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Techniques

Evaluation of Unbound Free Heme in Plant Cells by Differential Acetone Extraction

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Heme functions not only as a prosthetic group of hemoproteins but also as a regulatory molecule, suggesting the presence of 'free' heme. Classically, total non-covalently bound heme is extracted from plant samples with acidic acetone after removal of pigments with basic and neutral acetone. Earlier work proposed that free heme can be selectively extracted into basic acetone. Using authentic hemoproteins, we confirmed that acidic acetone can quantitatively extract heme, while no heme was extracted into neutral acetone. Meanwhile, a certain amount of heme was extracted into basic acetone from hemoglobin and myoglobin. Moreover, basic acetone extracted loosely bound heme from bovine serum albumin, implying that the nature of hemoproteins largely influences heme extraction into basic acetone. Using a highly sensitive heme assay, we found that basic and neutral acetone can extract low levels of heme from plant samples. In addition, neutral acetone quantitatively extracted free heme when it was externally added to plant homogenates. Furthermore, the level of neutral acetone-extractable heme remained unchanged by precursor (5-aminolevulinic acid) feeding, while increased by norflurazon treatment which abolishes chloroplast biogenesis. However, changes in these heme levels did not correlate to *genomes uncoupled* phenotypes, suggesting that the level of unbound free heme would not affect retrograde signaling from plastids to the nucleus. The present data demonstrate that the combination of single-step acetone extraction following a sensitive heme assay is the ideal method for determining total and free heme in plants.

Keywords: Free heme • Heme • Horseradish peroxidase (HRP) • HRP-based heme assay (HH assay).

Abbreviations: ALA, 5-aminolevulinic acid; BSA, bovine serum albumin; Cat, catalase; FC, ferrochelatase; HH assay, HRP-based heme assay; HRP, horseradish peroxidase; Hb, hemoglobin; Mb, myoglobin; MS, Murashige and Skoog; NF, norflurazon.

Introduction

Heme is an essential molecule that is responsible for crucial biological activities including oxygen metabolism and transfer, electron transfer and secondary metabolism. Heme is a tetrapyrrole that binds ferrous iron (Fe^{2+}) at four coordinated nitrogens in the protoporphyrin ring system and is incorporated into various apoproteins as a prosthetic group. The most common type of heme is heme *b*, which is formed from protoporphyrin IX in one step by ferrochelatase (FC). Other types of heme such as heme *a* and heme *c* are synthesized from heme *b*. Heme *a* and heme *b* are non-covalently bound to the hemoproteins, while heme *c* is covalently attached to cysteine residues of the hemoprotein, as observed in Cyt *c*.

In higher plants, heme *b* (hereafter referred to as 'heme') is synthesized in plastids and it shares a common biosynthetic pathway with Chl up to the intermediate protoporphyrin IX. These pathways diverge at the point of Fe^{2+} or Mg^{2+} insertion by the FC and magnesium chelatase, respectively. Heme is well known as a negative feedback inhibitor of 5-aminolevulinic acid (ALA), the first committed precursor of tetrapyrrole biosynthesis (Beale 1999). This step is considered to be the rate-limiting step for total tetrapyrrole biosynthesis and is regulated by endogenous levels of heme in both animal and plant systems (Beale 1999). Apart from these functions, recent evidence has identified novel roles for heme in regulatory processes such as transcription (Guarente and Mason 1983, Zitomer and Lowry 1992, Shan et al. 2004, Sun et al. 2004, von Gromoff et al. 2008), translation (Joshi et al. 1995), post-translational protein modification (Chen et al. 1989), protein translocation (Lathrop and Timko 1993) and ion-channel function (Tang et al. 2003). Recent transcriptome analysis also showed that in

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Chlamydomonas reinhardtii, heme has global impacts on gene expression (Voss et al. 2011).

More recently, heme has been particularly emphasized as a plastid-derived signal to coordinate nuclear gene expression. It is known that expression of photosynthesis-related nuclear genes is shut off when the function of the plastids is impaired by inhibitor treatments or under stressful conditions. Thus, it is hypothesized that the functional status of the plastids is transmitted via an unidentified signaling pathway to the nucleus (Larkin and Ruckle 2008). This hypothetical signaling pathway is called retrograde signaling and signaling molecules are referred to as 'plastid signals'. Susek et al. (1993) first identified five Arabidopsis *gun* (*genomes uncoupled*) mutants in which the retrograde signaling pathway is impaired. These mutants express nuclear-encoded photosynthesis genes even when chloroplast function is disrupted by treatment with norflurazon (NF), an inhibitor of carotenoid biosynthesis. Among the five *gun* mutants (*gun1*–*gun5*), four of them (*gun2*–*gun5*) have mutations in tetrapyrrole biosynthetic enzymes (Mochizuki et al. 2001), suggesting an involvement of tetrapyrrole metabolism in the retrograde signaling. Recently, Woodson et al. (2011) reported that overexpression of ferrochelatase isoform (FC1) also shows a *gun* phenotype. They propose that a specific heme pool that is produced by FC1 functions as a retrograde signal to coordinate nuclear gene expression.

As heme may exist as an intermediate for myriad metabolic fates, the existence of a 'free' heme pool has been considered (Duggan and Gassman 1974). Meanwhile, it is known that excess intracellular heme is highly toxic to cells because it can react with oxygen at one of the two uncoordinated binding sites of Fe^{2+}, oxidizing it to Fe^{3+} and producing a reactive oxygen species (Jones et al. 1973, Kumar and Bandyopadhyay 2005). Thus, the presence of free heme should be maintained at a low concentration to prevent oxidative stress through the uncontrolled oxidation of heme iron (Khan and Quigley 2011). It is assumed that given heme's low solubility in aqueous solution, free heme is probably dissolved in the membranes or adhered non-specifically to proteins (Thomas and Weinstein 1990). Thus, free heme can be defined as the heme that is not associated with hemoproteins acting as a prosthetic group, but adhering non-specifically to membranes or proteins, and functions as metabolic intermediates or second messengers of vital biological processes.

An assay that can accurately measure the differential pools of heme is thus important for the examination of the multiple cellular roles of heme. Determination of total heme is generally performed by sequential acetone extractions (Stillman and Gassman 1978, Weinstein and Beale 1983), which is widely adopted as the standard extraction procedure for heme (Masuda et al. 1990, Papenbrock et al. 1999, Peter and Grimm 2009). First, major pigments such as Chls and carotenoids, which interfere with spectroscopic determination of heme, are removed from plant tissues by basic acetone containing ammonia. After washing the precipitates with neutral 80% acetone, non-covalently bound heme is extracted from the precipitates into acidic acetone containing HCl. Usually, acidic acetone is 80% (v/v) acetone containing 20% (v/v) 0.6–2.1 M HCl (Schneegurt and Beale 1986, Stillman and Gassman 1978, Thomas and Weinstein 1990). On the other hand, it has been proposed that free heme can be extracted by basic acetone, while acidic acetone can extract total heme including non-covalently bound heme (Thomas and Weinstein 1990). Based on the hypothesis that loosely bound free heme can be extracted into basic acetone without denaturing hemoproteins, the free heme level in isolated chloroplasts and intact seedlings has been determined (Thomas and Weinstein 1990, Voigt et al. 2010). In contrast, previous studies reported that no heme was detectable in the initial basic or neutral acetone extractions (Castelfranco and Jones 1975, Stillman and Gassman 1978), implying that the presence of free heme in plant cells is still unclear.

Here, we evaluated whether unbound free heme can be determined by differential acetone extraction using a set of known heme-binding proteins. In addition, by using the recently developed sensitive and specific heme assay (Masuda and Takahashi 2006, Takahashi and Masuda 2009), we evaluated the extraction of free heme from plant cells. The present results show that the neutral acetone-extractable heme may represent an index of free heme which is not bound to proteins. Our data also suggest that the presence of unbound free heme is maintained at a very low concentration in plant cells as well as in mammalian cells (Khan and Quigley 2011). When the structural integrity of chloroplasts, which function as a major heme reservoir, is disrupted by NF, an increase in the endogenous free heme level was observed. However, changes in the free heme levels did not correlate to the *gun* phenotype, suggesting that if heme is functioning as an organelle-derived signal, such signaling heme may be transferred by binding to a specific trafficking system rather than in the unbound state.

Results

Solubility of heme and hemoproteins in neutral, basic and acidic acetone

We first determined whether the heme non-covalently bound to authentic hemoproteins can be extracted in neutral, basic or acidic acetone. In this experiment, we used 80% (v/v) acetone containing 20% (v/v) 1.6 M HCl as the acidic acetone. It is reported that non-covalently bound heme cannot be removed from holo-hemoproteins by neutral or basic acetone extraction, but is extractable into acidic acetone (Stillman and Gassman 1978). Thomas and Weinstein (1990) proposed that free heme that is dissolved in membranes or non-specifically adhered to proteins can be extracted into basic acetone without denaturing *b*-type hemoproteins. To test these hypotheses, we examined heme extraction of commercially available hemoproteins that have non-covalently bound heme. Hemoglobin (Hb) is a tetramer of four subunits, and myoglobin (Mb) is a monomer. The globular subunits (globin) of Hb (~16 kDa) and Mb

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(∼18 kDa) are homologous and each subunit binds one heme molecule. Catalase (Cat) is a tetramer of four subunits (60–80 kDa), where each binds one heme molecule. We dissolved bovine Hb containing 100 nmol of globin subunits, horse muscle Mb containing 100 nmol of globin subunits and *Aspergillus niger* Cat containing 10 nmol of subunits in Tris buffer, and separately extracted each with a total 1 ml of neutral, basic and acidic acetone. After centrifugation, the heme concentration in each supernatant was determined spectrophotometrically (Weinstein and Beale 1983, Thomas and Weinstein 1990). Heme contents in the acidic acetone extracts from Hb, Mb and Cat were 96.5 ± 1.7, 96.3 ± 0.9 and 10.1 ± 0.1 μM, respectively, showing that the heme non-covalently bound to these proteins was quantitatively extracted in each acidic acetone (**Fig. 1**). In contrast, no heme was detectable in neutral acetone extracts from any proteins. When extracted with basic acetone, no heme was detected from Cat; however, intermediate levels of heme were extracted at concentrations of 6.8 ± 0.0 and 12.0 ± 0.0 μM from Hb and Mb, respectively (**Fig. 1**).

To examine whether the level of detected heme in the acetone extracts is related to the protein solubility of each hemoprotein, we quantified the protein concentration of each acetone extract. Consistent with the heme content, the proteins showed very low solubility in neutral acetone. Moreover, Cat was insoluble not only in the basic acetone but also in the acidic acetone, showing that heme was completely dissociated from Cat and extracted into the acidic acetone without protein solubilization (**Fig. 1**). In contrast, 42.8% of Hb and 51.2% of Mb were dissolved in the acidic acetone extracts. These proteins are also dissolved in the basic acetone, and the levels of Hb (7.7 ± 1.7 μM) and Mb (11.1 ± 1.1 μM) dissolved in basic acetone were almost consistent with those of extracted heme, respectively (**Fig. 1**).

We then examined the efficiency of extraction from different amounts of Hb, since the concentration of solute has a significant impact on the yield of extraction. As shown in **Fig. 2A**, acidic acetone quantitatively extracted heme from Hb up to 200 μM with high linearity, while no heme was extracted in the neutral acetone (**Fig. 2A, B**). It was only at the lowest concentration of Hb that a small amount of heme was extracted (6%) into neutral acetone. In the basic acetone, high recovery of heme was observed at the lowest concentration of Hb (66% at 12.5 μM Hb). However, the efficiency of extraction decreased as the concentration of Hb increased (**Fig. 2B**), probably because of co-precipitation of heme with aggregated Hb (**Fig. 2A, B**). These results suggest that the heme concentration in the basic acetone reflects protein solubility, while all non-covalently bound heme can be extracted into the acidic acetone irrespective of the protein solubility.

Next, we investigated whether the heme non-specifically bound to protein is extractable by neutral or basic acetone solvents. In humans, it is reported that serum albumin has two heme-binding sites with different affinities of $K_1 = 7.8 \times 10^7\ M^{-1}$ and $K_2 = 2.3 \times 10^8\ M^{-1}$ (Rosenfeld and Surgenor 1950, Gattoni et al. 1996). We made a heme and bovine serum albumin (BSA) mixture with a molar ratio of 3.7 and then extracted heme from it with basic or neutral acetone. As shown in **Table 1**, free and bound hemes were extracted in these acetones, whereas BSA was quantitatively precipitated in these acetones. The molar ratios of the unrecovered portion of heme to co-precipitated BSA were calculated by subtracting the concentrations of extracted from initially added heme, which were 0.87 and 2.38 for basic and neutral acetones,

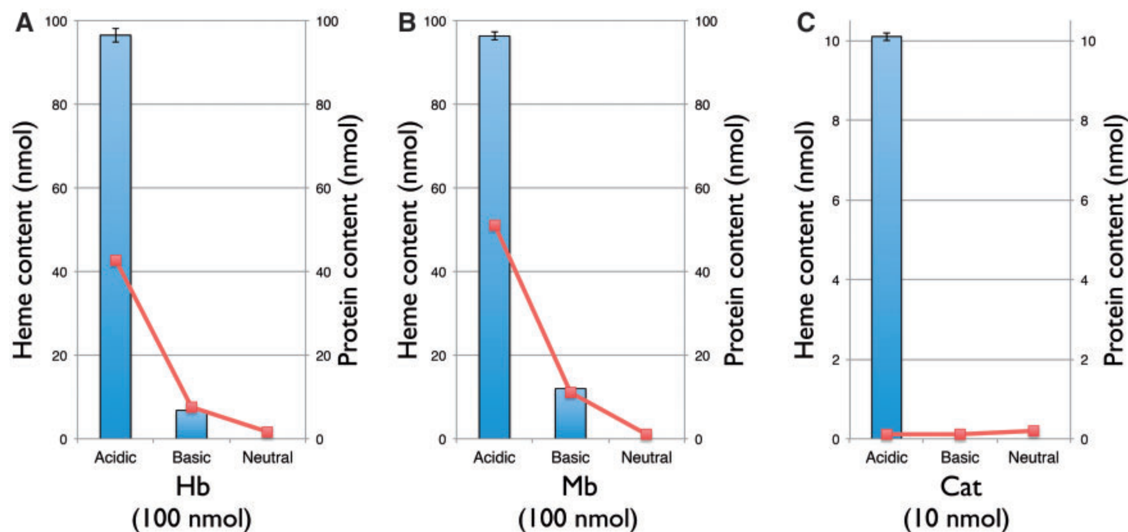

Fig. 1 Extraction of heme from hemoproteins. Bovine Hb containing 100 nmol of globin (A), horse muscle Mb containing 100 nmol of globin (B) and *Aspergillus niger* Cat containing 10 nmol of globin subunits (C) were directly extracted with a total of 1 ml of acidic, basic or neutral acetone. Bars indicate heme contents in these acetone extracts determined spectrophotometrically. Lines indicate protein contents in these extracts determined by the Lowry method. Data shown are the means ± SD ($n > 3$).

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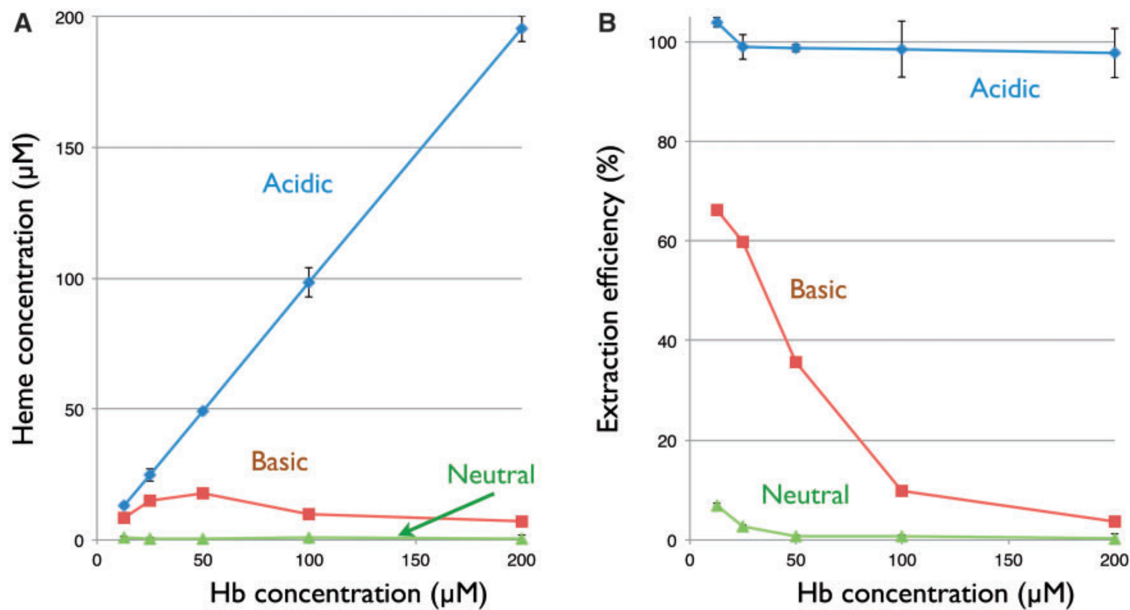

Fig. 2 Efficiency of heme extraction from Hb. (A) Different amounts of Hb were extracted with neutral, basic or acidic acetone. Heme concentrations in these acetone extracts were determined spectrophotometrically. Data shown are the means ± SD ($n > 3$). (B) Extraction efficiency was calculated by dividing the extracted heme concentration by the Hb concentration. Data shown are the means ± SD ($n > 3$).

Table 1 Heme extraction from the heme–BSA mixture

	Basic acetone	Neutral acetone
Heme (nmol)	22.96	22.96
BSA (nmol)	6.25	6.25
Heme : BSA ratio	3.7	3.7
Extracted heme (nmol)	17.48 ± 1.01	8.06 ± 0.67
Precipitated heme (nmol)	5.47	14.90
Precipitated BSA (nmol)	6.25	6.25
Precipitated heme : BSA ratio	0.88	2.38

Heme was mixed with BSA solution with molar ratio of 3.7. It was extracted from 50 μl of the heme–BSA mixture with 950 μl of neutral or basic acetone. Heme contents in these acetone extracts were determined spectrophotometrically. The precipitated heme level was calculated by subtracting the concentrations of extracted from initially added heme. Data shown are the means and SD ($n > 3$).

respectively. These results indicate that basic acetone can extract one of two bound heme molecules from BSA, while neutral acetone cannot extract any bound heme from BSA probably including heme non-specifically adhered to BSA. Thus, it revealed that basic acetone not only dissolves certain hemoproteins such as Hb, but also extracts heme molecules bound to non-hemoproteins, such as BSA, with low affinity. Meanwhile, neutral acetone can only extract heme not bound to proteins which may represent a major portion of free heme.

Oxidation state of heme extracted from Hb

Absorption spectra of all extracted heme showed the Soret absorption peak at 398 nm, suggesting that the heme molecules are air oxidized to the Fe^{3+} form. It is known that active Hb binds Fe^{2+} heme as oxygenated or deoxygenated Hb, while inactive methemoglobin binds Fe^{3+} heme. Absorption spectra of Hb in Tris buffer showed the Soret peak at 406 nm (**Supplementary Fig. S1**). Since horseradish peroxidase (HRP), which is used for the following heme assay, is active with Fe^{3+} heme, we determined the oxidation state of Hb used in this assay as described (Evelyn and Malloy 1938, Leahy and Smith 1960). Approximately 94% of Hb was present as methemoglobin, showing that most of Hb binds Fe^{3+} heme. We also examined whether extracted heme from Hb is air oxidized to the Fe^{3+} state. The Soret peak of the acetone extract from Hb matched with that of the authentic hemin solution (**Supplementary Fig. S1**). When dithionite was added to the authentic hemin to reduce Fe^{2+}, the Soret band shifted from 398 to 415 nm. This peak was readily reoxidized to Fe^{3+} hemin having the Soret peak at 398 nm. Thus, once heme is extracted from Hb, it is readily air oxidized to Fe^{3+} hemin.

Reconstitution of HRP with heme extracted from hemoproteins by acidic acetone

Recently, we developed a sensitive and specific heme determination method based on the ability of HRP apo-enzyme to reconstitute an active holo-enzyme with heme (Masuda and Takahashi 2006, Takahashi and Masuda 2009). Sensitive chemiluminescence detection of HRP activity enables high-throughput and extremely sensitive measurement of heme. This HRP-based heme assay is designated as the 'HH assay' and used for the following analysis. To examine whether heme extracted from the hemoproteins into acidic

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acetone can reconstitute the HRP apo-enzyme, we performed the HH assay using the acidic acetone extracts from Hb, Mb and Cat (**Supplementary Table S1**). Each hemoprotein containing 1.0 nmol of heme was extracted with acidic acetone and the heme concentration was determined by the HH assay. As shown in **Supplementary Table S1**, the heme content determined by the assay was approximately 1.0 nmol for all of the hemoproteins and the authentic heme extracts, showing that all of the heme extracted by the acidic acetone was used for the reconstitution of holo-HRP.

Heme extraction by neutral, basic and acidic acetone from plant tissues

By extraction with basic acetone, Thomas and Weinstein (1990) estimated the level of free heme in isolated chloroplasts as $\sim 47 \pm 17$ pmol heme mg^{-1} protein. Meanwhile, other studies reported that no heme was extractable from plant tissues by basic or neutral acetone extraction (Castelfranco and Jones 1975, Stillman and Gassman 1978). However, owing to the low sensitivity of the heme assay used in these studies (the pyridine–hemochromogen method) (Fuhrhop and Smith 1975), the possibility that they failed to detect low amounts of heme in the basic or neutral acetone cannot be excluded. To address whether heme is detectable in the neutral or basic acetone extracts from plant tissues, we performed the HH assay for heme determination in each acetone extract.

We first confirmed whether contamination of Chl or carotenoid pigments interferes with the chemiluminescent measurement of the HH assay. We mixed a definite amount of authentic heme solution (50 pM) and acetone extracts from Arabidopsis leaves containing Chl and carotenoid pigments with different molar ratios of Chl/heme. In general, the endogenous Chl/heme molar ratio in plant cells is approximately 100. As shown in **Supplementary Fig. S2**, HRP-emitted chemiluminescence was not significantly affected by the presence of Chl in the range of Chl/heme molar ratio 1 to 1,000. Thus, we concluded that co-extraction of Chls and carotenoids into acetone extracts does not interfere with the chemiluminescent measurement of heme.

Next, we performed the heme assay with plant samples by one-step acetone extraction following the HH assay. In this experiment, plant samples were first ground to powder in the presence of liquid nitrogen and were directly extracted by each form of acetone. As shown in **Fig. 3A**, the acidic acetone extract from 3-week-old Arabidopsis aerial tissues contained 24 nmol g^{-1} FW of heme, whereas low levels of heme was detectable in the neutral and basic acetone extracts. The levels of heme in neutral and basic acetone were 0.75 and 1.86 nmol g^{-1} FW, corresponding to 3.1 and 7.8% of total heme, respectively. To confirm the generality of this result, we also performed heme extraction from matured spinach and parsley leaves containing a high iron content (USDA National Nutrient Database for Standard Reference, Release 17, http://www.nal.usda.gov/fnic/foodcomp/Data/SR17/wtrank/sr17a303.pdf). As shown in **Fig. 3B** and **C**, the acidic acetone extract from matured spinach and parsley leaves contained 10.7 and 9.3 nmol g^{-1} FW of heme, respectively. In the neutral and basic acetone extracts, small amounts of heme were detected. The levels of neutral acetone-extractable heme were 0.4 and 0.3 nmol g^{-1} FW in spinach and parsley leaves, respectively, which correspond to 3.7 and 3.2% of total heme levels in these plant. The levels of neutral acetone-extractable heme were generally lower than those of basic acetone, although in parsley both levels were almost the same.

Heme extraction from a mixture of heme and plant samples

To ascertain whether neutral acetone can potentially extract free heme from plant samples, we added a defined concentration of exogenous authentic heme solution, representing free heme, to Arabidopsis leaf homogenates and performed the acetone extraction and the HH assay (**Fig. 4**). As a baseline, approximately 60 pmol of endogenous heme was detected from a defined amount of homogenate in the acidic acetone (equivalent to 2.2 mg of Arabidopsis sample) with no added heme. When 100 pmol of external heme was added to this homogenate before extraction, the extracted heme level increased to around 160 pmol, which corresponded to the sum of endogenous and exogenous heme. In contrast, only a small amount of heme was extracted by basic acetone from the same amount of homogenate, and undetectable levels of heme by the neutral acetone. Non-detection of neutral acetone-extractable heme in leaf homogenates indicates the possibility that during homogenization free heme is attached to non-specific proteins or lipids that are resistant to neutral acetone extraction. When 100 pmol of heme was exogenously added to samples, net increases of heme levels ($\sim$100 pmol) were observed in both neutral and basic acetone. The recovery of heme in neutral acetone was slightly lower but was not significantly different from that of exogenously added heme (100 pmol) at the level of $P < 0.05$. Although the possibility that a minute portion of added heme co-precipitated with the proteins in the neutral acetone cannot be excluded, our data suggest that neutral acetone can extract most of the free heme from plant samples.

Evaluation of unbound free heme level in Arabidopsis seedlings

As we showed that acidic acetone extracted quantitatively non-covalently bound heme from hemoproteins (**Fig. 1**), it is shown that total heme can be extracted from plant samples into acidic acetone (Stillman and Gassman 1978, Schneegurt and Beale 1986, Thomas and Weinstein 1990). Meanwhile, heme extractions from hemoproteins (**Fig. 1**) and leaf homogenates (**Fig. 4**) into neutral acetone indicate that only unbound free heme and not protein-bound heme can be extracted into neutral acetone. It should be noted that our results suggest a potential risk of losing heme if

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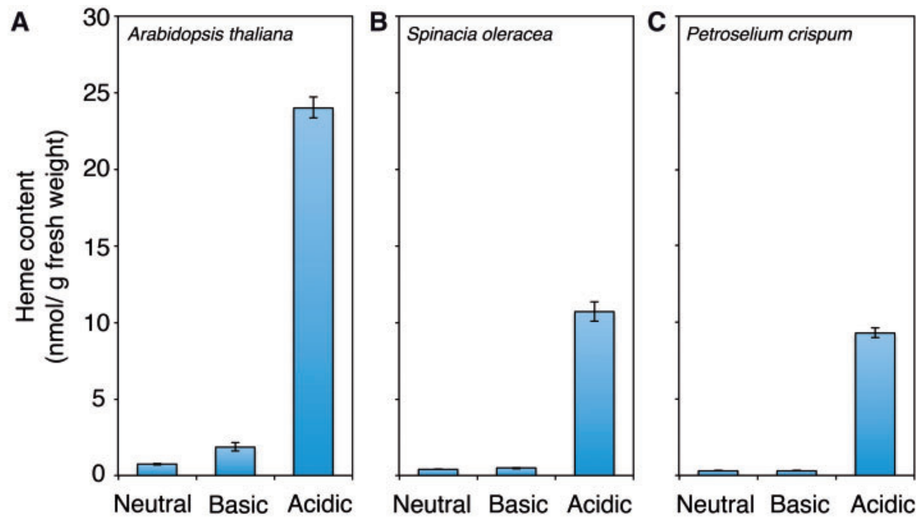

Fig. 3 Heme extraction from plant tissues with acidic, basic or neutral acetone. Arabidopsis, spinach (*Spinacia oleracea*) and parsley (*Petroselium crispum*) leaves were powdered in liquid nitrogen. Then, heme was extracted from the homogenate using neutral, basic or acidic acetone. These extracts were diluted with 10 mM KOH and heme contents were subsequently determined by the HH assay. Data shown are the means ± SD ($n > 3$).

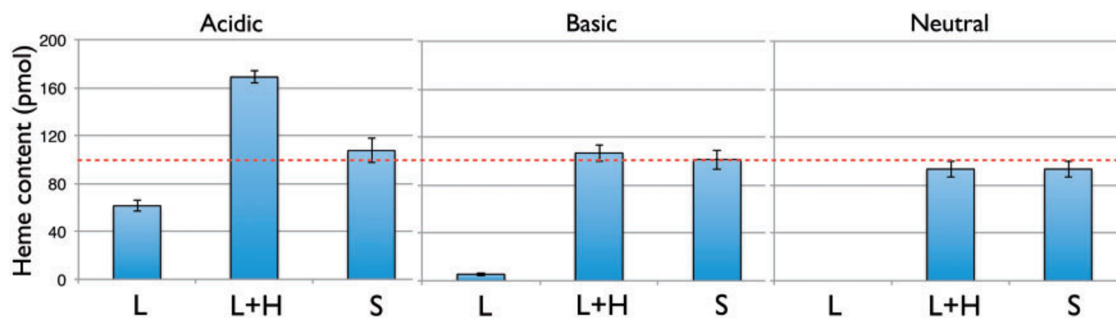

Fig. 4 Heme extraction from plant homogenates. Using acidic, basic or neutral acetone, heme was extracted from 50 µl of Arabidopsis leaf homogenates equivalent to approximately 2.2 mg FW of samples containing ∼60 pmol of heme, with or without the addition of 100 pmol of exogenous heme. Homogenates with or without exogenous heme were mixed with 950 µl of each acetone form. The HRP-based heme assay was performed to determine the heme content of each extract. (L) Contents of extracted heme from plant homogenates. (L+H) Contents of extracted heme from plant homogenate containing 100 pmol of externally added heme. (S) Calculated heme content by subtracting values of L from L+H. Data shown are the means ± SD ($n > 3$).

we follow the sequential extraction procedure (Stillman and Gassman 1978, Weinstein and Beale 1983). If we assume that the neutral and acidic acetone extracts represent the levels of 'free' and 'total' heme, respectively, the percentage of free heme among total heme levels in plant samples is about 3% (**Fig. 3A**).

Since free heme is proposed as a positive plastid-derived signal for nuclear-encoded photosynthesis-associated gene expression (Woodson et al. 2011), we first evaluated in Arabidopsis seedlings whether the level of neutral acetone-extractable heme is affected by treatment with ALA, which is the first committed precursor of tetrapyrroles. Treatment with 1 mM ALA in the dark significantly increased the total heme level in wild-type seedlings to about 1.3-fold (**Fig. 5B**), while the free heme level remained constant (**Fig. 5A**), suggesting that even though the total heme level increased, these hemes may attach to certain proteins that are resistant to neutral acetone extraction.

We then examined the effect of NF on total and free heme levels in Arabidopsis seedlings. NF disrupts chloroplast biogenesis through the inhibition of carotenoid biosynthesis, leading to down-regulation of photosynthesis-associated nuclear genes. Consistent with the previous report (Woodson et al. 2011), NF treatment significantly decreased the total heme level in the wild type (**Fig. 5B**). In contrast, the level of neutral acetone-extractable heme was increased 2-fold by NF (**Fig. 5A**).

Then, we examined the levels of neutral acetone-extractable heme in NF-treated *gun* mutants in which retrograde signaling is perturbed (Susek et al. 1993). In the *gun1* and *gun3* mutants, the levels of the neutral acetone-extractable heme were

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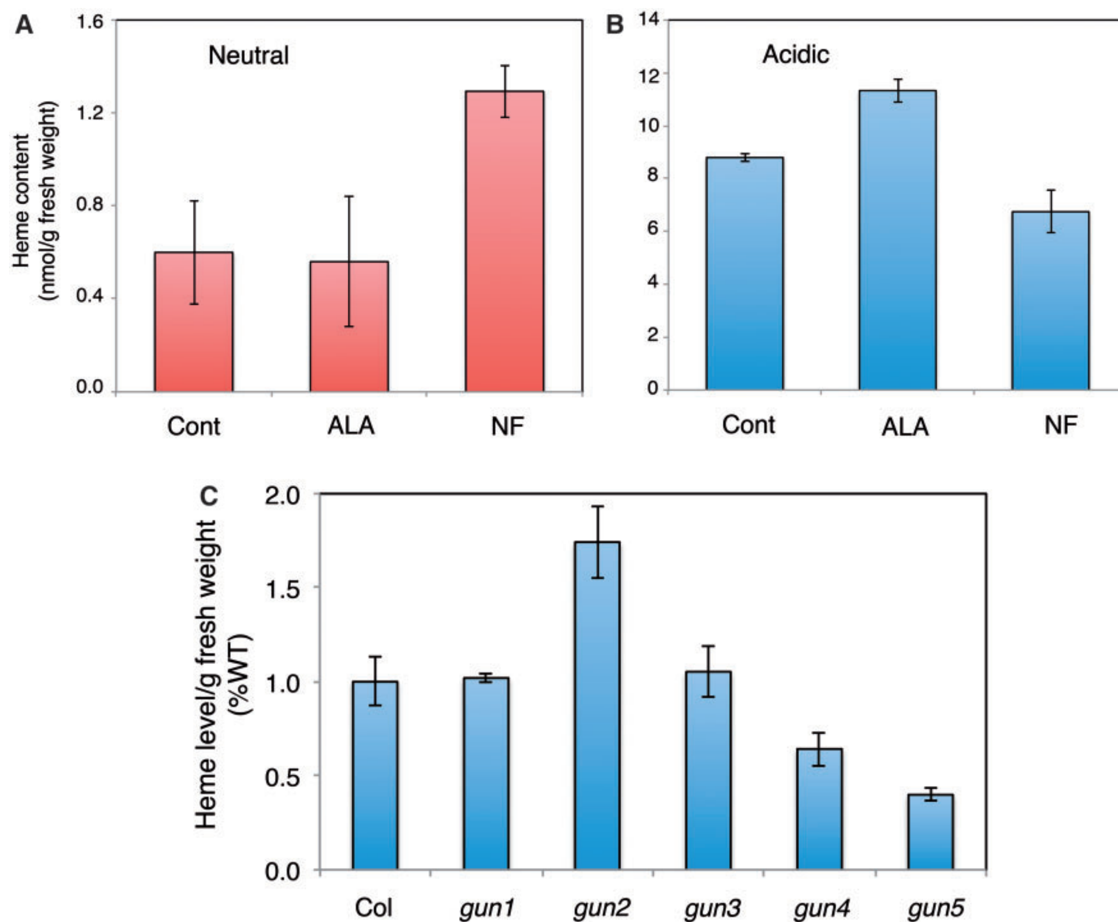

Fig. 5 Effects of 5-aminolevulinic acid (ALA) and norflurazon (NF) treatments on free (A) and total (B) heme levels. Arabidopsis seedlings were treated with 1 mM ALA in the dark for 24 h or with 2.5 μM NF for 4 d. From these samples, heme was extracted either in neutral or in acidic acetone. After extraction, heme contents were determined by the HRP-based heme assay. Data shown are the means ± SD ($n > 3$). (C) The wild type and *gun* mutants were grown on an NF-containing plate as described. From these seedlings, heme was extracted with neutral acetone. Heme levels per fresh weight relative to the wild type are shown. Data shown are the means ± SD ($n > 3$).

comparable with that in the wild type (**Fig. 5C**). While *gun* mutants deficient in the magnesium chelatase CHLH subunit (*gun5*) or its regulator (*gun4*) showed a decrease in the neutral acetone-extractable heme as compared with the wild type, the *gun2* mutant deficient in heme oxygenase 1 (HY1) showed an increase in that content (**Fig. 5C**). Considering the similar expression profile of photosynthesis-related nuclear genes in NF-treated *gun2*, *gun4* and *gun5* mutants (Woodson et al. 2011), it is apparent that the levels of neutral acetone-extractable heme in NF-treated plants are not correlated to the degree of *gun* phenotype.

Discussion

Although heme is important not only as a prosthetic group in hemoproteins but also as a cellular regulator in animal and plant cells, the direct measurement of heme and the investigation of its existing forms have been poorly performed. This is mainly because of the low sensitivity and technical difficulties inherent in previous assays. One of the major questions that remain to be solved is whether a pool of free heme exists in plant cells and, if present, what percentage of total heme exists as free heme and where it is located.

For total heme determination, heme was quantitatively extracted into acidic acetone, irrespective of protein solubility (**Fig. 1**). The extracted heme was readily air oxidized to Fe^{3+} hemin, which becomes an active prosthetic group of HRP. Moreover, contamination of hemoproteins did not affect the reconstitution and detection of HRP, as heme was quantitatively determined by the HH assay from defined amounts of hemoproteins (**Supplementary Table S1**). We have also confirmed that single extraction with the acidic acetone was sufficient for quantitative determination of heme contents in plant samples without any interference by the presence of Chl and carotenoid pigments (**Supplementary Fig. S2**). Thus, we propose that the combination of one-step acidic extraction and subsequent HH assay would be an ideal method to determine the total heme level from minute amounts of samples.

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Our data also suggest a substantial risk of losing heme if plant tissues are processed by the sequential acetone extraction method (Stillman and Gassman 1978, Weinstein and Beale 1983), which may result in imprecise determination. We have previously shown that HRP activity is not inhibited by 1% acetone (Masuda and Takahashi 2006). The extreme sensitivity of the HH assay enables precise and reproducible heme determination from minute amounts of tissue. In general, we detected around 10 and 30 nmol of heme from 1 g of 4-day-old seedlings and of 2-week-old mature leaves in Arabidopsis, respectively, indicating that the levels of heme increase during development. By extracting 1 mg samples with 1 ml of acidic acetone, an extract containing 10–30 nM heme would be obtained. Since the HH assay allows detection of 5–600 pM heme, the heme content can be directly measured by the HH assay if the extract is diluted >100-fold with 10 mM KOH. Following the extraction method of Weinstein and Beale (1983), Papenbrock et al. (1999) reported that the levels of heme in 4-week-old tobacco plants are 10–15 nmol g^{-1} FW and fluctuate diurnally following circadian rhythmic cycles. Peter and Grimm (2009) reported that wild-type tobacco leaves contain 19 ± 2 nmol g^{-1} FW of heme, while 12-day-old Arabidopsis seedlings grown under either dark/light or continuous light contain $\sim$10 nmol g^{-1} FW of heme. Although they might have lost some heme during extraction, these heme levels are basically consistent with our measurements.

Thomas and Weinstein (1990) proposed that heme extracted by basic acetone represents free heme dissolved in the membranes or non-specifically adhered to proteins. Based on this assumption, they estimated that $\sim$20% of the total heme exists as free heme in cucumber chloroplasts. Here, we show that the non-covalently bound heme of Hb and Mb can be extracted to some extent (7–12%) into basic acetone, mainly via solubilizing these proteins into the extract (**Figs. 1, 2**). Furthermore, the basic acetone can extract weakly bound heme from a heme–BSA mixture (**Table 1**). Although there are no available data regarding the solubility of plant hemoproteins in basic acetone, our data strongly suggest that certain hemoproteins do dissolve in the basic acetone. Thus, we conclude that there is no supporting evidence for the previous proposal (Thomas and Weinstein 1990) that the basic acetone can extract free heme.

Meanwhile, neutral acetone reproducibly extracted a small amount of heme from Arabidopsis, spinach and parsley. Considering that neutral acetone cannot extract non-covalently bound heme (**Fig. 1**) and heme superficially adhered to non-hemoproteins such as BSA (**Table 1**), it is possible that the heme extractable by neutral acetone may represent free heme presumably existing as a free form or dissolved in certain membranes. The level of neutral acetone-extractable free heme corresponds to 3% of the total heme in these plants. We are aware of the possibility that heme superficially attached to non-specific proteins is not taken into account for this assumption, but we propose that this level can be used as one index of the level of free heme in plant cells. We should also note that when heme was extracted from plant homogenates prepared in the buffer, the level of neutral acetone-extractable heme was certainly decreased. It is possible that the low levels of unbound heme may attach to non-specific proteins or lipids during homogenization and are resistant to neutral acetone extraction. Thus, for determination of neutral acetone-extractable heme, direct extraction of plant samples should be employed.

Although an increase in the total heme level was observed by exogenous ALA treatment, the level of neutral acetone-extractable heme remained constant. Thus, it is likely that the heme produced by ALA feeding exists in the bound state, which is resistant to neutral acetone extraction, inside and outside plastids. Interestingly, we found that the neutral acetone-extractable heme was increased by NF treatment while the total heme level was decreased. The reduction of the total heme level by NF treatment is consistent with a recent observation (Woodson et al. 2011). Meanwhile, Voigt et al. (2010) observed a similar increase in heme levels in basic acetone extraction. It is shown that NF-mediated photobleaching completely blocks chloroplast structural development (Susek et al. 1993). Thus, it is likely that chloroplasts are a major reservoir of total heme and, when disrupted, a portion of heme may be released as free heme although the majority of these hemes may also be degraded. Considering that *gun* mutants show de-repressed expression of photosynthesis-related genes in the case of chloroplast dysfunction by NF treatment, it is important to evaluate whether such released free heme acts as signaling heme under this condition or not.

Based on the proposal by Woodson et al. (2011), heme produced by FC1 in the plastid may function as a positive signal for control of photosynthesis gene expression. It is presumed that in *gun* mutants the level of signaling heme is maintained even in the presence of NF. Thus, we determined the NF-induced free heme levels in *gun* mutants. Several studies showed that the *gun* mutants exhibited similar de-repression pattern of photosynthesis-related genes in the presence of NF (Mochizuki et al. 2001, Mochizuki et al. 2008, Moulin et al. 2008, Woodson et al. 2011). In our data, however, the level of neutral acetone-extractable heme was higher in *gun2* and lower in *gun4* and *gun5* as compared with that in the wild type, clearly showing that the level of unbound free heme is not correlated to the *gun* phenotype. No correlation between heme levels and *gun* phenotypes was also reported by Voigt et al. (2010), although they analyzed the levels of basic acetone-extractable heme as 'free' heme in *gun* mutants. Since higher accumulation of total heme was observed in NF-treated *gun2* (Woodson et al. 2011), decreased heme catabolism may cause higher accumulation of total and unbound heme. In contrast, although mutations in *gun4* and *gun5* may lead to a flow of protoporphyrin IX into the heme branch of tetrapyrrole biosynthesis, the levels of free heme were rather decreased in these mutants. This also suggests that the levels of heme in plant cells are regulated through feedback inhibition and other mechanisms to maintain heme homeostasis. Currently, we do not know whether the free heme accumulates

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inside NF-treated plastids or not. The recent model showing that a specific heme pool produced by FC1 functions as a retrograde signal to coordinate nuclear gene expression is interesting (Woodson et al. 2011). However, if we assume that heme emitted from healthy chloroplasts acts as a positive signal for nuclear gene expression, such signaling heme must be different from protein-unbound free heme. It is possible that such signaling heme may be transferred by binding to a specific trafficking system, and NF may affect its transport from plastid to nucleus. Another detection technique such as molecular imaging will be required to show such a type of signaling heme.

Conclusion

In summary, by evaluating differential acetone extraction, we showed that acidic acetone extracts total heme, while neutral acetone can extract non-protein-bound free heme. We propose that a combination of a one-step extraction with acidic acetone and the HH assay is a simple, sensitive and high-throughput heme assay to determine total and free heme levels in plant cells. With this method, we have first succeeded in the determination of free heme levels in plant samples. Also, the level of free heme in plant cell is maintained at a very low concentration. To elucidate the signaling function of heme, the heme transport system should be examined in the future.

Materials and Methods

Heme determination from commercially available proteins

Bovine Hb, horse Mb and *A. niger* Cat were purchased from Nacalai Tesque. Based on the subunit concentration, Hb and Mb were dissolved in 10 mM Tris–HCl (pH 7.5) at 2 mM concentration and Cat was dissolved in the buffer at 0.2 mM concentration. A 50 µl aliquot of heme and these protein solutions was mixed with 950 µl of neutral, acidic and basic acetones to reach final concentrations of 80% (v/v) acetone (neutral acetone), 80% (v/v) acetone containing 20% (v/v) 10 mM NH_4OH (basic acetone) and 80% (v/v) acetone containing 20% (v/v) 1.6 M HCl (acidic acetone), respectively. After centrifugation (25,000×*g*) for 10 min at 4°C, acetone supernatants were used for heme measurement and protein determination.

To prepare authentic heme solution, bovine hemin (Nacalai Tesque) was dissolved in dimethylsulfoxide (DMSO) and then diluted in 10 mM KOH. Then, the solution was cleared by centrifugation (25,000×*g*) for 10 min and filtered with a sterile syringe filter (0.2 µm) (Corning). Concentrations were determined with the extinction coefficient of ε_{mM} = 144 at 398 nm (Weinstein and Beale 1983, Thomas and Weinstein 1990). A 50 µl aliquot of authentic heme solution was mixed with 950 µl of neutral, basic and acidic acetones as described. For neutral and basic acetones, the above extinction coefficient was applicable. This extinction coefficient was confirmed by the alkaline pyridine hemochromogen method using the extinction coefficient of ε_{mM} = 20.7 between 557 and 541 nm of the reduced minus oxidized spectrum (Falk 1964). For the acidic acetone extraction, a lower extinction coefficient (ε_{mM} = 96 at 398 nm) was required, probably because of protonation of propionic acid chains at positions C6 and C7. To determine the heme concentration in acetone extracts from hemoproteins, 100 µl of the extract was mixed with 900 µl of 66.5% ethanol, 17% acetic acid and 16.5% water (by vol.). Using the extinction coefficients at 398 nm (ε_{mM} = 144 for neutral and basic acetones and ε_{mM} = 96 for acidic acetone), heme concentrations were determined. The total protein content in the acetone extracts was quantified using a Protein Assay Lowry Kit (Nacalai Tesque) with BSA (Nacalai Tesque) as a standard.

For the experiment of the concentration dependency of Hb, various concentrations of Hb were prepared in 10 mM Tris–HCl (pH 7.5). The resulting Hb solutions (50 µl) were mixed with 950 µl of neutral, acidic and basic acetone, and heme concentrations were determined as described. The extraction efficiency was calculated by dividing the extracted heme concentration by the Hb concentration.

For determination of heme from hemoproteins by the HH assay, hemoproteins (Hb, Mb and Cat) were diluted to 20 µM with 10 mM Tris–HCl (pH 7.5). Authentic hemin solution at 20 µM concentration was prepared as described. From 50 µl aliquots containing 1.0 nmol of subunit or authentic hemin, heme was extracted by 950 µl of the acidic acetone. After incubation for 5 min at 4°C, acetone mixtures were centrifuged at 25,000×*g* for 10 min at 4°C. Supernatants were diluted with 10 mM KOH and used for the HH assay described below.

For heme extraction from the heme–BSA complex, BSA was dissolved in 10 mM Tris–HCl (pH 7.5) at 1.5 mM concentration. Hemin solution was prepared as described above at 10 mM concentration. For saturation of BSA with heme, hemin (22.96 nmol) was mixed with BSA (6.25 nmol) in 50 µl of 10 mM Tris–HCl (pH 7.5). This mixture was extracted with 950 µl of neutral and basic acetone. After centrifugation (25,000×*g*) for 10 min at 4°C, acetone supernatants were used for the spectrophotometric measurement of heme as described. Concentrations of precipitated heme were calculated by subtracting total minus extracted heme. The amount of BSA precipitates was quantified using the Protein Assay Kit. Molar ratios of heme to BSA in precipitates were estimated.

Plant samples and growth conditions

Seeds of wild-type Arabidopsis were germinated and grown on solidified Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar at 23°C under continuous white light (30 µmol photons m^{-2} s^{-1}). After 14 d, the first and second true leaves were collected, immediately frozen in liquid nitrogen, and stored at −80°C. For spinach and parsley leaves, vegetables were purchased from a local market.

For the ALA treatment experiment, Arabidopsis seedlings were grown in liquid MS medium containing 1% sucrose under continuous white light (30 µmol photons m^{-2} s^{-1}).

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After 7 d, ALA was added to the liquid medium at a final concentration of 1 mM. Arabidopsis seedlings were further incubated in the dark for 24 h and stored as described. For NF treatment experiments, plants were grown on MS medium supplemented with 1% sucrose and 2.5 μM NF (Sandoz Pharmaceutical) under continuous white light (100 μmol m^{-2} s^{-1}) for 4 d. These seedlings were collected, immediately frozen in liquid nitrogen, and stored at −80°C.

For *gun* mutants (*gun1*, *gun2*, *gun3*, *gun4* and *gun5*) (Susek et al. 1993, Mochizuki et al. 2001), seeds were grown on MS agar medium supplemented with 1% sucrose and 1.5 μM NF at 23°C under continuous light conditions. After 4 d, these seedlings were collected, immediately frozen in liquid nitrogen, and stored at −80°C.

Extraction of heme from plant samples

For heme extraction from plant samples, plant samples (approximately 4 mg) were powdered in liquid nitrogen and then directly extracted with 1 ml of neutral, acidic or basic acetone. After incubation at room temperature for 5 min, acetone mixtures were centrifuged at 25,000×*g* for 10 min. Supernatants were diluted 100- to 1,000-fold with 10 mM KOH, and 10 μl of diluted samples were used for the HH assay described below.

For the free heme recovery experiment, 26 mg of samples were powdered in liquid nitrogen and suspended with 0.5 ml of buffer (10 mM Tris–HCl, pH 7.5). A 5 μl aliquot of 20 μM authentic hemin solution or 10 mM KOH was externally added to 45 μl of each homogenized sample and incubated for 10 min at room temperature. Homogenates with or without exogenous heme were mixed with 950 μl of neutral, acidic or basic acetone. After incubation at room temperature for 5 min, acetone mixtures were centrifuged at 25,000×*g* for 10 min. Supernatants were diluted 100- to 1,000-fold with 10 mM KOH and were used for the HH assay described below.

HH assay

To reconstitute HRP holo-enzyme, 10 μl of diluted heme extract was mixed with 40 μl of apo-HRP (Biozyme) (6.25 nM apo-HRP, 250 mM Tris–HCl, pH 8.4) and incubated at room temperature for 30 min. To initiate the reaction, 50 μl of chemiluminescence reagent (Immobilon™ Western Chemiluminescent HRP Substrate) was added. After incubation at room temperature for 5 min, chemiluminescence was quantified using a microplate luminometer (GloMax Multi, Promega) with integrated time 0.5 s. This luminometer is equipped with a photomultiplier tube covering from 350 to 650 nm and can detect chemiluminescence from luminol having a wavelength maximum at 431 nm. In this assay condition, the detection limit of heme was approximately 5 pM, and a stable signal was obtained until 15 min after initiation of the reaction (Takahashi and Masuda 2009). A dynamic range of linearity was obtained up to 600 pM.

Methemoglobin determination

The level of methemoglobin in commercially purchased Hb was determined as described (Evelyn and Malloy 1938, Leahy and Smith 1960). In this assay, 5% (v/v) sodium cyanide is added to Hb solution. By conversion of methemoglobin into cyan-methemoglobin, the absorption maximum of methemoglobin (630 nm) was completely shifted to that of cyan-methemoglobin (540 nm). By measuring the difference in the A_{630}, which is proportional to the concentration of methemoglobin, we determined the level of methemoglobin in Hb.

Chl determination

Plant samples crushed into powder in liquid nitrogen were homogenized in 80% acetone and debris was removed by centrifugation at 10,000×*g* for 5 min. The absorbance of the supernatant at 720, 663, 647 and 645 nm was measured with an Ultrospec 2100 *pro* spectrophotometer (GE Healthcare Bioscience). The Chl (*a* and *b*) concentration of the samples was determined as described (Melis et al. 1987).

Supplementary data

Supplementary data are available at PCP online.

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