Figure S1. Characterization of the OsHO2 homozygous T-DNA insertion mutant (1C-07010). a. Os03g27770 gene structure and T-DNA insertion site. Exons and introns are represented by boxes and lines, respectively. b. Phenotypes of 10-day-old WT and 1c-07010 homozygous insertion plants. c. RT-PCR gene expression analysis using primers HO-F and HO-R. d. Different Chl precursors (Proto IX, Mg-Proto, PChlide, and Chlide) were measured in 1-week-old WT and 1c-07010 mutant plants. e. Levels of ALA. f. Quantitative RT-PCR expression analysis of HEMA, CHLD, CHLI, CHLH. Total RNA was extracted from leaves of 7-day-old plants. OsActin expression was used for normalization. All error bars = SD (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test).
Figure S2. Sequence analysis of HO2 homologs. Alignment of OsHO2 and related homologs was performed using CLUSTALW (http://www.ebi.ac.uk/clustalw). The predicted transit peptide cleavage site is indicated by an arrow and the HO signature sequence is boxed. The black line above the sequences indicates the region of the predicted HO-like domain. The heme-binding histidine residue conserved in bona fide HO1 sequences is replaced by an arginine residue (blue circle). On the other hand, the conserved histidine residues for protein stability (blue diamond) and ascorbic acid-binding (black diamond) are identified. The predicted ylc2 mutant protein sequence is also included.
Figure S3. Pairwise 3D structural alignment of OsHO2 WT and ylc2 mutant proteins. Primary sequences were first analyzed by the Phyre server (www.sbg.bio.ic.ac.hk/phyre). The PDB files generated were then submitted to the iPBA server (www.dsimb.inserm.fr/dsimb_tools/ipba) for the pairwise 3D structural comparison. The 3D models were constructed based on alignment of the 156-298th and 156-257th residues of the WT and mutant proteins, respectively, to the template d1wova1 (Synechocystis sp. HO2). In the superimposed image, the overlapping regions are shown in both red (WT) and green (mutant). Three C-terminal α-helices (boxed) present in WT OsHO2 are missing in the mutant protein.
Figure S4. Phylogenetic relationships of OsHO1, OsHO2 and their closely related homologs. The un-rooted phylogenetic tree was constructed by the maximum likelihood method using MEGA6. Bootstrapping with 1000 replications was performed to show the confident level of the branching. Scale bar represents 0.1 substitutions per site. Accession numbers: NP_001050290 for OsHO2 (rice), AF132477 for AtHO2 (Arabidopsis), AF320027 for SbHO2 (sorghum), NP_001234531 for SIHO2 (tomato), NP_001140654 for ZmHO2 (maize), XP_002282356 for VvHO2 (grape), AET97567 for BrHO2 (Brassica rapa), AB_021858 for AtHO1 (Arabidopsis), AF_320022 for AtHO3 (Arabidopsis), AF_320023 for AtHO4 (Arabidopsis), XP_002438642 for SbHO1 (sorghum), NP_001058011 for OsHO1 (rice), NP_001140654 for ZmHO1 (maize), AEI_69673 for NtHO1 (tobacco), respectively.
Figure S5. HO enzyme activity assays of recombinant OsHO2. a. SDS-PAGE gel image of OsHO2 expression in P. pastoris. The recombinant His-tagged protein was purified from the culture lysates by Ni\(^{2+}\) affinity chromatography. FT, column flow-through sample; W1 – W2, washing fractions. E1 – E4, elution fractions. M, molecular weight marker. b. LC-MS/MS analysis of the enzyme assay of HO using heme as a substrate. The reaction product BV (m/z = 583) was detected in a positive assay control using recombinant AtHO1. The identity was confirmed by MS/MS fragmentation of authentic BV standard (c) and the AtHO1 assay product (d). However, no BV was detected in assays containing purified OsHO2 (b). Therefore, OsHO2 does not function as a bona fide HO enzyme.