starting at residue 65) are shown. The dashed blue 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 H_4B-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 Ca^{2+}, 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 nitrosoglutathione 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,

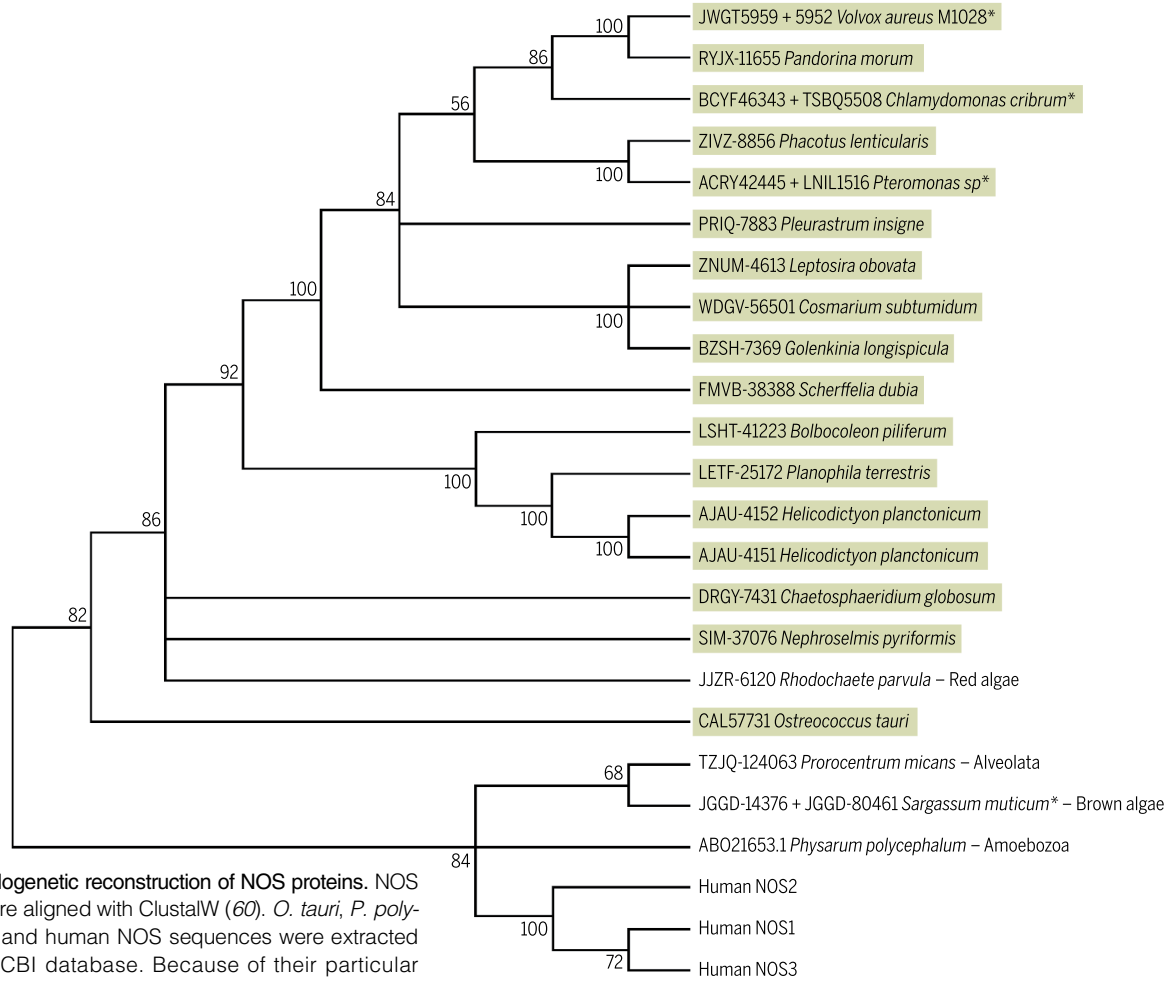

Fig. 3. Phylogenetic reconstruction of NOS proteins. NOS proteins were aligned with ClustalW (*60*). *O. tauri*, *P. polycephalum*, and human NOS sequences were extracted from the NCBI database. Because of their particular structures, prokaryotic NOSes were not included. The phylogenetic tree was constructed 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 1KP 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 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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Acknowledgments: We thank members of the Wendehenne laboratory for helpful comments on the manuscript. **Funding:** The 1KP initiative, led by G.K.-S.W., is funded by the Alberta Ministry of Innovation and Advanced Education, Alberta Innovates Technology Futures, Innovates Centre of Research Excellence, Musea Ventures, BGI-Shenzhen, and China National Genebank. S.J., D. Wipf, and D. Wendehenne are supported by La Région de Bourgogne (PARI AGRALE 8 project) and AgroSup Dijon. **Author contributions:** S.J., D. Wipf, and D. Wendehenne conceived the study and generated the hypotheses; S.J. carried the bioinformatic searches of NOSes and analyzed the data; D.J.S. and L.L. contributed to the analysis of the NOS domains of algal species; M.M., Z.T., Y.Z., E.J.C., and G.K.-S.W. generated the 1KP data and helped in interpreting the data; and S.J. and D. Wendehenne wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The NOS sequences are available upon request to S.J. (sjeandroz@dijon.inra.fr).

Submitted 15 September 2015
Accepted 5 February 2016
Final Publication 1 March 2016
10.1126/scisignal.aad4403
Citation: S. Jeandroz, D. Wipf, D. J. Stuehr, L. Lamattina, M. Melkonian, Z. Tian, Y. Zhu, E. J. Carpenter, G. K.-S. Wong, D. Wendehenne, Occurrence, structure, and evolution of nitric oxide synthase–like proteins in the plant kingdom. *Sci. Signal.* **9**, re2 (2016).

HOST-PATHOGEN INTERACTIONS

The adhesion GPCR BAI1 mediates macrophage ROS production and microbicidal activity against Gram-negative bacteria

Emily A. Billings *et al.* (James E. Casanova)

Citation
Sci. Signal. 02 Feb 2016:
Vol. 9, Issue 413, pp. ra14

10.1126/scisignal.aac6250

The detection of microbes and initiation of an innate immune response occur through pattern recognition receptors (PRRs), which are critical for the production of inflammatory cytokines and activation of the cellular microbicidal machinery. In particular, the production of reactive oxygen species (ROS) by the NADPH oxidase complex is a critical component of the macrophage bactericidal machinery. We previously characterized brain-specific angiogenesis inhibitor 1 (BAI1), a member of the adhesion family of G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors (GPCRs), as a PRR that mediates the selective phagocytic uptake of Gram-negative bacteria by macrophages. We showed that BAI1 promoted phagosomal ROS production through activation of the Rho family guanosine triphosphatase (GTPase) Rac1, thereby stimulating NADPH oxidase activity. Primary BAI1-deficient macrophages exhibited attenuated Rac GTPase activity and reduced ROS production in response to several Gram-negative bacteria, resulting in impaired microbicidal activity. Furthermore, in a peritoneal infection model, BAI1-deficient mice exhibited increased susceptibility to death by bacterial challenge because of impaired bacterial clearance. Together, these findings suggest that BAI1 mediates the clearance of Gram-negative bacteria by stimulating both phagocytosis and NADPH oxidase activation, thereby coupling bacterial detection to the cellular microbicidal machinery.

SYSTEMS IMMUNOLOGY

Comprehensive RNAi-based screening of human and mouse TLR pathways identifies species-specific preferences in signaling protein use

Jing Sun *et al.* (Iain D. C. Fraser)

Citation
Sci. Signal. 05 Jan 2016:
Vol. 9, Issue 409, pp. ra3

10.1126/scisignal.aab2191

Toll-like receptors (TLRs) are a major class of pattern recognition receptors, which mediate the responses of innate immune cells to microbial stimuli. To systematically determine the roles of proteins in canonical TLR signaling pathways, we conducted an RNA interference (RNAi)–based screen in human and mouse macrophages. We observed a pattern of conserved signaling module dependencies across species, but found notable species-specific requirements at the level of individual proteins. Among these, we identified unexpected differences in the involvement of members of the interleukin-1 receptor–associated kinase (IRAK) family between the human and mouse TLR pathways. Whereas TLR signaling in mouse macrophages depended primarily on IRAK4 and IRAK2, with little or no role for IRAK1, TLR signaling and proinflammatory cytokine production in human macrophages depended on IRAK1, with knockdown of IRAK4 or IRAK2 having less of an effect. Consistent with species-specific roles for these kinases, IRAK4 orthologs failed to rescue signaling in IRAK4-deficient macrophages from the other species, and only mouse macrophages required the kinase activity of IRAK4 to mediate TLR responses. The identification of a critical role for IRAK1 in TLR signaling in humans could potentially explain the association of IRAK1 with several autoimmune diseases. Furthermore, this study demonstrated how systematic screening can be used to identify important characteristics of innate immune responses across species, which could optimize therapeutic targeting to manipulate human TLR-dependent outputs.

HOST-PATHOGEN INTERACTIONS

Rosmarinic acid is a homoserine lactone mimic produced by plants that activates a bacterial quorum-sensing regulator

Andrés Corral-Lugo *et al.* (Tino Krell)

Citation
Sci. Signal. 05 Jan 2016:
Vol. 9, Issue 409, pp. ra1

10.1126/scisignal.aaa8271

Quorum sensing is a bacterial communication mechanism that controls genes, enabling bacteria to live as communities, such as biofilms. Homoserine lactone (HSL) molecules function as quorum-sensing signals for Gram-negative bacteria. Plants also produce previously unidentified compounds that affect quorum sensing. We identified rosmarinic acid as a plant-derived compound that functioned as an HSL mimic. In vitro assays showed that rosmarinic acid bound to the quorum-sensing regulator RhlR of *Pseudomonas aeruginosa* PAO1 and competed with the bacterial ligand *N*-butanoyl-homoserine lactone (C4-HSL). Furthermore, rosmarinic acid stimulated a greater increase in RhlR-mediated transcription in vitro than that of C4-HSL. In *P. aeruginosa*, rosmarinic acid induced quorum sensing–dependent gene expression and increased biofilm formation and the production of the virulence factors pyocyanin and elastase. Because *P. aeruginosa* PAO1 infection induces rosmarinic acid secretion from plant roots, our results indicate that rosmarinic acid secretion is a plant defense mechanism to stimulate a premature quorum-sensing response. *P. aeruginosa* is a ubiquitous pathogen that infects plants and animals; therefore, identification of rosmarinic acid as an inducer of premature quorum-sensing responses may be useful in agriculture and inform human therapeutic strategies.

PLANT BIOLOGY

Nitrate sensing and uptake in Arabidopsis are enhanced by ABI2, a phosphatase inactivated by the stress hormone abscisic acid

Sophie Léran *et al.* (Benoît Lacombe)

Citation
Sci. Signal. 05 May 2015:
Vol. 8, Issue 375, pp. ra43

10.1126/scisignal.aaa4829

Living organisms sense and respond to changes in nutrient availability to cope with diverse environmental conditions. Nitrate (NO3_) is the main source of nitrogen for plants and is a major component in fertilizer. Unraveling the molecular basis of nitrate sensing and regulation of nitrate uptake should enable the development of strategies to increase the efficiency of nitrogen use and maximize nitrate uptake by plants, which would aid in reducing nitrate pollution. NPF6.3 (also known as NRT1.1), which functions as a nitrate sensor and transporter; the kinase CIPK23; and the calcium sensor CBL9 form a complex that is crucial for nitrate sensing in *Arabidopsis thaliana*. We identified two additional components that regulate nitrate transport, sensing, and signaling: the calcium sensor CBL1 and protein phosphatase 2C family member ABI2, which is inhibited by the stress-response hormone abscisic acid. Bimolecular fluorescence complementation assays and in vitro kinase assays revealed that ABI2 interacted with and dephosphorylated CIPK23 and CBL1. Coexpression studies in *Xenopus* oocytes and analysis of plants deficient in ABI2 indicated that ABI2 enhanced NPF6.3-dependent nitrate transport, nitrate sensing, and nitrate signaling. These findings suggest that ABI2 may functionally link stress-regulated control of growth and nitrate uptake and utilization, which are energy-expensive processes.

CELL BIOLOGY

Proximity biotinylation provides insight into the molecular composition of focal adhesions at the nanometer scale

Jing-Ming Dong *et al.* (Ed Manser)

Citation
Sci. Signal. 14 Jun 2016:
Vol. 9, Issue 432, pp. rs4

10.1126/scisignal.aaf3572

Focal adhesions are protein complexes that link metazoan cells to the extracellular matrix through the integrin family of transmembrane proteins. Integrins recruit many proteins to these complexes, referred to as the "adhesome." We used proximity-dependent biotinylation (BioID) in U2OS osteosarcoma cells to label proteins within 15 to 25 nm of paxillin, a cytoplasmic focal adhesion protein, and kindlin-2, which directly binds β integrins. Using mass spectrometry analysis of the biotinylated proteins, we identified 27 known adhesome proteins and 8 previously unknown components close to paxillin. However, only seven of these proteins interacted directly with paxillin, one of which was the adaptor protein Kank2. The proteins in proximity to β integrin included 15 of the adhesion proteins identified in the paxillin BioID data set. BioID also correctly established kindlin-2 as a cell-cell junction protein. By focusing on this smaller data set, new partners for kindlin-2 were found, namely, the endocytosis-promoting proteins liprin β1 and EFR3A, but, contrary to previous reports, not the filamin-binding protein migfilin. A model adhesome based on both data sets suggests that focal adhesions contain fewer components than previously suspected and that paxillin lies away from the plasma membrane. These data not only illustrate the power of using BioID and stable isotope–labeled mass spectrometry to define macromolecular complexes but also enable the correct identification of therapeutic targets within the adhesome.