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Transcriptional control of ROS homeostasis under high N

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HNI9 and HY5 maintain ROS homeostasis under high nitrogen provision in Arabidopsis.

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One sentence summary

Excessive N nutrition leads to ROS accumulation, and requires the function of major transcriptional regulators to maintain plants under physiological conditions.

Author contributions:

An.M. and A.G. conceived research plans and supervised the experiments; F.B, Am.M., J.B., L.L., L.B. and An.M performed most of the experiments; F.B, Am.M., J.B., G.K., L.L., L.B. and An.M analyzed the data; An.M. wrote the article with contributions of all the authors.

1 **Summary**

2 Reactive Oxygen Species (ROS) can accumulate in cells at excessive levels, leading to

3 unbalanced redox status and to a potential oxidative stress, which can have damaging effects to

4 the molecular components of plant cells. Several environmental conditions have been described

5 as causing an elevation of ROS production in plants. Consequently, this requires the expression

6 of detoxification responses in order to maintain ROS homeostasis at physiological levels. In

7 case of mis-regulation of the detoxification systems, oxidative stress can lead ultimately to

8 growth retardation and developmental defects. Here, we demonstrate that Arabidopsis plants

9 growing under high nitrogen environment have to express a set of genes involved in

10 detoxification of ROS in order to maintain ROS at physiological levels. We show that the

11 chromatin factor HNI9 is an important actor of this response, required for the expression of

12 these detoxification genes. Mutation in HNI9 leads to elevated ROS levels, and to ROS-

13 dependent phenotypic defects under high but not low N provision. In addition, we identify HY5

14 as one of the major transcription factors also required for the expression of this detoxification

15 program under high N condition. Our results demonstrate the requirement of a balance between

16 N nutrition and ROS production, and identified the first major regulators required to control

17 ROS homeostasis under excessive N nutrition.

18

Introduction

19

20 Reactive Oxygen Species (ROS) are integral part of plant metabolism, generated as by-products

21 of a large range of enzymatic reactions. The dynamics of ROS in plant cells corresponds to two

22 distinct scenarios. First, ROS are involved in numerous signaling pathways, and thus their

23 dynamic affects a lot of developmental and physiological processes, like cell differentiation or

24 response to biotic and abiotic stresses (Noctor et al., 2018). In such cases, variations in ROS

25 are generally dynamic and transient, and occur at moderate concentration. However, on another

26 hand, ROS can also accumulate in cells at excessive and constant levels, leading to unbalanced

27 redox status and to potential oxidative stress (Schieber and Chandel, 2014). At the cellular level,

28 excessive accumulation of ROS can lead to oxidation and to damage of many essential

29 molecules (Moller et al., 2007). For instance, oxidation of enzymatic proteins often leads to

30 loss of activity, oxidation of DNA can lead to degradation or mutations, and oxidation of lipids

31 leads to disorganization of cellular membranes. The maintenance of ROS homeostasis is

32 therefore essential to keep cell integrity. ROS levels are also associated with a range of

33 physiological and developmental phenotypes. For instance, functioning of the shoot apical

34 meristem is largely influenced by the redox status (Schippers et al., 2016), and many

35 developmental processes imply interactions between ROS and phytohormones (Considine and

36 Foyer, 2014). Several reports have also demonstrated a role for ROS in root growth and

37 development (Tsukagoshi, 2016). As a consequence, mutations leading to excessive ROS

38 production or exogenous application of ROS at high concentration lead to retardation of root

39 growth in Arabidopsis (Dunand et al., 2007; Tsukagoshi et al., 2010; Zhao et al., 2016).

40 Altogether, these observations highlight that controlling the level of ROS is crucial for plant

41 growth, development and physiology.

42 Given the importance of ROS homeostasis, plants possess a large range of mechanisms that are

43 able to remove ROS from the cells. A main part of this antioxidant response corresponds to

44   enzymes, such as peroxidases, that use reducing power in oxidation/reduction reactions to

45   decrease the cell redox status (Moller et al., 2007). These enzymes work in complex systems

46   also involving essential antioxidant molecules, like ascorbic acid, glutathione or thiamin, that

47   are known to protect against oxidative stress (Tunc-Ozdemir et al., 2009; Ramírez et al., 2013).

48   At the molecular level, transcriptional induction of ROS-sensitive genes in plants constitutes a

49   major part of the response to ROS overproduction (Willems et al., 2016). However, in

50   agreement with the high number of genes coding for components of antioxidant responses in

51   Arabidopsis, a specificity of response seems to occur in function of the signals at the origin of

52   ROS production. Indeed, signaling pathways and genes involved in ROS homeostasis in

53   response to stress largely vary according to the type of stress encountered by the plant (Apel

54   and Hirt, 2004). However, many of the actors that drive each transcriptional response and its

55   specificity remain to be identified.

56   ROS production has been observed in plants in response to many environmental factors, with

57   both biotic interactions and abiotic stress often leading to ROS signaling (Baxter et al., 2014).

58   Concerning abiotic constraints, drought, salinity or heat stress are known to induce the

59   production of ROS (Choudhury et al., 2017). However, these responses correspond to cases

60   where ROS contribute to a signaling pathways, but do not accumulate and generate oxidative

61   stress. In opposition, several examples of abiotic stress have been directly linked to an elevation

62   of ROS, which is eventually perceived as a perturbation of the plant redox balance. This is the

63   case for nutrients deprivation, where potassium, nitrogen (N) or phosphorus starvation rapidly

64   leads to an accumulation of H_2O_2 in the roots (Shin and Schachtman, 2004; Shin et al., 2005).

65   This suggest that the redox status of plants can be very sensitive to changes in the nutritional

66   environment.

67   Previously, the chromatin factor HNI9 has been shown to be involved in the response to high

68   N provision in Arabidopsis. Mutations in *HNI9* leads to an increase in transcripts level

69   corresponding to the nitrate transporter *NRT2.1* under high N provision, where this gene is

70   normally strongly downregulated (Widiez et al., 2011). However, the direct relationship

71   between HNI9 and *NRT2.1* has not been demonstrated. Furthermore, several reports in plants,

72   animals and yeast demonstrated that HNI9 is a positive regulator of gene expression (Yoh et

73   al., 2008; Li et al., 2010; Chen et al., 2012; Wang et al., 2013; Wang et al., 2014), which is not

74   consistent with the hypothesis of a direct role of HNI9 on *NRT2.1* downregulation. Here, we

75   reexamined the function of HNI9 in the response of plants to high N provision, and we show

76   an unexpected role of HNI9 in the regulation of ROS homeostasis under high N provision,

77   through the induction of a subset of genes involved in detoxification. We also identify the

78   transcription factor HY5 as an important component of this response, and highlight a new

79   interaction between plant nutrition and ROS homeostasis.

80

81   **Results**

82   **HNI9 is required to upregulate the expression of a set of genes involved in redox processes**

83   **in response to high N provision.**

84   In order to reexamine the role of HNI9 in the response to high N provision, we compared

85   transcriptomic data of WT and *hni9-1* mutant plants under low nitrate and high N provisions

86   (Widiez et al., 2011). As HNI9 is a positive regulator of gene expression, we selected genes

87   that are induced by high N provision in WT, and looked for those that do not display such

88   induction in the *hni9-1* mutant line. Using these criteria, we obtained a list of 108 genes induced

89   under high N condition in a HNI9-dependent manner (Table S1). In order to determine which

90   biological functions are affected by the HNI9 mutation under high N provision, we performed

91   a gene ontology analysis using this list of 108 genes. Among the biological functions

92   overrepresented, we found that several categories related to redox processes, including

93   oxidoreductase activity, peroxidase activity, or antioxidant activity, were the most significantly

94   represented (Figure 1, Table 1). In addition, using further manual curation, we observed that

95   many other genes present in other GO categories might also encode for enzymes or proteins

96   associated with ROS detoxification or with antioxidant biosynthesis pathways (Table S1).

97   Therefore, we hypothesized that high N provision could challenge ROS homeostasis in plants,

98   and that HNI9 could be required in order to induce a set of genes involved in cellular

99   detoxification.

100

101   **HNI9 mutation leads to higher ROS levels and to ROS-dependent phenotypes under high**

102   **N provision.**

103   In order to assess the role of HNI9 in ROS homeostasis under high N provision, we measured

104   H_2O_2 and total ROS levels in WT and *hni9-1* mutant lines under low nitrate and high N

105   provisions. In WT line, H_2O_2 levels remained constant whatever N provision, whereas total

106   ROS levels slightly increase under high N provision, yet not significantly, showing that ROS

107   homeostasis is maintained from low to high N provision (Figure 2A, B). In contrast, ROS

108   accumulation displayed significant changes in the *hni9-1* mutant line. The levels of H_2O_2 and

109   total ROS in the *hni9-1* mutant line were identical to that of WT under low N provision, but

110   they increased significantly under high N provision (Figure 2A, B). To confirm these results,

111   we used an Arabidopsis line expressing the *GRX1-roGFP2* construct (hereafter mentioned as

112   *roGFP2*), in order to visualize ROS levels *in planta*. RoGFP2 is a redox-sensitive probe, which

113   allows to monitor plant redox status and in particular to address H_2O_2 levels *in vivo* (Meyer et

114   al., 2007; Marty et al., 2009). In WT plants under high N provision, fluorescence signal of

115   roGFP2 was very low, in agreement with our measurements of H_2O_2 and ROS levels (Figure

116   2C). In contrast, fluorescence signal of roGFP2 in *hni9-1* plants was strong, corresponding to

117   the elevation of H_2O_2 and ROS levels measured in this line (Figure 2D). Quantification of

118   roGFP2 signals in each lines validates a strongly significant increase in ROS levels in *hni9-1*

119   mutant line as compared to the WT (Figure S1). Altogether, these results led us to conclude that

120   HNI9 contributes to prevent ROS overaccumulation under high N supply, likely through the

121   induction of a set of genes required to detoxify ROS produced under high N condition.

122   We next looked whether upregulation of *NRT2.1* expression under high N supply, which is a

123   main phenotype described for the *hni9-1* mutant line, could be linked with ROS

124   overaccumulation in this mutant. Therefore, we investigated the expression of *NRT2.1* under

125   two conditions associated with elevated ROS levels. First, we measured *NRT2.1* transcript

126   levels in WT plants treated with menadione, a redox-active quinone which causes an elevation

127   of ROS in plant roots (Lehmann et al., 2009). Our results showed that this treatment indeed

128   results in an increase in *NRT2.1* transcript levels (Figure 3A). Then we measured *NRT2.1*

129   transcript levels in the ascorbate-deficient mutant *vtc2*, which is affected in ROS detoxification

130   processes and displays significantly higher H_2O_2 levels than in WT plants (Kotchoni et al.,

131   2009). In comparison to WT, *NRT2.1* transcripts levels were significantly higher in the *vtc2*

132   mutant line, as we observed for the *hni9-1* mutant (Figure 3B). These observations suggest that

133   *NRT2.1* is induced by ROS, and thus lend support to the hypothesis that the previously reported

134   *hni9-1* phenotype under high N condition may be due to elevation of ROS levels in this mutant.

135   Conversely, we investigated whether well-known phenotypes associated with ROS

136   overaccumulation were also observed in *hni9-1* plants under high N supply. In particular,

137   elevated ROS levels have been shown to alter root growth, and thus plants growing under

138   elevated ROS levels display a reduced primary root length (Dunand et al., 2007; Tsukagoshi et

139   al., 2010). Therefore, we asked whether elevated ROS levels in *hni9-1* mutant line under high

140   N provision are correlated with an alteration of root growth. We used the *vtc2* mutant line,

141   affected in ROS detoxification processes, as a positive control to confirm that elevated ROS

142   levels lead to a reduction of root growth under our experimental conditions. Indeed, the *vtc2*

143   mutant line showed a reduction of primary root length as compared to the WT, regardless of

144   the level of N provision; however, the reduction of root growth was significantly higher under

145   high N than under low N (Figure 4). The *hni9-1* mutant line also displays a reduction of root

146   growth in comparison to the WT (Figure 4), which is consistent with the general role of HNI9

147   on plant growth and development that has been previously described (Li et al., 2010). However,

148   the reduction of root growth was also significantly higher under high N condition than under

149   low N condition (Figure 4). Again, this suggests that phenotypic changes due to HNI9 mutation

150   under high N supply may be explained by ROS overaccumulation. Finally, we tested whether

151   external application of antioxidant molecule could complement the *hni9-1* root growth

152   phenotype. We therefore measured root growth of WT and *hni9-1* lines under high N

153   conditions, supplemented or not with thiamin, an antioxidant known to mitigate growth

154   retardation caused by oxidative stress (Tunc-Ozdemir et al., 2009). Indeed, we observed that

155   primary root growth defect of *hni9-1* was fully abolished in the presence of thiamin (Figure 5).

156   This demonstrates that the presence of antioxidant can complement *hni9-1* root growth

157   phenotype, further indicating that HNI9 affects root development under high N supply

158   specifically through altered ROS homeostasis.

159   **HNI9 is required to achieve full levels of H3K4me3 at the locus of detoxification genes.**

160   HNI9 is part of a large protein complex, including elongation and chromatin remodeling factors,

161   which affects the histone modification state of genes (Yoh et al., 2008; Li et al., 2010). Indeed,

162   several reports in plants and animals demonstrated that HNI9 operates at the switch between

163   transcriptional repression and activation, in cooperation with H3K27me3 demethylases, and

164   H3K36 methyl-transferases (Li et al., 2013; Wang et al., 2014). Therefore, we tested the

165   hypothesis that HNI9 controls the expression of ROS detoxification genes by modulating their

166   chromatin state in response to high N condition. To do so, we assayed the level of 3 active

167   chromatin marks (H3K4me3, H3K9ac and H3K36me3) and 1 repressive chromatin mark

168   (H3K27me3) at the locus of several HNI9-dependent genes involved in ROS detoxification in

169   response to high N condition. Here, the results showed that the level of H3K27me3 and

170   H3K36me3 were not changed in the *hni9-1* mutant at the locus of HNI9-dependent

171   detoxification genes (Figure 6). In addition, the levels of H3K9ac, associated with active

172   transcription were also generally similar between WT and *hni9-1*. However, we observed that

173   H3K4me3 levels were globally lower in the *hni9-1* mutant at the locus of HNI9-dependent

174   detoxification genes (Figure 6). We therefore conclude that HNI9 may induce the expression

175   of ROS detoxification genes by regulating the profile of H3K4me3.

176   **Analysis of HNI9-dependent genes involved in ROS detoxification under high N condition**

177   **reveals the role of HY5 in the control of ROS homeostasis.**

178   HNI9 is a generic regulator of gene expression, as it does not provide by itself any sequence

179   specificity to its target loci. Specificity of response thus requires the action of transcription

180   factors, to drive chromatin remodeling complexes to target loci (Li et al., 2010). Therefore, we

181   tested whether promoter sequence analysis of genes involved in ROS detoxification under high

182   N could reveal the implication of transcription factors. We submitted the promoter sequences

183   from the 108 genes induced by high N and dependent on HNI9 to MEME software in order to

184   find putative conserved cis regulatory elements (CREs). This analysis reveals a significant

185   enrichment of 2 CREs showing significant homology with the binding site consensus of the

186   transcription factor HY5 defined by DAP-seq (O'Malley et al., 2016) (Figure 7A, B). In total,

187   45 out of 108 genes involved in ROS detoxification under high N contained a putative HY5

188   binding site (Table S2 and S3). Interestingly, it has been shown previously that HY5 is involved

189   in the control of ROS production in response to light or temperature treatments (Catala et al.,

190   2011; Chen et al., 2013; Chai et al., 2015). To first validate the implication of HY5 in the

191   regulation of genes involved in ROS detoxification under high N condition, we aimed to

192   investigate the binding of HY5 to the putative consensus sequences identified in part of the

193   ROS detoxification genes. To this end, we performed chromatin immunoprecipitation followed

194   by quantitative PCR on a subset of genes with or without putative HY5 binding sites. The results

195   showed that HY5 indeed binds to the promoter of detoxification genes containing a putative

196   HY5 binding site, whereas no significant binding was observed for genes without putative HY5

197   binding site, although they are also involved in detoxification processes under high N provision

198   (Figure 7C). Next, in order to test the effect of HY5 mutation, we measured the expression of

199   these genes (with or without HY5 binding sites) in WT and in *hy5-215* mutant lines. Every gene

200   we tested, except one, were statistically down-regulated in the *hy5-215* mutant line under high

201   N provision (Figure 7D), strongly supporting a major role of HY5 in the activation of the

202   detoxification program under high N provision. Finally, to test the effect of the mis-regulation

203   of the detoxification program under high N following HY5 mutation, we measured the levels

204   of ROS and H_2O_2 in WT and in *hy5-215* mutant lines. The results clearly showed that ROS and

205   H_2O_2 levels were higher in *hy5-215* than in WT, demonstrating the functional importance of

206   HY5 in the process of detoxification under high N provision (Figure 8). Altogether, our results

207   demonstrate the induction of a transcriptional program needed for ROS detoxification under

208   high N provision, which is driven in most part by the chromatin remodeler HNI9 and the

209   transcription factor HY5.

210

211   **Discussion**

212   Plants, like every living organism, have to cope with environmental constraints. ROS

213   accumulation has been shown to be involved in numerous signaling pathways or to cause

214   oxidative stress in response to different environmental challenges (Baxter et al., 2014;

215   Choudhury et al., 2017; Noctor et al., 2018). The main finding of our work is that high N

216   provision leads to the generation of ROS in plant roots, which are managed by the

217   transcriptional induction a specific detoxification program. Interestingly, increasing N

218   provision is viewed positively and as favorable conditions for plant growth, but we

219   demonstrated here that excessive N nutrition could in fact be detrimental for the plant. Indeed,

220   even if efficient detoxification systems exist and maintain ROS homeostasis, we can reasonably

221   think that their induction and functioning imply a cost for plants, and that their efficiency can

222   be limited under certain circumstances. In spite of the demonstration that high N nutrition leads

223   to ROS accumulation, the physiological cause of ROS production under high N conditions

224   remains to be determined. Nitrate assimilation and to a lower extent ammonium assimilation

225   pathways can be candidate, as they consume a lot of reducing power (Hachiya and Sakakibara,

226   2017), and may in consequence alter the cellular redox balance of plants. However, it may also

227   reflect a more general link between nutrition and redox balance. Indeed, several reports have

228   described that excessive nutrition in animals leads to ROS production and perturbation of the

229   cellular redox status (Sies et al., 2005; Samoylenko et al., 2013; Gorlach et al., 2015). In the

230   case of plants, nutrient starvation has also been identified as a condition that generates oxidative

231   stress through the production of ROS (Shin and Schachtman, 2004; Shin et al., 2005). In

232   addition, recent reports demonstrated that ROS accumulate early during N starvation response,

233   and most probably contribute to regulate nitrate-responsive genes like *NRT2.1* (Jung et al.,

234   2018; Safi et al., 2018). Therefore, these studies and our present work suggest the existence of

235   thresholds of N provision inside which the cellular redox balance is optimal. This situation is

236   reminiscent of what has been described for iron (Fe) homeostasis. Indeed, excessive Fe

237   provision can also lead to an accumulation of ROS, and specific mechanisms have been

238   described to limit this negative effect (Briat et al., 2010). Nutrient deficiency or excess (at least

239   for N and Fe) would correspond to conditions in which ROS production is above a physiological

240   limit and alter plant redox status. Again, it is interesting to observe that the same analysis is true

241   for animals (Gorlach et al., 2015).

242   In our work, we highlight the role of HNI9 in the transcriptional induction of this detoxification

243   program. In Arabidopsis, HNI9 has been mainly associated to brassinosteroids (BRs) signaling

244   (Li et al., 2010; Wang et al., 2014). In response to BRs, HNI9 is associated with the chromatin

245   remodeling factors ELF6, REF6 and SDG8, and with the transcription factor BES1. ELF6 and

246   REF6 are responsible for removing of the repressive chromatin mark H3K27me3, and SDG8 is

247   supposed to catalyze the trimethylation of H3K36, which promotes elongation of transcription.

248   This suggests a role for HNI9 in complexes associated with transcriptional switch, from

249   repression to activation, as demonstrated in animals (Chen et al., 2012; Wang et al., 2013). In

250   our work, HNI9 is mainly linked with differences in H3K4me3 levels. This suggests that HNI9

251   could be associated with other chromatin complexes or other steps of transcriptional regulation.

252   Nevertheless, it identifies another transcriptional pathway in which HNI9 is essential for the

253   induction of expression of a large set of genes.

254   In addition, our work identifies HY5 as a major component for the induction of the gene

255   network involved in ROS detoxification under high N provision. HY5 is a master regulator of

256   gene expression in Arabidopsis, at the center of several transcriptional networks. It is notably

257   involved in the responses to photosynthesis, light, temperature or hormones (Gangappa and

258   Botto, 2016). Interestingly, it has been also implicated in the control of ROS homeostasis in

259   response to light or cold treatments (Catala et al., 2011; Chen et al., 2013; Chai et al., 2015). In

260   each cases, HY5 is required to suppress ROS accumulation during stressful conditions.

261   However, the set of genes induced by HY5 to balance ROS levels seems different from one

262   stimulus to the others. This suggests that other transcription factors, in addition to HY5, could

263   specify the response according to the environmental signal. Interestingly, we found that the

264   expression of every gene of the ROS detoxification network in response to high N that we tested

265   was dependent on HY5, whatever the presence of a HY5 binding site in their promoter. This

266   may be due to the existence of several layers in this gene network, and suggests that HY5 would

267   be at the top of the network.

268   In conclusion, we identified how a detoxification program is induced in order to maintain plant

269   redox status under physiological conditions even under high nutritional provision. However,

270   usage of detoxification processes is certainly not costless for plants. Thus, this work

271   demonstrates that excessive N input is not appropriate for plant physiology and development,

272   and lends support to the occurrence of an optimum between nutrition and ROS production in

273   physiology.

274

275   **Material and methods**

276   **Plant material and growth conditions**

277   The *Arabidopsis thaliana* accession used in this study was Col-0. Mutant alleles and transgenic

278   plants used in this study are *hni9-1* (Widiez et al., 2011), *vct2 (Collin et al., 2008)*, *hy5-215*

279   (Oyama et al., 1997), and *GRX1-roGFP2* (Meyer et al., 2007). Experiments were performed

280   using roots from 7 days-old seedlings grown under a long-day photoperiod (16 h light and 8 h

281   dark) on vertical MS/2 plates without N (PlantMedia) supplied with 0.8 % agar, 0.1 % of

282   sucrose, 0.5 g/L MES and the appropriate concentration of N as described in figures legend or

283   in the main text.

284   **Analysis of gene expression by quantitative PCR**

285   Root samples were frozen and ground in liquid nitrogen, and total RNA was extracted using

286   TRI REAGENT (MRC), DNase treated (RQ1 Promega), and reverse transcription was achieved

287   with M-MLV reverse transcriptase (RNase H minus, Point Mutant, Promega) using an anchored

288   oligo(dT)20 primer. Transcript levels were measured by qRT-PCR (LightCycler 480, Roche

289   Diagnostics) using the TB Green^{TM} Premix Ex Taq^{TM} (Tli Rnase H plus) Bulk (TaKaRa). Gene

290   expression was normalized using *ACT2* as an internal standard. Sequences of primers used in

291   qPCR for gene expression analysis are listed in Supplementary file 2.

292   **ChIP quantitative PCR**

293   ChIP experiments were performed as previously described (Bellegarde et al., 2018). Chromatin

294   was precipitated with 2.5 µg of antibodies against H3 (Abcam 1791), H3K27me3 (Millipore

295   07-449), H3K4me3 (Diagenode C15410030), H3K36me3 (Abcam 9050), H3K9ac (Agrisera

296   AS163198), or HY5 (Agrisera AS12 1867). Immunoprecipitated DNA was purified by phenol-

297   chloroform and ethanol-precipitated, and resulting DNA was analyzed by qPCR analysis. For

298   chromatin marks analyses, ChIP experiments were quantified using H3 level as an internal

299   standard, and normalized using *ACT7 (*H3K4me3, H3K9ac), *ACT2* (H3K36me3) or *LEC2*

300   (H3K27me3) enrichment. For HY5 binding sites analyses, ChIP experiments were normalized

301   to the input levels. Sequences of primers used in qPCR for ChIP experiments are listed in

302   Supplementary file 2.

303   **ROS and H_2O_2 assays**

304   Root samples were frozen and ground in liquid nitrogen, and ROS and H_2O_2 were extracted

305   using about 30 mg of plant material in 200 µL of phosphate buffer (20 mM K2HPO4, pH 6.5).

306   For ROS measurements, 50 µL of supernatant were mixed with 50 µL of 20 µM DFFDA

307   (Molecular Probes). Reactions were incubated 30 minutes at ambient temperature, and

308   fluorescence was detected using 492/527 nm as excitation/emission parameters. H_2O_2 was

309   measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), as

310   described in (Brumbarova et al., 2016).

311   **Microscopy**

312   Confocal imaging of Arabidopsis root cells expressing *GRX1-roGFP2* were performed using a

313   Leica SP8 Confocal Microscope (Leica, Germany). GFP quantifications were done with an

314   Axiovert 200 M microscope (Zeiss, Germany) and images were analyzed with ImageJ analysis

315   software. The data represent the fluorescence quantification values measured in the root tip.

316   **Root growth analyses**

317   Vertical agar plates containing plants were scanned after 7 days of growth and root length was

318   analyzed using ImageJ analysis software. For experiments with thiamin, plants were grown on

319   vertical plates with MS/2 without N (PlantMedia) supplied with 0.8 % agar, 0.1 % of sucrose,

320   0.5 g/L MES and 10 mM NH_4NO_3 for 4 days, and transferred to the same medium with or

321   without 100 μM thiamin. Position of the root tip was marked on the back of the new plates, and

322   root length was analyzed 2 days after transferred by measuring growth from the mark to the

323   root tip.

324   **Determination of conserved cis regulatory sequences**

325   Putative cis regulatory sequences were identified using the MEME suite (Bailey et al., 2009).

326   500 base pairs of promoter sequences were used as primary input, with the following

327   parameters: 0-order background model, classic discovery mode, 0 or one occurrence per

328   sequence, motif width between 6 and 50 nucleotides.

329   **Analysis of gene ontology**

330   Gene ontology analysis was performed using BINGO under Cytoscape environment, using

331   Biological Process file, and a significance level of 0.05.

332   **Statistical analysis**

333   Mean ± SE is shown for all numerical values. Statistical significance was computed using a

334   two-tailed Student's t-test. Significance cutoff: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

335

336

337   **Competing interests**

338   The authors declare no competing financial interests.

339   **Acknowledgments**

346

347   **Table 1:** Gene ontology enrichment identified in HNI9-dependent genes induced by high N

348   conditions (10 mM NH_4NO_3). The gene ontology term enrichment analysis was performed

349   under Cytoscape environment by BINGO software using the Molecular Function ontology file.

350   Details of the analysis are provided in Supplementary File 1.

351

GO-ID	p-value	Number of genes in the GO category	Description
3824	6.5259E-4	50	catalytic activity
16491	6.5259E-4	17	oxidoreductase activity
51739	6.5259E-4	2	ammonia transmembrane transporter activity
15200	6.5259E-4	2	methylammonium transmembrane transporter activity
16684	1.5486E-3	5	oxidoreductase activity, acting on peroxide as acceptor
4601	1.5486E-3	5	peroxidase activity
50284	2.2260E-3	2	sinapate 1-glucosyltransferase activity
16209	3.4940E-3	5	antioxidant activity
15250	5.4994E-3	3	water channel activity
5372	5.4994E-3	3	water transmembrane transporter activity
20037	1.5347E-2	6	heme binding
22838	1.5347E-2	4	substrate-specific channel activity
22803	1.5347E-2	4	passive transmembrane transporter activity
15267	1.5347E-2	4	channel activity
9055	1.6121E-2	7	electron carrier activity
46906	1.6121E-2	6	tetrapyrrole binding
19825	1.8955E-2	5	oxygen binding
5506	2.5391E-2	6	iron ion binding
34074	2.7983E-2	1	marneral synthase activity
22820	2.7983E-2	1	potassium ion symporter activity
46905	2.7983E-2	1	phytoene synthase activity
16767	2.7983E-2	1	geranylgeranyl-diphosphate geranylgeranyltransferase
80014	2.7983E-2	1	thalianol hydroxylase activity
9674	2.7983E-2	1	potassium:sodium symporter activity
8171	4.5083E-2	2	O-methyltransferase activity
80041	4.6532E-2	1	ADP-ribose pyrophosphohydrolase activity
8909	4.6532E-2	1	isochorismate synthase activity
249	4.6532E-2	1	C-22 sterol desaturase activity
16866	4.6532E-2	2	intramolecular transferase activity

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353

Figure Legends

Figure 1: Genes induced by high N provision in HNI9 dependent manner are associated with redox processes. Functional network realized from the list of 108 genes induced specifically under high N condition (10 mM NH_4NO_3) in a HNI9 dependent manner. Orange and yellow circles correspond to p-values lower than 0.01 and 0.05, respectively (see also Table 1 for details). Insert in the right corresponds to the central hub of the network with biological function associated with antioxidant activities.

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Figure 3: *NRT2.1* expression is sensitive to ROS homeostasis. A. Relative expression of *NRT2.1* in the presence of 100 μM menadione in roots of WT plants. Plants were grown under MS/2 medium containing 1 mM NO_3^-, and transferred on the same medium with or without menadione for 4 hours. B. Relative expression of *NRT2.1* in roots of WT, *hni9-1* and *vtc2* mutants. Plants were grown under MS/2 medium containing high N provision (10 mM NH_4NO_3). Statistical significance was computed using a two-tailed Student's t-test. Significance cutoff: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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380 **Figure 5: External application of antioxidant molecule complement the *hni9-1* root**

381 **growth phenotype**. Plants were grown under high N condition (10 mM NH_4NO_3) for 4 days,

382 and transferred to the same medium with or without 100 μM thiamin. Primary root growth of

383 WT and *hni9-1* lines was measured after 2 days of growth. Statistical significance was

384 computed using a two-tailed Student's t-test. Significance cutoff: $*p < 0.05$, $**p < 0.01$, $***p$

385 < 0.001.

386 **Figure 6: Mutation in HNI9 is associated with reduction of H3K4me3 at the loci of**

387 **detoxification genes.** ChIP analysis of H3K4me3, H3K27me3, H3K36me3 and H3K9ac in *WT*

388 and *hni9-1* roots of 7 days-old plants grown under high N provision (10 mM NH_4NO_3).

389 Quantification by qRT-PCR is shown as the percentage of H3K4me3, H3K36me3, H3K27me3

390 or H3K9ac over H3 at target loci, normalized by the percentage of H3K4me3, H3K36me3,

391 H3K27me3 or H3K9ac over H3 at positive controls (*ACT2* for H3K4me3, H3K36me3, and

392 H3K9ac, *LEC2* for H3K27me3). Data are presented relative to the WT level. Error bars

393 represent standard errors of the mean based on at least 3 biological replicates.

394 **Figure 7: HY5 binds to and regulates the expression of genes involved in detoxification**

395 **under high N provision.** A, B. Comparison of 2 conserved motifs discovered by MEME

396 analysis in the promoters of HNI9-dependent genes induced under high N provision (10 mM

397 NH_4NO_3) with HY5 consensus binding site identified by DAP-seq. C. ChIP analysis of HY5

398 enrichment in WT roots at the loci of HNI9-dependent genes induced under high N provision.

399 Quantification by qRT-PCR is shown as the percentage of input. Error bars represent standard

400 errors of the mean based on at least 3 biological replicates. Statistical significance was

401 computed using a two-tailed Student's t-test (significance cutoff: $*p < 0.05$, $**p < 0.01$, $***p$

402 < 0.001), in comparison to a negative control (Neg. Ct.: *AT4G03900*, showing no relation with

403 N or HY5 signaling). D. Relative expression of genes involved in detoxification induced under

404   high N provision in roots from WT and *hy5-215* lines. Statistical significance was computed

405   using a two-tailed Student's t-test. Significance cutoff: *p < 0.05, **p < 0.01, ***p < 0.001.

406   **Figure 8: ROS levels are higher in *hy5-215* under high N provision.** Measurement of H_2O_2

407   (A) and ROS (B) in roots of WT and *hy5-215* lines under high N (10 mM NH_4NO_3) provision.

408   Statistical significance was computed using a two-tailed Student's t-test. Significance cutoff:

409   *p < 0.05, **p < 0.01, ***p < 0.001.

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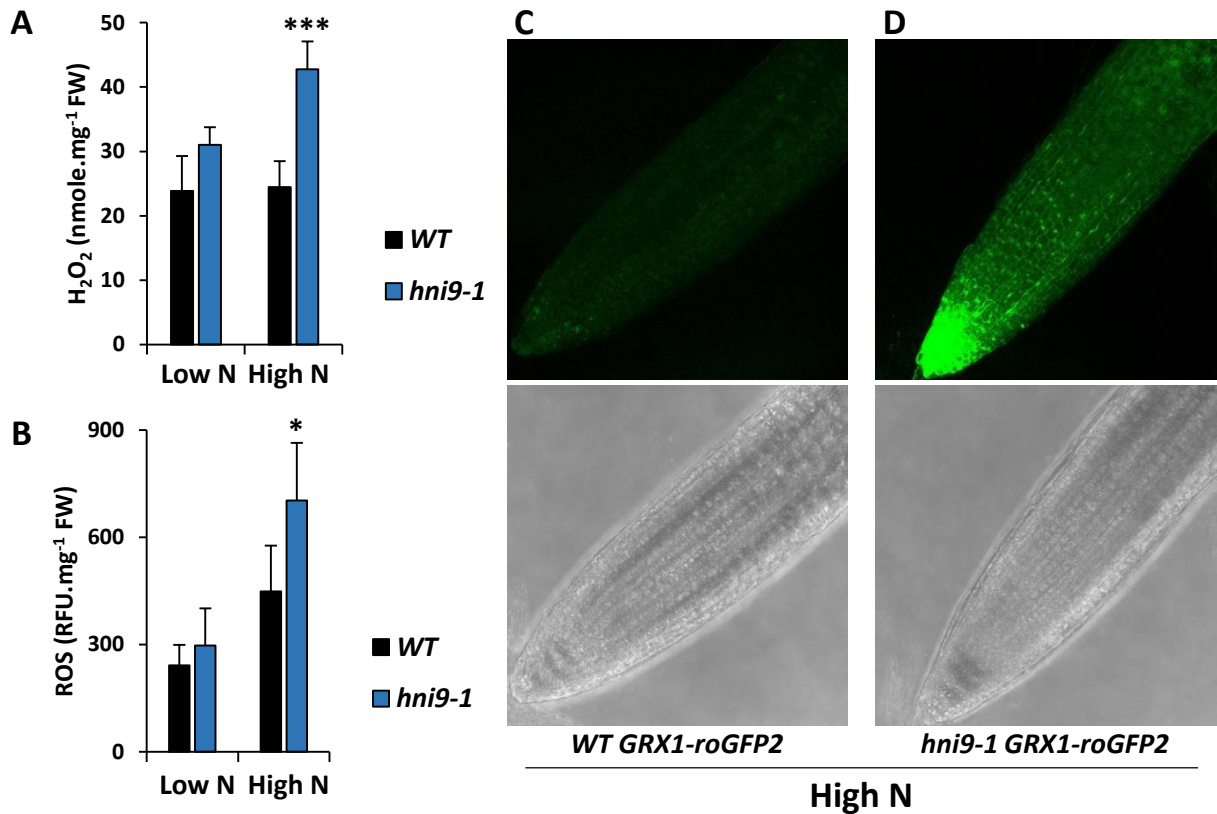534   modulating reactive oxygen species homeostasis in Arabidopsis. Sci Rep **6:** 28683

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Figure 2 : ROS levels are higher in *hni9-1* under high N provision. Measurement of H_2O_2 (A) and ROS (B) in roots of WT and *hni9-1* lines under low (0.3 mM NO_3^-) and high N (10 mM NH_4NO_3) provision. Statistical significance was computed using a two-tailed Student's t-tes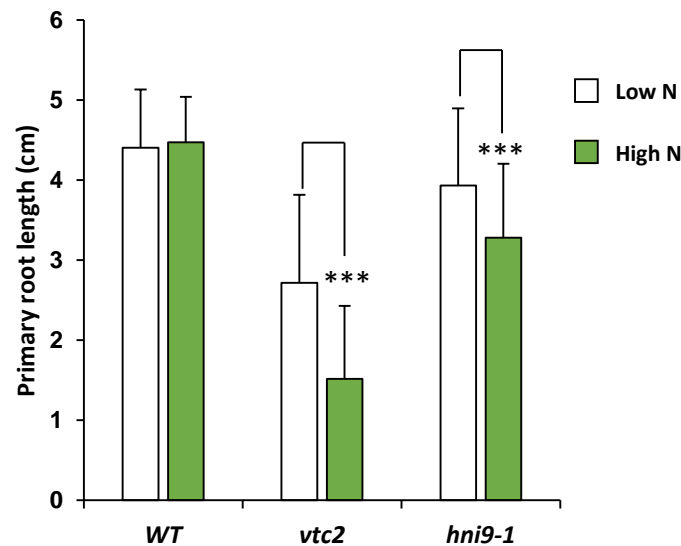t. Significance cutoff: *p < 0.05, **p < 0.01, ***p < 0.001.Visualization of H_2O_2 levels *in vivo* using *GRX1-roGFP2* probe in WT (C) or *hni9-1* (D) lines under high N conditions.

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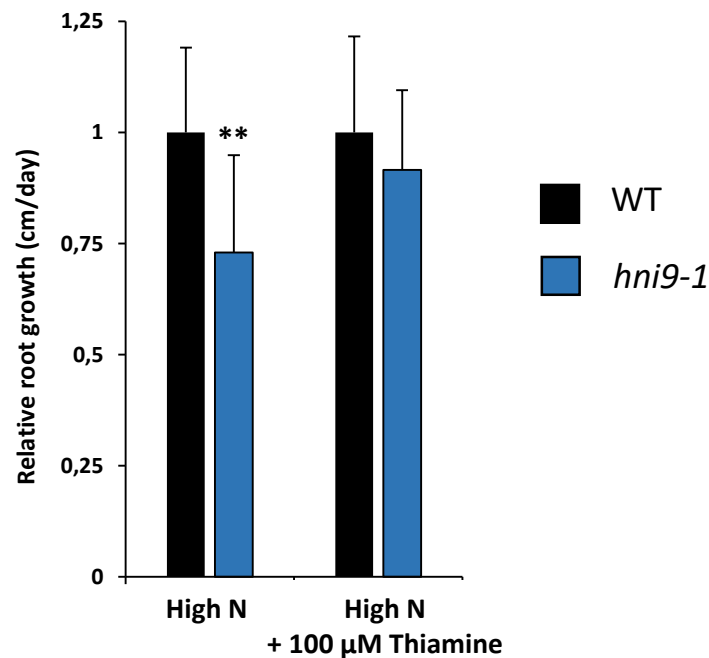
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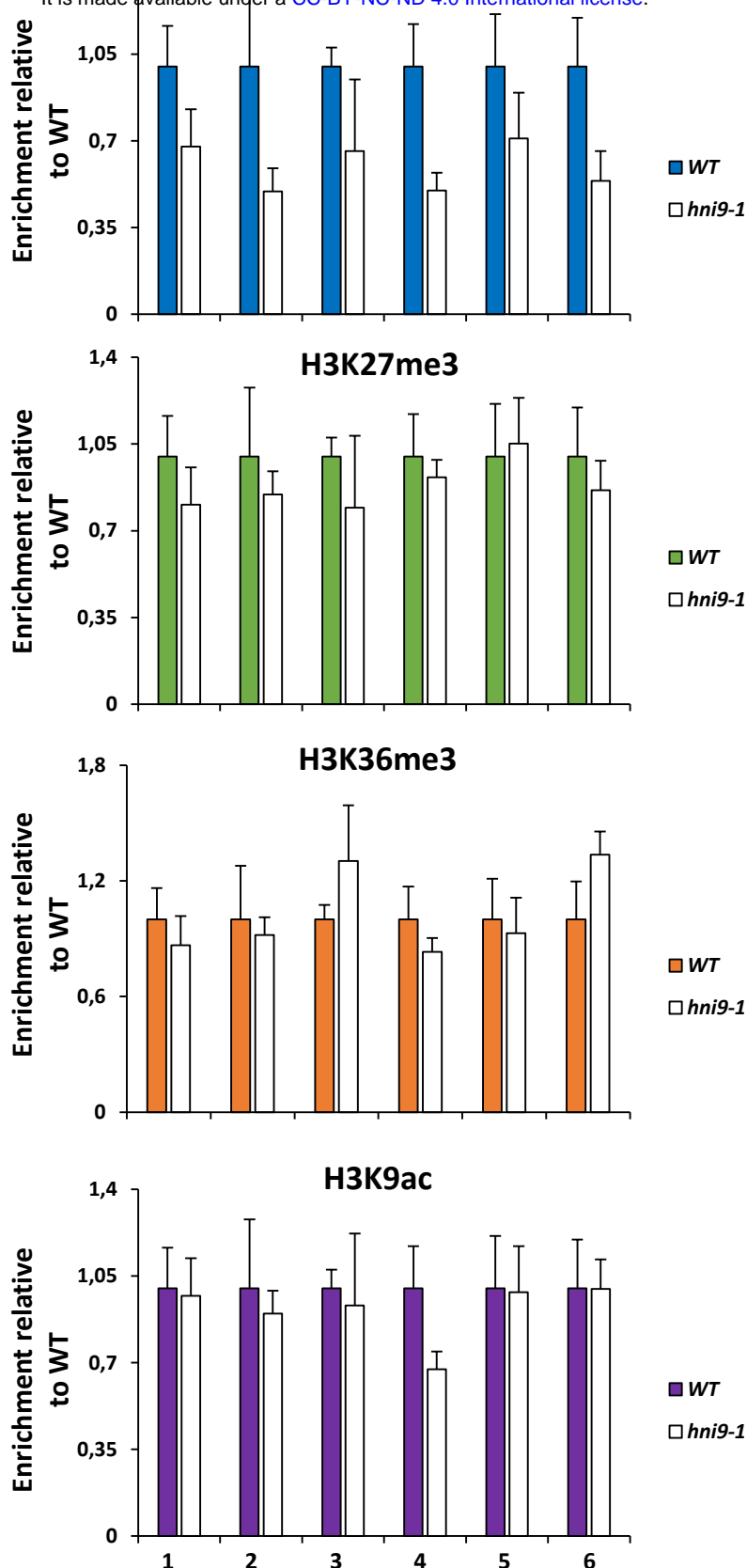
1. *THALIANOL HYDROXYLASE (AT5G48000)*
2. *2OG/Fe(II)-DPT OXYGENASE (AT1G52820)*
3. *CYTOCHROME P450 (AT2G42250)*
4. *2OG/Fe(II)-DPT OXYGENASE (AT2G36690)*
5. *IRON SULFUR CLUSTER (AT2G29630)*
6. *PEROXIDASE (AT2G37130)*

Figure 6 : Mutation in HNI9 is associated with reduction of H3K4me3 at the loci of detoxification genes. ChIP analysis of H3K4me3, H3K27me3, H3K36me3 and H3K9ac in *WT* and *hni9-1* roots of 7 days-old plants grown under high N provision (10 mM NH_4NO_3). Quantification by qRT-PCR is shown as the percentage of H3K4me3, H3K36me3, H3K27me3 or H3K9ac over H3 at target loci, normalized by the percentage of H3K4me3, H3K36me3, H3K27me3 or H3K9ac over H3 at positive controls (*ACT2* for H3K4me3, H3K36me3, and H3K9ac, *LEC2* for H3K27me3). Data are presented relative to the WT level. Error bars represent standard errors of the mean based on at least 3 biological replicates.
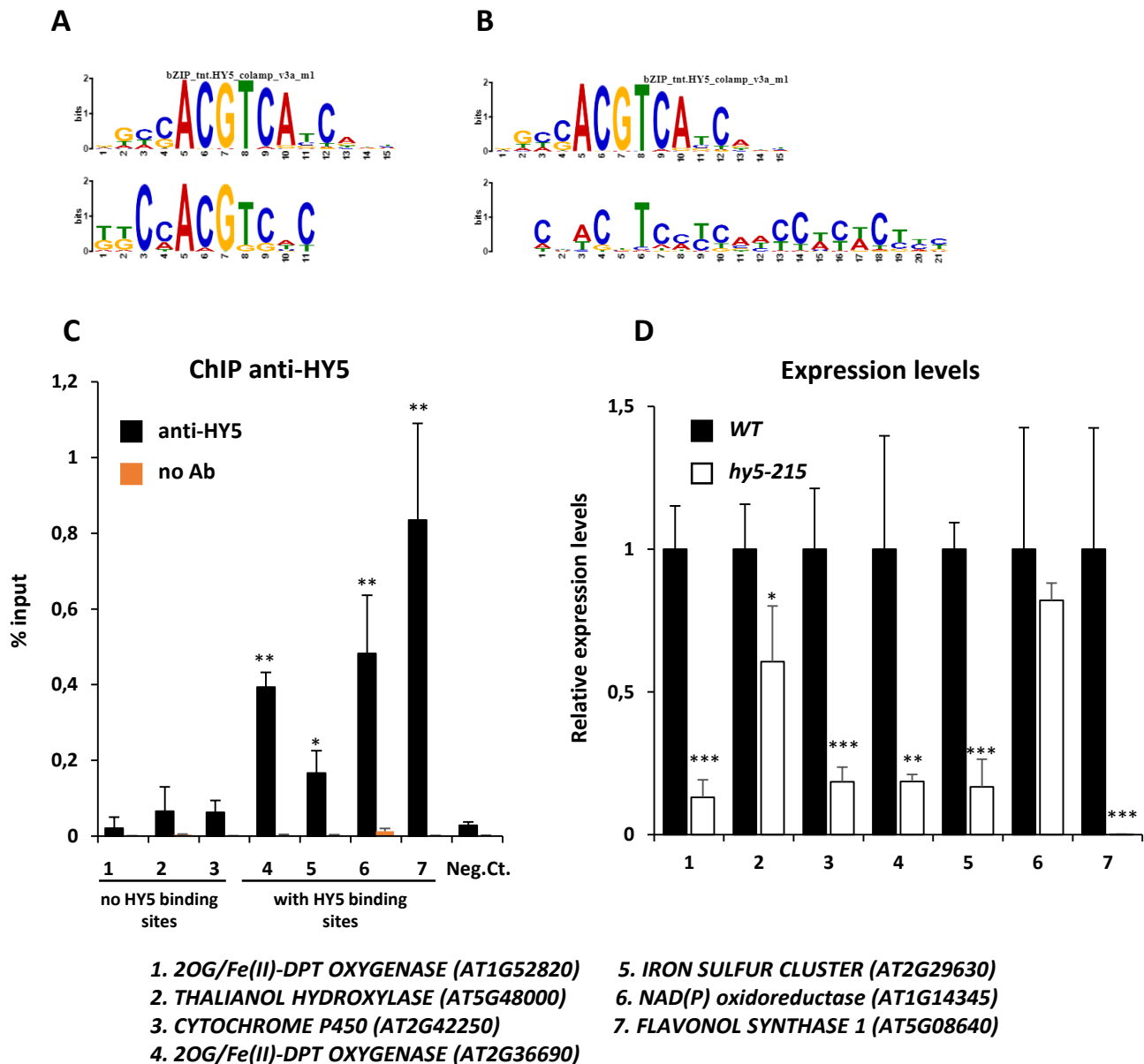
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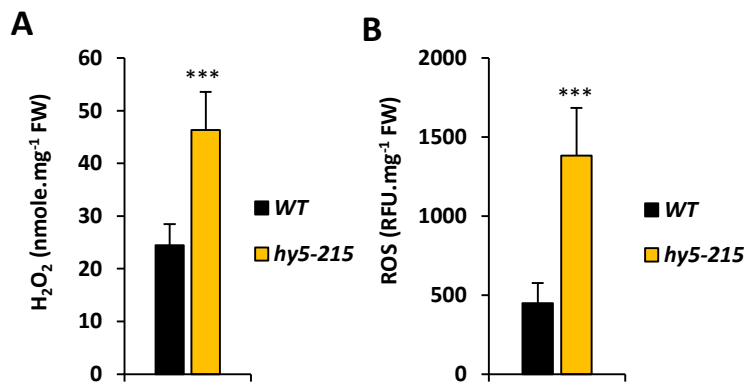
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