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Identification of a novel *Arabidopsis thaliana* nitric oxide-binding molecule with guanylate cyclase activity *in vitro*

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ABSTRACT

While there is evidence of nitric oxide (NO)-dependent signalling via the second messenger cyclic guanosine 3′,5′-monophosphate (cGMP) in plants, guanylate cyclases (GCs), enzymes that catalyse the formation of cGMP from guanosine 5′-triphosphate (GTP) have until recently remained elusive and none of the candidates identified to-date are NO-dependent. Using both a GC and heme-binding domain specific (H-NOX) search motif, we have identified an Arabidopsis flavin monooxygenase (At1g62580) and shown electrochemically that it binds NO, has a higher affinity for NO than for O_2 and that this molecule can generate cGMP from GTP *in vitro* in an NO-dependent manner.

1. Introduction

In higher plants, cyclic guanosine 3′,5′ monophosphate (cGMP) functions as second messenger in many physiological processes [1–9] including nitric oxide (NO)-dependent signalling [6,8], biotic [5–7] and abiotic stress responses [2,4,10–12], NaCl and drought stress [10–12], ozone [13] and pathogen challenge [5–7,14,15]. In tobacco, cGMP is an essential downstream signalling molecule in NO-mediated pathogen defence responses and is required for the induction of defence-related genes such as phenylalanine ammonia lyase (*PAL*) as well as for the activation of PAL enzyme activity [6] which generates precursors for phenylpropanoid and thus salicylic acid (SA) biosynthesis [16]. It has also been shown that both the virulent and avirulent strains of *Pseudomonas syringae* induce an increase in plant endogenous cGMP generation, with the avrB strain inducing a more rapid and bigger response [15].

In higher plants, the first GC was discovered using a search motif strategy based on conserved and functionally assigned amino acid residues in the catalytic centre of eukaryotic GCs [17]. Site directed mutagenesis has since lead to the discovery of additional GCs [7,18]. However, none of these GCs have been shown to be NO-dependent or indeed to contain a heme-binding site.

With a view to discover NO-dependent GCs in plants, we have used both a GC search motif as well as a motif that we deduced from the heme-NO and oxygen-binding (H-NOX) domains [19] to identify a candidate protein that binds NO. The identified protein binds NO with higher affinity than O_2 and generates cGMP from guanosine 5′ triphosphate (GTP) in an NO-dependent manner *in vitro*.

2. Materials and methods

2.1. Cloning and expression of AtNOGC1

Total RNA from Arabidopsis seedlings was extracted with RNeasy Plant Mini Kit (Qiagen GmbH, Germany) and 200 ng was used for first strand cDNA synthesis (reverse primer: 5′-gct ag<u>a</u>

att ctc agc ttg tat att ctc ttt cga-3′) using the Omniscript RT Kit (Qiagen GmbH, Germany). The AtNOGC1 gene was PCR-amplified using the forward primer (5′-gat gga tcc atg gta cca gca gta aat cct-3′) and the reverse primer and cloned into pETSUMO vector (Invitrogen).

AtNOGC1 was expressed in BL21 Star (pLysS) *Escherichia coli* cells (Invitrogen, Carlsbad, USA) and was purified as a His-tagged SUMO-fusion using a Ni-NTA affinity chromatography (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Electrochemical procedures and apparatus

Electrochemical experiments were carried out at 25 °C using a BAS Epsilon electrochemical workstation (BioAnalytical Systems, West Lafayette, IN) and voltammograms were recorded with a computer interfaced to the workstation. A 0.071 cm^2 glassy carbon electrode (GCE) (BioAnalytical Systems, West Lafayette, IN, USA) was used as the working electrode, Ag/AgCl with a 3 M NaCl salt bridge electrode served as reference and a platinum wire as the auxiliary electrode in a 20 mL cell. For anaerobic conditions, solutions were purged with argon for 20 min and maintained under an argon blanket. The GCE was polished with 1.0, 0.3 and 0.05 μm alumina (Buehler, IL, USA) and washed with distilled water before ultrasonication for 5 min in distilled water and 5 min in ethanol. The GCE was dried in a stream of N$_2$ for 10 s before drop coating with protein.

2.3. Preparation of AtNOGC1 bioelectrode and voltammetric measurements

The bioelectrode was prepared by immobilizing an *AtNOGC1*-didodecyldimethylammonium bromide (DDAB)-BSA film on the polished GCE using a method described previously [20]. NO was generated from copper granules and 5 M HNO$_3$ [21,22] and voltammetric experiments were performed in phosphate buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). Voltammograms were recorded in the presence and absence of substrate at a potential scan rate of 2 mV s^{-1} from initial potential, E_i = +300 mV to a switch potential, E_λ = −800 mV (for O$_2$-containing experiments) or −500 mV (for experiments containing only NO). Amplitude of 25 mV and a frequency of 15 mV were used for square wave voltammetry.

2.4. Guanylate cyclase activity assays

GC activity was assessed *in vitro* as detailed previously [18] using an immunoassay kit following the acetylation protocol as described in the supplier's manual (Sigma–Aldrich, Saint Louis, Missouri; code CG-201). All methods are more extensively detailed in a Supplementary file.

3. Results and discussion

3.1. Identification of AtNOGC1

Searching for H-NOX domains in Arabidopsis, we have identified a candidate protein with a conserved heme-binding motif (Hx{12}Px{14,16}YxSxR) that is annotated as flavin-dependent monooxygenase (AtNOGC1; At1g62580) (Fig. 1) and also contains the 14 amino acid catalytic centre found in experimentally tested plant GCs [7,17,18].

Assessment of O$_2$-binding by AtNOGC1 with our bioelectrode using cyclic voltammetry showed a cathodic response with a cathodic peak potential (E_{pc}) value of −500 mV under aerobic conditions (Fig. 2), indicating catalytic coupling of the reduction of Fe^{3+}
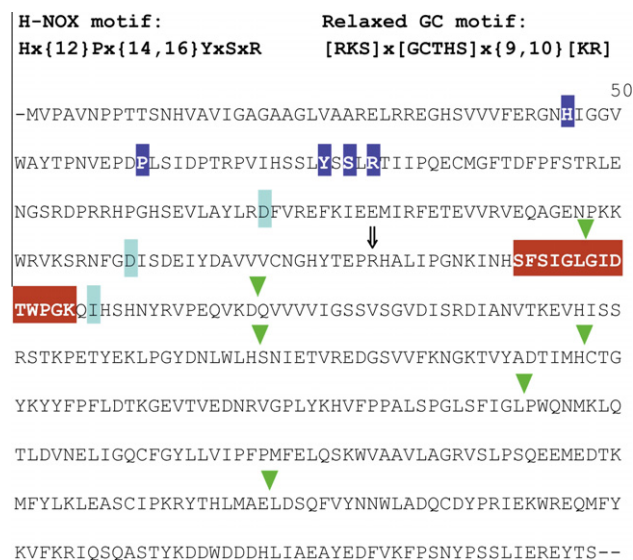

Fig. 1. Sequence of the *Arabidopsis thaliana* NO-binding protein (AtNOGC1). The H-NOX (H-x{12}-P-x{14,16}-Y-x-S-x-R) motif (curly brackets indicate the gap sizes) is highlighted in blue and the GC catalytic centre is highlighted in red. The putative metal-binding residues are highlighted in aquamarine. The black arrow points to the PPi-binding residue and the green arrows indicate exon boarders.
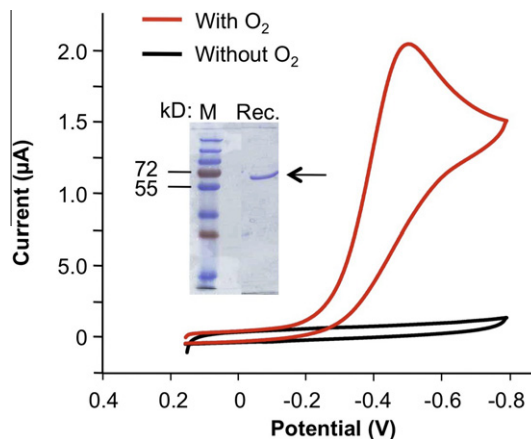

Fig. 2. Cyclic voltammogram showing the response of an AtNOGC1 in phosphate buffer in the presence or absence of O$_2$. For the anaerobic conditions (black), the oxygen in the buffer was purged with argon (flow rate of 10 l/min). Under aerobic conditions, the buffer was equilibrated with air to achieve maximum O$_2$ saturation (red). The scan shows a reduction peak (corresponding to the binding of oxygen) as the voltage applied to the system reaches about −0.5 V, and no species were oxidised in the reverse scan. The potential ranged from +0.15 to −0.8 V and the scan rate was 2 mV s^{-1}. Inset: SDS–PAGE gel showing the recombinant AtNOGC1 protein (∼67.7 kDa) used as bio-sensor.

to the oxygenation of Fe^{2+}. The cathodic peak current (2.2 μA) under aerobic conditions is larger by three orders of magnitude than that under anaerobic conditions (0.005 μA). During the cathodic scan, Fe^{2+} was generated and used up in a fast follow-up oxygenation reaction making it unavailable for re-oxidation during the anodic scan. The peak potential (E_p) for the binding of oxygen to the protein occurs at −500 mV while the AtNOGC1-Fe$^{III/II}$ reduction process occurs at −140 mV. Large shifts to higher reduction potential under aerobic conditions mean that the monooxygenation is a catalytic redox process involving several reaction steps and the irreversible cyclic voltammograms suggest binding of molecular oxygen to the Fe^{2+}-heme redox centre.

Further, we investigated the responses of the bioelectrode in the absence and presence of NO. In the absence of NO, anodic and