Investigation of genes for heavy metal [e.g. nickel (Ni) and zinc (Zn)] absorption and detoxification in green algae is of great importance because some of the metals have become one of the major contaminants in the aquatic ecosystem. In plants, overload of heavy metals modifies many aspects of biological processes. However, the mechanisms by which heavy metals exert detrimental effects are not fully understood. The present study identified a biological role for HISN3 (the gene coding for phosphoribosylformimino-5-aminimidazole carboxamide ribonucleotide isomerase) in regulating the response of Chlamydomonas reinhardtii, a unicellular green alga, to Ni toxicity. In higher plants, HISN3 encodes an enzyme catalyzing the fourth step in the histidine biosynthesis pathway, but its functional importance is yet to be identified. Transgenic algae overexpressing HISN3 in C. reinhardtii showed high tolerance to excess Ni, with a 48.3–57.4% increase in cell population and moderate histidine accumulation compared with the wild type. HISN3 overexpression improved accumulation of Chl and photosynthesis efficiency, but suppressed Ni-induced generation of reactive oxygen species (ROS) (Baccouch et al. 2012). At the physiological level, overload of Ni in plant cells disrupts many metabolic processes including photosynthesis, protein function, enzyme activities and mineral homeostasis (Cempel and Nikel 2006, Yusuf et al. 2011). The adverse effects of Ni are closely linked to the oxidative stress caused by metal-induced generation of reactive oxygen species (ROS) (Baccouch et al. 2001, Gonnelli et al. 2001, Hao et al. 2006). Previous studies have shown that in the Ni hyperaccumulator Alyssum lesbiacum, the free histidine concentrations are constitutively high in the xylem (Ignle et al. 2005). In the closely related Ni-sensitive non-accumulator plants Brassica juncea and Alyssum montanum, administration of exogenous free histidine conferred Ni tolerance to whole plants (Kerkeb and Krämer 2003). Furthermore, expression of the genes coding for the first enzyme [ATP-phosphoribosyltransferase (ATP-PRT)] in the histidine biosynthetic pathway in Arabidopsis was sufficient to

**Introduction**

Nickel (Ni) is a naturally occurring trace metal that acts as an essential micronutrient or a toxicant for plants depending on its concentration (Marschner 1995). Over the last decades, Ni has become an environmental contaminant due to its natural release and anthropogenic activities (Han et al. 2002). As soil Ni exists in the form of Ni(II), it is of high mobility through irrigation into aquatic environments and thus has adverse effects on aquatic organisms. Great concern has arisen recently from the effect of the metal on aquatic microorganisms (Kukier and Chaney 2004, Cempel and Nikel 2006, Jian et al. 2009). Higher plants can accumulate higher quantities of Ni from the soil (Krämer et al. 1996, Ingle et al. 2005). This suggests that as promising passive biomarkers, green algae have the potential to be used for monitoring phytotoxic effects of Ni in water on aquatic plants.

Many studies have been carried out to investigate the biological adaptation of microalgae to metals or toxic materials (Morlon et al. 2005, Zhang et al. 2008, Bi et al. 2012, Wei et al. 2012). At the physiological level, overload of Ni in plant cells disrupts many metabolic processes including photosynthesis, protein function, enzyme activities and mineral homeostasis (Cempel and Nikel 2006, Yusuf et al. 2011). The adverse effects of Ni are closely linked to the oxidative stress caused by metal-induced generation of reactive oxygen species (ROS) (Baccouch et al. 2001, Gonnelli et al. 2001, Hao et al. 2006). Previous studies have shown that in the Ni hyperaccumulator Alyssum lesbiacum, the free histidine concentrations are constitutively high in the xylem (Ignle et al. 2005). In the closely related Ni-sensitive non-accumulator plants Brassica juncea and Alyssum montanum, administration of exogenous free histidine conferred Ni tolerance to whole plants (Kerkeb and Krämer 2003). Furthermore, expression of the genes coding for the first enzyme [ATP-phosphoribosyltransferase (ATP-PRT)] in the histidine biosynthetic pathway in Arabidopsis was sufficient to
increase the pool of free histidine in shoot tissue of plants with a negligible effect on any other amino acid (Wycisk 2004, Ingle 2005, Rees 2009). Recently, a study on Caenorhabditis elegans demonstrated that elevated levels of histidine in the hal-1 mutant were able to prevent zinc (Zn) and Ni toxicity by acting as a low molecular weight chelator (Murphy et al. 2011). These results indicate that increased endogenous free histidine can reduce Ni toxicity in plants.

The histidine biogenesis pathway comprises 10 reactions catalyzed by eight enzymes, which are in turn encoded by nine genes (Rees et al. 2009). The gene HISN3 encodes phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase (EC 5.3.1.16), catalyzing the fourth step in the histidine biosynthesis pathway. This step includes an amadori rearrangement that isomerizes the aminoaldose moiety of PRFAR [N-(5-phospho-D-ribosylformimino)-5-amino-1-(5-phosphoribosyl)-4-imidazolocarboxamide] to the aminoketose of PROFAR [N-(5-phospho-D-1-ribulosylformimino)-5-amino-1-(5-phospho-ribosyl)-4-imidazolocarboxamide] (Margolies and Goldberger 1967). Based on the description by Muralla et al. (2007) HISN3 in Arabidopsis was named HIS6. The corresponding gene HISN3 was called HisA in bacteria and archaea, while BBMII isomerase in Arabidopsis thaliana has also been named. The BBMII knockout mutant isomerase-apg10 displays embryo lethality, pale green cotyledons and true leaves at the juvenile stage (Noutoshi et al. 2005). The majority of the genes encoding the eight enzymes involved in the histidine biosynthesis pathway have been characterized from higher plants, including Arabidopsis, tobacco and Thlaspi goesingense, but their homologs in green algae such as Chlamydomonas reinhardtii have not been identified. In one of our previous studies, we observed that modification of HISN3 expression resulted in the change in accumulation of several metals [e.g. Ni and iron (Fe)]. This prompted us to investigate whether HISN3 was involved in histidine accumulation and its relationship to metal homeostasis. The green microalgal C. reinhardtii is a model system that is frequently used to characterize functional genes (Wei et al. 2011, Zhang et al. 2011). It has been recently employed to test ecotoxicological responses to hazardous materials including heavy metals (Zhang et al. 2008, Lavoie et al. 2009, Wei et al. 2011) and other toxicants (Chua et al. 2008, Zhang et al. 2011, Jin et al. 2012). The genome of C. reinhardtii has been sequenced (Merchant et al. 2007). This makes gene identification and analysis easy on a genome scale. To understand whether C. reinhardtii HISN3 is involved in the algal response to Ni toxicity, we functionally identified the role of HISN3 in Ni absorption and detoxification. Thus, the objective of this study is to gain an understanding of how HISN3 regulates algal adaptation to Ni toxicity.

**Results**

**Effect of Ni on algal growth**

The growth response of C. reinhardtii to a wide range of Ni concentrations was analyzed. A linear negative relationship was found between the growth response and Ni concentrations (Fig. 1A). However, cell growth was not significantly affected by Ni at 30–90 μM. Rather, a slight increase in cell growth with 60 μM Ni was observed. Only with concentrations of Ni up to 120–150 μM was the algal growth decreased significantly. The inhibitory rate was calculated and the 50% growth inhibition at 6 d was about 125 μM, which could be represented by an EC_{50} (half-maximal effective concentration) value (OECD 2002, US EPA 2002). Furthermore, the highest concentration that does not cause a statistically significant effect can be represented by the no observed effect concentration (NOEC), whereas the lowest concentration that causes a statistically significant effect is represented by the lowest observed effect concentration (LOEC). Based on the US EPA method (2002), the 6 d NOEC was calculated and shown to be 90 μM, and the LOEC was 120 μM. The Chl accumulation was determined and showed a similar pattern to the cell population, with a progressive decrease with Ni concentrations up to 90 μM; at 90 and 125 μM Ni, the Chl content was decreased to 85.3% and 26.7% of that of the control, respectively (Fig. 1B).

**Identification of HISN3 and its transgenic lines of C. reinhardtii**

The genomic sequence of C. reinhardtii HISN3 (Gene ID: 5726103) is 3,158 bp long, and comprises eight exons interrupted by seven introns; the CDS (coding sequence) contains an open reading frame coding for a protein of 305 amino acid

![Fig. 1 Effect of Ni on the growth (A) and accumulation of Chl (B) of C. reinhardtii. Algae were grown for 3 d and transferred to fresh culture with Ni at the indicated concentrations for 6 d. After that, the cell population and Chl were quantified. Vertical bars represent the SD (n = 3). Asterisks indicate that the mean values are significantly different between the Ni treatment and control (P < 0.05).](https://academic.oup.com/pcp/article-abstract/54/12/1951/1838567)
residues (Supplementary Figs. S1, S2A). Alignment of the deduced amino acid sequence of HISN3 revealed several regions that are highly conserved in Volvox carteri, A. thaliana, Oryza sativa and Saccharomycyes cerevisiae (Supplementary Fig. S2B). Phylogenetic analysis showed that HISN3 could be grouped with its ortholog from V. carteri (XP_002956910.1) (Supplementary Fig. S3). Comparison of the deduced amino acid sequence showed the highest identity with V. carteri HISN3 (VcHISN3) (84%), followed by Chlorella variabilis HISN3 (79%). The schematic representation of the histidine biosynthetic pathways for yeast (S. cerevisiae), Arabidopsis and C. reinhardtii is comparatively summarized in Supplementary Fig. S4.

To identify whether HISN3 plays a role in plant tolerance to Ni toxicity, we constructed HISN3 overexpression vectors driven by the 35S Cauliflower mosaic virus (CaMV) promoter and introduced it into C. reinhardtii using the method described previously (Wei et al. 2011). We simultaneously constructed HISN3-silenced transgenic algae (35S::AntiHISN3) using antisense HISN3 sequences. Semi-quantitative PCR analysis revealed that expression of HISN3 in 35S::HISN3 algae was higher than that of the wild type, whereas expression of HISN3 in 35S::AntiHISN3 algae was lower than in the wild type (Fig. 2A). Real-time quantitative reverse transcription–PCR (qRT–PCR) also demonstrated that 35S::HISN3 algae had a 3.8- to 16.4-fold increase in HISN3 expression compared with the wild type; in contrast, HISN3 transcripts in 35S::AntiHISN3 algae were reduced to 54.3–68.6% of those of the wild type (Fig. 2C).

We next analyzed the HISN3 expression in wild-type, 35S::HISN3 and 35S::AntiHISN3 algae under Ni exposure. For wild type, expression of HISN3 was relatively higher in Ni-exposed cells than in Ni-free cells, with 1.6- to 2.4-fold increases in HISN3 transcripts induced by Ni (Fig. 2B, D), suggesting that treatment with Ni can moderately regulate HISN3 expression. Because in transgenic cells expression of HISN3 and antisense HISN3 was constitutive, the transcript abundance of HISN3 in 35S::HISN3 cells was considerably higher than that in the wild type, whereas the expression of HISN3 in 35S::AntiHISN3 cells was relatively lower.

**Overexpression of HISN3 elevated levels of histidine**

HISN3 encodes an enzyme in the histidine biosynthesis pathway. Thus, manipulation of HISN3 may alter the level of histidine. Free histidine was quantified in algae with or without Ni exposure. Under the Ni-free condition, HISN3-overexpressing algae had slightly but significantly higher levels of histidine than the wild type; in contrast, 35S::AntiHISN3 algae had much lower levels of histidine than the wild type (Fig. 3A). Under Ni exposure, however, much more histidine was accumulated in 35S::HISN3 algae than in the wild type. Compared with wild type in which the histidine level was increased by 37.8% under Ni stress, histidine in 35S::HISN3 algae was increased by 48.9–59.2%. These results indicate that HISN3-overexpressing algae could accumulate more histidine under Ni stress.

**Overexpression of HISN3 conferred algal tolerance to Ni stress**

The observation on histidine accumulation in 35S::HISN3 cells led us to hypothesize that an elevated histidine level may confer cellular Ni tolerance. To address the question, we first measured algal growth. The population of HISN3-overexpressing algae was 1.49- to 1.59-fold higher than the control in the presence of 125 μM Ni (Fig. 3B). Conversely, the 35S::AntiHISN3 algae had a reduced cell population under Ni stress. Chl is one of the most important parameters for C. reinhardtii growth (Zhang et al. 2011) and is also a biomarker of metal toxicity (Ekmeckci et al. 2008). Examination of Chl accumulation showed that 35S::HISN3 algae accumulated 1.47- to 1.56-fold higher levels of Chl than the wild type, whereas the 35S::AntiHISN3 algae accumulated much less Chl than the wild type (Fig. 3C).

**Overexpression of HISN3 improved Chl fluorescence in Ni-exposed algae**

The Fv/Fm ratio is one of the major parameters representing Chl fluorescence emission of photosynthetic activity, which originates mainly from a Chl a molecule of PSII and is frequently used as an indicator of heavy metal stress (Mallicka and Mohn 2003, Wei et al. 2011). Under normal conditions, the Fv/Fm value remained unchanged between the transgenic and wild-type algae (Fig. 4A). Treatment with 125 μM Ni for 24 h resulted in a general decrease in Fv/Fm in all cells. However, compared with wild-type cells, a significantly higher Fv/Fm ratio was observed in HISN3-overexpressing algae, whereas a drastically lower Fv/Fm ratio was detected in 35S::AntiHISN3 algae.

Photosynthetic electron transport rates (ETRs) are derived from Chl fluorescence parameters and used for studying adaptation to light intensity, photosynthetic productivity and responses to abiotic stresses (White and Critchley 1999, Wei et al. 2011). Alpha (the initial slope of the rapid light curve) represents the efficiency of utilization of light energy (Kohl and Nicklisch 1988). We compared the maximal electron transport rate (ETRmax) and the alpha value from the rapid light curve between the transgenic and wild-type algae exposed to 125 μM Ni for 24 h. HISN3-overexpressing algae showed a relatively higher photosynthetic ETR and higher alpha value than the wild type. In contrast, a lower level was observed in 35S::AntiHISN3-transformed algae when compared with wild-type algae (Fig. 4B, C). These results indicate that parameters such as the Fv/Fm ratio, ETRs and the alpha value were very sensitive to Ni toxicity, and the photosynthesis efficiency was impaired in 35S::AntiHISN3-transformed algae.

**Overexpression of HISN3 improved tolerance to Ni-induced oxidative stress**

It is well known that excess metals in plants induce ROS production (Schützendübel and Polle 2002, Wang and Yang 2005, Shen et al. 2011). To investigate whether HISN3 has an antioxidative capability, we determined the contents of the major ROS O2•− and H2O2. As shown in Fig. 5, transgenic cells overexpressing
HISN3 showed low levels of O$_2^-$ and H$_2$O$_2$, whereas 35S::AntiHISN3 cells accumulated a high abundance of these ROS. Because overgeneration of ROS is closely related to oxidative damage to plant cells (Zhou et al. 2008, Bi et al. 2012), the lipid peroxides were determined in terms of thiobarbituric acid-reactive substances (TBARS). Exposure to Ni at 90–135 μM significantly increased the accumulation of TBARS in wild-type algae (Fig. 6A). To determine further the antioxidative capacity in the transgenic algae, the TBARS were quantified after cell exposure to 125 μM Ni for 6 d. The TBARS accumulation was decreased by 34.6–38.9% in HISN3-overexpressing algae compared with the wild type (Fig. 6B). Meanwhile, the 35S::AntiHISN3 algae showed a relatively higher peroxide accumulation.

The non-protein thiol (NPT) compounds consisted of several acid-soluble sulfhydryl components such as cysteine, γ-glutamylcysteine, glutathione (GSH) and phytochelatin; these compounds are able to chelate toxic metals [e.g. cadmium (Cd) and mercury (Hg)] and detoxify them (Cobbett and Goldsborough 2002). In wild-type algae, treatment with Ni (125 μM) for 3 d induced NPT accumulation (Fig. 7A). Likewise, accumulation of NPT in 35S::AntiHISN3-transformed algae was also induced by Ni. However, no NPT accumulation was observed in 35S::HISN3 algae. Both proline and ascorbate (ASC) are antioxidants against metal toxicity (Siripornadulsil et al. 2002, Zhang et al. 2008, Zhou et al. 2008). The pattern of proline accumulation under Ni exposure was similar to that of NPT in wild-type, 35S::HISN3 and 35S::AntiHISN3 algae (Fig. 7B). Because NPT and proline accumulated less in 35S::HISN3 cells than in the wild type, it is possible that generation of NPT and proline was not the major mechanism for Ni detoxification. The ASC content was higher in 35S::HISN3 algae than in the wild type under the Ni stress (Fig. 7C). Conversely, ASC accumulated less in 35S::AntiHISN3 algae compared with the wild type, suggesting that overexpression of HISN3 can alter accumulation of ASC in algae.

**Effect of HISN3 overexpression on metal accumulation in algae**

Determination of the Ni content in algae showed that Ni was accumulated in 35S::HISN3 algae (Fig. 8). To evaluate whether treatment with Ni affected accumulation of other essential
metals, Cu, Fe, Zn, magnesium (Mg) and manganese (Mn) were quantified. Interestingly, these metals were also enriched in Ni-treated 35S::HISN3 cells (Fig. 8). In 35S::AntiHISN3 algae, however, accumulation of Ni, Cu and Mg was decreased under Ni stress compared with the wild type. There was no change in Fe accumulation. For Zn and Mn, only a slight increase was observed in 35S::AntiHISN3 algae. Finally, a comparative analysis was carried out on the total cellular concentrations of histidine and Ni in the wild-type and transgenic cells. There was no change in Fe accumulation. For Zn and Mn, only a slight increase was observed in 35S::AntiHISN3 algae. Finally, a comparative analysis was carried out on the total cellular concentrations of histidine and Ni in the wild-type and transgenic cells. Although more histidine was accumulated in 35S::HISN3 algae than in the wild type, these cells simultaneously accumulated more Ni than the wild type. As a result of this, the ratio of histidine over Ni for 35S::HISN3 algae was lower than that of the wild type (Supplementary Table S1). Compared with the wild type, 35S::AntiHISN3 cells always had a lower ratio of histidine to Ni.

**Discussion**

We used genetic analysis to characterize the function of *C. reinhardtii* HISN3 and showed that this gene was involved in detoxification of excess Ni in the algal cells. HISN3 overexpression conferred algal tolerance to Ni toxicity, in terms of increased cell population and Chl content. Chl fluorescence parameters such as maximum PSII activity and electron transport efficiency were also improved under Ni stress. Furthermore, the 35S::HISN3 algae had low levels of ROS and lipid peroxides, implying that HISN3 overexpression can counteract Ni-induced oxidative stress. In contrast, HISN3 silencing resulted in enhanced sensitivity to Ni toxicity.

We analyzed histidine, the final product with which HISN3 is involved. Compared with the wild type, the abundance of histidine was higher in 35S::HISN3 algae, whereas its content was
lower in 35S::AntiHISN3 algae. These results suggest that manipulation of HISN3 can modify the endogenous pool of free histidine. Because the histidine level was increased in 35S::HISN3 cells, this should be responsible for the attenuation of Ni toxicity. In Arabidopsis, enhancement of the first enzymatic step in the histidine biosynthesis pathway increased the free histidine pool and Ni tolerance (Wycisk et al. 2004). The histidine biosynthesis pathway consists of 10 reactions catalyzed by eight proteins, starting with the condensation of ATP and phosphor-ibosyl pyrophosphate catalyzed by ATP-PRT (Ingle et al. 2005). ATP-PRT (encoded by HIS1) is considered as the first rate-limiting enzyme in the histidine biosynthesis pathway, because its activity is subject to allosteric feedback inhibition by free histidine, a typical feature of key enzymes (Ohta et al. 2000, Rees et al. 2009). Transgenic Arabidopsis expressing a Salmonella typhimurium ATP-PRT enzyme resistant to feedback inhibition by histidine produced 2-fold higher histidine concentrations than the wild type (Wycisk et al. 2004). Furthermore, a recent study has shown that constitutive overexpression of two isoforms of ATP-PRT in Arabidopsis resulted in the shoot histidine level being up to 42-fold higher than that of the wild type. These results indicate that control of the pool of free histidine largely depends on ATP-PRT. In this study, overexpression of HISN3 resulted in high levels of HISN3 transcripts (3.8- to 16.4-fold increases) and promoted the abundance of histidine in C. reinhardtii, but accumulation of extra histidine was moderate in the transgenic plants. This may be attributed to the fact that the step catalyzed by HISN3 is not rate limiting. Also, it is possible that there are other regulatory mechanisms that influence histidine accumulation in the cells of C. reinhardtii.

When plants are exposed to excess heavy metals, they tend to reprogram gene expression and alter their physiological state to acclimate to their adverse environment (Herbette et al. 2006, Sobrino-Plata et al. 2009, Zhou et al. 2012, Zhang et al. 2013). Chlamydomonas reinhardtii is a eukaryotic green microalga. Its chloroplast is highly susceptible to oxidative stress due to the elevated metal concentration in the environment (Ekmekci et al. 2008, Zhang et al. 2008, Elbaz et al. 2010, Wei et al. 2011). Metal-induced oxidative stress is a constant threat to photosynthetic microorganisms. Under metal exposure, superoxide anion (O$_2^-$) is generated, and diffusion of O$_2^-$ into the stroma results in its dismutation to oxygen and hydrogen peroxide (H$_2$O$_2$). Both O$_2^-$ and H$_2$O$_2$ constitute strong oxidants damaging several biomolecules (Okamoto et al. 2001). The
observed increase in cellular H$_2$O$_2$ and O$_2^-$ was attributed to the excess Ni (Fig. 5). Thus, the Ni-enhanced ROS most possibly triggered the cellular lipid peroxidation and plasma membrane injury (Fig. 6). However, the Ni-induced ROS and oxidative stress could be prevented by HISN3 overexpression. It is obvious that the increased abundance of HISN3 transcripts and histidine is necessary for tolerance to Ni-induced oxidative stress. Previous studies show that cellular anti-oxidative capacity is closely associated with histidine. In mammals, histidine is considered as an anti-inflammatory and anti-oxidant factor. Histidine could alleviate 1-methyl-4-phenylpyridinium ion (MPP$^+$)-induced hydroxyl radical generation in the rat striatum (Obata et al. 2001). Several amino acids such as histidine, cysteine, glutamine and threonine were reported collectively to protect astrocytes from Zn toxicity, but histidine is the most potent among the four (Ralph et al. 2010). In fission yeast, endogenous oxidative damage was observed during histidine starvation (Nemoto et al. 2010). With regard to Ni-induced oxidation, the content of ASC was reduced. The HISN3-overexpressing cells accumulated more ASC compared with the wild type, whereas HISN3 silencing resulted in loss of ASC. The elevated level of ASC in 35S::HISN3 algae is most probably attributable to the histidine-improved antioxidative capacity. Accumulation of NPTs is considered favorable for chelation and detoxification of Cd and other heavy metals (Na et al. 2011, Zhang et al. 2013). However, no NPT accumulation was observed in 35S::HISN3 algae, suggesting that induction of NPTs is not the mechanism by which algae detoxify Ni. Under metal stress, NPTs are induced in some plants. In this case, NPT is often thought of a biomarker indicating the degree of metal stress (Na et al. 2011). The low level of NPTs in 35S::HISN3 algae suggests a low degree of Ni stress. The same thing might be true for proline accumulation under Ni exposure in the cells. Overall, the above data indicate that free histidine can confer tolerance of cells to Ni toxicity, which supports

Fig. 5 Effect of Ni on the generation of O$_2^-$ (A) and H$_2$O$_2$ (B) in the 35S::HISN3 and 35S::AntiHISN3 algae. Algae were grown for 3 d and transferred to fresh culture containing 125 M Ni for 8 h. O$_2^-$ and H$_2$O$_2$ content were then quantified. Vertical bars represent the SD (n = 3). Asterisks indicate that mean values are significantly different between the wild type (WT) and 35S::HISN3/35S::AntiHISN3 (P < 0.05).

Fig. 6 Effect of Ni on the accumulation of lipid peroxides in the 35S::HISN3 and 35S::AntiHISN3 algae. (A) Wild-type (WT) algae were grown for 3 d and transferred to fresh culture with Ni at 0–135 μM for 6 d. (B) Transgenic algae were grown for 3 d and transferred to fresh culture with 125 μM Ni for 6 d. After that, TBARS were quantified. Vertical bars represent the SD (n = 3). Asterisks indicate that mean values are significantly different between the Ni treatment and control (P < 0.05) (A) and between the WT and 35S::HISN3/35S::AntiHISN3 (P < 0.05) (B).
Fig. 7 Effect of Ni on the accumulation of ASC (A), non-protein thiol (NPT) (B) and proline (C) in the 35S::HISN3 and 35S::AntiHISN3 algae. The transgenic algae were grown for 3 d and transferred to fresh culture containing 125 μM Ni for 48 h. The ASC, NPT and proline contents were then quantified. Vertical bars represent the SD (n = 3). Asterisks indicate that mean values are significantly different between the wild type (WT) and 35S::HISN3/35S::AntiHISN3 (P < 0.05).

Fig. 8 Metal accumulation in the 35S::HISN3 and 35S::AntiHISN3 algae under Ni exposure. Algae were grown for 3 d and transferred to fresh culture containing 125 μM Ni for 3 d. After that, total cellular Ni (A), Cu (B), Mg (C), Fe (D), Zn (E) and Mn (F) were quantified by coupled plasma-atomic emission spectrometry (ICP-AES). Vertical bars represent the SD (n = 3). Asterisks indicate that mean values are significantly different between the wild type (WT) and 35S::HISN3/35S::AntiHISN3 (P < 0.05).
the notion that HISN3 is an antioxidant gene responsible for the detoxification of Ni.

It was reported that increased histidine concentrations in ATP-PRT-overexpressing Arabidopsis and other plants failed to increase Ni uptake under hydroponic conditions (Wycisk et al. 2004, Ingle et al. 2005, Rees et al. 2009). In this study, however, the elevated histidine concentrations led to significantly high levels of Ni accumulation in 3SS::HISN3 algae. We calculated the proportion of the cellular histidine and Ni concentrations and found that the histidine/Ni ratios in wild-type, 3SS::HISN3 and 3SS::AntiHISN3 cells were significantly different, with the highest values in the wild type, followed by 3SS::HISN3, and the lowest in 3SS::AntiHISN3 cells, suggesting that the model of Ni accumulation can be altered by HISN3 manipulation. The reason for this is currently unknown, but it may be the result of the different Ni uptake and detoxification mechanisms between the unicellular and pluricellular organisms. This suggests that Arabidopsis may lack the transport proteins required for efficient xylem loading of histidine-Ni. In contrast, *Chlamydomonas* is a unicellular organism and no transport of Ni between tissue types is required. Therefore, the increased histidine content alone may be sufficient to allow increased Ni uptake.

The elevated histidine level due to HISN3 overexpression also promoted accumulation of other essential trace metals including Cu, Mg, Fe, Zn and Mn. Histidine may increase metal availability for transporters. For example, Nishida et al. (2011) recently showed that a primary iron uptake transporter AtIRT was induced by Ni and mediated excess Ni accumulation in the root of Arabidopsis. Ni could be competitively taken up via the Fe uptake system. In this study, the increased absorption of the metals might be attributed to the following reasons: (i) metals such as Zn and Mn can be also bind to histidine with a certain affinity and (ii) under excess Ni conditions, uptake of Zn, Fe or other metals may be required to balance the cellular Ni and other metals. Thus, the increased amount of metals such as Zn and Fe in 3SS::HISN3 algae is possibly a mechanism for metal homeostasis or a combination of protection mediated by HISN3.

In conclusion, the biological identification of HISN3 is fundamentally important because it allowed us to understand the primary role of HISN3 in molecular regulation of plant resistance to nickel ecotoxicology through the histidine biosynthetic pathway. Ni-induced generation of ROS and lipid peroxidation were attenuated by HISN3 expression. This is likely to be the result of higher levels of free histidine in 3SS::HISN3 cells, which severed as an Ni-binding ligand and an antioxidant depressing Ni-induced oxidative stress. Importantly, HISN3 overexpression led to more accumulation of Ni and other metals in the cells. Increased uptake of the metals in the transgenic algae may help to remove the metals from water media, if they are contaminated with excess metals. Thus, the present work also provided an example for molecular breeding of algae designed to improve tolerance to metal toxicity and develop techniques for environmental restoration of metal-contaminated aquatic ecosystems.

### Materials and Methods

#### Algae culture and treatment

*Chlamydomonas reinhardtii* algae (strain, CC-503 cw92 mt+) were grown in TAP medium [Tris-acetate-phosphate, pH 7.0; Culture Collection of Cryptophyta Algae (http://cccryo.ntr.io/sources/files/medien/TAP.pdf)] under conditions of 72 μmol photons m⁻² s⁻¹ photosynthetically available radiation in a 12:12 h light/dark cycle at 25 ± 2 °C, as described previously (Wei et al. 2011).

The heavy metal NiSO₄ was used for treatment of cells. The exponentially growing cells were used for all experimental analyses. For cell counting, algae were fixed with Lugol’s iodine solution and counted with a hemocytometer under a light microscope (AxioImager. A1, Zeiss). The data for the growth rate (algal cell density) after 6 d of exposure to Ni were expressed as the EC₅₀ (Jin et al. 2012). The logistic model of probit analysis was performed by estimating the EC₅₀ and associated 95% confidence interval (OECD 2002, US EPA 2002, Choa et al. 2008, Bi et al. 2012). The NOEC and LOEC were calculated using a hypothesis test approach by the algal cell number after 6 d exposure to Ni (US EPA 2002).

#### Transformation

A 1,467 bp HISN3 sequence was amplified by PCR using the sense primer 5'-CATGCCATGTGAAGGCGCCGCGCGC-3' and anti-sense primer 5'-GAAGATCTCTGGAAAGCAACTTACAGAAT-3'. The PCR product was cloned into the pCAMBIA1304 vector driven by a CaMV 35S promoter.

For construction of the 3SS::AntiHISN3 vector, a 425 bp fragment of the HISN3 CDS was amplified by PCR using sense 5'-GA CTaggtgcctgctagtgaagAggaACTTACAGAAT-3' and anti-sense 5'-GAAGATCTCTGGAAAGCAACTTACAGAAT-3' primers. The PCR product was cloned into the BglII/SpeI sites of the pCAMBIA1304 vector with a GUS (β-glucuronidase) reporter gene driven by the 35S CaMV promoter. Genes in *C. reinhardtii* were transformed by the glass bead method (Kindle et al. 1990).

#### Analysis of cis-elements in the *C. reinhardtii* HISN3 promoter region

To analyze cis-elements in the *C. reinhardtii* HISN3 promoter region, 2,000 bp DNA sequences upstream of the HISN3 were retrieved. The motifs were predicted based on the publicly available cis-acting regulatory DNA elements database PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

#### Determination of free histidine

The free histidine concentration was determined based on the modified Pauly reaction (Macpherson 1942). The treated cells were collected by centrifugation at 3,000 × g for 10 min. The collected algae were homogenized with 6 ml of water and sonicated at 80 W for 180 s. The homogenate was centrifuged at 12,000 × g at 4 °C for 10 min. A 2 ml aliquot of the supernatant was mixed with 0.2 ml of 1% sulfanilic acid and 0.2 ml of 5% NaNO₂. After
Determination of Chl and PSII fluorescence

Cells were collected by centrifugation at 3,000 × g for 10 min. Chl was extracted with 10% methanol and quenched by reading the absorbance at 650 and 665 nm. Chl fluorescence of PSII was determined using a PHYTO-PAM Phytoplankton Analyzer (Wei et al. 2011). Cells were kept in darkness for 5 min. This allowed cells to reduce PSII to a constant fluorescence level (F0). After measuring the initial fluorescence (F0), the maximal fluorescence (Fm) was determined using a saturating pulse of 8,000 μmol m−2 s−1 for 0.7 s. The variable fluorescence (Fv) was calculated from the formula, Fv = Fm − F0. The maximum quantum yield (Fv/Fm) was calculated. In comparison, the same absorption was assumed for a phytoplankton suspension: the ETR = Yield × PAR × 0.5 × 0.84 (μmol electrons m−2 s−1). ETRmax represents the maximal ETR. Alpha (the initial slope of the rapid light curve) represents the efficiency of utilization of light energy. However, before measurements were taken, the background signal of the filterate (offset) was measured and the absolute values for different measurements were calculated by subtracting the measured values of filtrate from the measured values of algal suspension.

Determination of oxidative metabolites

O2− was quantified based on the hydroxylamine oxidation method (Wang and Luo 1990). The collected algae were homogenized with 3 ml of ice-cold potassium phosphate buffer solution (50 mM, pH 7.8) and sonicated at 80 W for 180 s. The homogenate was centrifuged at 12,000 × g at 4 °C for 20 min. Then, 0.1 ml of the supernatant was mixed with 0.4 ml of phosphate-buffered saline (PBS) (50 mM, pH 7.8) and 0.5 ml of 1 mM hydroxylamine hydrochloride, and after 1 h reaction at 25 °C, 0.25 ml of 17 mM 4-aminobenzene sulfonic acid and 0.25 ml of 7 mM 1-naphthalenamine were added. The mixture was centrifuged at 12,000 × g for 20 min and the absorbance of the supernatant was measured at 530 nm. H2O2 content was determined based on H2O2–titanium complex formation, and the absorption at 408 nm was measured (Nag et al. 2000). Lipid peroxide, in terms of TBARS, was determined using a phytoplankton suspension: the ETR = Yield × PAR × 0.5 × 0.84 (μmol electrons m−2 s−1). ETRmax represents the maximal ETR. Alpha (the initial slope of the rapid light curve) represents the efficiency of utilization of light energy. However, before measurements were taken, the background signal of the filterate (offset) was measured and the absolute values for different measurements were calculated by subtracting the measured values of filtrate from the measured values of algal suspension.

Metal determination

The dried cells were digested with nitric acid and hydrogen peroxide (HNO3 : H2O2, 4 : 1, v/v). Total amounts of Ni, Zn, Mg, Mn, Fe and Cu were quantified using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optimal 2100DV, Perkin Elmer Instruments).

Determination of non-enzymatic antioxidants

Collected algae were homogenized with 3 ml of 5% (w/v) trichloroacetic acid solution and sonicated at 80 W for 180 s. The homogenate was centrifuged at 12,000 × g at 4 °C for 20 min. The amount of ASC was determined based on the method described previously (Zhou et al. 2008). NPTs were extracted by homogenizing treated algae in 3 ml of of ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at 12,000 × g at 4 °C for 20 min, the supernatant was collected, and the amount of NPTs was determined (Zhou et al. 2008). Free proline was extracted by homogenizing treated algae in sulfosalicylic acid and estimated using ninhydrin reagent (Zhang et al. 2008).

Analysis of transcripts

Total RNA was extracted from cells using Trizol (Invitrogen), followed by DNase I digestion to remove DNA. Reverse transcription was performed at 42 °C for 60 min and 70 °C for 15 min. The mixture (20 μl) consisted of 2 μg of RNA, 0.5 μM oligo(dT) primers, 1 mM dNTP mixture, 20 U of RNase inhibitor and 200 U of Super MLV (Biouniquer). The first cDNA was used as a template for semi-quantitative reverse transcription–PCR (sqRT–PCR) and qRT–PCR amplification to analyze the transcript level. The following primers were used for sqRT–PCR amplification: HISN3 (sense: 5′-TGAGGCGCGCAGCCTTGA-3′) and (antisense: 5′-TGTTGAAA GCACTTACAGAAPG-3′), 1,467 bp, 35 cycles; 18S RNA (sense: 5′-ATGGTGCGAAAGCTGAA-3′) and (antisense: 5′-GCTAAACGC CAATGCTCC-3′), 288 bp, 29 cycles. 18S rRNA was used for cDNA normalization. The PCR products were subjected to 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The following primers were used for qRT–PCR amplification: HISN3 (sense: 5′-TACAGAAGACTGCCTAATGC-3′) and (antisense: 5′-TCCCTACCGCGTGGACC-3′), 184 bp, 40 cycles; 18S RNA (sense: 5′-ACACCGGAAGATTACAGA-3′) and (antisense: 5′-AGGGCAACCCACACAACTAAG-3′), 85 bp, 40 cycles. The qRT–PCR amplification was run according to the following protocol: 94 °C for 30 s; 40 cycles of 94 °C for 5 s, 60 °C for 30 s; followed by a dissociation cycle. For each gene, a common threshold setting applied to each of the three biological replicates determined the threshold cycle. The relative abundance of each gene was determined by the 2−ΔΔCt method (Livak and Schmittgen 2001). Fold change and SE were log-transformed for graphical representation.

Statistical analyses

The study was carried out with at least three independent experiments. The data shown in the figures were the mean of three biological replicates determined the threshold cycle. The relative abundance of each gene was determined by the 2−ΔΔCt method (Livak and Schmittgen 2001). Fold change and SE were log-transformed for graphical representation.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


