On-line Supporting Information

A twin-track approach has optimised proton and hydride transfer by dynamically-coupled tunnelling during the evolution of protochlorophyllide oxidoreductase.

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Supplementary tables

Table S1. The rate constants and thermodynamic parameters for the hydride transfer step for the various POR enzymes in the presence of either *pro-S* NADPH or *S*-NADP^{2}H (*S*-NADPD). All rate constants were measured at 25°C as described in the *Methods*. The enthalpies of activation, $\Delta H^{\ddagger}$, and the entropies of activation, $\Delta S^{\ddagger}$, have been calculated by fitting the temperature dependence data to the Eyring equation.

	Thermosyn. elongatus	***Synechocystis sp.* PCC6803**	***Gloeobacter violaceus***	***Arabidopsis* POR B**
k_{H^-} (s^{-1} x10^6)	1.87 ± 0.17	1.86 ± 0.18	1.86 ± 0.12	1.81 ± 0.24
k_{D^-} (s^{-1} x10^6)	0.98 ± 0.14	0.98 ± 0.13	0.95 ± 0.18	0.96 ± 0.11
KIE	1.91 ± 0.17	1.90 ± 0.16	1.94 ± 0.13	1.91 ± 0.23
$\Delta H^{\ddagger}$ (kJ mol^{-1})	9.3 ± 0.4	11.7 ± 0.2	13.3 ± 0.5	13.6 ± 0.7
(< -27ºC)	(27.2 ± 0.5)	(35.0 ± 1.0)	(33.1 ± 1.0)	(33.7 ± 1.0)
$\Delta\Delta H^{\ddagger}$ (kJ mol^{-1})	8.2 ± 0.6	7.0 ± 0.3	6.9 ± 0.7	7.6 ± 0.9
(< -27ºC)	(0.4 ± 1.0)	(0.4 ± 1.5)	(0.5 ± 1.2)	(0.3 ± 1.2)
$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	-94.4 ± 1.2	-85.9 ± 0.5	-78.6 ± 1.1	-79.4 ± 1.7
(< -27ºC)	(-21.8 ± 0.3)	(10.0 ± 0.2)	(0.3 ± 0.1)	(2.8 ± 0.1)
ln A'	12.4 ± 0.2	13.4 ± 0.1	14.3 ± 0.2	14.2 ± 0.3
(< -27ºC)	(21.1 ± 0.3)	(25.0 ± 0.5)	(23.8 ± 0.5)	(24.1 ± 0.5)
A'_H/A'_D	0.08 ± 0.02	0.11 ± 0.01	0.15 ± 0.05	0.08 ± 0.04
(< -27ºC)	(3.6 ± 2.0)	(3.4 ± 2.7)	(3.1 ± 1.9)	(3.5 ± 2.3)

Table S2. The rate constants and thermodynamic parameters for the proton transfer step for the various POR enzymes in either H_2O or ^2H_2O (D_2O) buffers. All rate constants were measured at 25°C as described in the *Methods*. The enthalpies of activation, $\Delta H^\ddagger$, and the entropies of activation, $\Delta S^\ddagger$, have been calculated by fitting the temperature dependence data to the Eyring equation.

	Thermosyn. elongatus	***Synechocystis sp.* PCC6803**	***Gloeobacter violaceus***	***Arabidopsis* POR B**
k_{H^+} (s^{-1} x10^3)	27.4 ± 1.0	30.4 ± 0.6	152.0 ± 5.7	123.3 ± 3.7
k_{D^+} (s^{-1} x10^3)	13.6 ± 0.8	16.9 ± 0.7	98.9 ± 2.7	82.0 ± 1.9
SIE	2.01 ± 0.14	1.80 ± 0.08	1.54 ± 0.07	1.50 ± 0.07
$\Delta H^\ddagger$ (kJ mol^{-1})	53.7 ± 1.6	52.1± 2.1	43.9 ± 0.8	36.7 ± 1.3
$\Delta\Delta H^\ddagger$ (kJ mol^{-1})	15.6 ± 3.1	17.6 ± 3.7	2.6 ± 2.4	1.0 ± 1.9
$\Delta S^\ddagger$ (J mol^{-1} K^{-1})	19.4 ± 0.5	14.4 ± 0.5	1.7 ± 0.1	-24.3 ± 0.6
ln A'	26.1 ± 0.6	25.5 ± 0.8	24.0 ± 0.3	20.8 ± 0.5
A'_H/A'_D	0.004 ± 0.005	0.002 ± 0.003	0.55 ± 0.57	0.90 ± 0.70

Table S3. Rate constants of the product release steps for the various POR enzymes. All values were measured at 25°C as described in the *Methods*. n/a means not applicable as no rate constant is measurable. Standard errors are within 5% of the measured values for the rate constants. These are approximate rate constants for each kinetic phase fitted by using a single exponential expression to each resolved phase as previously described (2), rather than fitting to a more complex sequential model. Data for *T. elongatus* is taken from (2).

Product release event	***Thermosyn. elongatus***	***Synechocystis sp*. PCC6803**	***Gloeobacter violaceus***	***Arabidopsis* POR B**
Conformational change	350 s^{-1}	350 s^{-1}	n/a	n/a
$NADP^+$ release	45 s^{-1}	45 s^{-1}	10 s^{-1}	65 s^{-1}
NADPH rebinding	16 s^{-1}	n/a	1 s^{-1}	11 s^{-1}
Chlide release	1.7 s^{-1}	0.2 s^{-1}	0.04 s^{-1}	1.4 s^{-1}

Supplementary Figures

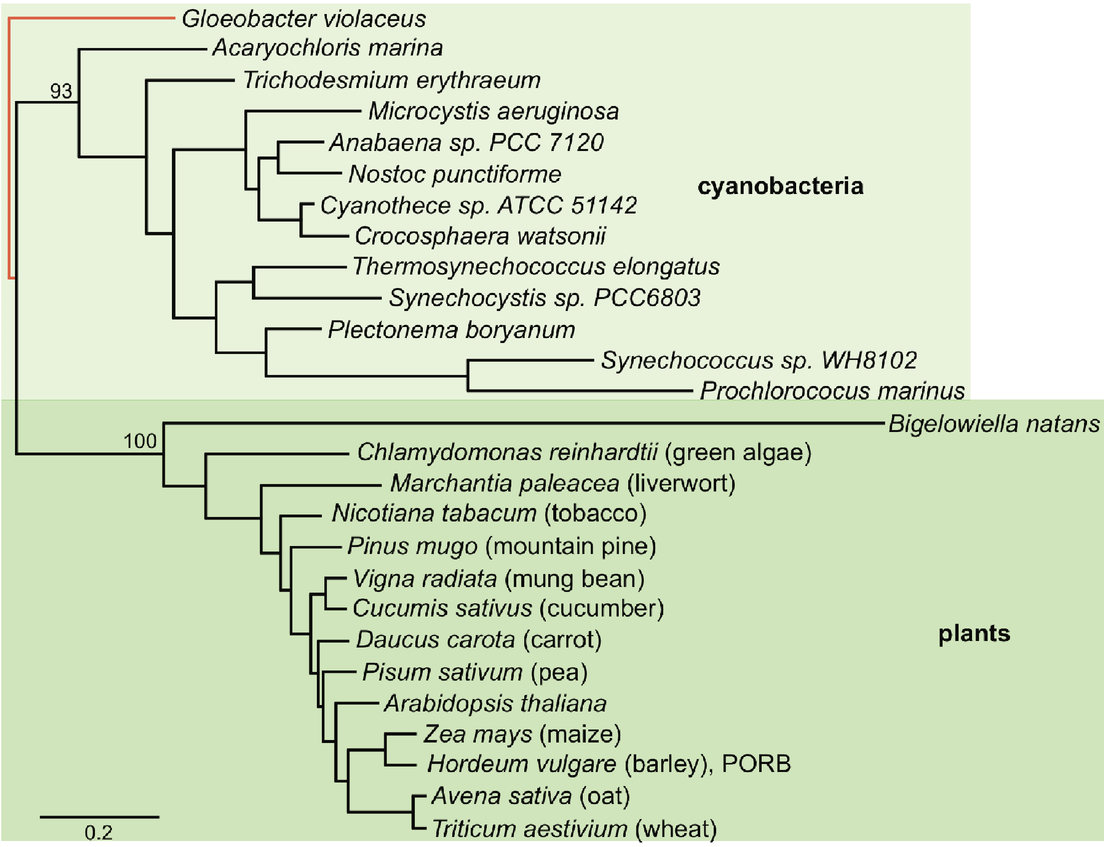
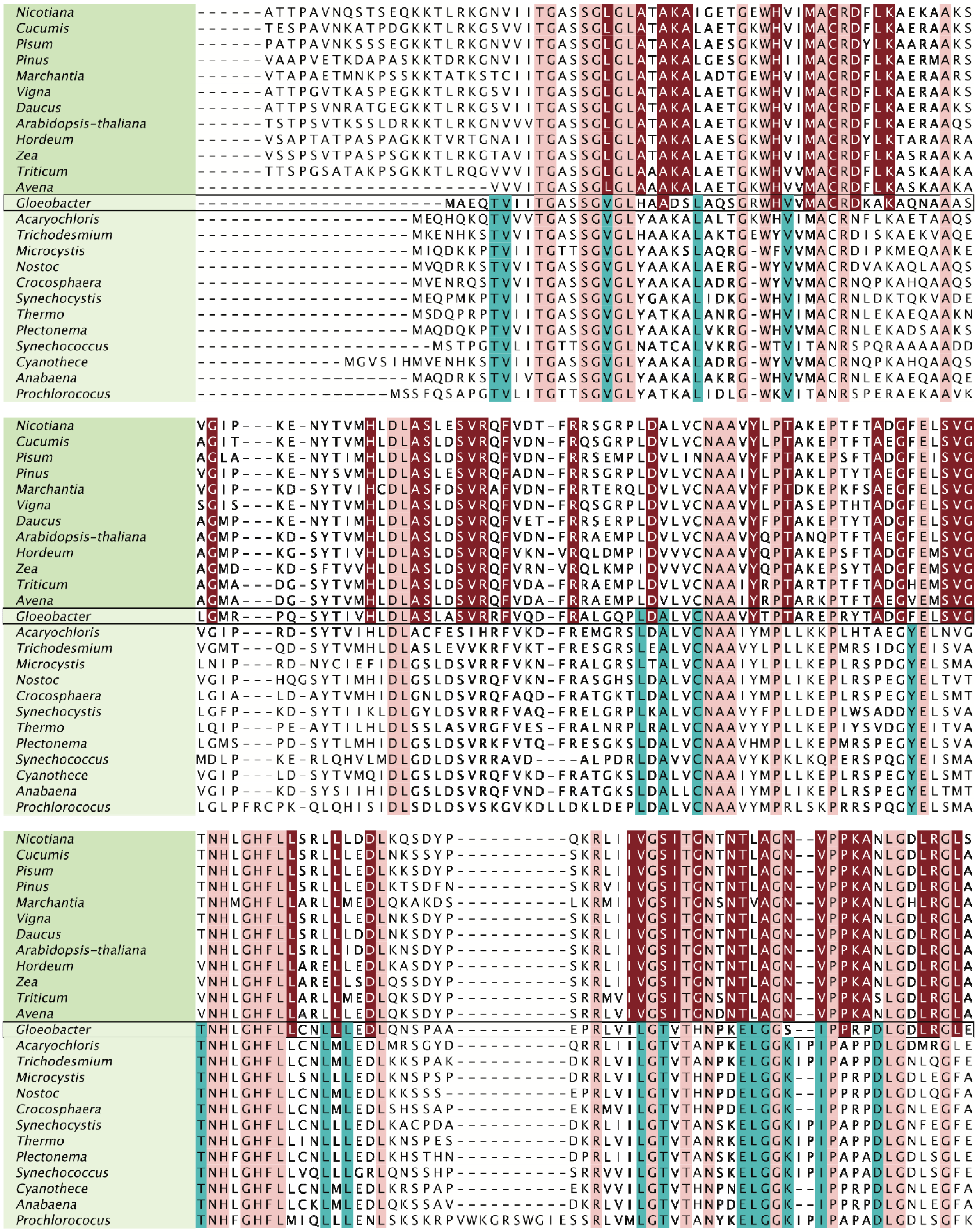


Figure S1. Inferred evolutionary history of plant and cyanobacteria PORs based on the alignment in panel B. The scale bar represents amino acid replacements per site. Numbers next to nodes correspond to % bootstrap replicates; only shown for the main cyanobacteria and plant clades.



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Cucumis ------TESPAVNKATPDGKKTLRKGSVVITGASSGLGLATAKALAETGKWHVIMACRDFLKAERAAKS
Pisum ------PATPAVNKSSSEGKKTLRKGNVVITGASSGLGLATAKALAESGKWHVIMACRDYLKAARAAKS
Pinus ------VAAPVETKDAPASKKTDRKGNVIITGASSGLGLATAKALGESGKWHIIMACRDFLKAERMARS
Marchantia ------VTAPAETMNKPSSKKTATKSTCIITGASSGLGLATAKALADTGEWHVIMACRDFLKAERAARS
Vigna ------ATTPGVTKASPEGKKTLRKGSVIITGASSGLGLATAKALAETGKWHVIMACRDFLKAERAAKS
Daucus ------ATTPSVNRATGEGKKTLRKGSVIITGASSGLGLATAKALAETGKWHVIMACRDFLKAERAAKS
Arabidopsis-thaliana ------TSTPSVTKSSLDRKKTLRKGNVVVTGASSGLGLATAKALAETGKWHVIMACRDFLKAERAAQS
Hordeum ------VSAPTATPASPAGKKTVRTGNAIITGASSGLGLATAKALAESGKWHVIMACRDYLKTARAARA
Zea ------VSSPSVTPASPSGKKTLRKGTAVITGASSGLGLATAKALAETGKWHVIMACRDFLKASRAAKA
Triticum ------TTSPGSATAKPSGKKTLRQGVVVITGASSGLGLAAAKALAETGKWHVVMACRDFLKASKAAKA
Avena --------------------------VVVITGASSGLGLAAAKALAETGKWHVVMACRDFLKASKAAKA
Gloeobacter ----------------------MAEQTVIITGASSGVGLHAADSLAQSGRWHVVMACRDKAKAQNAAAS
Acaryochloris -------------------MEQHQKQTVVVTGASSGVGLYAAKALALTGKWHVIMACRNFLKAETAAQS
Trichodesmium -------------------MKENHKSTVVITGASSGVGLHAAKALAKTGEWYVVMACRDISKAEKVAQE
Microcystis -------------------MIQDKKPTVIITGTTSGVGLYAAKSLAQRG-WFVVMACRDIPKMEQAAKE
Nostoc -------------------MVQDRKSTVVITGASSGVGLYAAKALAERG-WYVVMACRDVAKAQLAAQS
Crocosphaera -------------------MVENRQSTVVITGASSGVGLYAAKALADRG-WYVVMACRNQPKAHQAAQS
Synechocystis -------------------MEQPMKPTVIITGASSGVGLYGAKALIDKG-WHVIMACRNLDKTQKVADE
Thermo -------------------MSDQPRPTVIITGASSGVGLYATKALANRG-WHVIMACRNLEKAEQAAKN
Plectonema -------------------MAQDQKPTVVITGASSGVGLYAAKALVKRG-WHVVMACRNLEKADSAAKS
Synechococcus ---------------------MSTPGTVLITGTTSGVGLNATCALVKRG-WTVITANRSPQRAAAAADD
Cyanothece -------------MGVSIHMVENHKSTVIITGASSGVGLYAAKALADRG-WYVVMACRNQPKAHQAAQS
Anabaena -------------------MAQDRKSTVIVTGASSGVGLYAAKALAKRG-WHVVMACRNLEKAEQAAQE
Prochlorococcus -----------------MSSFQSAPGTVLITGTTSGVGLYATKALIDLG-WKVITANRSPERAEKAAVK

Nicotiana VGIP---KE-NYTVMHLDLASLESVRQFVDT-FRRSGRPLDALVCNAAVYLPTAKEPTFTADGFELSVG
Cucumis AGIT---KE-NYTVMHLDLASLDSVRQFVDN-FRQSGRPLDVLVCNAAVYLPTAKEPTFTAEGFELSVG
Pisum AGLA---KE-NYTIMHLDLASLDSVRQFVDN-FRRSEMPLDVLINNAAVYFPTAKEPSFTADGFEISVG
Pinus VGIP---KE-NYSVMHLDLASLESVRQFADN-FRRSGRPLDVLVCNAAIYLPTAKLPTYTAEGFELSVG
Marchantia VGIP---KD-SYTVIHCDLASFDSVRAFVDN-FRRTERQLDVLVCNAAVYFPTDKEPKFSAEGFELSVG
Vigna SGIS---KE-NYTVMHLDLASLDSVRQFVDN-FRQSGRPLDVLVCNAAVYLPTASEPTHTADGFELSVG
Daucus AGMP---KE-NYTIMHLDLASLDSVRQFVET-FRRSERPLDVLVCNAAVYFPTAKEPTYTADGFELSVG
Arabidopsis-thaliana AGMP---KD-SYTVMHLDLASLDSVRQFVDN-FRRAEMPLDVLVCNAAVYQPTANQPTFTAEGFELSVG
Hordeum AGMP---KG-SYTIVHLDLASLDSVRQFVKN-VRQLDMPIDVVVCNAAVYQPTAKEPSFTADGFEMSVG
Zea AGMD---KD-SFTVVHLDLASLDSVRQFVRN-VRQLKMPIDVVVCNAAVYQPTAKEPSYTADGFEMSVG
Triticum AGMA---DG-SYTVMHLDLASLDSVRQFVDA-FRRAEMPLDVLVCNAAIYRPTARTPTFTADGHEMSVG
Avena AGMA---DG-SYTVMHLDLASLDSVRQFVDA-FRRAEMPLDVLVCNAAIYRPTARKPTFTAEGVEMSVG
Gloeobacter LGMR---PQ-SYTIVHLDLASLASVRRFVQD-FRALGQPLDALVCNAAVYTPTAREPRYTADGFELSVG
Acaryochloris VGIP---RD-SYTVIHLDLACFESIHRFVKD-FREMGRSLDALVCNAAIYMPLLKKPLHTAEGYELNVG
Trichodesmium VGMT---QD-SYTVMHLDLASLEVVKRFVKT-FRESGRSLEALVCNAAVYLPLLKEPMRSIDGYEISVA
Microcystis LNIP---RD-NYCIEFIDLGSLDSVRRFVKN-FRALGRSLTALVCNAAIYLPLLKEPLRSPDGYELSMA
Nostoc VGIP---HQGSYTIMHIDLGSLDSVRQFVKN-FRASGHSLDALVCNAAIYMPLIKEPLRSPEGYELTVT
Crocosphaera LGIA---LD-AYTVMHIDLGNLDSVRQFAQD-FRATGKTLDALVCNAAIYMPLLKEPLRSPEGYELSMT
Synechocystis LGFP---KD-SYTIIKLDLGYLDSVRRFVAQ-FRELGRPLKALVCNAAVYFPLLDEPLWSADDYELSVA
Thermo LQIP---PE-AYTILHLDLSSLASVRGFVES-FRALNRPLRALVCNAAVYYPLLKEPIYSVDGYEITVA
Plectonema LGMS---PD-SYTLMHIDLGSLDSVRKFVTQ-FRESGKSLDALVCNAAVHMPLLKEPMRSPEGYELSVA
Synechococcus MDLP---KE-RLQHVLMDLGDLDSVRRAVD----ALPDRLDAVVCNAAVYKPKLKQPERSPQGYEISMA
Cyanothece VGIP---LD-SYTVMQIDLGSLDSVRQFVKD-FRATGKSLDALVCNAAIYMPLLKEPLRSPEGYELSMT
Anabaena VGIP---KD-SYSIIHIDLGSLDSVRQFVND-FRATGKSLDALLCNAAIYMPLIKEPLRSPEGYELTMT
Prochlorococcus LGLPFRCPK-QLQHISIDLSDLDSVSKGVKDLLDKLDEPLDALVCNAAVYMPRLSKPRRSPQGYELSMA

Nicotiana TNHLGHFLLSRLLLDDLKQSDYP----------QKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLS
Cucumis TNHLGHFLLSRLLLEDLNKSSYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Pisum TNHLGHFLLSRLLLEDLKKSDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Pinus TNHLGHFLLSRLLLEDLKTSDFN----------SKRVIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Marchantia TNHMGHFLLARLLMEDLQKAKDS----------LKRMIIVGSITGNSNTVAGN--VPPKANLGHLRGLA
Vigna TNHLGHFLLSRLLLEDLNKSDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Daucus TNHLGHFLLSRLLLDDLNKSDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Arabidopsis-thaliana INHLGHFLLSRLLIDDLKNSDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Hordeum VNHLGHFLLARELLEDLKASDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Zea VNHLGHFLLARELLSDLQSSDYP----------SKRLIIVGSITGNTNTLAGN--VPPKANLGDLRGLA
Triticum VNHLGHFLLARLLMEDLQKSDYP----------SRRMVIVGSITGNSNTLAGN--VPPKASLGDLRGLA
Avena VNHLGHFLLARLLLEDLQKSDYP----------SRRLVIVGSITGNDNTLAGN--VPPKANLGDLRGLA
Gloeobacter TNHLGHFLLCNLLLEDLQNSPAA----------EPRLVILGTVTHNPKELGGS--IPPRPDLGDLRGLE
Acaryochloris TNHLGHFLLCNLMLEDLMRSGYD----------QRRLIILGTVTANPKELGGKIPIPAPPDLGDMRGLE
Trichodesmium TNHLGHFLLCNLMLEDLKKSPAK----------DKRLVILGTVTANPKELGGKIPIPAPPDLGNLQGFE
Microcystis TNHLGHFLLSNLLLEDLKNSPSP----------DRRLVILGTVTHNPDELGGK--IPPRPDLGDLEGFA
Nostoc TNHLGHFLLCNLMLEDLKKSSS-----------EPRLVILGTVTHNPDELGGK--IPPRPDLGDLQGFA
Crocosphaera TNHLGHFLLCNLMLEDLSHSSAP----------EKRMVILGTVTHNPDELGGK--IPPRPDLGNLEGFA
Synechocystis TNHLGHFLLCNLLLEDLKACPDA----------DKRLIILGTVTANSKELGGKIPIPAPPDLGNFEGFE
Thermo TNHLGHFLLINLLLEDLKNSPES----------DKRLVILGTVTANRKELGGKIPIPAPPDLGNLEGFE
Plectonema TNHFGHFLLCNLLLEDLKHSTHN----------DPRLIILGTVTANSKELGGKIPIPAPADLGDLSGLE
Synechococcus TNHLGHFLLVQLLLGRLQNSSHP----------SRRVVILGTVTANSKELGGKIPIPAPADLGDLSGFE
Cyanothece TNHLGHFLLCNLMLEDLKRSPAP----------EKRVVILGTVTHNPNELGGK--IPPRPDLGNLEGFA
Anabaena TNHLGHFLLCKLMLEDLQKSSAV----------DKRLVILGTVTHNPDELGGK--IPPRPDLGNLEGFA
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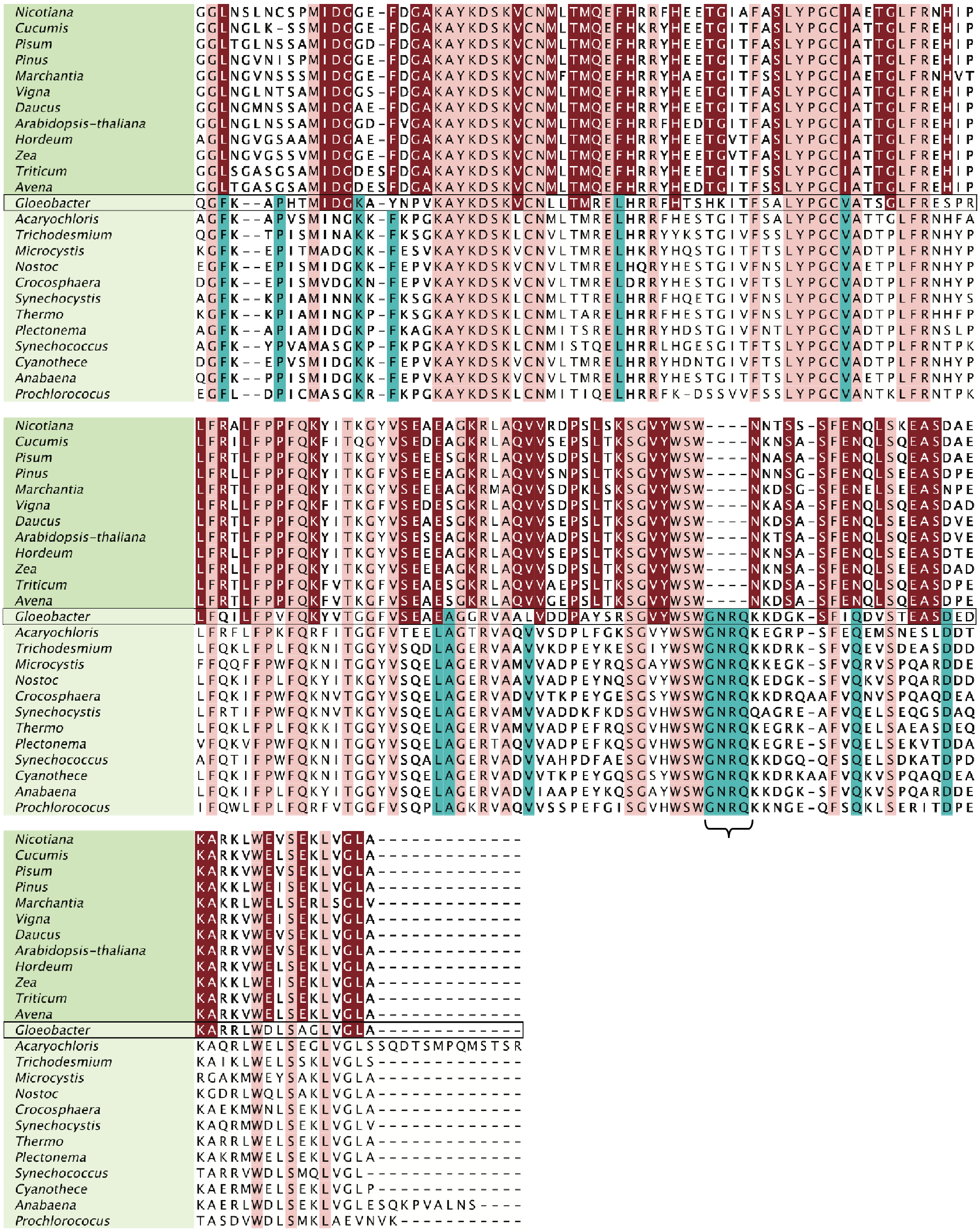

Figure S2. Sequence alignment highlighting total conservation (pink) and differential conservation in the cyanobacterial (green) and plant PORs (red). *Gloeobacter violaceus* (black box) shows a mixture of conservation of features relating to both groups of POR. Species 'Thermo' represents the sequence from *Thermosynechococcus elongatus*. The bracket indicates the additional 4 residues at position 281 (numbering in *T. elongatus* POR) in the cyanobacteria PORs referred to in the main manuscript.

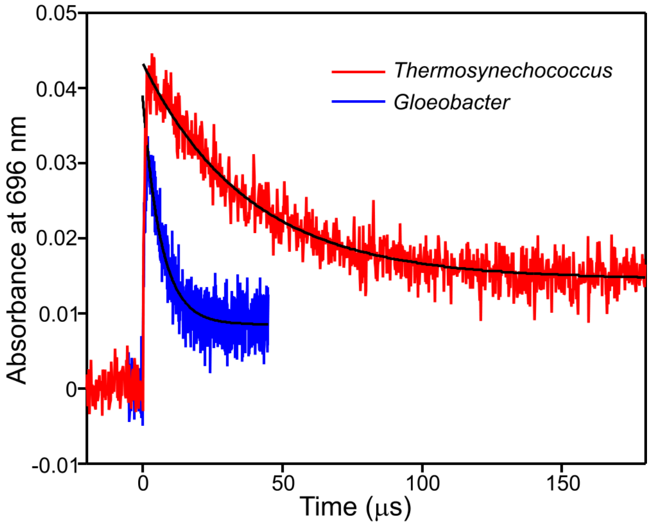

Figure S3. Kinetic transients showing the absorbance changes at 696 nm for POR from *Thermosynechococcus elongatus* and *Gloeobacter violaceus* following laser photoexcitation at 450 nm. The initial increase in absorbance represents hydride transfer (1) and the slower decrease in absorbance represents the proton transfer step (1). Transients were collected at 25°C as described in the *Materials and Methods*.

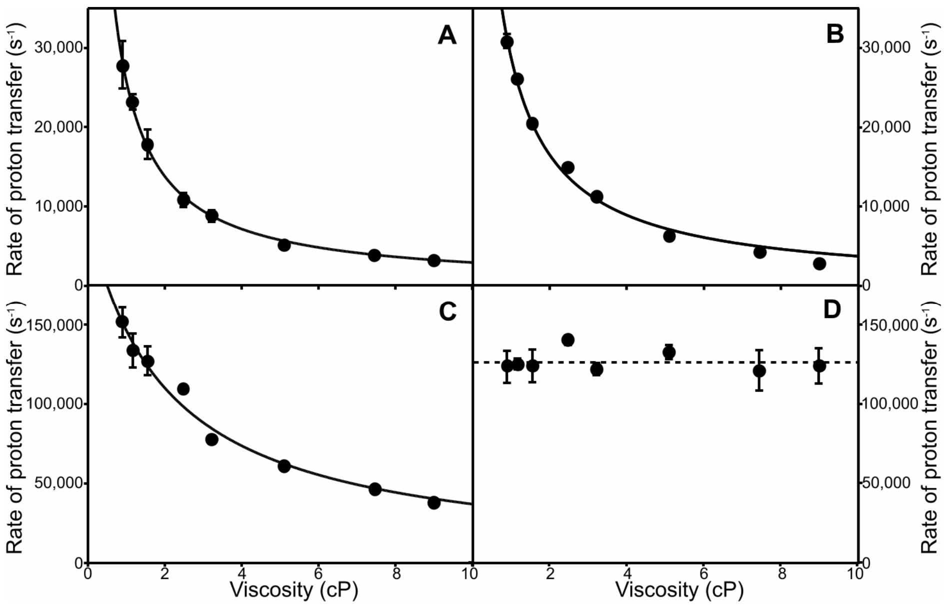

Figure S4. The viscosity-dependence of the proton transfer reaction. The viscosity dependence of the rate constant for the decrease in absorbance at 696 nm, representing proton transfer, is shown for POR from *Thermosynechococcus elongatus* (**A**), *Synechocystis* sp. PCC6803 (**B**), *Gloeobacter violaceus* (**C**) and *Arabidopsis thaliana* POR B (**D**). All measurements were recorded over a range of timescales as described in the *Methods*. The error bars were calculated from the average of at least 3 traces.

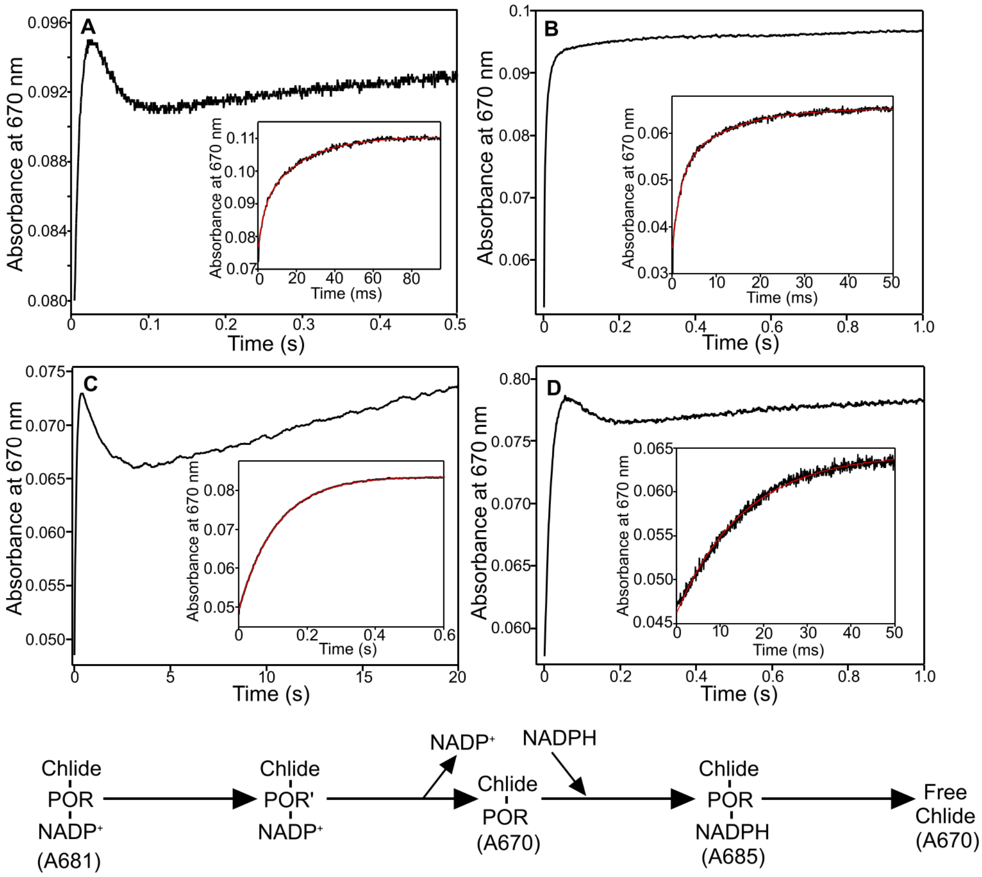

Figure S5. Kinetic transients showing product release steps following laser photoexcitation at 450 nm. Transients, showing all three spectroscopic changes associated with the product release and NADPH re-binding events, were measured at 670 nm for POR from *Thermosynechococcus elongatus* (**A**), *Synechocystis* sp. PCC6803 (**B**), *Gloeobacter violaceus* (**C**) and *Arabidopsis thaliana* POR B (**D**) (2). All measurements were recorded over a range of timescales at 25°C as described in the *Methods*. A scheme of the product release steps is shown at the bottom to indicate the spectral change associated with each phase (2). The rates of each product release step are shown in Table S3. Panel A is taken from (2).

Supplementary References

1. Heyes, D. J., Sakuma, M., De Visser, S. & Scrutton, N. S. (2009) Nuclear quantum tunneling in the light-activated enzyme protochlorophyllide oxidoreductase. *J. Biol. Chem.* **284:** 3762-3767.
2. Heyes, D. J., Sakuma, M. & Scrutton, N. S. (2007) Laser excitation studies of the product release steps in the catalytic cycle of the light-driven enzyme, protochlorophyllide oxidoreductase. *J. Biol. Chem.* **282:** 32015–32020.