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Photochemical Characterization of a Novel Fungal Rhodopsin from

Phaeosphaeria nodorum

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¹Abbreviations: RDs – rhodopsins; ORPs – opsin-related proteins; BR – bacteriorhodopsin; LR – *Leptosphaeria* rhodopsin; NR – *Neurospora* rhodopsin; PhaeoRD1 – LR-like rhodopsin from *Phaeosphaeria*; PhaeoRD2 – the second (auxiliary) rhodopsin from *Phaeosphaeria*; PCR – polymerase chain reaction; DDM – N-dodecyl- β -D-maltoside; FTIR – Fourier-transform infrared

Abstract

Eukaryotic microbial rhodopsins are widespread bacteriorhodopsin-like proteins found in many lower eukaryotic groups including fungi. Many fungi contain multiple rhodopsins, some significantly diverged from the original bacteriorhodopsin template. Although few fungal rhodopsins have been studied biophysically, both fast-cycling light-driven proton pumps and slow-cycling photosensors have been found. The purpose of this study was to characterize photochemically a new subgroup of fungal rhodopsins, the so-called auxiliary group. The study used the two known rhodopsin genes from the fungal wheat pathogen, *Phaeosphaeria nodorum*. One of the genes is a member of the auxiliary group while the other is highly similar to previously characterized proton-pumping *Leptosphaeria* rhodopsin. Auxiliary rhodopsin genes from a range of species form a distinct group with a unique primary structure and are located in carotenoid biosynthesis gene cluster. Amino acid conservation pattern suggests that auxiliary rhodopsins retain the transmembrane core of bacteriorhodopsins, including all residues important for proton transport, but have unique polar intramembrane residues. Spectroscopic characterization of the two yeast-expressed *Phaeosphaeria* rhodopsins showed many similarities: absorption spectra, conformation of the retinal chromophore, fast photocycling, and carboxylic acid protonation changes. It is likely that both *Phaeosphaeria* rhodopsins are proton-pumping, at least *in vitro*. We suggest that auxiliary rhodopsins have separated from their ancestors fairly recently and have acquired the ability to interact with as yet unidentified transducers, performing a photosensory function without changing their spectral properties and basic photochemistry.

1. Introduction

Microbial rhodopsins are typical membrane proteins with seven transmembrane helical bundle similar to that of G-protein-coupled receptors [1-3]. Microbial rhodopsins are photosensitive, with all-*trans*-retinal as chromophore, covalently bound via the Schiff base to a Lys sidechain. Retinal photoisomerization triggers functionally important conformational changes in the protein (opsin) moiety. Since the last century, our perception of the functional, taxonomic, and ecological diversity of microbial rhodopsins has undergone a revolutionary change. Previously regarded as an eclectic mix of halobacterial light-driven proton and chloride pumps and related photosensory receptors, they have emerged as a large, widespread, multi-functional group found not only in *Archaea*, but in many *Bacteria* and *Eukarya*, including numerous fungal and algal species [2,4-7]. New functions were defined, including new types of photosensors, light-gated ion channels, and light-activated enzymes. We now recognise that many prokaryotic and eukaryotic species possess multiple rhodopsin (RD) and opsin-related protein (ORP) genes, which may have arisen both via gene duplication (often, multiple) and by lateral gene transfer [2,4].

There is clear evidence that fungal rhodopsins evolved via gene duplication and neofunctionalisation [6,8,9]. Fungal rhodopsins are clearly related to archaeal, rather than eubacterial, ancestors, most probably originating from the light-driven halobacterial proton pump, bacteriorhodopsin (BR) [2,10]. Some fungal opsins conserve the original haloarchael BR-like protein template and its proton pumping ability, whilst others lost the chromophore-binding lysine (these are not true opsins but opsin-related proteins (ORPs)), with a range of divergent forms in between [1,6,8]. The recent flood of genome sequences has shown that numerous fungal species possess multiple RDs and ORPs. However, few have been functionally characterised, and

their photobiological role is largely unknown.

The first identified homologs of BR in fungi were ORPs from yeast and basidiomycetes. On the basis that they were expressed during stress it was suggested they act as chaperones [11,12]. Their discovery was followed by the detection [13] and *in vitro* photochemical characterization [14] of *Neurospora crassa* rhodopsin (NR), which coexists with its ORP. Photochemical characterization of NR expressed in *Pichia pastoris* revealed a slow photocycle suggesting its role is photosensory rather than proton-pumping [14-16]. Phenotypic characterization of the knock-out mutants of NR (*nop*) (or its close homolog in *Fusarium fujikuroi* (*opsA*)) did not reveal an obvious function for NR, but implied participation in carotenoid biosynthesis regulation [17,18]. In contrast, the closely related rhodopsin from *Leptosphaeria maculans* (LR) [19] had a fast photocycle and could pump protons like BR [20,21]. Site-directed mutagenesis showed that one of the key differences responsible for the dramatically different photochemical behavior of NR and LR originated from a seemingly innocuous Asp/Glu replacement at the key position of the cytoplasmic proton donor to the retinal Schiff base [22,23]. Recent electrophysiological studies of NR (along with its close homolog in *Podospora anserina*) and LR expressed in neurons confirmed their drastically different proton-pumping abilities [24].

Thus, even the limited biochemical and physiological analysis available so far suggests multiple functions of fungal rhodopsins. Additionally, genomic information from several fungal species shows the existence of a third group of fungal rhodopsins; these have overall sequence resemblance to ORPs, but conserve all the key residues of the BR-like template [6,10]. We have tentatively called this group the auxiliary ORP-like rhodopsins, referring to their co-existence with other rhodopsin forms in the same species [10]. Auxiliary rhodopsins have been found in

many fungal species, but their expression pattern has been analyzed only in *Fusarium fujikuroi* [25] and *Bipolaris oryzae* [26] (plus distant homologs from basidiomycete *Ustilago maydis* [27]). A knock-out mutant of the *Fusarium fujikuroi* gene (*carO*) produced no phenotypical alterations under laboratory conditions. It may be linked to carotenoid metabolism as it is found in the carotenoid biosynthesis gene cluster [25,28]. So far, no auxiliary rhodopsin have been characterised physiologically or photochemically. Thus, one may only speculate about their role(s); a photosensory function tuned to a distinct spectral region is perhaps the most plausible hypothesis.

Here, we present photochemical characterization of an auxiliary rhodopsin using the protein from *Phaeosphaeria (Stagonospora) nodorum* (PhaeoRD2) and compare it with the LR-like homolog (PhaeoRD1) [29]. Both rhodopsins were expressed in *Pichia pastoris* and characterized spectroscopically. The two rhodopsins have similar absorption spectra, disproving the idea that the auxiliary species are needed to respond to light stimuli of different wavelengths. Spectroscopic and mutational data suggest that the auxiliary PhaeoRD2 may have some proton-pumping ability, similar to LR and PhaeoRD1.

2. Materials and Methods

2.1. Protein expression

Similar to our previous work on NR and LR [16,20,30], the two *Phaeosphaeria* rhodopsins were heterologously expressed in methylotrophic yeast (*Pichia pastoris*, strain GS115) with a yield of ~5 mg of purified protein per litre of culture. The *Phaeosphaeria* rhodopsin genes *Ops1* (SNOG_00807, Gene ID: 5968425, renamed PhaeoRD1) and *ops2* (SNOG_00341, Gene ID: 5967674, renamed PhaeoRD2) were cloned between the *EcoRI* and *XbaI* sites of the pPICZ α A vector. The coding sequences were truncated (*ops1* to 795 bp and

ops2 to 822 bp) to remove most of the putative extramembrane parts of the N termini using sequence alignments with NR and LR. Such replacement of the native N-terminus with the yeast signal sequence produced robust expression and good membrane targeting in the past [14,20,30]. *EcoRI* site was created at the 5' ends of the rhodopsin genes, while *XbaI* site was at the 3' ends, and 6-His-tag coding sequence was added at the C-terminus by performing the polymerase chain reaction (PCR) with the following primers: PhaeoRD1 forward (5'GCGAATTCGAATCTGGCCAGAAGACCCTC3') and reverse (5'GCTCTAGATTAATGGTGATGGTGATGGTGCGCGCCGTCATCCTCACCGAG3'), and PhaeoRD2 forward (5'GCGAATTCGACCATGGCTCAGACTTG3') and reverse (5'GCTCTAGATTAATGGTGATGGTGATGGTGAACGTTGGCGGGGCCATCGAG3').

The pPICZ α A-PhaeoRD1 and pPICZ α A-PhaeoRD2 vectors were propagated in DH5 α strain of *E. coli* in low salt LB medium with 25 μ g/ml zeocin, isolated using Qiagen kit (QIAprep Spin Miniprep), and transformed into *P. pastoris* GS115 cells by electroporation according to the manual of the *Pichia* expression kit (Invitrogen). The transformed colonies were isolated from the YPDS/zeocin plates and screened for high expression levels of rhodopsins in small-scale cultures, similar to what was done with LR [20,23]. The cells were grown in 25 ml of BMGY medium in 250 ml baffled flasks, shaking at 30°C, 300 rpm for 1-2 days. As OD₆₀₀ reached ~10, 2.5 ml of culture was centrifuged at 1,500g for 5 min at 4°C, resuspended in 25 ml of BMMY medium, and grown by shaking at 240 rpm, 30°C. After 24 h, additional 175 μ l of 100% methanol (final concentration 0.7%) and 6.25 μ l of 10 mM all-*trans*-retinal (isopropanol stock, final concentration 2.5 μ M) were added into the culture. At different time points (24 h, 40 h, 48 h, and 52 h), 1 ml of the expression culture was taken and centrifuged at 1,500g for 5 min at 4°C. The expression level of the protein was evaluated by the intensity of the color of the yeast

pellet, and the colonies showing the most intense red color were selected for a large-scale expression.

The large-scale protein expression followed the established shake-flask protocol of the *Pichia* expression kit (Invitrogen) with small modifications. Briefly, a small amount of cells from a colony with the highest expression level of rhodopsins in small-scale cultures was inoculated into 25 ml of BMGY in a sterile 250 ml baffled flask. This seed culture was grown, shaking at 30°C (300 rpm) for 18–24 h, until the OD₆₀₀ exceeded 2, and inoculated into a sterile 2 L baffled flask containing 250 ml of BMGY. This culture was shaken at 29–30°C (270 rpm) for 18–24 h, until the OD₆₀₀ reached 3.6. To induce rhodopsin expression, the cells were pelleted in sterile containers at 1,500g for 5 min at 4°C, and gently resuspended in 0.8 L of BMMY, which was placed into 2.8 L Fernbach flask and shaken at 29–30°C (240 rpm). 10 mM isopropanol stock of all-*trans*-retinal (Sigma, final concentration 5 µM) and 100% filtered methanol (final concentration 0.7%) were added to the growth medium after 24 h of induction. The red-colored cells were collected by centrifugation at 1,500g for 5 min at 4°C after 40 h of induction, as the protein yield was found to be lower upon longer (48–52 h) and shorter (24 h) incubation times. The cell pellet was washed with MilliQ water twice and stored frozen at -20°C for later use.

D126N mutant of PhaeoRD2 was expressed analogously to the wild-type. To produce the mutant, two primers containing DNA for the desired mutation and high-fidelity thermostable Pwo polymerase were employed in a single-step PCR from the wild-type construct. To set up the polymerase chain reaction (PCR), 5'CCTTTGCTCCTGACCAACCTCATGCTCACCGC3' and 5'GCGGTGAGCATGAGGTTGGTCAGGAGCAAAGG3' primers were used.

2.2. Protein purification and lipid reconstitution

The cell breakage and protein purification protocols were based on those used for LR

[20,22,30] with small modifications. Cell pellets collected from the 800 ml of culture were re-suspended in one pellet volume of buffer A (7 mM NaH₂PO₄ at pH 6.5, 7 mM EDTA, 7 mM DTT, and 1 mM PMSF), incubated in the dark at room temperature for 3 h with 5 mg of lyticase (from *Arthrobacter luteus*, Sigma) for digestion of the cell walls, and additional 25 μM of all-*trans*-retinal to ensure complete rhodopsin regeneration. The cells were then centrifuged at 1,500g for 5 min at 4°C and resuspended in one pellet volume of buffer A. Half of the pellet volume of ice-cold acid-washed glass beads (Fisher) (420–600 μm diameter) was added, and the cells were disrupted with four 1 min pulses using vigorous vortexing. The cell debris were removed by centrifugation at 700g for 5 min at 4°C and the cell lysate was collected. An additional half pellet volume of buffer A was added to resuspend the cell debris, and vortexing and centrifugation steps were repeated several times to achieve complete breakage of the cells. All cell supernatants containing the membrane fraction were combined and centrifuged at 40,000g for 30 min (or at 150,000g for 50 min for smaller membrane fragments) at 4°C, and the membrane pellets were stored at -20°C for later use.

For visible spectroscopy experiments in the fungal membrane environment, the membrane pellets were washed with N-dodecyl-β-D-maltoside (DDM) to decrease the size of the membrane fragments and remove peripheral proteins and cell walls. The suspension was incubated at room temperature for 5 min and centrifuged at 5,000g for 5 min at 4°C. The maximal final DDM concentration in the mixture was 0.5%, as higher DDM concentrations solubilized the membranes fully. The colored supernatant was collected and centrifuged at 20,000g for 30 min at 4°C, the solubilized protein in the supernatant was discarded, and only the membrane-embedded proteins from the pellet were used to prepare rhodopsin-loaded polyacrylamide gels for spectroscopic measurements. The protein gels were equilibrated with the

desired buffer for at least 2 h before the measurements. As the D126N mutant of PhaeoRD2 was unstable after the DDM treatment, its photocycle (along with the wild-type controls) was measured in gels incorporating untreated yeast membranes.

To purify rhodopsins for reconstitution into liposomes needed for vibrational spectroscopy, we used 6-His tag affinity resin (Ni-NTA agarose, Qiagen). We estimated the quantity of solubilized proteins spectroscopically (Cary 50, Varian), assuming the molar extinction similar to that of BR. Due to different biochemical properties and stability of the two rhodopsins, the conditions for purification were different. To purify PhaeoRD1, the pellets of frozen membranes were resuspended with solubilization buffer (1% DDM, 20 mM KH_2PO_4 , 1 mM PMSF, pH 7.5), and stirred in the dark at 4°C for 3-4 h, then centrifuged at 40,000g for 30 min at 4°C to remove unsolubilized material. The membrane pellets of PhaeoRD2 were resuspended in solubilization buffer (1% Triton X-100, 20 mM KH_2PO_4 , 0.3 M NaCl, 10 mM β -mercaptoethanol, 1 mM PMSF, pH 7.5), and stirred in the dark at 4°C overnight, then centrifuged at 38,000 rpm for 50 min at 4°C. Solubilized rhodopsins were mixed with 6-His tag affinity resin and incubated in the dark at room temperature with gentle agitation to allow complete binding (usually 3 h). The clear supernatant containing other solubilized proteins was removed after centrifugation at 4,000g at 4°C for 2 min. The resin was washed with increasing concentrations of imidazole (0.25% DDM, 50 mM KH_2PO_4 , 400 mM NaCl, up to 35 mM imidazole, pH 7.5 for PhaeoRD1, and 0.25% Triton-X100, 50mM KH_2PO_4 , 400 mM NaCl, up to 35 mM imidazole, 1 mM DTT, pH 7.5 for PhaeoRD2) until the spectral cytochrome band at 410 nm disappeared from the wash spectrum. The purified proteins were eluted from the resin with the elution buffers of the same composition as the respective wash buffers, but with 250 mM imidazole. Addition of *Pichia* lipid extract (at 0.2 mg/ml) was needed to stabilize solubilized

PhaeoRD2, similar to what was found for NR [15].

The lipid reconstitution protocol followed that used for LR [30]. The dry powder lipids (DMPC: DMPA = 9:1 w/w, Avanti lipids) were first dissolved and mixed in warm chloroform, which was thoroughly removed by evaporation under vacuum to yield a thin lipid film. The dry lipids were rehydrated by 50 mM KH_2PO_4 , 100 mM NaCl, pH 7.5 and agitated to obtain lipid suspension at high concentration (usually, 10 mg/ml). Purified solubilized rhodopsins were added to the preformed liposomes, which were semi-solubilized (as judged by the drop in turbidity) with Triton X-100 at protein/lipids/detergent (w/w/w) ratio of 1:3:1.5, and stirred for 15 min at room temperature. The resultant semi-transparent mixture became turbid after removal of detergent by adding 400 mg of Bio-beads SM-2 (Biorad) per 1 ml of the mixture and incubation with stirring at 4°C in the dark. The proteoliposomes were collected by centrifugation at 20,000g for 30 min at 4°C.

2.3. Visible and vibrational spectroscopy measurements

The static visible spectroscopy was performed with a Cary 50 spectrophotometer. The time-resolved visible spectra were collected using custom-built flash-photolysis equipment [20,31], with 7 ns excitation pulses of the second harmonic of a Nd-YAG laser at 532 nm (Continuum Minilite II). Light-induced absorption changes at different wavelengths were averaged (usually, several hundreds of traces) and converted into a quasilogarithmic time scale using in-house software.

Time-resolved difference FTIR spectra were gathered at 4 cm^{-1} resolution in a rapid-scan mode as described previously [20], using a Bruker IFS66vs apparatus with a temperature-controlled sample holder (Harrick) connected to a circulating water bath (Fisher). The photocycle was initiated by the laser pulses as described above. The films of hydrated DMPA:DMPC

liposomes were compressed between two CaF₂ windows with 6 μm spacer, and data acquisition was controlled by the OPUS software (Bruker). Static Raman spectra were collected using FRA106/s accessory to the IFS66vs spectrometer, with excitation at 1024 nm, at 2 cm⁻¹ resolution.

3. Results and Discussion

3.1. Sequence-based Analysis

The *Phaeosphaeria nodorum* genome annotation [29] included two rhodopsins. Ops 1 (or PhaeoRD1) is very similar to LR, while the second rhodopsin (PhaeoRD2) belongs to a new subgroup, not characterized spectroscopically [10]. We called this subgroup “auxiliary”, because most of its members were found in addition to other rhodopsin forms. Since then, many new fungal genomes have become publicly available, so that the placement of *Phaeosphaeria* rhodopsins, as well as clustering of fungal rhodopsins in general, can be reevaluated with much greater confidence. Thus, we first compared amino acid sequences of the two *Phaeosphaeria* opsins to the sequences of opsins (full sequences only, excluding ORPs) from other ascomycetes, using publicly available genome databases (<http://blast.ncbi.nlm.nih.gov/>; <http://genome.jgi-psf.org/>; <http://www.broadinstitute.org/>).

The results of CLUSTALW analysis confirm our earlier suggestion [10] that auxiliary rhodopsins form a very distinct branch on the fungal rhodopsin tree (boxed in Fig. 1). The analyzed fungal rhodopsins can be divided into two large subgroups. Within each of the subgroups, the rhodopsins align with species phylogenies [32]. The first subgroup includes previously characterized putative photosensors and proton pumps such as NR and LR. Many of the fungal species found in this first group, especially those from *Pleosporomycetidae*,

Dothideomycetidae, *Helotiales*, and *Hypocreomycetidae*, have additional, second rhodopsin forms in the second (auxiliary) subgroup. Additionally, the auxiliary subgroup contains third and fourth forms of rhodopsins of *Dothideomycetidae* and a few standalone (if we disregard ORPs) rhodopsins, e.g., from several species of *Colletotrichum* and *Verticillium* (Fig. 1). It is clear that, as suggested before, PhaeoRD1 is the closest homolog of LR (79% identity, 91% similarity), and as such belongs to the first subgroup, while PhaeoRD2 is a member of the auxiliary cluster, with distinct amino acid sequence (35% identity, 53% similarity to LR). This analysis suggested that the photochemical and functional properties of PhaeoRD1 would be LR-like, while those of PhaeoRD2 were unknown.

In view of the earlier finding that the auxiliary rhodopsin from *Gibberella* (*Fusarium*) *fujikuroi*, *carO*, was found in a carotenoid biosynthesis cluster that also contains a carotene oxygenase *carX*, phytoene synthase/cyclase *carRA* and phytoene desaturase *carB* [25], we explored the genomic context for the members of this group. The cluster structure is preserved in *Helotiales* (*Botrytis* and *Sclerotinia*) and *Hypocreomycetidae* (*Gibberella zeae*, *Gibberella moniliformis*, *Fusarium oxysporum*, *Nectria*), and selected *Dothideomycetes* (*Mycosphaerella graminicola* and *Rhizidhysterium rufulum*). In the *Pleosporomycetidae* (*Phaeosphaeria*, *Leptosphaeria*, *Pyrenophora*, *Alternaria*, *Cochliobolus*, *Setosphaeria*) gene orders and orientations are shuffled (as seen in many fungal gene clusters, e.g. [29]), in a local reflection of mesosynteny [33]. The clustering of auxiliary rhodopsins with carotenoid biosynthesis genes is strongly suggestive of a carotenoid-related physiological role and expression regulation as described for *carO* [18,28].

To gain further insight into the structural differences between the two major rhodopsin subgroups, we have aligned amino acid sequences of the representative members of the auxiliary

subgroup (restricted to second rhodopsin forms, including PhaeoRD2) (Fig. 2) and compared the conservation pattern in the last six transmembrane helices (most conserved in microbial rhodopsins) with that known for BR and LR [10,19,34]. The full-length alignment (except for the non-conserved termini) of a broader selection of sequences is available in the supplementary data file. The first general trend observed from the alignment is a very high degree of conservation of the BR-like template (shown yellow on black) in fungal rhodopsins of both subgroups. The conserved residues include most of the retinal-binding pocket and the majority of amino acids implicated in the light-driven proton transport (BR's T46, Y57, R82, D85, T89, T90, D96, D115, W182, Y185, W189, E194, E204, D212, and many others). This suggests that auxiliary rhodopsins may possess proton pumping ability similar to that observed for LR [20], as they conserve all major proton donors and acceptors of BR. It must be noted that the primary proton donor (homolog of BR's D96) is strictly conserved as Asp in the auxiliary subgroup, as it is known that its conservative replacement by Glu can strongly impede the proton transport in NR and mutant LR [22]. From the conservation pattern of the BR template in fungi, it is impossible to reliably predict which one of the subgroups is evolutionary closer to the archaeal ancestor, as there is almost equal number of cases of exclusive conservation of BR residues in each subgroup. On the other hand, our CLUSTALW analysis of the full-length opsin sequences (not shown) places BR somewhat closer to the first subgroup, in agreement with the previous analysis [18].

Next, we analyzed distribution of the residues uniquely conserved in the auxiliary subgroup (highlighted purple in Fig. 2) relative to the putative membrane core of these proteins, as defined by homology to BR structure. While most of the unique residues are located at the ends of the helices in the membrane interfacial regions, there are notable exceptions, the most striking of which is helix D. Even though there are several uniquely conserved residues in the

middle of the helices E and F, they do not change the overall character of those helices, being mere changes in size of the affected hydrophobic sidechains. On the contrary, there must be a dramatic change in the properties of the helix D, as a result of the introduction of a polar residue with hydrogen bonding ability into the middle of the transmembrane domain, corresponding to position 116 of BR, along with a number of other changes (Fig. 2). The polar residue in the middle of helix D of fungal rhodopsins from the auxiliary group is usually represented by Glu, and sometimes by Trp, and follows the super-conserved homolog of Asp-115 of BR. This puts severe constraints on the possible sidechain orientation of this new polar residue. As Asp-115 is hydrogen-bonded to Thr-90 from helix C in BR, and this pair is preserved in all fungal rhodopsins, one may expect that the following Glu-116 will face the core of the lipid bilayer. This is highly unlikely, unless it is used to interact with a protein partner (either an unknown transducer or another rhodopsin molecule, leading to oligomerization). From this tentative analysis, one may speculate that rhodopsins of the auxiliary subgroup have preserved their proton-pumping ability, but have also acquired capacity to interact with membrane-bound transducers. This is reminiscent of the evolutionary relationship between BR and halobacterial sensory rhodopsins, which preserved rudimentary proton-pumping ability in the absence of their transducers and use the same conformational changes as proton pumps to perform signaling [35,36].

3.2. Photochemical Characterization

Both *Phaeosphaeria* opsins expressed in *Pichia pastoris* formed red pigments upon addition of all-*trans*-retinal, which were stable both in the yeast membranes and upon reconstitution of the purified proteins into synthetic lipids. The dark states of the obtained

chromoproteins were first characterized by the visible and Raman spectroscopies (Fig. 3). The maxima of the visible absorption spectra of both proteins were similar and close to that observed for LR (542 nm in yeast membranes [20]). Purified solubilized proteins have absorption maxima at 540 nm for PhaeoRD1 and 535 nm for Phaeo RD2 (Fig. 3, left panel), and the respective maxima are at 545 nm and 538 nm in yeast membranes (not shown). No apparent light- or dark-adaptation was observed, similar to the case of LR [20,21]. According to the Raman spectroscopy results (Fig. 3, right panel), which report mostly on the retinal chromophore, the dark states contain predominantly all-*trans*-retinal. This is obvious from the prominent pair of C-C stretching vibrations around 1202 and 1168 cm^{-1} , similar to those of light-adapted BR and LR [20,37]. The location of the major ethylenic C=C stretches (at 1533 cm^{-1} for PhaeoRD1 and at 1537 cm^{-1} for PhaeoRD2) is consistent with their visible maxima, where higher frequency correlates with more blue-shifted visible absorption [38]. From the characterization of the dark states, we can conclude that it is unlikely that these two rhodopsin forms exist solely to respond to different wavelength of visible light, as their absorption maxima are very close to each other and both fall into the green region. It can be also argued that the retinal-binding pockets of both *Phaeosphaeria* opsins must be similar to that of LR, which is expected from the conservation of their transmembrane regions (Fig. 2), as they show very close visible maxima and similar vibrational spectra of the chromophore.

Next, we characterized the photochemical cycles of both *Phaeosphaeria* rhodopsins using time-resolved spectroscopy in the visible range. As expected from the high degree of sequence identity of PhaeoRD1 and LR, their photochemistry was very similar (Fig. 4, lower panel). At neutral pH, the photocycle of PhaeoRD1 is quite fast, finishing in a few tens of milliseconds, as expected for proton pumps [1,10]. It has a well-defined M intermediate with the

deprotonated retinal Schiff base (observed at 400 nm), which forms on a submillisecond time scale and decays in a pH-dependent manner (Fig. 4, upper panel), again, similar to LR [20], but with somewhat stronger pH-dependence (see below). At lower pH, the reprotonation of the Schiff base (M decay) is fast (a few ms), and the M intermediate is followed by a red-shifted intermediate, which disappears at higher pH, when the M decay becomes slow. The only notable difference in the photocycle kinetics of PhaeoRD1 and LR is a higher accumulation of the early red-shifted intermediate along with the early M intermediate on the tens of microseconds time scale, which may point at a somewhat shifted protonation equilibrium between the Schiff base and the primary proton acceptor. From the early parts of the 460 nm kinetics, it is also obvious that an L-like intermediate accumulates in equilibrium with the K-like and the early M states. But this difference does not affect the later parts of the photocycle of PhaeoRD1, which is consistent with the expected LR-like photochemistry of a light-driven proton pump.

The photocycle kinetics of PhaeoRD2, on the contrary, appears to be quite different from those of PhaeoRD1 and LR (Fig. 5). On the one hand, the overall kinetics of the photocycle is quite fast, with the turnover characteristic time of a few tens of ms at neutral pH, which is consistent with a proton-pumping rhodopsin behavior, similar to PhaeoRD1. On the other hand, kinetics of the rise and decay, as well as relative concentrations of photointermediates, differ dramatically for the two *Phaeosphaeria* rhodopsins (Fig. 5, lower panel). The most striking feature of the photocycle of PhaeoRD2 is an extremely fast deprotonation of the retinal Schiff base, as observed by the rise of the M intermediate at 400 nm. The plateau of the M intermediate concentration is reached in less than 10 microseconds, as opposed to the sub-millisecond plateau in PhaeoRD1, LR, and BR. Such extremely fast pH-independent deprotonation of the retinal Schiff base is typical for BR mutants with perturbed protonation equilibria between the Schiff

base nitrogen and Asp-85, especially as found in mutants involving Arg-82 [39]. While homologs of Arg-82, as well as of other important members of the extracellular hydrogen-bonded network (Tyr-57, Glu-194, Glu-204) [40], are conserved in all auxiliary fungal rhodopsins, there are many unique residues in the extracellular loops and interfacial regions (Fig. 2 and supplementary file). These unique residues could interact with the sidechain of the homolog of Arg-82 in PhaeoRD2 and change its position, affecting the pK_a of the primary proton acceptor (homolog of Asp-85) via the well-described coupling mechanism [41-43]. Additionally, even though the kinetics of the Schiff base reprotonation (the M decay at 400 nm) are similarly fast and pH-dependent for PhaeoRD1 and PhaeoRD2, the accumulation of the late red-shifted intermediate (observed at 620 nm) is much higher in PhaeoRD1, possibly due to its faster decay in PhaeoRD2. It should be noted that the pH-dependence of the Schiff base reprotonation in both proteins (Fig. S1) is much more strongly pH-dependent than that in BR (and even LR). The persistence of the fast phase of the Schiff base reprotonation in BR is usually explained by the internal nature of its proton donor, Asp-96. The absence of such phenomenon can be interpreted as a sign of a lower pK_a of its homologs in the N-like intermediates in *Phaeosphaeria* rhodopsins.

The fast reprotonation of the Schiff base along with the rapid photocycle turnover in PhaeoRD2 hints at the possibility that it may have some proton-pumping ability. This would be consistent with the sequence analysis presented above, which showed the presence of the conserved homolog of Asp-96 of BR, Asp-126, possibly serving as an internal cytoplasmic proton donor to the Schiff base, ensuring its fast reprotonation. To verify that idea, we replaced the putative cytoplasmic proton donor Asp-126 with non-protonatable Asn and studied the photocycle of the D126N mutant (Fig. 6). If Asp-126 is indeed the primary proton donor for the Schiff base of PhaeoRD2, one would expect a dramatically slower Schiff base reprotonation (M

decay at 400 nm), similar to what was observed for LR [20,22]. Consistent with these expectations, we observed extremely slow (on the seconds time scale) pH-dependent M decay (Fig. 6, upper panel, and Fig. S1). While such dramatic deceleration of the Schiff base reprotonation is indicative of the proton-donating role of the replaced Asp-126, there is a possibility that it may occur through the global conformational effect of the D126N mutation. The latter hypothesis can be easily disproved by checking the effect of a common artificial proton shuttle, sodium azide (NaN_3), which is known to accelerate the Schiff base reprotonation in the homologous mutants of microbial rhodopsins [20,44]. Addition of 1 mM NaN_3 (Fig. 6, lower panel) restored the wild-type-like kinetics of the Schiff base reprotonation (millisecond time scale), confirming the proton-donating role of Asp-126.

Taken together, the photocycle kinetics data obtained by visible spectroscopy on the wild-type and mutant PhaeoRD2 strongly argue for its proton-pumping ability, even though we could not verify it directly, due to the instability of PhaeoRD2 under continuous illumination in liposomes. At the same time, it is conceivable that the photocycle of PhaeoRD2 (as well as its proton-pumping ability) are different *in vivo*, upon interaction with its putative transducer (in the case it is a photosensory rhodopsin as hinted by the sequence analysis). As dramatic changes in the photochemistry and ion transport are known for halobacterial sensory rhodopsins [45-47], *in vitro* kinetic data should be treated with caution.

To obtain further insight into the molecular details of light-induced proton transfers and conformational changes of the retinal chromophore and the opsin moiety of the *Phaeosphaeria* rhodopsins, we employed time-resolved difference Fourier-transform infrared (FTIR) spectroscopy. Figure 7 compares difference FTIR spectra of PhaeoRD1 and PhaeoRD2 taken at a few ms after the excitation. From the results of the visible spectroscopy, both spectra were

expected to be dominated by the M intermediate, with some contribution from a later red-shifted intermediate in the case of PhaeoRD1. This is indeed the case, as can be observed from the C-C stretching vibrations region (fingerprints), which shows only negative bands [48,49] corresponding to all-*trans*-retinal of the dark state for PhaeoRD2 (1201 and 1168 cm⁻¹, lower panel), with a weak positive band of 13-*cis*-retinal of a late photointermediate at 1188 cm⁻¹ for PhaeoRD1 (upper panel). The latter band becomes prominent in the PhaeoRD1 spectra taken at 25 ms delay after the flash (Fig. S2), consistent with the expected rise of the late intermediate and decay of M. Overall, the FTIR difference spectra of PhaeoRD1 corresponding to the M intermediate (Fig. 7, upper panel), as well as to the late intermediate (not shown), are very similar to the corresponding spectra of LR [20]. Among the most typical and important opsin bands observed both for PhaeoRD1 and LR, one should mention those of protonation of the primary proton acceptor (homolog of D85 of BR) at 1759 cm⁻¹, and the perturbation of the homolog of BR's D115 at 1741/1736 cm⁻¹. At a later delay (25 ms, Fig. S2), an additional negative band assigned to the deprotonation of the homolog of the primary proton donor D96 was observed at 1745 cm⁻¹. Additionally, prominent bands at 1390/1381 cm⁻¹ recently assigned to deprotonated carboxylic acids in isotope-labeled LR [30] were observed in PhaeoRD1 as well. Most retinal bands were identical or very similar between LR and PhaeoRD1, consistent with the Raman data (Fig. 3), including C=C stretches at 1533 cm⁻¹, C-C stretches (with other contributions) at 1250, 1201, 1169 cm⁻¹, and putative Schiff base vibrations at 1643/1620 cm⁻¹.

Surprisingly, the FTIR difference spectra of PhaeoRD2, dominated by the M intermediate (Fig. 7, lower panel) were very similar to those obtained for PhaeoRD1 and LR. Some minor differences in the FTIR spectra of *Phaeosphaeria* rhodopsins originate from the different mixtures of intermediates (almost pure M for PhaeoRD2 and mixture of M with a later

intermediate in PhaeoRD1). It should be noted, that at higher pH, the appearance of an N-like signatures of 13-*cis*-retinal and deprotonated homolog of Asp-96 of BR can be observed (Fig. S2), similar to those in PhaeoRD1 at longer delay times. Overall, in spite of the differences in the photocycle kinetics and the amino acid sequences, all the major vibrational bands of retinal and carboxylic acids discussed above for PhaeoRD1 were observed for PhaeoRD2 as well. This points to the high degree of conservation of the transmembrane core of BR in the auxiliary rhodopsin group and that the light-induced isomerization of retinal and ensuing proton transfers are very similar for the LR-like PhaeoRD1 and auxiliary PhaeoRD2. There are a number of differences between the two rhodopsins in several opsin bands in the range of Amide I and Asn/Gln sidechain vibrations ($1700\text{-}1600\text{ cm}^{-1}$), which may reflect the differences in the conformational changes of the proteins' interfacial regions expected from the differences in the primary structures, but at this point we can not assign them.

3.3. Conclusions

We studied a new subgroup of fungal rhodopsins (termed the auxiliary group [10]), using sequence analysis of the fungal genomic data and photochemical comparison of two representative rhodopsins from *Phaeosphaeria nodorum* [29]. The bioinformatic analysis confirms that the auxiliary subgroup forms a very distinct cluster on the rhodopsin tree (Fig. 1) due to the unique primary structure of its members (Fig. 2), which are usually present in addition to other rhodopsin forms in their host species. Evidently, the auxiliary group diverged from the other rhodopsins early in the history of the ascomyota, some 400 Mya [50]. Analysis of the genomic context shows that auxiliary rhodopsins may be linked to the carotenoid biosynthesis cluster of genes. Structural analysis of the conserved regions suggests that auxiliary rhodopsins

preserved the common transmembrane core of BR and LR, but have some polar residues on the hydrophobic protein periphery, which may suggest interactions with a putative transducer or other membrane partner.

Spectroscopic analysis by the visible, Raman, and FTIR spectroscopy reveals some characteristic photocycle features for the auxiliary rhodopsin of *Phaeosphaeria*, but also confirms conservation of the main BR-like characteristics. Close similarity of the absorption spectra of the two *Phaeosphaeria* rhodopsins (LR-like and auxiliary) implies that they are not designed to interact with different wavelengths of light. The photocycles of both rhodopsins are fast, and show photointermediates and proton transfer steps typical for proton-pumping rhodopsins. Taken together with the distinct phenotype of the auxiliary rhodopsin in which the cytoplasmic Schiff base proton donor is disabled, it suggests that auxiliary rhodopsins preserved their proton-pumping ability, at least in the absence of their putative transducers. We suggest that this may point to a fairly recent evolutionary separation of these putative photosensors. Whether the auxiliary rhodopsins indeed serve as photosensors remains to be seen by the future *in vivo* experiments.

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Figure Legends

Figure 1. *Phaeosphaeria* rhodopsins as representatives of the two major subgroups of fungal rhodopsins. Unrooted guide tree of fungal rhodopsin sequences from ascomycetes (excluding OPRs) produced from the CLUSTALW [51] alignment and plotted using TREEVIEW [52]. Numbers after the names of fungal species indicate multiple forms of rhodopsins found in the same species. The scale bar represents number of substitutions per site (0.1 indicates 10 nucleotide substitutions per 100 nucleotides). *Phaeosphaeria* rhodopsins studied in this work are highlighted yellow, previously characterized rhodopsins are highlighted purple, and the auxiliary subgroup of rhodopsins is boxed.

Figure 2. Conservation of the BR template in fungal rhodopsins and unique structural features of the auxiliary subgroup. CLUSTALW alignment of partial sequences of representative members of the auxiliary subgroups (highlighted purple), restricted to the conserved transmembrane regions of the last six helices (helices B-G, see supplementary file for the full-length alignment). Sequences of BR, LR, and PhaeoRD1 are given for comparison. The residues conserved in BRs are yellow on black, residues most important for proton transport are numbered using BR sequence, and the residues unique for the auxiliary group are highlighted purple. Abbreviations: Leptos. – *Leptosphaeria maculans*, Pyrenoph. – *Pyrenophora tritici-repentis*, Altern. – *Alternaria brassicicola*, Bipolar. – *Bipolaris oryzae*, Dothistr. – *Dothistroma septosporum*, Mycosph. – *Mycosphaerella graminicola*, Gibber. – *Gibberella zeae*, Fusar. – *Fusarium oxysporum*, Hyster. – *Hysterium pulicariae*, Sclerot. – *Sclerotinia sclerotiorum*.

Figure 3. Characterization of the dark states of *Phaeosphaeria* rhodopsins. (Left panel) Visible spectra of solubilized purified rhodopsins at room temperature, pH 7.5. PhaeoRD1 was solubilized in 0.25% DDM, 50 mM KH_2PO_4 , 400 mM NaCl, 250 mM imidazole, and PhaeoRD2 in 0.25% Triton X-100, 50 mM KH_2PO_4 , 400 mM NaCl, 250 mM imidazole, 0.2 mg/ml *Pichia* lipids. (Right panel) Raman spectra of liposome-reconstituted dark-adapted rhodopsins suspended in 0.05 M KH_2PO_4 , 0.1 M NaCl, pH 7, at room temperature, accumulated for 14 h with 1024 nm excitation.

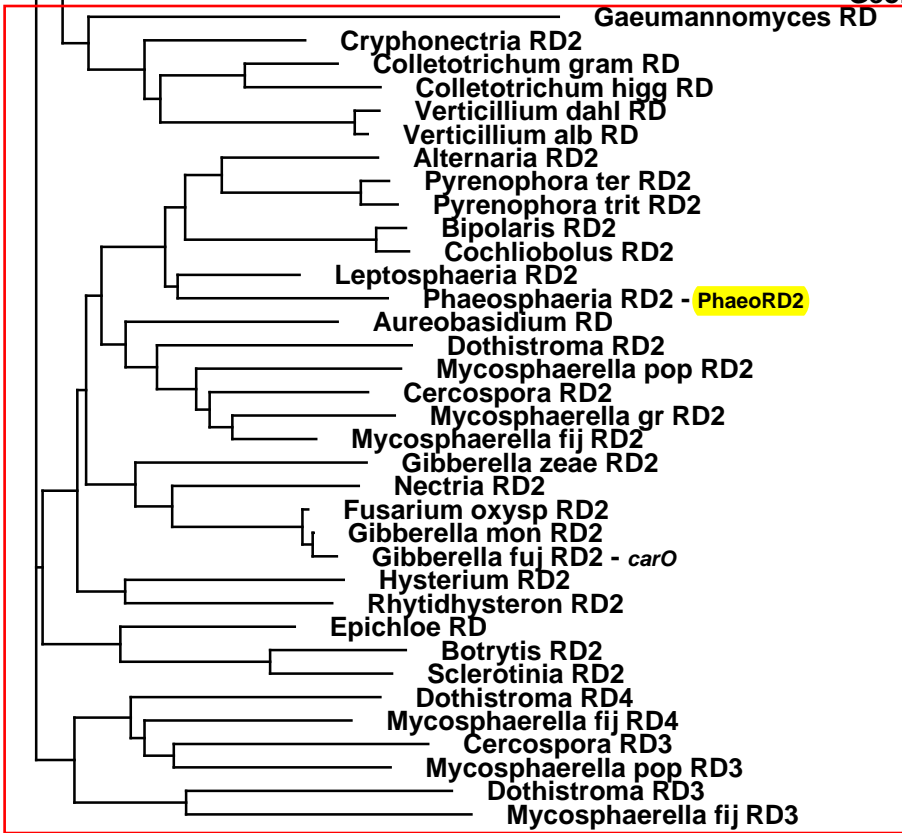
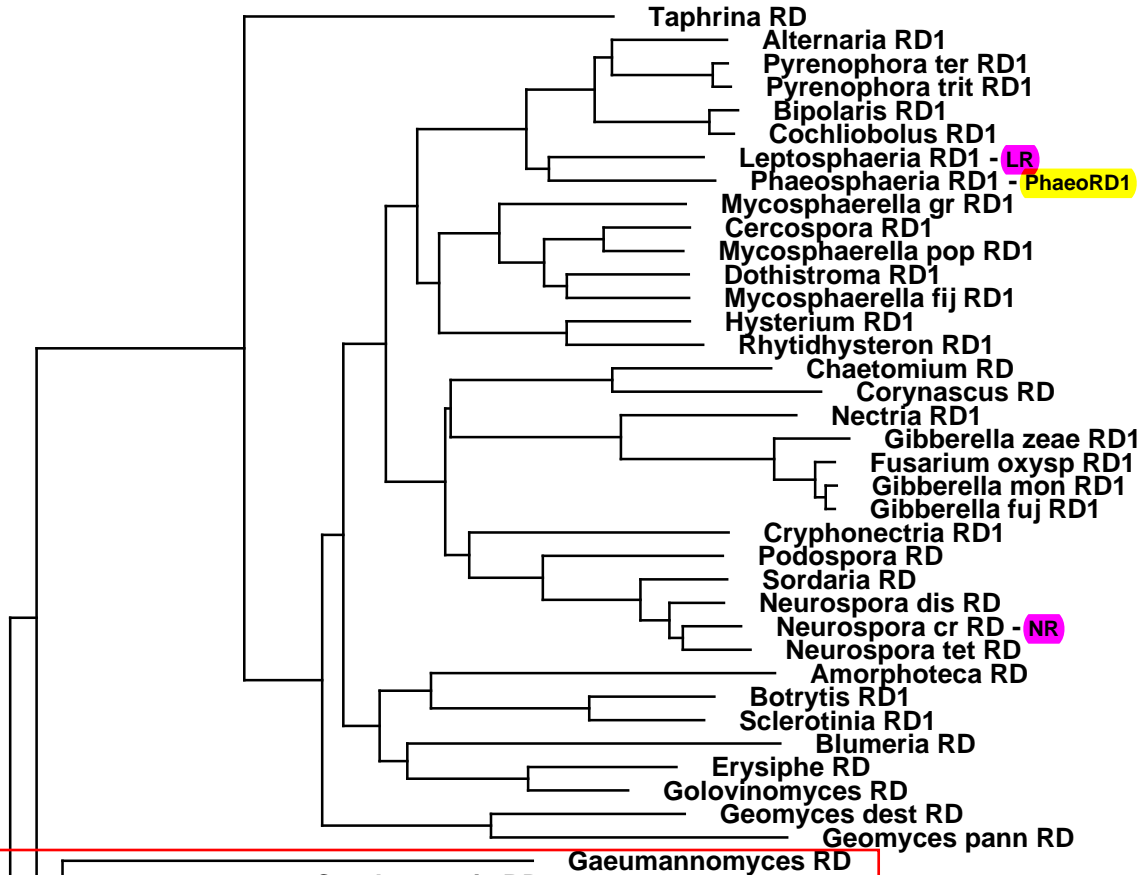
Figure 4. The laser flash-induced photocycle kinetics of PhaeoRD1 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH_2PO_4 and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle kinetics measured at pH 5-9 at characteristic wavelength: 620 nm – red, 540 nm – green, 460 nm – cyan, 400 nm – blue. (Lower panel) Comparison of the photocycle kinetics of PhaeoRD1 (color-coded as in the upper panel) and LR (black) at pH 6, normalized at the minimum of the 540 nm signal. The LR data are taken from the earlier multi-wavelength dataset [20].

Figure 5. The laser flash-induced photocycle kinetics of wild-type PhaeoRD2 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH_2PO_4 and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle

kinetics measured at pH 5-9 at characteristic wavelength: 620 nm – red, 540 nm – green, 460 nm – cyan, 400 nm – blue. (Lower panel) Comparison of the photocycle kinetics of PhaeoRD2 (color-coded as in the upper panel) and PhaeoRD1 (black, taken from Fig. 4) at pH 6, normalized at the minimum of the 540 nm signal.

Figure 6. The laser flash-induced photocycle kinetics of PhaeoRD2 D126N mutant in yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes (not treated with DDM) were encased in polyacrylamide gels equilibrated with 0.05 M KH_2PO_4 and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris. (Upper panel) Photocycle kinetics measured at pH 5- 8 at characteristic wavelength: 620 nm – red, 540 nm – green, 460 nm – cyan, 400 nm – blue. (Lower panel) Comparison of the normalized photocycle kinetics at 400 nm, representing the reprotonation of the retinal Schiff base, of PhaeoRD2 wild-type (black) and the D126N mutant at pH 5 with (red) and without (blue) 1 mM NaN_3 .

Figure 7. Time-resolved laser flash-induced difference FTIR spectra of *Phaeosphaeria* rhodopsins reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH_2PO_4 , 0.1 M NaCl, pH 7, and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 12°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see text for details. (Upper panel) PhaeoRD1; (Lower panel) PhaeoRD2.



AUXILIARY

0.1

	Helix B	Helix C
BR	AKK F YAITTLVPA I AFTMYL S MLLG	EQNP I Y W ARYADWLF T TP L LLLL D L L LV D
LR	RRL Y HVITTT I ITLTAALS Y FAMAT G	VYRQ V Y Y ARYIDWAI T TP L LLLL D L G LL A G
PhaeoRD1	KRL Y HTITTT M ITIFAALS Y FAMAT G	VHRQ V F W ARYVDW S V T TP L LLLL D L G LL A G
PhaeoRD2	DR I F H YLTAAV V FVA A IAYFT M GS N	NYRS I Y V RYIDW V I T TP L LL L D L ML T AG
Leptos.RD2	DR V F H YLTAAV V FVA A IAYF S MGS N	NFRG I F Y VRYIDW V I T TP L LL L D L LL T AG
Pyrenoph.RD2	DR L F H YLTAAV V FVA A IAYFT M GS N	TYRA V Y Y ARYIDW F I T TP L LL L D L LL T AG
Altern.RD2	DR L F H YITAS V V F V A CIAYFT M GS N	TYRS V Y V RYIDW F I T TP L LL L D L LL T AG
Bipolar.RD2	NR I F H YITCG V V F V A AIA Y FT M GAN	TYRA I Y Y ARYVDW F I T TP L LL L D L LL T AG
Dothistr.RD2	DR I F H YITAG V VM V AIA Y FT M ASH	QTRE I Y V RYIDW V I T TP L LL L D L LL T AA
Mycosph.RD2	DR I F H YITAS V TM V AIA Y F S MA H	LTRE I F Y VRYIDW F I T TP L LL L ID L ML T AA
Gibber.RD2	HR V F H YITAS I TM V AIA Y FT M GAN	NYRE I F Y VRYIDW F I T TP L LL L D L LL T AG
Nectria RD2	DR I F H YITGG I TM I A A IS Y F S MAS N	VYRE I F Y VRYIDW F I T TP L LL L D L LL T AG
Fusar.RD2	DR I F Q YITAG I TM I A S IAYFT M AS N	IYRE I F Y ARYIDW F I T TP L LL L ID L LL T AG
Hyster.RD2	HR I F N YITAG I TM V A F IAY S MAS N	MYRE I F Y VRYIDW F I T TP L LL L D L LL T AG
Botrytis RD2	KRV F HYITAA T MT A SIA Y FT M AS N	VYRE I F Y VRYIDW V I T TP L LL L D L LL T AG
Sclerot.RD2	KRI F HYITAA T MT A IA Y FT M AS N	VTRE I F Y VRYIDW V I T TP L LL L D L LL T AG

46

57

82

85

96

	Helix D	Helix E
BR	DQ G T I LALV G AD G IM I GT L VGAL T	SYRF V W W A I ST A AM L Y I LY V LFF G F
LR	SGAH I FMA I VAD L IM V L T GLFAAF G	PQ K W G W Y TIACIAY I F V W H L V L N G
PhaeoRD1	SGGH I MA I VAD L IM L T L GLFAAF G	PQ K W G W Y TIACIAY I F V I W H L AL N G
PhaeoRD2	PWP S I L W T I I V D EIM I I T GLV G AL V	KY K W G Y F AFGNL A L V Y I I Y Q L V W ES
Leptos.RD2	PWP T I F V I L D EIM I V T GLV G AL V	SY K W G F F AF G CAAL V Y V Y Q L V W ES
Pyrenoph.RD2	PWP T MF V I A V D EIM I I T GL I GAL I	RY K W A Y F V F G C VAL F Y I V Y H L V W ES
Altern.RD2	PWP T L W V I M V D E IM I V T GL I GAL I	RY K W A Y F V F G C VAL F Y I V Y Q L A W ES
Bipolar.RD2	PWP T L W V I L V D E IM I V T GL I GAL I	IY K W P F F V F G C VAL F Y I V F Q L T W E A
Dothistr.RD2	PWP T L W A I L V D E VM I I T GLV G AL V	SY K W G Y F V F G C VAM F W I I Y I L V W E A
Mycosph.RD2	PWP T L F V V L V D E VM I I T GLV G AL V	SY K W G Y F T F G C VAL V Y I V Y V L V W E A
Gibber.RD2	PWP T V L Y V I L V D EIM I V T GLV G AL V	SY K W G Y F T I G C VAL V Y I V Y Q L A W E A
Nectria RD2	PWP T V L W V I L V D W V M I V T GLV G AL V	SY K W G Y F AF G CAAL A Y I V Y Q L A W E A
Fusar.RD2	PWP T V L W V I L V D W V M I V T GLV G SL V	SY K W G Y F AF G CAAL A Y I V Y V L A W E A
Hyster.RD2	PWP T V M W I I L V D EVM I V T GLV G AL V	RY K W G Y F V F G C AA L A Y I M Y H L A W ES
Botrytis RD2	PWP T I L F T I F L D EIM I I T GLV G AL V	SY K W G Y F V F MA A L F G I A W N I L F V G
Sclerot.RD2	PWP T I L F T I F L D EVM I I T GLV G AL V	SY K W G Y F V F MA F A L F G I A W N I L F V G

115

	Helix F	Helix G
BR	E K V L R N V T V V L W S A Y P V V W L I G S E G A G I V P L N I E T L L F M V L D V S A K V G F G L I L L R S R	
LR	F V A I G A Y T L I L W T A Y P I V W G L - A D G A R K I G V D G E I I A Y A V L D V L A K G V F G A W L L V T H	
PhaeoRD1	F V A I G G Y T L L L W T A Y P M V W G L - A D G S R K I G V D G E V I A Y A I L D V L A K G V F G A W L L I T H	
PhaeoRD2	F L M C G S L T A F L W I L Y P V A W G V - A E G G N V I S P D S E A I F Y S I L D F L A K P V F G A L L I W H G	
Leptos.RD2	F L L C G S L T S F L W I L Y P V A W G L - C E G G N V I S P D S E A V F Y G V L D F L A K P I F G A L L I W H G	
Pyrenoph.RD2	F L M C G S L T A F L W L L Y P I A W G V - C E G A N L V A P D S E A V F Y G V L D F L A K P I F G A L L L W H G	
Altern.RD2	F M M C G S L T T L L W I L Y P V A W G V - C E G A N L I A P D S E A V F Y G V L D F L A K P C F G A L L L W H G	
Bipolar.RD2	F L M C G S L T A F L W I L Y P I A W G L - S E G G N V I A P D S E A V F Y G V L D F L A K P V F G A L L L W H G	
Dothistr.RD2	F V I C G S L T A F M W T L Y P I A W G L - S E G G N V I S S D G E A A F Y G V L D I A K P V F G A L L I W H	
Mycosph.RD2	F L Y C G T L T A F L W T L Y P I A W G V - A E G G N I I A P D S E A V F Y G I L D V L A K P V F G A L L I W H G	
Gibber.RD2	F L W C G S L T A V V W I L Y P I A W G V - C E G G N L I S P D S E A V F Y G I L D I I A K P V F G A I L L F G H	
Nectria RD2	F L A C G T I T L I V W I C Y P I A W G V - C E G G N I I A P D S E A V F Y G I L D L L A K P V F G A I L L W H G	
Fusar.RD2	F V M C G S L T A V V W I L Y P I A W G V - C E G G N L I A P D S E A V F Y G I L D L I A K P V F G A L L L W H G	
Hyster.RD2	F L M C G S L T L V V W V L Y P I A W G V - C E G G N V I S P D S E A V F Y G I L D F I A K P V F G T M L L L G H	
Botrytis RD2	Y W T C G G V T M F L W F L Y P I A W G L - S E G G N V I A P D S E A V F Y G V L D V L A K I G F G S L L L F G H	
Sclerot.RD2	Y W M C G G I T M F L W F L Y P I A W G L - S E G G N I I A P D S E A V F Y G V L D V L A K I G F G I L L L N G H	

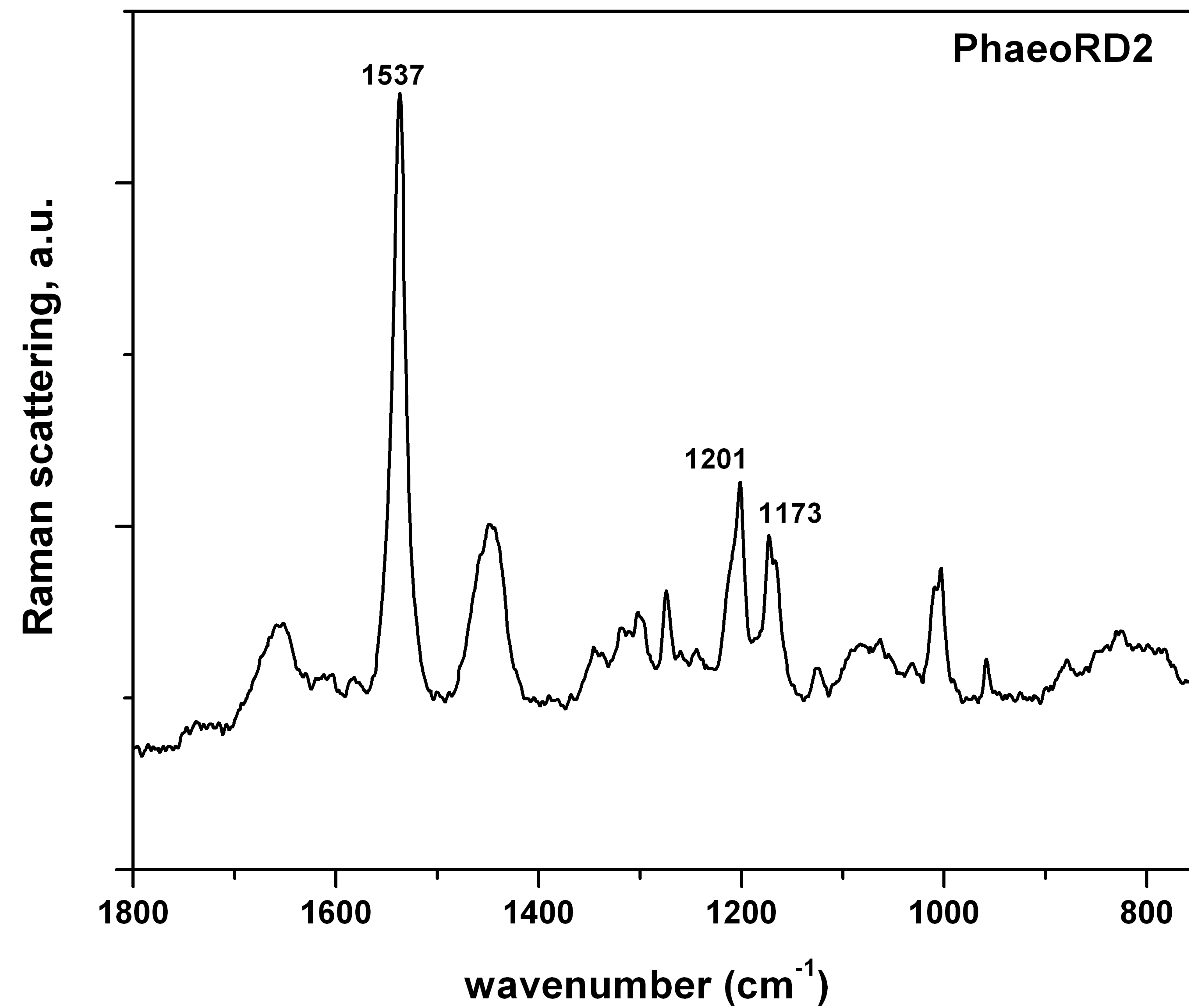
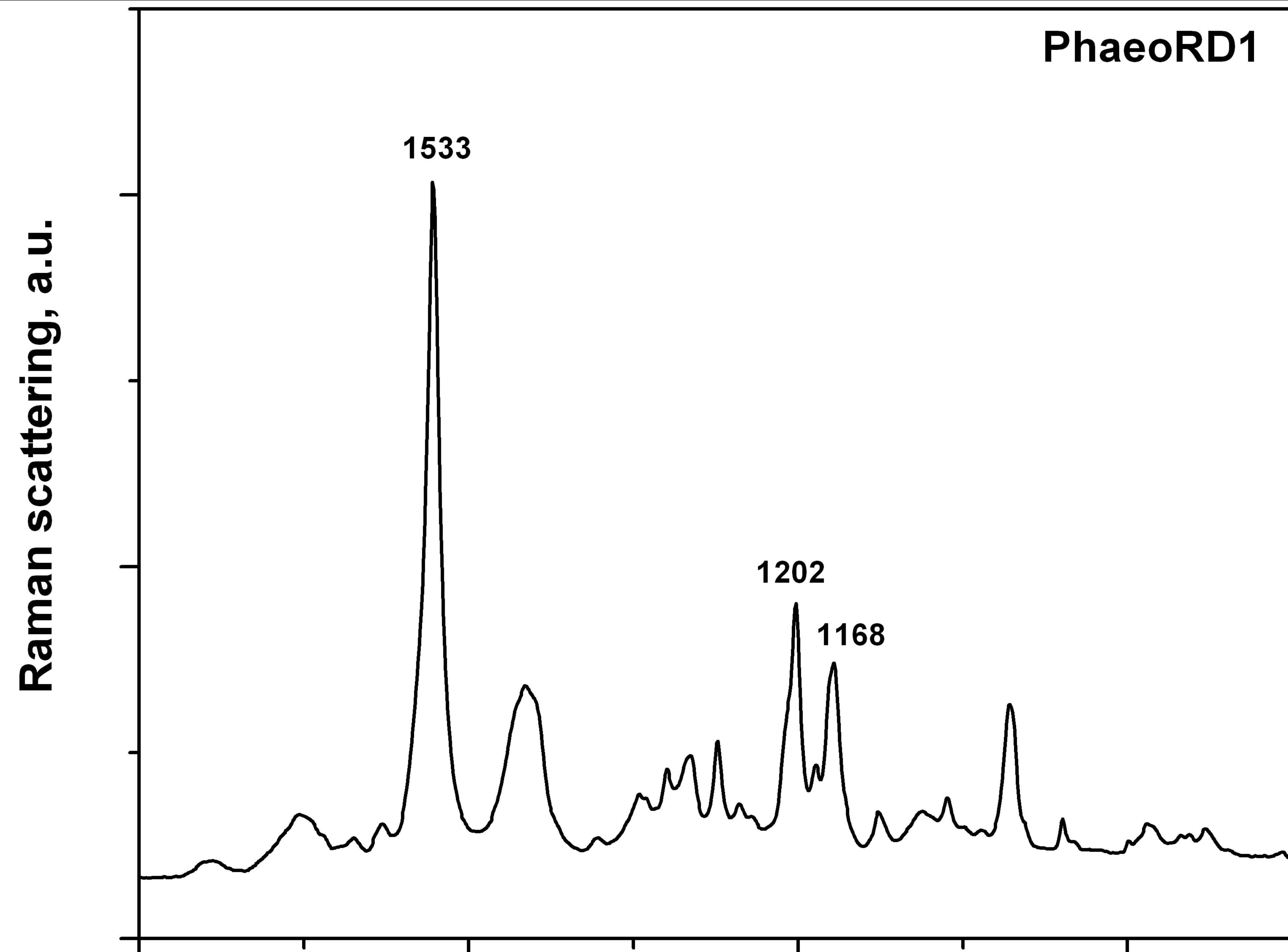
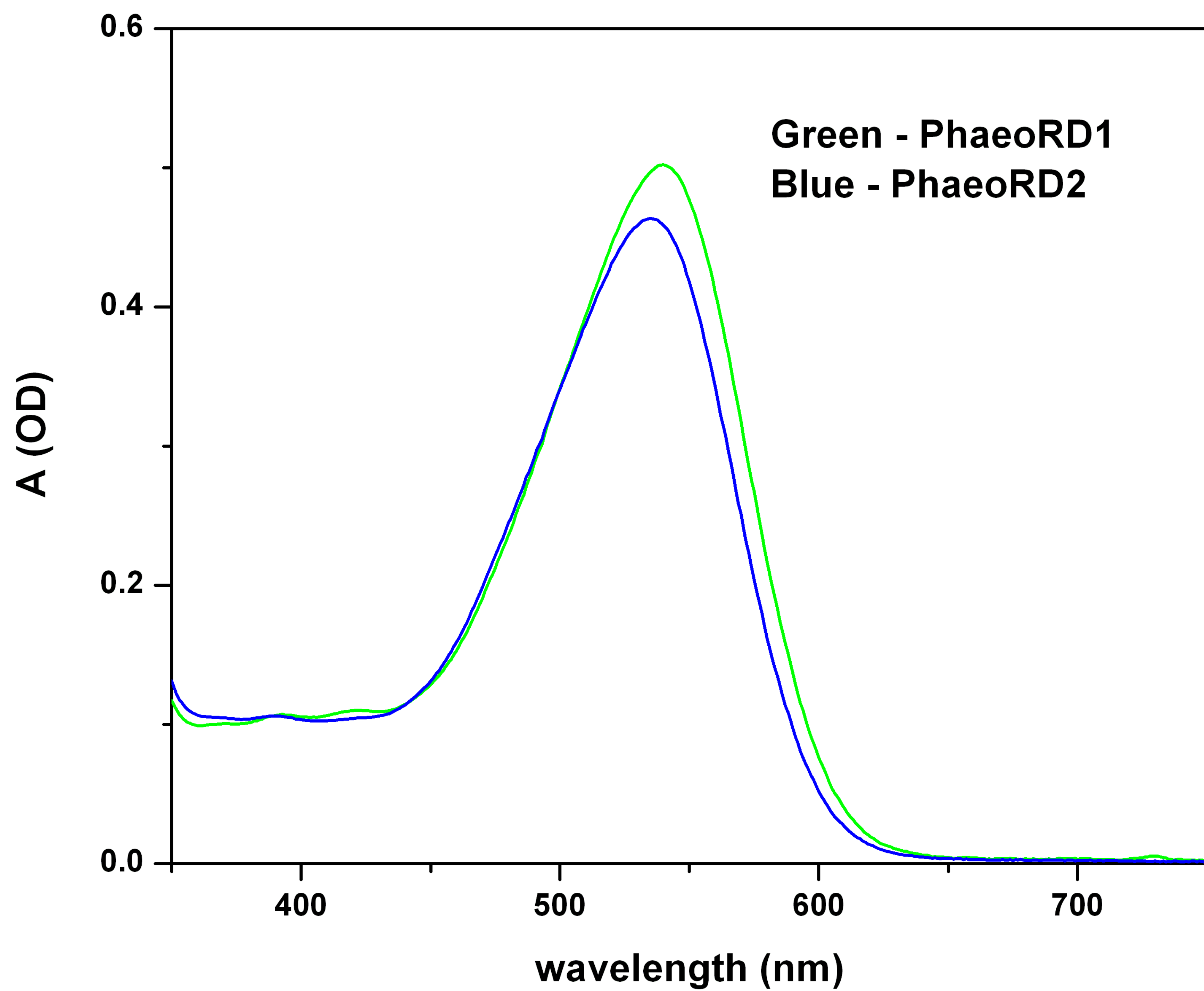
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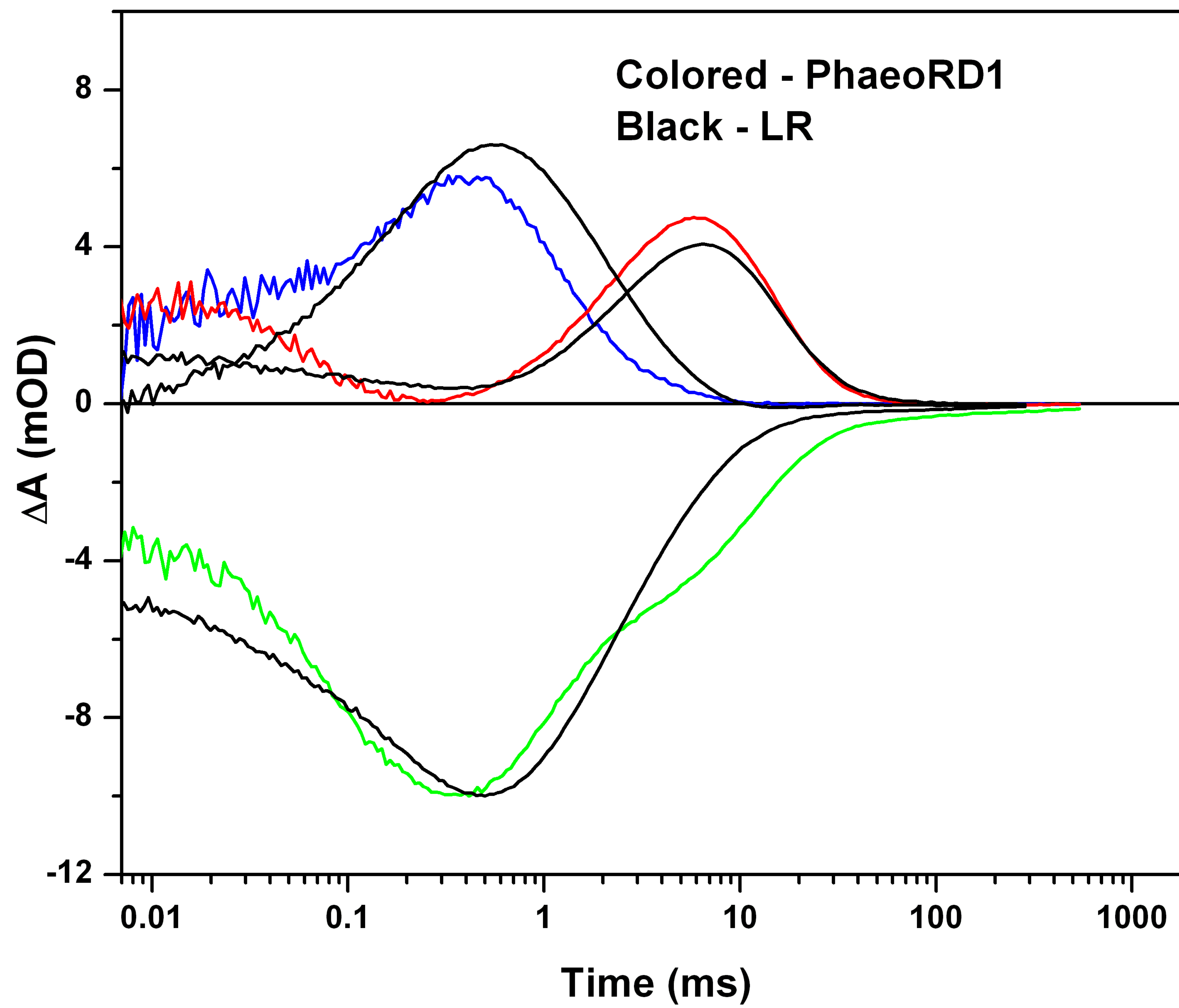
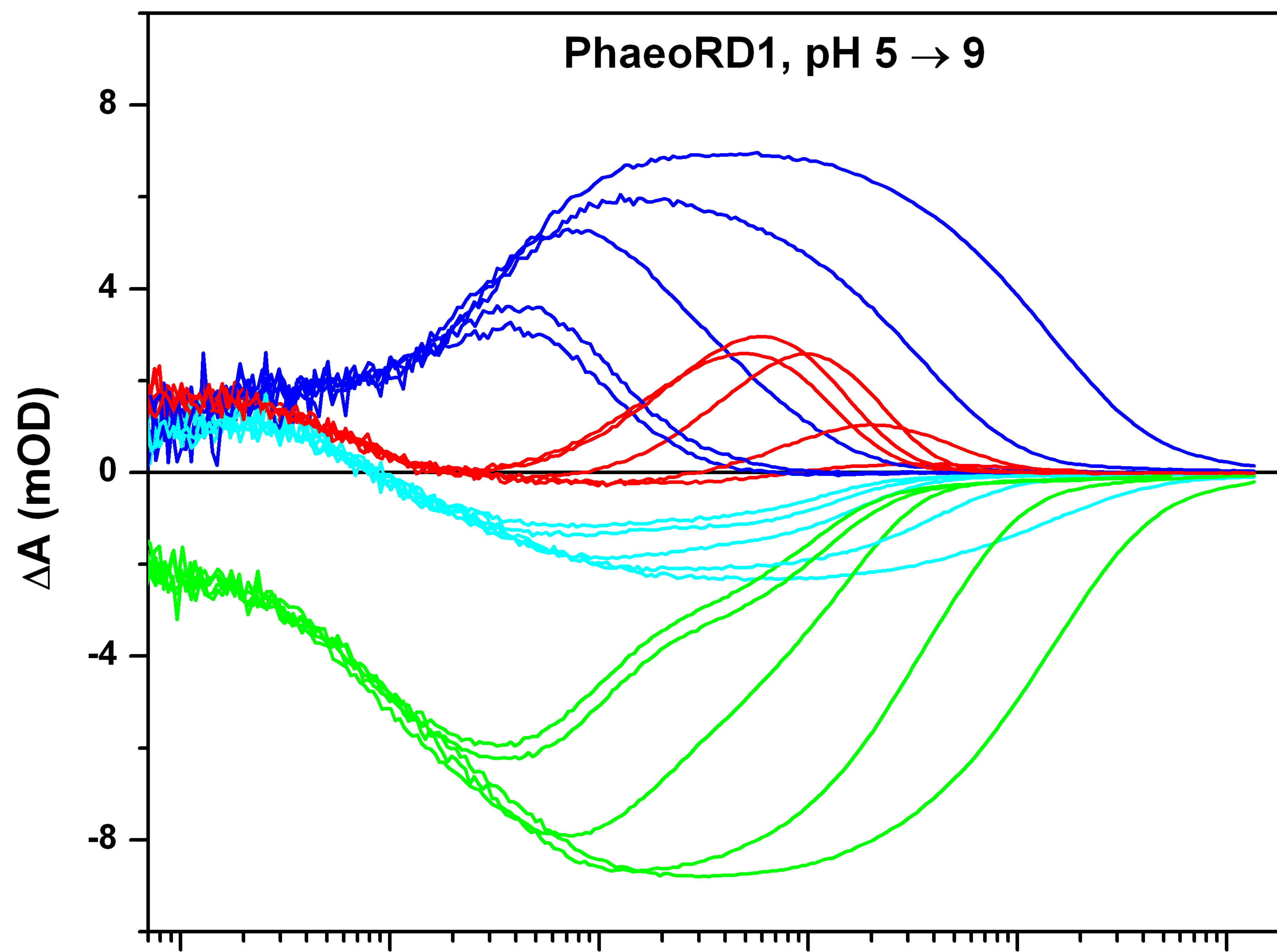
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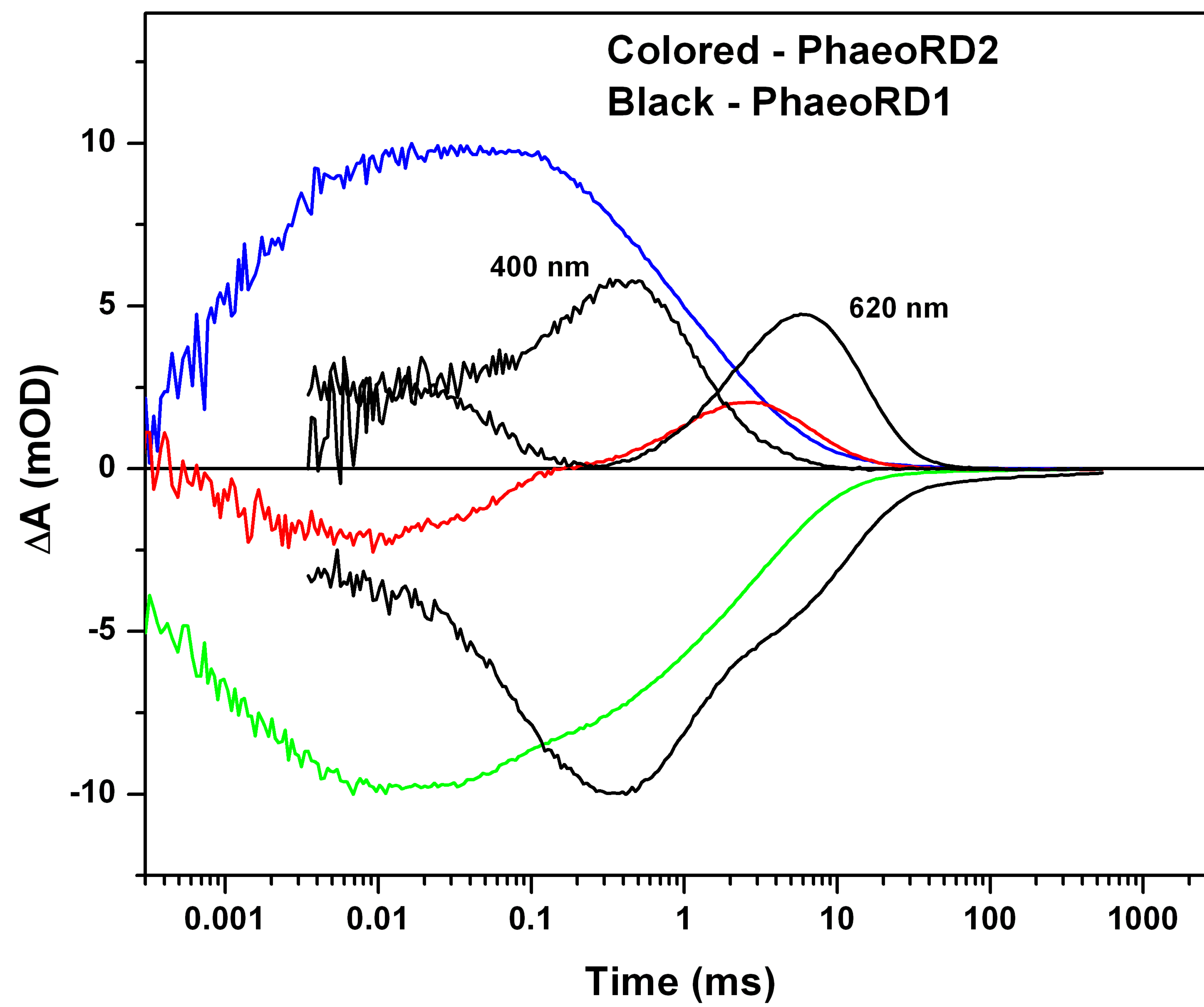
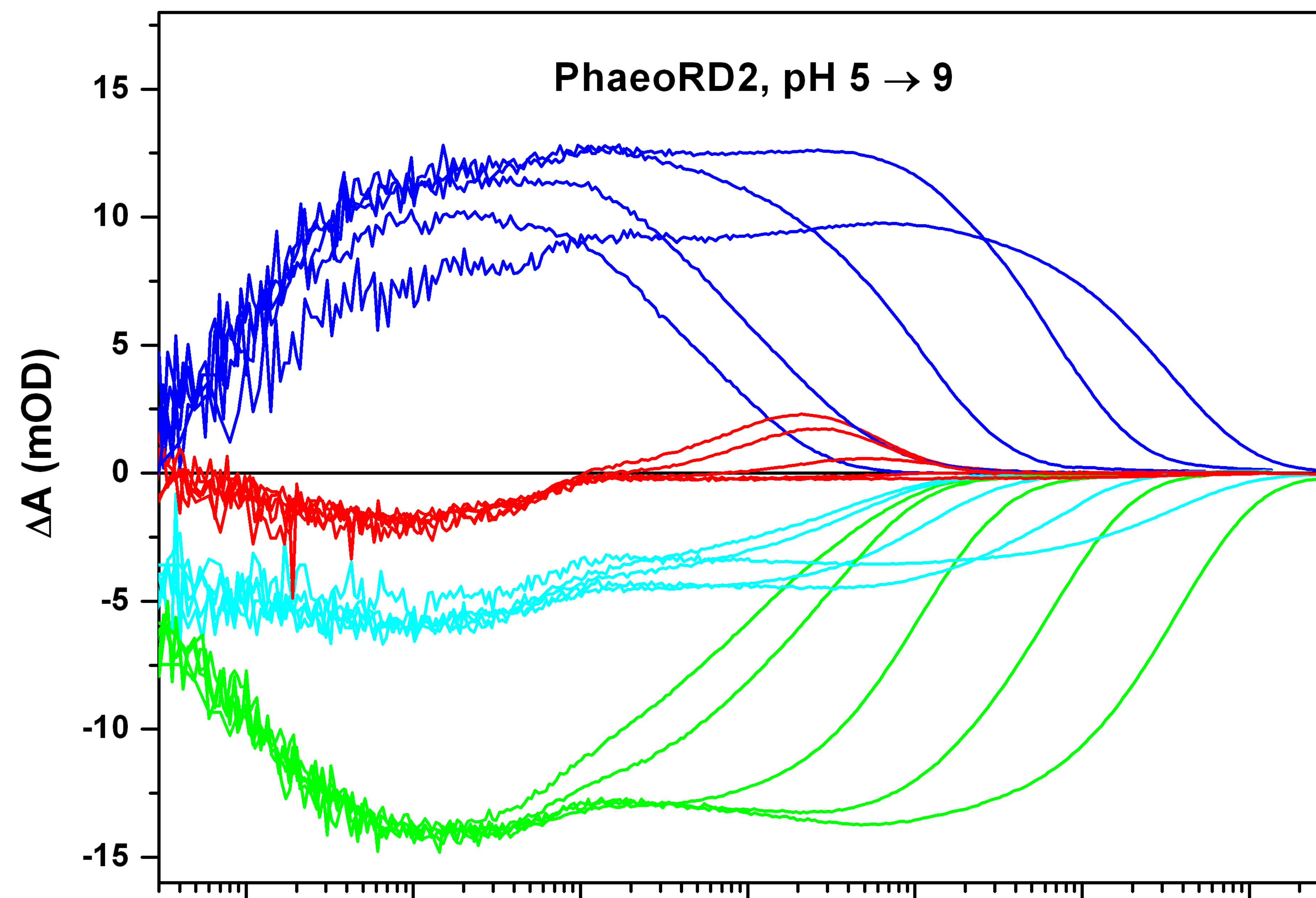
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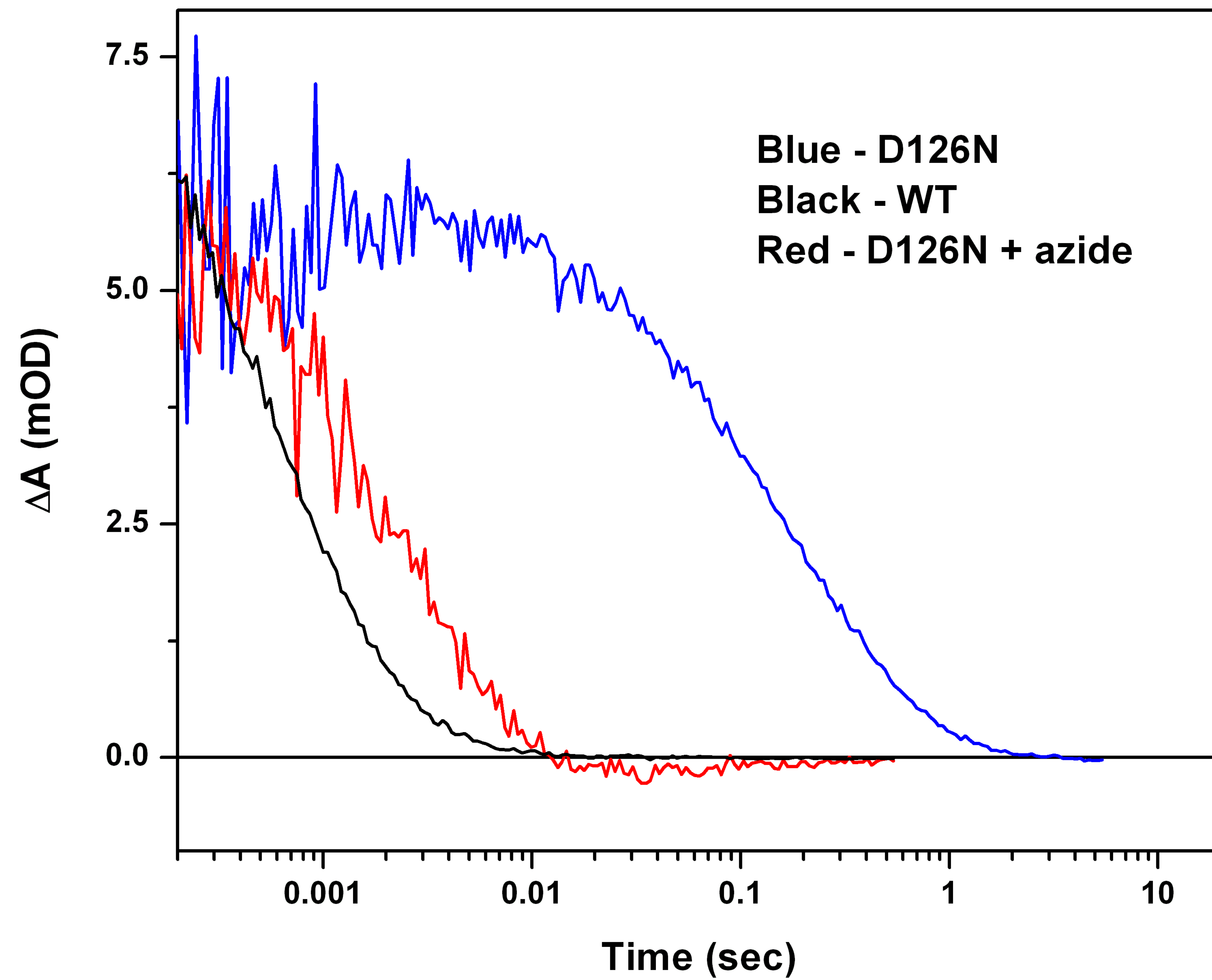
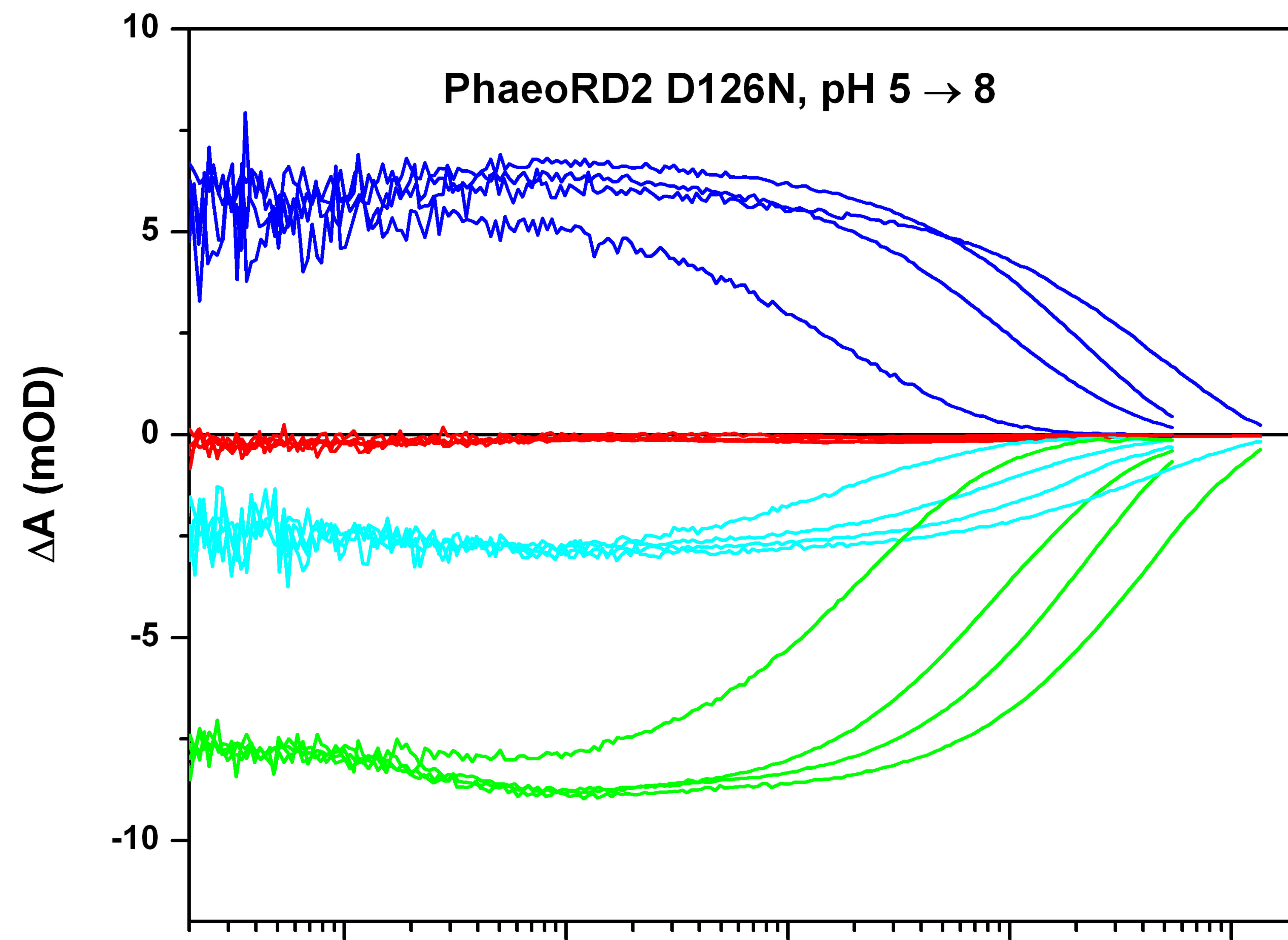
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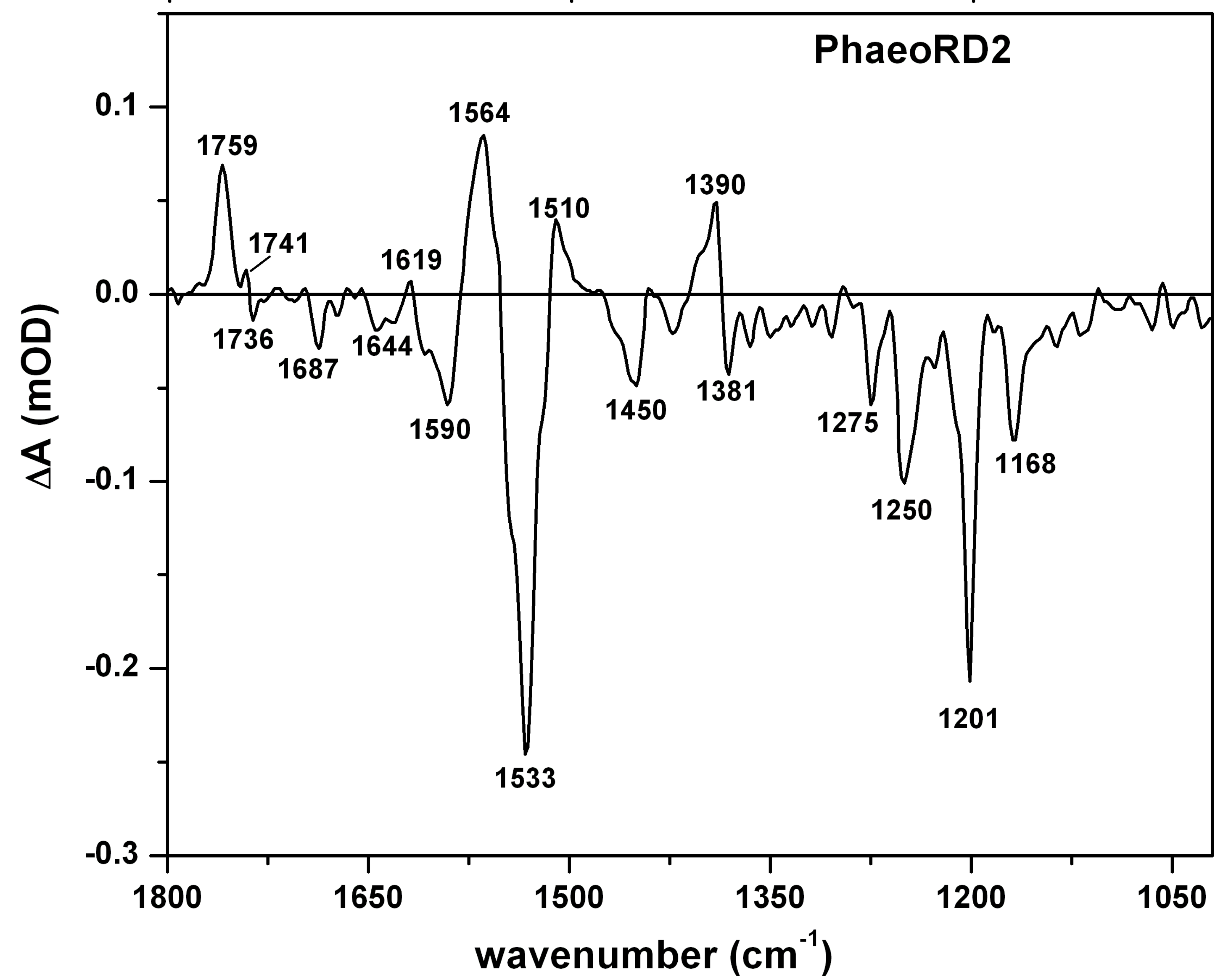
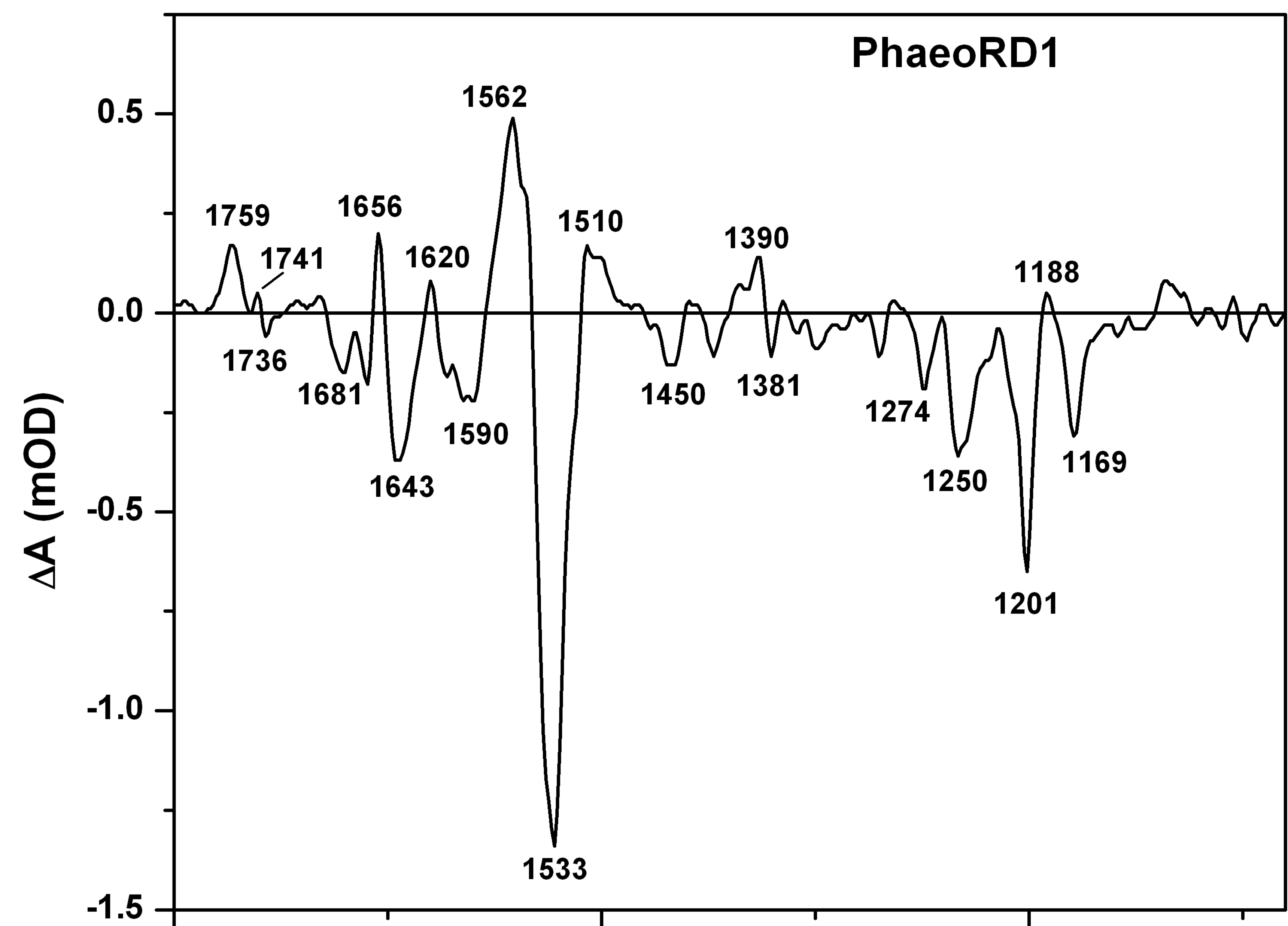
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Photochemical Characterization of a novel Fungal Rhodopsin from *Phaeosphaeria nodorum*

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Supplementary data

1. Sequence alignment of rhodopsins from ascomycetes produced by CLUSTALW. Members of the auxiliary group are shown on top, LR and PhaeoRD1 are shown at the bottom for comparison.

	Helix A	Helix B	
Pyrenophora_ter_RD2	RTADIAITTHGSDFYFAICSVMGFAGFAFIILGQRKARRDRLFHYLTA	AV	69
Pyrenophora_trit_RD2	RTADIAITTHGSDFYFAICAVMGFAGFAFVILGQRKARRDRLFHYLTA	AV	69
Alternaria_RD2	ATADIAITTHGSDFYFAICAVMGFSGFCFLGLAYRKQRRDRLFHYITAS	V	55
Bipolaris_RD2	RTADIGLTTTHGSDNFYFSICAVMTVAGFVFAAMSRYRIERRNRLFHYITCG	V	69
Cochliobolus_RD2	RTADIGLTTTHGSDNFYFSICVMTVAGFAFAAMSRYRIERRNRLFHYITCG	V	69
Leptosphaeria_RD2	RTADINITS DGSSFYFAICAVMGASGF AFLFLASRRKRTDRVFHYLTA	AV	45
Phaeosphaeria_RD2	RTADIAITDHGSDLYFAICAAMTVSGFVFIGLGMRKQRRDRIFHYLTA	AV	70
Mycosphaerella_gr_RD2	NPPDIGITVRGSDWYWAVCAIMTVATFAFVGLSITKPRQDRIFHYITAS	V	68
Mycosphaerella_fij_RD2	GSAQIGITVRGSDWYWTVCAMTVATFAFIALSATKPRQDRIFHYITAG	V	53
Cercospora_RD2	ATADIALTVRGSDWYWAVCAIMTVATFAFLGMGRKPRHDRIFHYITAS	V	70
Mycosphaerella_pop_RD2	GVAQIAITTHGSDFYFAVCAVMTVSTFAFLALGQMKPRAERIFYYITAS	V	65
Dothistroma_RD2	ATADVALTTHGSDWYWAVCAVMTCSAFAMGLAYTKPRRDRIFHYITAG	V	73
Aureobasidium_RD	KTADIAITVRGSDWYWTVCAMTTATFVFLGLGITKPRQHRIFHYITAA	I	70
Gibberella_mon_RD2	QRSDINITVRGSDWYWAVCAVMTVSTFAFLGLGMRKPRTRDRIFHYITAG	I	52
Gibberella_fuj_RD2	QRSDINITVRGSDWYWAVCAVMTVSTFAFLGLGMRKPRTRDRIFHYITAG	I	72
Fusarium_oxysp_RD2	QRSDINITVRGSDWYWAVCAVMTVSTFAFLGLGMRKPRTRDRIFQYITAG	I	52
Nectria_RD2	QSSDINLTTRGSDWYWAVCAVMTVATIAFIGTAWTKPRTRDRIFHYITGG	I	72
Gibberella_zeae_RD2	ISTQINITPRGSDWYFTVCAMTVSSIVFVGMGLRKPRTTHR VFHYITASI		74
Hysterium_RD2	QTAEIAITVRGSDFYWAI CSAMGLATLLFLAHAFTKPRSHRIFNYITAG	I	69
Rhytidhysterion_RD2	RRAEIAITIRGSDFYWAI CSLMGFATLCFLGLSLTKPRTHRIFHYITAG	I	59
Botrytis_RD2	ASSDISITTHGSDVYWAITAAMAFATIVFLALSFRVPRSKRVFHYITAA	I	70
Sclerotinia_RD2	TSTDIAITTRGSDVYWAITAAMAFATLCFLAWSFRIPRKRIFHYITAA	I	71
Leptosphaeria_RD1	TPIYETVGDGSKTLWVVFVLM L IASAAFTALSWKIPVNRRLYHVITTI	I	90
Phaeosphaeria_RD1	TPELQFIGESGQKTLWVVFVLM I IASAGFTALSWRVPLSKRLYHTITMI	I	85

Helix B

Pyrenophora_ter_RD2 VFVAAIAYFTMGSNLGFTPIRVEFFRSDPKVSG-----TYRAVYY 109
 Pyrenophora_trit_RD2 VFVAAIAYFTMGSNLGFTPIRVEFFRSDSVVRG-----TYRAVYY 109
 Alternaria_RD2 VVACIAYFTMGSNLGFTPIAVEFARSDPKIAG-----TYRSVYY 95
 Bipolaris_RD2 VFVAAIAYFTMGANLGFPIEVEFRRSDPVVRG-----TYRAIYY 109
 Cochliobolus_RD2 VFVAAIAYFTMGANLGFPIEVEFRRNNPVVRG-----TYRAVYY 109
 Leptosphaeria_RD2 VVACIAYFSMGSNLGFTPIEVEYKRSDPVVRG-----NFRGIFY 85
 Phaeosphaeria_RD2 VFVAAIAYFTMGSNLGFTPIEVEFKRNNPVVRG-----NYRSIYY 110
 Mycosphaerella_gr_RD2 TMVAAIAYFSMAAHLGWTEIDVEFVRS DPRVAG-----LTREIFY 108
 Mycosphaerella_fij_RD2 TMVAAIAYFTMGSHLGFPIIDVEFARSGPKVAG-----VNREIYY 93
 Cercospora_RD2 TMVAAIAYFSMGSNLGWTPINVEFERS DPRVAG-----LNREIYY 110
 Mycosphaerella_pop_RD2 TMVAAIAYFTMGSHLGFPIIDVEYQRSNSRVAG-----VNREIYY 105
 Dothistroma_RD2 VMVAAIAYFTMASHLGWTPIVIEFQRSNPVVRG-----QTREIYY 113
 Aureobasidium_RD TMVAAIAYFSMGSNLGWTPIDVEFSRNNPVVRG-----VNREIFY 110
 Gibberella_mon_RD2 TMIASIAYFTMASNLGWTPIAVEFQRS DHRVAG-----IYREIFY 92
 Gibberella_fuj_RD2 TMIASIAYFTMASNLGWTPIAVEFQRSNHRVAG-----IYREIFY 112
 Fusarium_oxysp_RD2 TMIASIAYFTMASNLGWTPIAVEFQRS DHRVAG-----IYREIFY 92
 Nectria_RD2 TMIAAISYFSMASNLGWTPIAVQFRRSDHRVAG-----VYREIFY 112
 Gibberella_zeae_RD2 TMVAAIAYFTMGANLGWAPTEVEFHRRDHEVAG-----NYREIFY 114
 Hysterium_RD2 TMVAFIAYYSMASNLGWTPIQVEYQRS DHRVSG-----MYREIFY 109
 Rhytidhysterion_RD2 TMVAFIAYFSMAANLGWVPIAVEFSRSDPKVAG-----AYREIFY 99
 Botrytis_RD2 TMTASIAYFTMASNLGYASIIQEFQRS DPKVSG-----VYREIFY 110
 Sclerotinia_RD2 TMTAAIAYFTMASNLGYASIIQEFQRGNPKVRG-----VTREIFY 111
 Leptosphaeria_RD1 TLTAALS YFAMATGHGVALNKIVI-RTQHDHVPD TYETVYRQVYY 134
 Phaeosphaeria_RD1 TIFAALS YFAMATGHGVS VQKIIV-REQHDHVPD TTFTEVHRQVFW 129

Helix C

Helix D

Pyrenophora_ter_RD2 ARYIDWFITTPLLLLDLLLLTAGTPWPTTLFVIAIDEIMIVTGLIGALVD 158
 Pyrenophora_trit_RD2 ARYIDWFITTPLLLLDLLLLTAGTPWPTTMFVIAVDEIMIITGLIGALID 158
 Alternaria_RD2 VRYIDWFITTPLLLLDLLLLTAGMPWPTLLWVIMVDEIMIVTGLIGALID 144
 Bipolaris_RD2 ARYVDWFITTPLLLLMDLLLLTAGMPWPTILWVILVDEIMIVTGLIGALIQ 158
 Cochliobolus_RD2 ARYVDWFITTPLLLLMDLLLLTAGMPWPTILWVILVDEIMIVTGLIGALIQ 158
 Leptosphaeria_RD2 VRYIDWVITTPLLLLDLLLLTAGMPWPTIIFVILIDEIMIVTGLVGLV 134
 Phaeosphaeria_RD2 VRYIDWVITTPLLLLDMLMLTAGMPWPSILWTIIVDEIMIITGLVGLV 159
 Mycosphaerella_gr_RD2 VRYIDWFITTPLLLLIDLMLTAAMPWPTTLFVVLVDEVMIITGLVGLVS 157
 Mycosphaerella_fij_RD2 VRYIDWFITTPLLLLDMLMLTAAMPWPTTAWVILVDEVMIITGLVGLVS 142
 Cercospora_RD2 VRYIDWFITTPLLLLADLMLTAAMPWPTTAFVILVDEVMIITGLVGLVS 159
 Mycosphaerella_pop_RD2 ARYIDWVITTPLLLLIDLMLTAAMPWPSILFVILVDEVMIITGLIGALVA 154
 Dothistroma_RD2 VRYIDWVITTPLLLLMDLLLLTAAMPWPTILWAILVDEVMIITGLVGLVA 162
 Aureobasidium_RD VRYIDWFITTPLLLLMDLLLLTAAMPWPTLLFVVLVDEVMIVTGLVGLVR 159
 Gibberella_mon_RD2 ARYIDWFLTTPLLLTDLLLLTAGMPWPTVLWVILVDWVMIVTGLVGLVK 141
 Gibberella_fuj_RD2 ARYIDWFLTTPLLLTDLLLLTAGMPWPTVLWVILVDWVMIVTGLVGLVK 161
 Fusarium_oxysp_RD2 ARYIDWFLTTPLLLTDLLLLTAGMPWPTVLWVILVDWVMIVTGLVGLVK 141
 Nectria_RD2 VRYIDWFITTPLLLLMDLLLLTAGMPWPTVLWVILVDWVMIVTGLVGLVK 161
 Gibberella_zeae_RD2 VRYIDWFITTPLLLLMDLLLLTAGMPWPTVLYVILVDEIMIVTGLVGLVT 163
 Hysterium_RD2 VRYIDWFITTPLLLLDLLLLTAGMPWPTVMWIIILVDEVMIVTGLVGLVR 158
 Rhytidhysterion_RD2 VRYIDWFITTPLLLLDLLLLTAGMPWPTVLWVILVDWAMIVTGLVGLVQ 148
 Botrytis_RD2 VRYIDWVTTPLLLLDILLTAGLPWPTILFTIFLDEIMIITGLVGLVA 159
 Sclerotinia_RD2 VRYIDWVTTPLLLLDILLTAGLPWPTILFTIFLDEVMIITGLVGLVA 160
 Leptosphaeria_RD1 ARYIDWAIITTPLLLLDLGLLAGMSGAHIFMAIVADLIMVLTGLFAAFGS 183
 Phaeosphaeria_RD1 ARYVDWSVTTPLLLLDLGLLAGMSGGHIIMAIIVADLIMILTGLFAAFGE 178

	Helix E	Helix F	
Pyrenophora_ter_RD2	--NRYKWAYFVFGCVALFYIVYHLVWESRLQAKKFGRDVERCFLMCGSLT		206
Pyrenophora_trit_RD2	--NRYKWAYFVFGCVALFYIVYHLVWESRLQAKKFGRDVERCFLMCGSLT		206
Alternaria_RD2	--NRYKWAYFVFGCVALFYIVYQLAWESRIHAKSFGRDVERTFMMCGSLT		192
Bipolaris_RD2	--SIYKWPFVFGCVALFYIVFQLTWEARIHSKTFGRDVERTFLMCGSLT		206
Cochliobolus_RD2	--SIYKWPFVFGCAALFYIVFQLTWEARIHSKTFGRDVERTFLMCGSLT		206
Leptosphaeria_RD2	--SSYKWGFFAFGCAALVYVVYQLVWESRRHSKFFGRDVERTFLLCGSLT		182
Phaeosphaeria_RD2	--SKYKWGYFAFGNLALVYI IYQLVWESRTHARHFGRDVERTFLMCGSLT		207
Mycosphaerella_gr_RD2	--SSYKWGYFTFGCVALVYIVYVLVWEARKHANGVSSDAGKAFLYCGTLT		205
Mycosphaerella_fij_RD2	--SSYKWGYFVFGCVALIWIIVYVLVWEARKHAYGVSSDAGKAFMFCGSLT		190
Cercospora_RD2	--SSYKWGYFTFGCVALGYIVYVLWWEARLHANGISSDAGKAFLYCGSLT		207
Mycosphaerella_pop_RD2	--SSYKWGYFVFGCVALVYIVYVLVWEARKHANGVSSDAGKTFLYCGSLT		202
Dothistroma_RD2	--SSYKWGYFVFGCVAMFWI IYILVWEARIHANAISTDAGRAFVICGSLT		210
Aureobasidium_RD	--SSYKWGYFVFGCVALFYVWVVLVWEARRHANALGSDVGRAFTICGSLT		207
Gibberella_mon_RD2	--SSYKWGYFAFGCAALAYIVYVLWWEARLHAKHVGPVGRTFVMCGSLT		189
Gibberella_fuj_RD2	--SSYKWGYFAFGCAALAYIVYVLWWEARLHAKHVGPVGRTFVMCGSLT		209
Fusarium_oxysp_RD2	--SSYKWGYFAFGCAALAYIVYVLWWEARLHAKHVGPVGRTFVMCGSLT		189
Nectria_RD2	--SSYKWGYFAFGCAALAYIVYQLAWEARIHANRIGNDVGRVFLACGTIT		209
Gibberella_zeae_RD2	--TSYKWGYFTIGCVALVYIVYQLAWEARIHANHVGPVGRVFLWCGSLT		211
Hysterium_RD2	--TRYKWGYFVFGCAALAYIMYHLAWESRRNASRLGNDIGRVFLMCGSLT		206
Rhytidhysterion_RD2	--SRYKWGYFTFGCVALFYI IYQLAWEARRHATKLGEDVGRAFLYCGSLT		196
Botrytis_RD2	--SSYKWGYFVFAMAALFGIAWNILFVGAQHAKALGSEVNKVYWTCCGGVT		207
Sclerotinia_RD2	--SSYKWGYFVFAMFALFGIAWNILFVGARHAKSLGTEVNKTYWMCGGIT		208
Leptosphaeria_RD1	EGTPQKWGWYTIACIAYIFVWHLVLNNGGANARVKGEKLRSFVVAIGAYT		233
Phaeosphaeria_RD1	EGTPQKWGWYTIACIAYIFVIWHLALNNGGANATSKGPKLRSFVVAIGGYT		228

	Helix F	Helix G	
Pyrenophora_ter_RD2	AFLWILYPIAWGICEGANLIAPDSEAVFYGVLDLFLAKPIFGALLLWGHR		255
Pyrenophora_trit_RD2	AFLWLLYPIAWGVCEGANLVAPDSEAVFYGVLDLFLAKPIFGALLLWGHR		255
Alternaria_RD2	TLLWILYPIAWGVCEGANLIAPDSEAVFYGVLDLFLAKPCFGALLLWGHR		241
Bipolaris_RD2	AFLWILYPIAWGLSEGGNVIAPDSEAVFYGVLDLFLAKPVFGALLLWGHR		255
Cochliobolus_RD2	AFLWILYPIAWGLSEGGNVIAPDSEAVFYGVLDLFLAKPVFGALLLWGHR		255
Leptosphaeria_RD2	SFLWILYPIAWGLCEGGNVIAPDSEAVFYGVLDLFLAKPIFGALLIWGHR		231
Phaeosphaeria_RD2	AFLWILYPIAWGVAEGGNVIAPDSEAVFYGVLDLFLAKPVFGALLIWGHR		256
Mycosphaerella_gr_RD2	AFLWTLYPIAWGVAEGGNI IAPDSEAVFYGILDVLAKPVFGALLIWGHR		254
Mycosphaerella_fij_RD2	ALLWTLYPIAWGVSEGGNVIAPDSEAVFYGILDILAKPFGALLLWGHR		239
Cercospora_RD2	AFLWILYPIAWGVCEGGNVIAPDSEAVFYGILDLLAKPVFGALLIWGHR		256
Mycosphaerella_pop_RD2	AFLWILYPIAWGVSEGGNI IAPDSEAVFYGILDLLAKPLFGALLIWGHR		251
Dothistroma_RD2	AFMWTLYPIAWGLSEGGNISSDGEAAFYGVLDLIAKPVFGALLIWGHR		259
Aureobasidium_RD	TFLWILYPLAWGLCEGGNVIAPDSEAVFYGILDLLAKPVFGALLIWGHR		256
Gibberella_mon_RD2	AVVWILYPIAWGVCEGGNLIAPDSEAVFYGILDLIAKPVFGALLLWGHR		238
Gibberella_fuj_RD2	AVVWILYPIAWGVCEGGNLIAPDSEAVFYGILDLIAKPVFGALLLWGHR		258
Fusarium_oxysp_RD2	AVVWILYPIAWGVCEGGNLIAPDSEAVFYGILDLIAKPVFGALLLWGHR		238
Nectria_RD2	LIVWICYPIAWGVCEGGNI IAPDSEAVFYGILDLLAKPVFGAILLWGHR		258
Gibberella_zeae_RD2	AVVWILYPIAWGVCEGGNLIAPDSEAVFYGILDIIAKPVFGAILLFGHR		260
Hysterium_RD2	LVVWVLYPIAWGVCEGGNVIAPDSEAVFYGILDIIAKPVFGTMLLLGHR		255
Rhytidhysterion_RD2	LVLWICYPIAWGVCEGGNVIAPDSEAVFYGVLDLFLAKPVFGALLMFGHR		245
Botrytis_RD2	MFLWFLYPIAWGLSEGGNVIAPDSEAVFYGVLDVLAKIGFGSLLLFGR		256
Sclerotinia_RD2	MFLWFLYPIAWGLSEGGNI IAPDSEAVFYGVLDVLAKIGFGILLNHR		257
Leptosphaeria_RD1	LILWTAYPIVWGLADGARKIGVDGEI IAYAVLDVLAKGVFGAWLLVTHA		282
Phaeosphaeria_RD1	LLLWTAYPMVWGLADGSRKIGVDGEVIAYAILDVLAKGVFGAWLLITHA		277

Pyrenophora_ter_RD2	DIDPARLGLAIRDYDG-DAVVHEKVKP-----AQNHSAAPP--VDATV	295
Pyrenophora_trit_RD2	DIDPARLGLAIRDYDG-DAVVHEKVKP-----AQNHSAAPP--VDATV	295
Alternaria_RD2	GIDPARLGLSITDYDG-DAMVHEKRNP-----TVNNDQTATHPDGYNGTA	286
Bipolaris_RD2	NIDPARLGLQIRDYND-DTMLQEKRKPD----AAAHNGQNVVNPPhDGPA	300
Cochliobolus_RD2	NIDPARLGLQIRDYND-DTMLQEKRKPD----AAAHNGQNVVNPPhDGPA	300
Leptosphaeria_RD2	NVDPARLGLAIRDYGDADAVVHEKRAP-----AVHPDGPINN	240
Phaeosphaeria_RD2	NIDPARLGLAIKDYDH-DTSVSEKRKPD----VAPGTAAHNPPPhDGPA	300
Mycosphaerella_gr_RD2	NISPAQLGLTIRDYNGTDAVIHEKRTGV----ANGNTSNVTHENAAAN--	298
Mycosphaerella_fij_RD2	NISPAQLGLSIRDYDGTDPVIHEKR-----	264
Cercospora_RD2	NITPAQLGLTIHDYGGDDPVVHHEKSAG----APGHTGHPSENPALHNNG	302
Mycosphaerella_pop_RD2	NISPAQLGLTIHDYGGEDPVIHEKNTGI----SHGHTGHPGDNPANVGV	297
Dothistroma_RD2	NISPADLGLAIHDYGADEPIFHEKNHRN----GNGPLADHSGATRPINSA	305
Aureobasidium_RD	GIDPARLGLYIHDYDEKDPVVKDKVG-----APGPNVHPNTNNAAT--	298
Gibberella_mon_RD2	NIDPARLGLRIRDIDER-IFPDGP-----NNKAASGHGARNDTA-	276
Gibberella_fuj_RD2	NIDPARLGLRIRDIDER-IFPDGP-----NNKVASGHGARNDTAT	297
Fusarium_oxysp_RD2	NIDPARLGLRIRDIDER-IFPDGP-----NNKATSGHGARNDTA-	276
Nectria_RD2	NIDPARLGLRIRDVTEGPPVYPEGPGAQK----RSVNEPAVGAGANGTQNP	304
Gibberella_zeae_RD2	NIDPARLGLRIRDVNER-IVPEGP-----NVKPGQQRNAGNVNAP	299
Hysterium_RD2	NIDPARLGLQIRDYDEDLSVHGGL-----GRGEKRTPNAPLDGPA	295
Rhytidhysterion_RD2	NIDPGRLGLRIRDYDEDPSIHGGVSGREKALHQNGTNEAQAVGGVTDGAT	295
Botrytis_RD2	NIDPAHLGLHIRDYNEQPRTFNDKDVGHNGAHHDGAHVPTNNGYREGQ	306
Sclerotinia_RD2	NIDPAHLGLHIRDYNEQPGSFHEKNS-YANGAAGS-SSAPVTG-----	298
Leptosphaeria_RD1	NLRESDELNGFWANGLNREGAIRIGEDDGA-----	313
Phaeosphaeria_RD1	KLRESDELNGFWSNGLNSEGAVRLGEDDGA-----	308

2. Supplementary figures

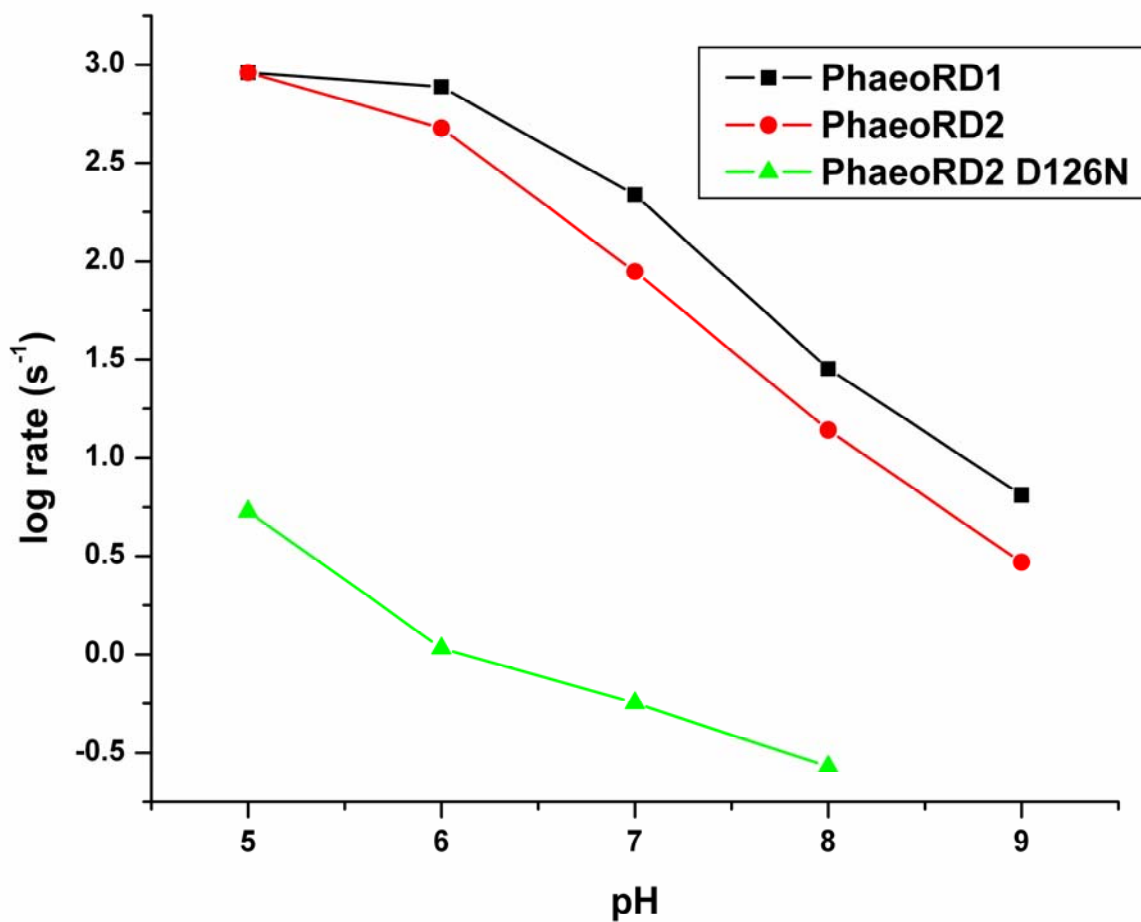
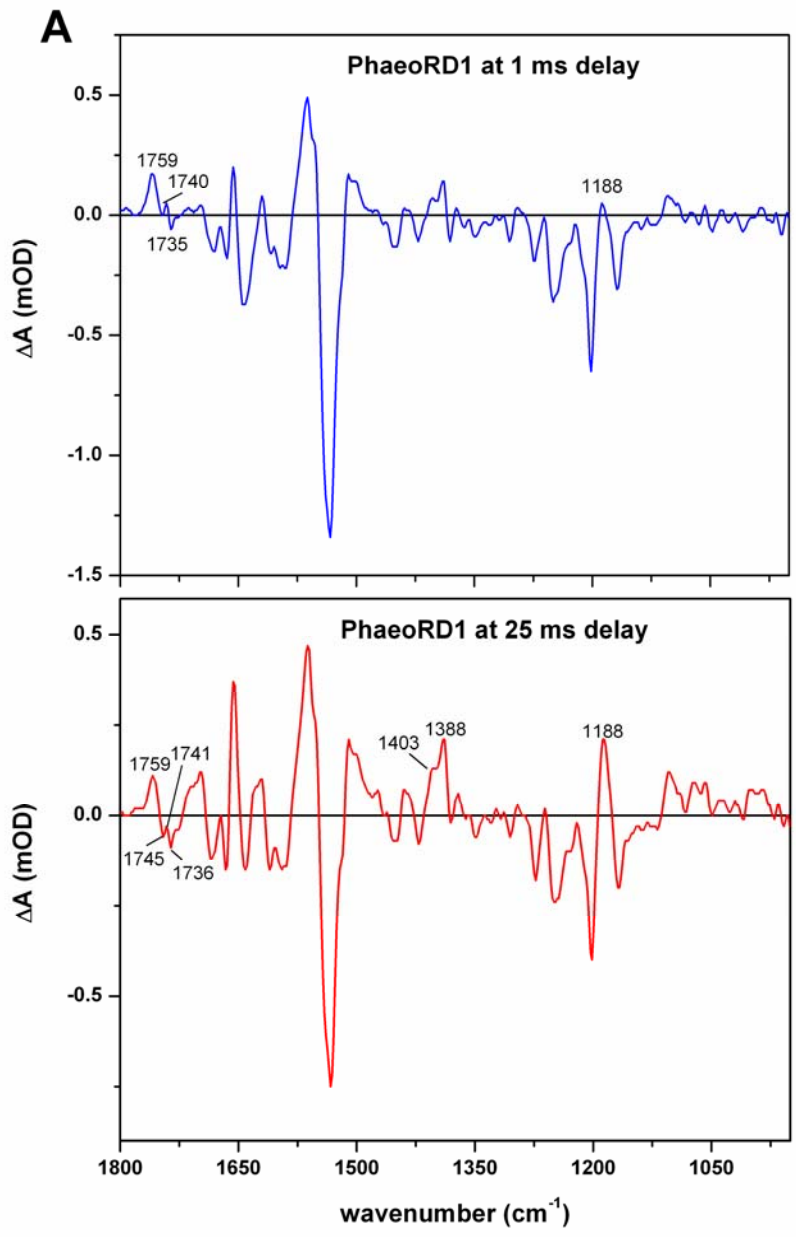


Figure S1. pH-dependence of the rates of reprotonation of the Schiff base (M decay) obtained by the exponential fitting of the data shown in Figs. 4-6.



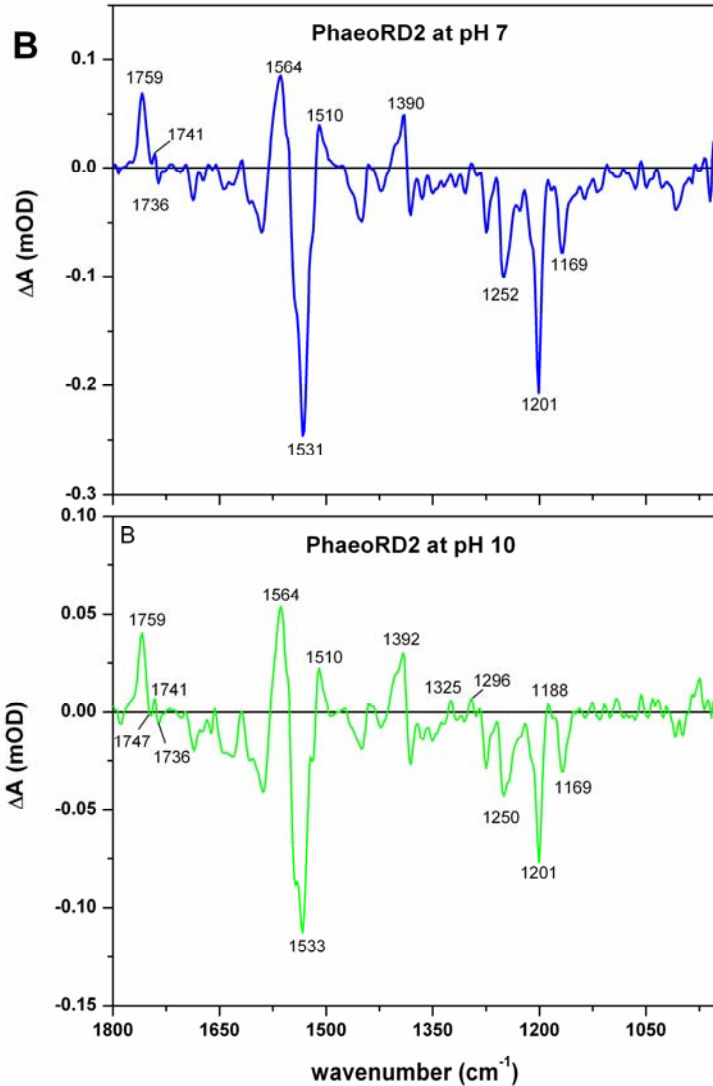


Figure S2. Time-resolved laser flash-induced difference FTIR spectra of *Phaeosphaeria* rhodopsins reconstituted into DMPC/DMPA liposomes under the conditions promoting accumulation of the late intermediates. Difference spectra of the mixtures dominated by the M intermediates from Fig. 7 are given for the comparison. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. (A) PhaeoRD1 liposomes hydrated with 0.05 M KH_2PO_4 , 0.1 M NaCl, pH 7, and measured at 1 ms (upper panel, from Fig. 7) and 25 ms (lower panel) delays after the flash (but note the 12 ms full interferogram acquisition time) at 12°C. (B) PhaeoRD2 liposomes measured at 1 ms delay after the flash, hydrated with 0.05 M KH_2PO_4 , 0.1 M NaCl, pH 7 (upper panel, from Fig. 7) and with 0.05 M KH_2PO_4 , 0.1 M NaCl, 0.05 M CHES, pH 10 (lower panel).