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Enhancement of Thiamin Content in *Arabidopsis thaliana* by Metabolic Engineering

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Thiamin is an essential nutrient in the human diet. Severe thiamin deficiency leads to beriberi, a lethal disease which is common in developing countries. Thiamin biofortification of staple food crops is a possible strategy to alleviate thiamin deficiency-related diseases. In plants, thiamin plays a role in the response to abiotic and biotic stresses, and data from the literature suggest that boosting thiamin content could increase resistance to stresses. Here, we tested an engineering strategy to increase thiamin content in Arabidopsis. Thiamin is composed of a thiazole ring linked to a pyrimidine ring by a methylene bridge. THI1 and THIC are the first committed steps in the synthesis of the thiazole and pyrimidine moieties, respectively. Arabidopsis plants were transformed with a vector containing the THI1-coding sequence under the control of a constitutive promoter. Total thiamin leaf content in THI1 plants was up approximately 2-fold compared with the wild type. THI1-overexpressing lines were then crossed with pre-existing THIC-overexpressing lines. Resulting THI1 × THIC plants accumulated up to 3.4- and 2.6-fold more total thiamin than wild-type plants in leaf and seeds, respectively. After inoculation with *Pseudomonas syringae*, THI1 × THIC plants had lower populations than the wild-type control. However, THI1 × THIC plants subjected to various abiotic stresses did not show any visible or biochemical changes compared with the wild type. We discuss the impact of engineering thiamin biosynthesis on the nutritional value of plants and their resistance to biotic and abiotic stresses.

Keywords: Arabidopsis • Engineering • Plants • Thiamin • Vitamin B_1.

Abbreviations: AIR, 5-aminoimidazole ribonucleotide; APX, ascorbate peroxidase; CaMV, *Cauliflower mosaic virus*; CAT, catalase; CFU, colony-forming unit; GR, glutathione reductase; HET-P, 4-methyl-5-β-hydroxyethylthiazole phosphate; HMP-P, 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate; HMP-PP, 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate; MS, Murashige and Skoog; NBT, nitro blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TDPK, thiamin diphosphokinase; TH1, HMP-P kinase/ThMP pyrophosphorylase; ThDP, thiamin diphosphate; ThMP, thiamin monophosphate; THI1, HET-P synthase; THIC, HMP-P synthase.

Introduction

Thiamin diphosphate (ThDP), also known as vitamin B_1, is an essential nutrient in the human diet, and is often referred to as the 'energy vitamin'. ThDP serves as an enzymatic cofactor for several thiamin-dependent enzymes involved in glucose metabolism, the Krebs cycle and branched-chain amino acid biosynthesis (Goyer 2010). Thiamin helps to promote healthy nerves, improves mood, strengthens the heart and decreases heartburn (Fardet 2010), and also is an antioxidant (Lukienko et al. 2000, Huang et al. 2010). Unlike plants and microorganisms, humans are not able to synthesize thiamin de novo and must obtain it from the diet, mostly from plant sources. Severe thiamin deficiency leads to a lethal disease known as beriberi, and is classically associated with diets that are low in thiamin and rich in carbohydrates (Lonsdale 2006). Unfortunately, some of the most consumed crop-based foods such as rice, wheat and maize are poor sources of thiamin (Fitzpatrick et al. 2012). Consequently, thiamin deficiency is prevalent among populations whose diet is largely based on these low thiamin/high carbohydrate foods (Rindi 1996, WHO 1999, Lonsdale 2006), especially in south-east Asia. In several industrialized countries, thiamin fortification has been implemented for many years in low-cost staple food products such as flour and bread (Backstrand 2002), leading to the eradication of severe thiamin deficiency. However, marginal deficiency remains a real health concern (deCarvalho et al. 1996, Harper 2006, Lonsdale 2006). In addition, food fortification programs require sustained investments year after year which can be difficult in developing countries (Bouis 2002). Meanwhile, biofortification of staple foods via genetic engineering or plant breeding is a sustainable strategy to reduce micronutrient malnutrition. Recent studies also show the benefits of higher thiamin intake on various health issues. A cross-sectional study of 2,900 Australian men and women, 49 years of age and older, found that those in the highest quintile of thiamin intake were 40% less likely to have nuclear cataracts than those in the lowest quintile (Cumming et al. 2000). In addition, a recent study of 408 US women found that higher dietary intakes of thiamin were inversely associated with 5-year change in lens opacification (Jacques et al. 2005). High dose thiamin therapy also may help reverse microalbuminuria in patients with type 2 diabetes (Rabbani et al. 2009).

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Thiamin also plays a role in plants in the response to abiotic and biotic stresses. Pools of thiamin and its phosphate esters increased in plants subjected to abiotic stresses such as osmotic, salt and oxidative stress, and exposure to cold, heat and high light conditions (Rapala-Kozik et al. 2008, Tunc-Ozdemir et al. 2009). Moreover, exogenous application of thiamin to plants and the subsequent increase in intracellular thiamin content confer some degree of resistance to salt and oxidative stress (Sayed and Gadallah 2002, Tunc-Ozdemir et al. 2009, Kaya et al. 2015). Similarly, application of thiamin, as well as its phosphate esters thiamin monophosphate (ThMP) and ThDP, triggers defense responses in plants (Ahn et al. 2005, Ahn et al. 2007, Bahuguna et al. 2012, Boubakri et al. 2012, Zhou et al. 2013) and systemic acquired resistance through elicitation competency (Graham and Graham 1994) or priming (Conrath et al. 2002), and protects plants against infection by diverse pathogens. In addition, two thiamin biosynthesis genes were shown to be essential in the response to pathogens. First, the rice gene *OsDR8* (Os070529600) which encodes THI1, a protein involved in the synthesis of the thiazole moiety of thiamin (Belanger et al. 1995) (**Fig. 1**), co-localizes with a quantitative trait locus for blast resistance that explains 24% of the phenotypic variance of resistance (Wang et al. 1994, Wen et al. 2003) and *OsDR8* transcript levels increase after pathogen inoculation (Wen et al. 2003). Transgenic rice plants that repress expression of *OsDR8* had reduced resistance or susceptibility to bacterial leaf blight and rice blast pathogens (Wang et al. 2006). The transgenic plants had significantly lower levels of thiamin than the control plants (1.4- to 2.5-fold lower) and their compromised defense responses could be reversed by the exogenous application of thiamin. Secondly, a gene encoding thiamin diphosphokinase (TDPK) which pyrophosphorylates thiamin to ThDP (**Fig. 1**) is a positive regulator of XA21-mediated immunity (Lee et al. 2011). The *XA21* gene confers broad-spectrum, robust resistance to *Xanthomonas oryzae* (Nishino et al. 1973, Bechtold et al. 1993, Mollier et al. 1995, Song et al. 1995). Altogether, these results suggest that engineering thiamin metabolism may enhance resistance to abiotic and biotic stresses.

In plants, thiamin is synthesized from condensation of a pyrimidine moiety, 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP), and a thiazole moiety, 4-methyl-5-β-hydroxyethylthiazole phosphate (HET-P), in chloroplasts (**Fig. 1**) to form ThMP (Goyer 2010). ThMP is then dephosphorylated to thiamin by a so far uncharacterized enzyme whose subcellular location also remains unknown, although broad specificity phosphatases have been suggested (Rapala-Kozik et al. 2009). Thiamin is then pyrophosphorylated to ThDP in the cytosol by TDPKs (Ajjawi et al. 2007). The first enzymes in pyrimidine and thiazole syntheses are HMP-P synthase (THIC) and HET-P synthase (THI1), respectively, and, as such, are obvious targets for thiamin engineering. The THIC gene contains a ThDP riboswitch located in its 3′-untranslated region (UTR) that negatively regulates THIC gene expression and protein production when the ThDP level increases (Bocobza et al. 2007, Wachter et al. 2007). This mechanism seems crucial to prevent thiamin accumulation. However, Arabidopsis plants carrying a deficient riboswitch had a modest increase of total thiamin levels in leaves and seeds (<1.6- and 1.2-fold increase, respectively) (Bocobza et al. 2013). Likewise, plants that constitutively overexpressed THIC under the control of the UBIQUITIN1 or *Cauliflower mosaic virus* (CaMV) 35S promoters had a <1.5-fold increase in total leaf thiamin content (Kong et al. 2008, Bocobza et al. 2013). A possible reason for limited thiamin accumulation may be an insufficient supply of HET-P to form a complete molecule of thiamin. Regulation of THI1 gene expression seems to exert some control on thiamin pools as well. Indeed, the increase of thiamin levels in plants subjected to abiotic stress correlates with the accumulation of THI1 mRNA transcripts (Tunc-Ozdemir et al. 2009), and the THI1 promoter was shown to be responsive to stress conditions as shown by THI1 promoter–GUS (β-

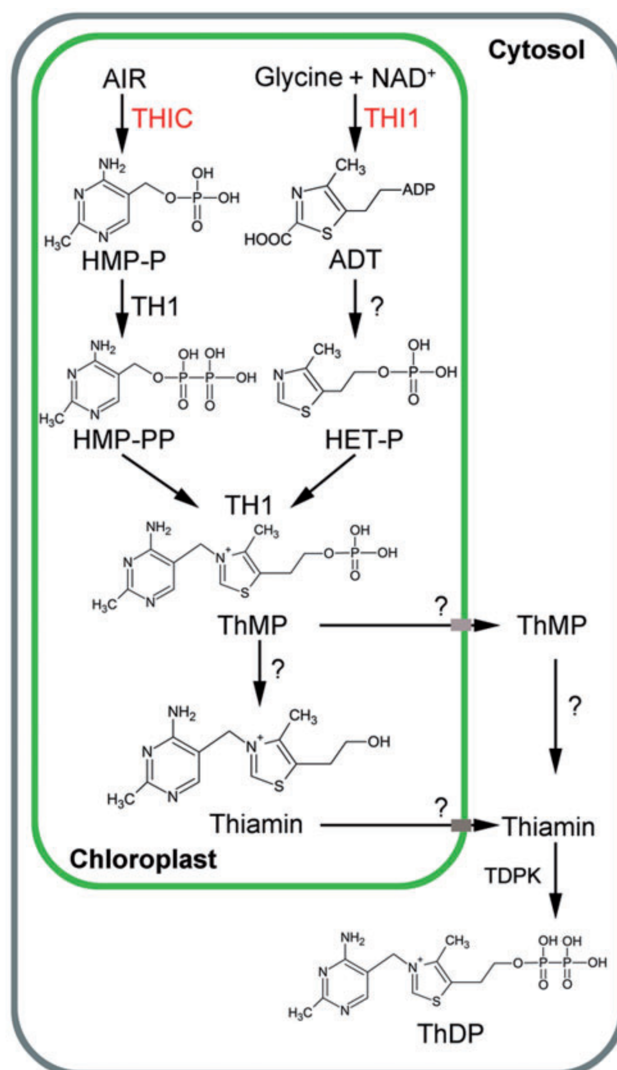

Fig. 1 Structure and biosynthesis of thiamin in plants. Engineered enzymes are in red. AIR, 5-aminoimidazole ribonucleotide; HMP-P, 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate; HMP-PP, 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate; HET-P, 4-methyl-5-β-hydroxyethylthiazole phosphate; ThMP, thiamin monophosphate; ThDP, thiamin diphosphate; THI1, HET-P synthase, THIC; HMP-P synthase; TDPK, thiamin diphosphokinase; TH1, HMP-P kinase/ThMP pyrophosphorylase.

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glucuronidase) fusion experiments (Ribeiro et al. 2005). Inversely, rice plants that repress expression of *OsDR8*, a THI1 homolog in rice, had significantly lower levels of thiamin than the control plants (Wang et al. 2006). In addition, THI4, the homolog of THI1 in yeast, is a suicide enzyme that catalyzes only a single turnover (Chatterjee et al. 2011). These data prompted us to investigate the effect of engineering thiazole synthesis alone or thiazole and pyrimidine syntheses together on thiamin pools. In this study, we report on the enhancement of thiamin content in Arabidopsis leaves and seeds by overexpressing both THI1 and THIC, and show that the increased thiamin pools have important consequences on the seed nutritional value and plant resistance to pathogens, but not on the response to abiotic stresses.

Results

Overexpression of HET-P synthase (THI1) increases thiamin levels

The HET-P synthase (THI1) coding sequence was cloned in an *Agrobacterium* binary vector under the control of the constitutive CaMV 35S promoter and introduced into Arabidopsis plants. Thirty-four independent T_1 plants that were positive upon selection on kanamycin were transferred to soil and allowed to self-pollinate. T_2 seeds were sown on selective medium containing kanamycin to determine the number of insertion events. Twenty-four lines showed a 3 : 1 segregation ratio that is indicative of a single insertion event and were used for further analysis. Resistant T_2 plants were allowed to self-pollinate, and T_3 seeds were sown on selective medium as above to identify homozygous lines (i.e. 100% seeds that are resistant) that were further used for thiamin screening. Total leaf thiamin concentrations increased up to 2-fold in THI1 transgenic plants, from 1.5 nmol g^{-1} FW in the wild-type control to 3 nmol g^{-1} FW in some THI1 lines (**Fig. 2A**), as determined by microbiological assay. In accordance with thiamin increase, thiamin-accumulating THI1 lines showed an increase in THI1 transcript levels as shown by real-time quantitative reverse transcription–PCR (RT–PCR) (**Fig. 2B**) and THI1 protein levels as determined by Western blot (**Fig. 2C**).

Crosses between THI1 and THIC transgenic lines boost thiamin levels

Two T_3 THI1 lines (8-6 and 19-1) were crossed with two THIC-overexpressing lines (THIC-1 and THIC-2) that contain the THIC coding sequence under the control of the ubiquitin promoter as described by Bocobza et al. (2013). F_1 seeds obtained from THI1 × THIC crosses were sown on soil and treated with BASTA to identify hybrid plants that contain the THIC construct, and were allowed to self-pollinate. F_2 plants which contain the THIC and THI1 constructs were identified by BASTA selection or PCR genotyping, respectively, and were used for thiamin determination by HPLC. Total thiamin content increased up to 3.4-fold in leaves of THI1 × THIC hybrid lines (i.e. line #7-5) compared with the wild type (**Fig. 3A**, **C**). Although both thiamin and ThDP levels increased (ThMP could not be detected), thiamin levels increased more drastically than ThDP levels (611% and 243%, respectively), leading to a decrease in the ratio ThDP/thiamin from approximately 3 in wild-type Arabidopsis leaves to approximately 1.1 in THI1 × THIC hybrid lines. F_2 plants homozygous for both transgenes were selected and allowed to self-pollinate. F_3 seeds from homozygous THI1 × THIC F_2 plants were used for thiamin determination. Total thiamin accumulated up to 31.2 nmol g^{-1}, under the thiamin form (**Fig. 3B**, **D**), a 2.6-fold increase compared with the Columbia control. Although ThDP could be detected in some samples (**Fig. 3D**), levels were below the limit of quantification.

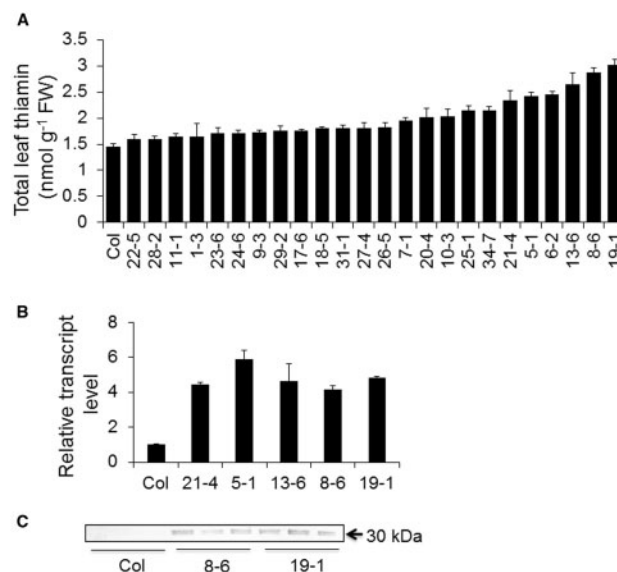

Fig. 2 THI1 overexpression increases thiamin content in Arabidopsis leaves. (A) Total leaf thiamin content in 24 T_3 THI1 transformants. Data are means ± SEM of at least three independent biological replicates. (B) Real-time quantitative RT–PCR analysis of THI1 gene expression in T_3 leaves of selected THI1 transformants. Data are means ± SEM of at least three independent biological replicates. (C) Western blot analysis of THI1 protein expression in T_3 leaves of two THI1 lines, 8-6 and 19-1.

Thiamin does not accumulate in chloroplasts of THI1 × THIC plants

That thiamin was the predominant form to accumulate in THI1 × THIC lines suggested that ThMP, which is synthesized in chloroplasts (**Fig. 1**), is freely converted to thiamin and that a bottleneck exists in the conversion of thiamin to ThDP. One can hypothesize that two steps in the thiamin biosynthesis pathway may be limiting: transport of thiamin out of chloroplasts for conversion of thiamin to ThDP by TDPK, assuming that ThMP to thiamin conversion occurs in the chloroplast, or thiamin to ThDP conversion by TDPK. To test these hypotheses, thiamin profiles were determined in chloroplasts isolated from THI1 × THIC line #7-5 and wild-type control. If transport of thiamin out of the chloroplasts became limiting in thiamine-accumulating plants, then we would observe an accumulation of thiamin inside the chloroplasts. Interestingly, thiamin content in chloroplasts of line #7-5 increased only 2.3-fold compared with the wild type (**Fig. 4**).

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Fig. 3 HPLC analysis of thiamin, ThMP and ThDP in leaves (A) and seeds (B) of homozygous THI1 × THIC plants along with parent lines and wild-type controls. Representative HPLC chromatograms in leaf (C) and seeds (D) are shown (dotted line, wild-type control; solid line; THI1 × THIC #7-5 plant). Retention times for thiamin, ThMP and ThDP were 3.6, 11.2 and 13.8 min, respectively. Lines #8-6 and #19-1 are THI1 parent lines; THIC-1 and THIC-2 are THIC parent lines; lines #7-3 and #7-5 are F_3 homozygous plants from crosses between THIC-1 and #19-1; lines #8, #10-3, #10-5, #12-5, and 13 are F_3 homozygous plants from crosses between THIC-2 and #19-1.

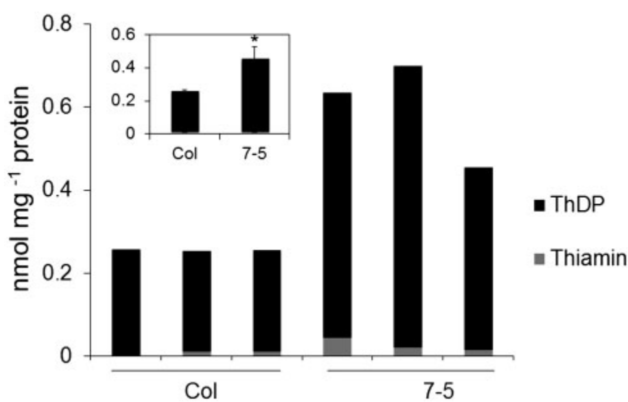

Fig. 4 Thiamin profiles of purified wild-type and line #7-5 leaf chloroplasts. Data are means ± SEM from nine determinations. The inset graph shows means ± SEM of all determinations per genotype. The asterisk indicates a significant difference ($P < 0.05$).

ThDP content increased 1.9-fold and ThMP was below the level of detection. These results show that thiamin accumulates to a much lower extent in chloroplasts than in whole leaf tissue (6.1-fold) and suggest that thiamin (or ThMP) is quickly transported out of the chloroplast. Altogether, these results indicate that TDPK, more than transport out of the chloroplast, may be the main limiting step in the conversion of thiamin to ThDP.

Thiamin-accumulating plants have lower populations of *Pseudomonas syringae*

THI1 × THIC plants were tested for resistance to *Pseudomonas syringae* in two independent experiments (**Fig. 5**). None of the leaves inoculated with the non-pathogenic HrcC mutant exhibited symptoms of tissue collapse in either experiment. Two days after inoculation, leaves infiltrated with the pathogenic strain DC3000 showed initial symptoms of chlorosis. By 4 d after inoculation, tissue collapse was noted on all pathogen-inoculated genotypes. The mean population sizes of DC3000 in leaves of wild-type Columbia ranged from 9.3×10^7 to 1.1×10^8

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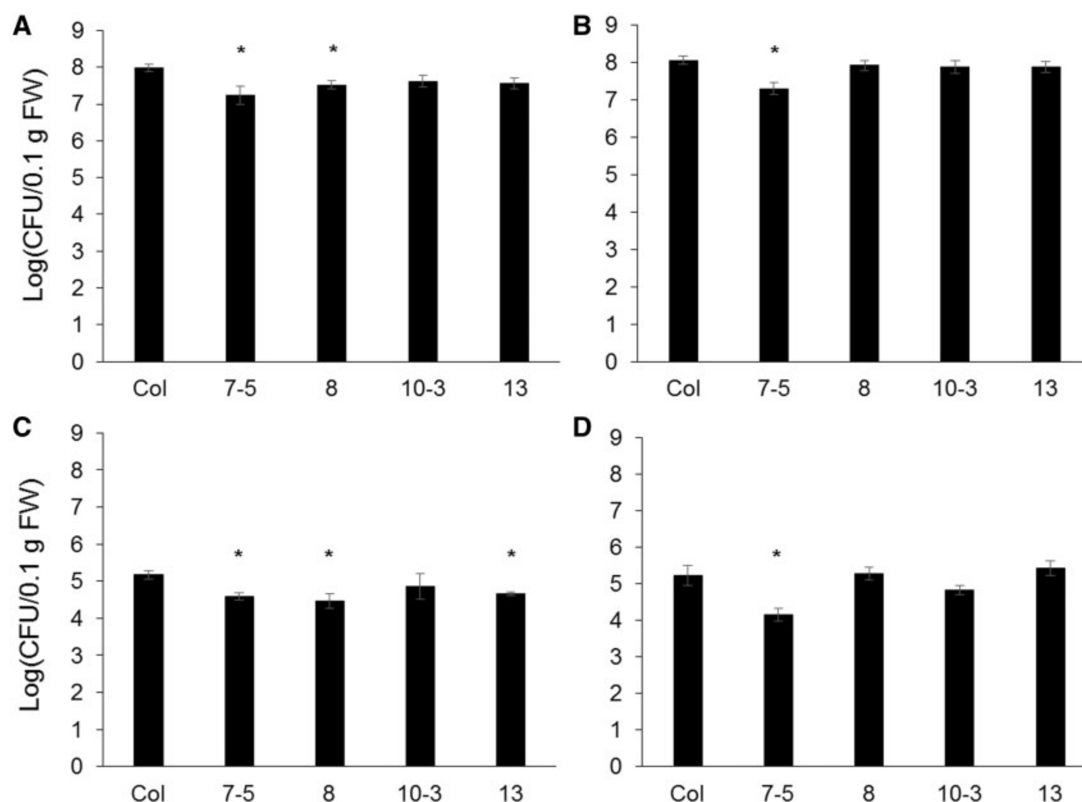

Fig. 5 Mean populations (CFU per 0.1 g FW) of pathogenic *P. syringae* pv tomato strain DC3000 (A, B) and non-pathogenic derivative strain HrcC (C, D) 4 d after inoculation in leaves of Columbia wild type and THI1 × THIC lines in repeated experiments. Data are means ± SEM from five replicate samples. An asterisk above a bar indicates that bacterial population sizes in leaves from a THI1 × THIC line differed significantly from populations in Columbia leaves determined with a two-tailed *t*-test at $P < 0.05$.

colony-forming units (CFU) per 0.1 g FW (Fig. 5A, B). In repeated experiments, the population sizes of DC3000 were significantly lower ($P = 0.024$ and $P = 0.005$) in leaves of line #7-5 compared with populations established in the wild-type Columbia. Population sizes of DC3000 were significantly lower ($P = 0.015$) in leaves of line #8 compared with Columbia in one experiment (Fig. 5A), but not in the second experiment (Fig. 5B). Population sizes of DC3000 established in leaves of lines #10-3 and #13 were not significantly lower ($P < 0.05$) compared with populations in Columbia in either experiment (Fig. 5A, B). The population sizes of the non-pathogenic HrcC mutant of DC3000 ranged from 1.5×10^5 to 1.6×10^5 CFU per 0.1 g FW in leaves of Columbia (Fig. 5C, D). The population sizes of the HrcC mutant were significantly lower ($P = 0.004$ and $P = 0.013$) in leaves of line #7-5 compared with populations established in Columbia in repeated experiments. In one experiment, populations of the HrcC mutant were significantly lower in leaves of lines #8 and #13 ($P = 0.015$ and $P = 0.002$) compared with Columbia (Fig. 5C), but they were not significantly lower when the experiment was repeated (Fig. 5D). Population sizes of the HrcC mutant established in leaves of line #10-3 were not significantly lower ($P < 0.05$) compared with populations in Columbia in either experiment (Fig. 5C, D). In summary, population sizes of DC3000 and the HrcC mutant were significantly lower in line #7-5 compared with Columbia in both experiments, were significantly lower in line #8 than those in Columbia in one of the two experiments, and were not significantly lower in line #10-3 than those of Columbia in either experiment. Population sizes of the HrcC mutant were significantly lower in line #13 in one of the two experiments. Interestingly, total thiamin accumulation was the highest in line #7-5 (Fig. 3A) (note that leaf thiamin content in line #13 was analyzed in an independent experiment and was found not to be significantly different from that of the Columbia control). These results show that accumulation of thiamin in the plant decreases the development of *P. syringae* populations.

THI1 × THIC overexpressors showed no increase in resistance to abiotic stresses

We investigated resistance of THI1 × THIC line #7-5, which accumulated the most total thiamin (~3.4-fold), and line #10-3 which accumulated total thiamin approximately 1.4-fold compared with the control, to oxidative stresses. Wild-type Columbia was used as control. Root growth of transgenic plants grown in vitro on medium containing salt, sorbitol or paraquat, or grown at low temperature was compared with that of the wild type control (Fig. 6A–D). Seedlings of transgenic plants did not show significant differences in relative root length and root elongation rate from control plants subjected to either treatment. Transgenic plants grown in soil containing salts under long-day conditions (14 h light) appeared similar to

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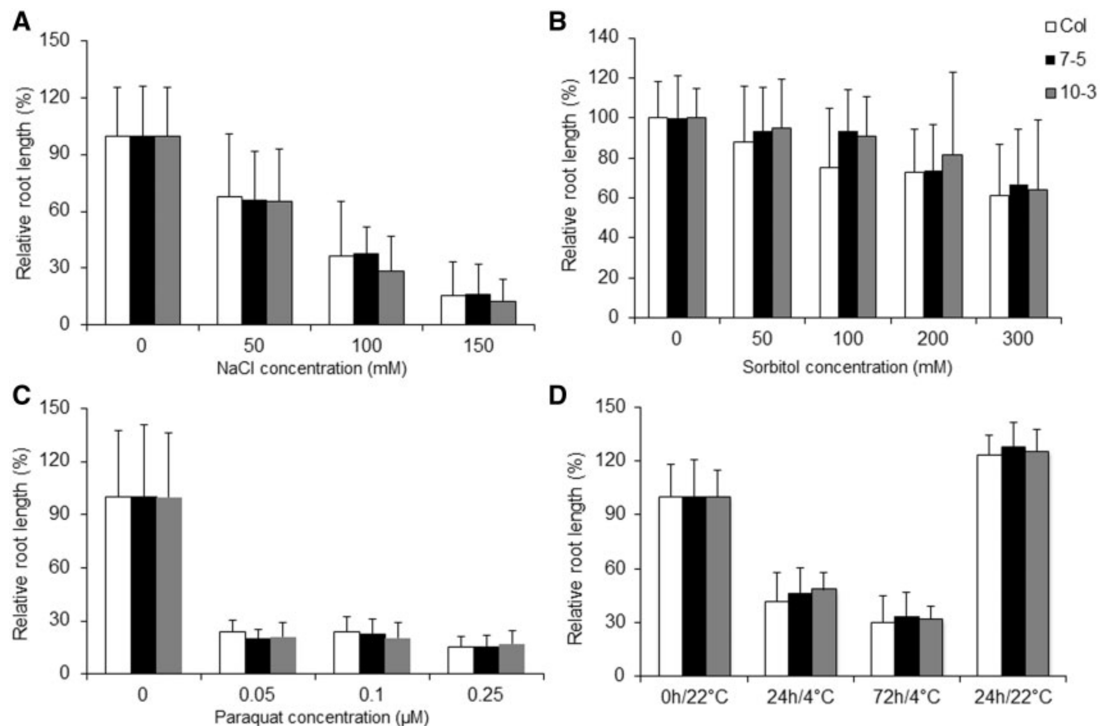

Fig. 6 Root growth of THI1 × THIC seedlings grown on medium supplemented with NaCl, sorbitol or paraquat, or subjected to cold treatment. Wild-type Columbia and THI1 × THIC (lines #7-5 and #10-3) seedlings were grown on MS medium containing the indicated concentrations of NaCl (A), sorbitol (B) and paraquat (C), or were subject to cold treatment at 4°C for 24 and 72 h, with 24 h recovery (D).

the wild-type control (**Supplementary Fig. S1**). Transgenic plants grown in soil from which watering was withdrawn for 22 d under long-day conditions (14 h light) had a slight chlorotic phenotype compared with the wild-type Columbia (**Supplementary Fig. S2**), similar to the chlorotic phenotype observed in plants grown under short-day conditions (10 h light) (data not shown) and as previously reported for THIC-1 plants (Bocobza et al. 2013). However, total leaf biomass of line #7-5 was the same as that of the control. Seeds of line #7-5 did not have any visible phenotype compared with wild-type Columbia seeds (**Supplementary Fig. S3**).

Next, we measured reactive oxygen species (ROS) content, total antioxidant capacity (TAC), which represents both enzymatic and non-enzymatic antioxidants, and specific enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) in line #7-5 and the wild-type control under normal growth conditions or after 7 d of salt treatment (**Table 1**). ROS content in the wild-type Columbia was identical under both stress and no-stress conditions, while ROS content in line #7-5 was significantly higher under no-stress conditions than under stress conditions. However, there were no significant differences between line #7-5 and the wild-type Columbia under either condition. TAC was higher under stress conditions for both genotypes, but there were no significant differences between line #7-5 and the wild-type Columbia under either condition. SOD, CAT and GR activities were not significantly different between genotypes and growth conditions. APX activities were lower under stress conditions for both genotypes, but there were no significant differences between line #7-5 and the wild-type Columbia under either condition. Altogether, these data are in agreement with the lack of any particular visible phenotype in line #7-5 grown under stress conditions.

Finally, we compared thiamin profiles in line #7-5 and the wild-type Columbia between no-stress and salt stress conditions (**Fig. 7**). Although there were no significant changes in thiamin and ThDP content in the wild-type Columbia subjected to salt stress, line #7-5 accumulated 1.7- and 1.3-fold more thiamin ($P < 0.05$) and ThDP ($P > 0.05$), respectively, under salt stress conditions than under no-stress conditions.

Discussion

Our results show that a 'pushing' strategy based on overexpression of both THI1 and THIC genes led to a significant increase in total thiamin pools in leaves and seeds. To our knowledge, this is the first report of such thiamin increases, in particular in plant seeds. It has been estimated that thiamin content should be increased 3.9-, 5.7- and 3.0-fold in wheat, rice and corn, respectively, to reach the recommended daily allowance if any one of these crops represents 80% of the daily intake of calories (Fitzpatrick et al. 2012). The increase of thiamin content obtained in our study (3.4- and 2.6-fold in leaves and seeds, respectively) shows that engineering THI1 and THIC expression is a first step towards those goals, and such increases in food crops may be sufficient to supply the daily needs of populations that suffer marginal thiamin deficiency.

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Plants overexpressing both THI1 and THIC accumulated significant amounts of thiamin, while plants overexpressing THI1 or THIC singly did not (**Fig. 3A**, **B**), which supports our initial hypothesis that a limitation to increased thiamin accumulation in THI1 lines is an insufficient supply of the pyrimidine moiety. Likewise, a limitation to additional thiamin accumulation in THIC lines is an insufficient supply of the thiazole moiety. The extent of accumulation of these precursors in THI1 and THIC plants could not be determined because of the current lack of methods to quantify thiazole and pyrimidine precursors in plant tissues. Nevertheless, in our initial thiamin screening of THI1 lines with a microbiological assay, growth readings were higher in some THI1 lines such as 8-6 and 19-1 than in the wild-type control (**Fig. 2A**), even though HPLC analysis showed that 8-6 and 19-1 plants did not accumulate thiamin (**Fig. 3A**). This discrepancy between the two assays may be due to interference of thiamin precursors with bacterial growth and suggests that the thiazole precursor did accumulate in THI1 lines. Although some studies reported that *Lactobacillus viridescens* (Deibel et al. 1957, Hankin and Squires 1959) could not utilize thiamin precursors, unlike *Lactobacillus fermentum* (Sarett and Cheldelin 1944), thiamin concentrations used in our assay were 10–100 times lower than those used in these studies, and this may have promoted the use of the thiazole moiety for growth.

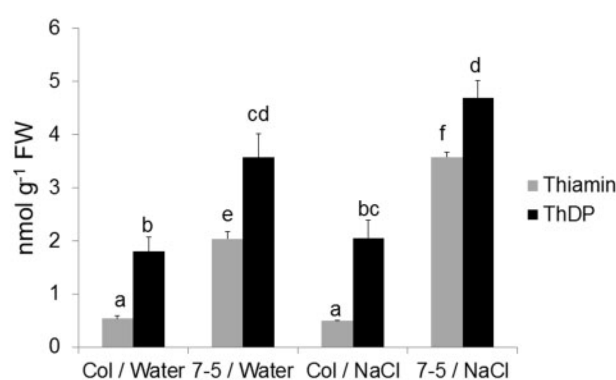

Fig. 7 HPLC analysis of thiamin and ThDP in leaves of line #7-5 and wild-type Columbia subjected to salt stress. Three-week-old wild-type Columbia (Col) and line #7-5 plants were irrigated with either water or 150 mM NaCl for 7 d under 14 h daylight. Rosette leaves were harvested and used for determination of thiamin, ThMP and ThDP. Note that ThMP was not detected. Data are means ± SE of three independent determinations. Identical letters indicate that there was no significant difference ($P > 0.05$) between samples as determined by ANOVA and Tukey test.

Despite the significant accumulation of thiamin obtained by engineering THI1 and THIC, future research should focus on identifying bottlenecks for further thiamin accumulation. There are several reasons which can explain why thiamin pools did not accumulate beyond those observed in this study. First, the precursors of thiamin's thiazole and pyrimidine moieties, namely NAD, 5-aminoimidazole ribonucleotide (AIR) and glycine (**Fig. 1**), could become limiting. In particular, it was reported that NAD concentrations in leaves of 3- to 4-week-old Arabidopsis plants were approximately 9 nmol g^{-1} FW (Schippers et al. 2008), which is only twice the amount of total thiamin in leaves of Arabidopsis line #7-5. Assuming a distribution of NAD between subcellular compartments, NAD pools available for thiazole synthesis in chloroplasts may not be sufficient for further thiamin increase. This scenario seems less likely in the case of AIR and glycine. AIR is an intermediate of purine biosynthesis which leads to the synthesis of purine nucleotides (van der Graaff et al. 2004). Purine nucleotides and purine-nucleotide-derived cofactors are found in larger quantities than thiamin in the cells of living organisms. For instance, Hung et al. (2004) reported levels of ATP, ADP, GTP and GDP of 60, 30, 9 and 5.5 nmol g^{-1} FW in Arabidopsis leaves. Likewise, Goyer et al. (2004) found glycine concentrations of 140 nmol g^{-1} FW in Arabidopsis leaves. Secondly, the ThMP synthase activity of TH1 (**Fig. 1**) could become rate limiting in THIC-overexpressing lines. Supporting this, the ThMP synthase activity of recombinant HMPPK/ThMP-PPase from maize was shown to be inhibited by excess HMP-PP (Rapala-Kozik et al. 2007). Analytical methods for the quantification of thiazole and pyrimidine precursors will need to be developed to test whether these compounds accumulate in large amounts in engineered plants. Thirdly, our data show that free thiamin was the main thiamin form to accumulate in leaves (up to 6.1-fold). A similar increase in free thiamin (~8-fold) was reported in wild-type Arabidopsis seedlings supplemented with both HET and HMP, while total thiamin content increased only approximately 1.2-fold (Pourcel et al. 2013). These results suggest a limiting rate in the conversion of thiamin to ThDP. Thiamin profiling in chloroplasts shows that thiamin pools increased only 2.3-fold in this organelle, which suggests that an exporter

Table 1 Reactive oxygen species, total antioxidant capacity and activities of major antioxidative enzymes

	ROS (nmol g^{-1} FW)	TAC (mM UAE mg^{-1} protein)	SOD (U mg^{-1} protein)	CAT (μmol min^{-1} mg^{-1} protein)	GR (nmol min^{-1} mg^{-1} protein)	APX (nmol min^{-1} mg^{-1} protein)
Col/water	24.0 ± 0.6^{ab}	12.1 ± 0.1^{a}	28.4 ± 2.9^{a}	66.6 ± 1.7^{a}	42.8 ± 4.5^{a}	140.1 ± 8.5^{a}
#7-5/water	29.3 ± 2.9^{a}	12.0 ± 0.2^{a}	30.6 ± 0.8^{a}	53.9 ± 2.6^{a}	37.2 ± 2.8^{a}	127.4 ± 6.5^{a}
Col/NaCl	24.1 ± 0.9^{ab}	13.1 ± 0.2^{b}	28.2 ± 1.7^{a}	63.4 ± 2.0^{a}	53.2 ± 10.0^{a}	111.8 ± 13.5^{ab}
#7-5/NaCl	18.8 ± 2.0^{b}	13.8 ± 0.2^{b}	31.5 ± 1.7^{a}	57.9 ± 5.0^{a}	45.5 ± 2.8^{a}	84.9 ± 4.9^{b}

Three-week-old wild-type Columbia (Col) and cross #7-5 (#7-5) plants were irrigated with either water or 150 mM NaCl for 7 d under 14 h daylight. Rosette leaves were harvested and used for determination of reactive oxygen species (ROS), total antioxidant capacity (TAC) and enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX).
Data are means ± SE of at least three independent determinations.
Identical superscript letters indicate that there was no significant difference ($P > 0.05$) between samples as determined by ANOVA and Tukey test.

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of thiamin from the chloroplast to the cytosol is unlikely to be the main limiting step. This hypothesis is supported by thiamin feeding experiments in Arabidopsis seedlings that showed that, despite a large accumulation of thiamin, presumably in the cytosol, ThDP content only increased by 10%. One limiting factor for accumulation of ThDP, and possibly total thiamin content, could be the activity of the cytosolic TDPK, as Pourcel et al. (2013) suggested, in which case a 'pulling' strategy based on overexpression of TDPK could potentially further increase total thiamin production by releasing the clog. Another potential factor which limits ThDP accumulation may be the enhanced activity of ThDP phosphatase (Pourcel et al. 2013). ThDP phosphatase can dephosphorylate ThDP back to thiamin (Rapala-Kozik et al. 2009), and a balance between the activities of ThDP phosphatase and TDPK may regulate ThDP homeostasis. It must be noted that humans are able to convert free thiamin into the ThDP cofactor, and that the storage form of thiamin in seeds is free thiamin. Therefore, from a human nutrition perspective, conversion of thiamin to ThDP probably does not matter, and engineering of thiamin in cereal grains should focus on the overproduction of free thiamin.

Our results also show for the first time that plants accumulating higher levels of thiamin in vivo are able to maintain lower bacterial populations, in this case *P. syringae*, than the wild-type control. Although the effect was relatively mild (~10% decrease or 0.7–1 log unit) and *P. syringae* populations remained high enough to trigger necrosis, the fact that line #7-5, which accumulated the highest amounts of thiamin amongst the lines tested in this study, was the only line to show lower *P. syringae* populations consistently suggests that there is a minimum threshold of thiamin levels that must be reached to significantly suppress bacterial populations. This also suggests that higher thiamin levels could further decrease bacterial populations, but how much remains unclear. Millimolar concentrations of thiamin have been applied to observe an effect on disease progress in laboratory settings (Ahn et al. 2005, Bahuguna et al. 2012, Boubakri et al. 2012, Zhou et al. 2013). However, how much of the applied thiamin enters the cell to trigger host defense mechanisms remains unknown. It also remains unclear what the target of thiamin is and where it is located during priming of plants for pathogen defense, although it was recently shown that activation of NADPH oxidase may be implicated (Zhou et al. 2013).

Foliar application of thiamin or growth on thiamin-supplemented medium enhances stress tolerance and alleviates damage caused by abiotic stress (Sayed and Gadallah 2002, Rapala-Kozik et al. 2008, Tunc-Ozdemir et al. 2009, Kaya et al. 2015). Therefore, it was surprising that thiamin-accumulating plants of line #7-5 did not show increased resistance to abiotic stresses (salt, cold, osmotic and oxidative) and that their redox status, as determined by ROS content, TAC and enzymatic activities of SOD, CAT, GR and APX, was essentially the same as that of the wild-type Columbia under either no-stress or salt stress conditions. These results show that modulation of intracellular thiamin levels alone is not sufficient to trigger increased resistance of the plants to abiotic stress, and suggest that stress resistance conferred by external thiamin application depends on additional parameters other than the subsequent increase of cellular thiamin level. For instance, one can hypothesize that, before entering the cell, thiamin interacts with extracellular components to trigger the resistance mechanism. It is also unclear how much of the applied thiamin remains in its native form. It is possible that thiamin undergoes degradation or oxidation after application, and that the products of these reactions induce resistance. Further investigation will be needed to understand the resistance mechanism triggered by external application of thiamin.

It has also been shown that total thiamin content increases in Arabidopsis plants subjected to abiotic stress (up to 2.9-fold under high-light stress, and ~2-fold under salt stress), mostly under the diphosphate form, as a result of the up-regulation of the de novo thiamin biosynthesis genes THI1, THIC, TH1 and TDPK (Tunc-Ozdemir et al. 2009). Similar increases (up to ~2-fold) in total thiamin content were reported in maize under water, salt and oxidative stress, although in this case free thiamin was the predominant accumulating form (Rapala-Kozik et al. 2008). It has been suggested that increases in thiamin content are necessary to regenerate damaged metabolic pathways that involve the ThDP-dependent enzymes transketolase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and 2-deoxy-D-xylulose-5-phosphate synthase, and to provide antioxidants and stress protectants (Rapala-Kozik 2011). Both wild-type and line #7-5 plants recovered similarly after salt stress (**Supplementary Fig. S1**), suggesting that thiamin pools present in the wild type are already sufficient to regenerate any damaged pathways. Further accumulation of thiamin levels in line #7-5 under salt stress conditions indicate that even higher thiamin levels do not increase resistance to salt stress. Thiamin and its phosphate esters are prone to oxidative damage under stress conditions, and damaged forms of ThDP, such as oxo-ThDP, are toxic to the cell and must be quickly converted to non-toxic forms to prevent inhibition of ThDP-dependent enzymes (Goyer et al. 2013). It is therefore likely that both detoxification of the damaged cofactor and increased thiamin production are necessary to protect the plant from major impairment.

In conclusion, we successfully overexpressed the thiazole synthesis enzyme THI1 in Arabidopsis plants for increased content of thiamin. Thiamin accumulation was further increased in both leaves and seeds by crossing THI1 transgenics with THIC-overexpressing plants. To our knowledge, this is the first report to express two thiamin biosynthesis genes together in plants. Plants that accumulated the most thiamin showed lower populations of *P. syringae* after mechanical inoculation. Engineering of thiamin biosynthesis by overexpression of both THI1 and THIC should now be attempted in crops of agricultural importance for increased nutritional value and, with further improvement, disease resistance.

Materials and Methods

Chemicals and reagents

Thiamin, ThMP, ThDP, NADPH, oxidized glutathione (GSSG), nitro blue tetrazolium (NBT), ascorbic acid, riboflavin, methionine and hydrogen peroxide

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were from Sigma. Acetonitrile was from EMD Chemicals Inc. Restriction enzymes, Phusion DNA polymerase, Antarctic phosphatase and T4 DNA ligase were from New England Biolabs.

Plant expression vector

The coding sequence of the Arabidopsis HET-P synthase *THI1* gene (At5g54770) was obtained from the Arabidopsis Biological Resource Center (clone U14240) and was verified by sequencing. The coding sequence was amplified by PCR with Phusion DNA polymerase using the following primers: forward primer 5′-TTTTTTCTCGAGATGGCTGCCATAGCTTCTACT-3′ and reverse primer 5′-TTTTTTTCTAGATTAAGCATCTACGGTTTCAGCT-3′. The PCR product was digested with *Xho*I and *Xba*I, and ligated into *Xho*I/*Xba*I-digested pKannibal vector with T4 DNA ligase. The CaMV35S-*THI1*-OCS cassette was amplified by PCR using the following primers: forward primer 5′-ACTCACTATAGGGAGCTCGTC-3′, and reverse primer 5′-AGTGCCAAGCTGACTTGGTCA-3′. The PCR amplicon was digested with *Not*I and ligated into *Not*I-digested pMOA33 (Barrell and Conner 2006) dephosphorylated with Antarctic phosphatase. All constructs were verified by sequencing.

Plant growth, transgenic plants and crosses

Arabidopsis plants (ecotype Columbia-0, *THI1*, *THIC*, *THI1* × *THIC* transgenic lines) were grown on soil (Sunshine Mix # 1, Sun Gro Horticulture, Inc.) in a greenhouse. The *THI1* construct was used to transform Arabidopsis plants. The construct was introduced into the electrocompetent *Agrobacterium tumefaciens* ABI strain, and Arabidopsis plants were transformed using the floral dip method (Clough and Bent 1998). Transformants (T_1) were selected on media containing 30 mg l^{-1} kanamycin. Homozygous T_2 plants were identified by analyzing segregation of the progeny on selective medium containing kanamycin. Two *THIC* transgenic lines (*THIC-1* and *THIC-2*) (Bocobza et al. 2013) were obtained from Dr. Asaph Aharoni (Weizmann Institute of Science, Israel). *THIC* seeds were planted in soil, and plants carrying the *THIC* construct were selected with glufosinate ammonium (200 mg l^{-1}). The presence of the construct in *THIC* plants was confirmed by PCR using the following primers: forward primer 5′-TCAAATCTCAAGGCATAAGGTAG-3′, and reverse primer 5′-CGACGATCAATCCACTCCTT-3′. Two independent homozygous T_2 *THI1* lines (#8-6 and #19-1) were then crossed with the two *THIC* lines in a reciprocal cross design. Seedlings of hybrid progeny were screened for the presence of both constructs using the primers mentioned above.

Abiotic stress experiments

For in vitro stress experiments, seeds were sown on plates (150 × 150 mm) with medium containing 1 × Murashige and Skoog (MS) mineral salts, 0.8% agar, 1% sucrose, 1 × B5 vitamins supplemented with various concentrations of paraquat (0, 0.05, 0.1 and 0.25 µM), sodium chloride (0, 50, 100 and 150 mM) or sorbitol (0, 50, 100, 200 and 300 mM), and placed at 4°C for 48 h. Plates were then transferred to a growth chamber (21°C, 12 h daylight, 120 µmol m^{-2} s^{-1}) and maintained vertically. Root length was measured every 2 d for 10 d. For the in vitro cold stress experiment, 10-day-old seedlings were grown on 1 × MS agar plates, and placed at 4°C for 24 and 72 h. After recovery for 24 h at 21°C, root length was measured and compared with the control.

For salt stress experiments in soil, seedlings were either grown on medium as described above for 2 weeks before being transferred to soil (Sunshine Mix # 1, Sun Gro Horticulture Inc.) in a greenhouse or directly planted in soil. One week after transfer to soil or 3 weeks after planting, plants were irrigated with 0, 50, 100 and 150 mM NaCl once every other day for the time indicated. For drought stress experiments, plants were grown in a growth chamber for 18 d, then water irrigation was withheld for 22 d. After drought treatment, plants were watered and allowed to recover for 5 d. Leaf biomass was determined from five replicates of 10 plants each.

RNA isolation and expression analysis

Leaf samples (50–100 mg) were collected and immediately frozen in liquid nitrogen. Samples were ground in liquid nitrogen with a mortar and pestle, and total RNA was isolated by using an RNeasy Plant Mini Kit (Qiagen). RNAs (1 µg) were reverse-transcribed to cDNAs with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies). A 1 µl aliquot of cDNAs was used as template in 25 µl PCRs containing the SYBR Green QPCR Master Mix (Agilent Technologies) and 150 nM of forward and reverse primers. PCRs were performed on an Mx3005P instrument (Agilent Technologies). PCR conditions were: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 72°C for 60 s. A dissociation step (1 min at 95°C, ramping down to 55°C and up to 95°C) was added at the end of the amplification cycles to check for primer specificity. The housekeeping gene *Actin 2* (At3g18780) was used as control for quantitative PCR analysis. Primer sequences were as follows: actin forward primer 5′-CTTGCACCAAGCAGCATGAA-3′, actin reverse primer 5′-CCGATCCAGACACTGTACTTCCTT-3′, THI1 forward primer 5′-CGCTATTGTGAGGTTGACCAGA-3′, THI1 reverse primer 5′-CAAAAGTTGGTCCCATTCTCG-3′. Primer efficiency was determined using the protocol described in Schmittgen and Livak (2008). Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

Protein extraction from leaf tissue and Western blot analysis

Plant leaf tissues (100 mg) were ground into a fine powder with a mortar and pestle in liquid nitrogen. After addition of 500 µl of extraction buffer [50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 10% glycerol, 0.2% Triton X-100, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM dithiothreitol], samples were vortexed and chilled on ice for 10 min. After centrifugation at 13,000 r.p.m. for 15 min at 4°C, the supernatant was collected, frozen in liquid nitrogen and stored at −80°C until analysis. Concentrations of proteins were determined by using the Bradford Protein Assay kit (Bio-rad) with bovine serum albumin as a standard. Equal amounts of proteins (30 µg) were separated by SDS–PAGE (Life Technologies), and transferred to a nitrocellulose membrane (GenScript). Protein detection was carried out according to the instructions of the One-Hour Western Detection Kit (GenScript) using polyclonal antibodies raised against Arabidopsis THI1. THI1 antibodies were raised by immunization of rabbits with the synthesized peptide CEVAEIDGAPRMGPT as antigen (GenScript). Recombinant rice THI1 protein which contains the same peptide used to raise antibodies as its Arabidopsis homolog was used as positive control.

Thiamin analysis

Thiamin was analyzed by microbiological assay or HPLC. For microbiological assay, thiamin was extracted from 250 mg of leaf tissue and determined as described before (Goyer and Haynes 2011). For HPLC assay, thiamin and its phosphate esters were extracted as previously described (Bocobza et al. 2013) with the following modifications. Leaf tissue (50–100 mg) and seeds (20 mg) were ground in liquid nitrogen with a mortar and pestle. After addition of 4 ml of 0.1 N HCl, samples were sonicated for 30 min in a water bath, and a 1 ml aliquot was centrifuged for 15 min at 13,000 r.p.m. Supernatants (300 µl) were used for thiamin oxidation into thiochrome as described before (Bocobza et al. 2013). Thiochrome forms of thiamin, ThMP and ThDP were separated on a Capcell Pak NH_2 column (5 µm, 4.6 × 150 mm i.d.) (Shiseido) equipped with an NH_2 guard column (10 × 4.0 mm) (Shiseido) using a 4 : 6 (v/v) solution of 90 mM potassium phosphate buffer, pH 8.2, and acetonitrile as mobile phase. The analyses were performed using an UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a WPS-3000TSL autosampler, a TCC-3000 column compartment set at 25°C, an LPG-3400SD quaternary analytical pump and an FLD-3000 fluorescence detector. Chromatograms were integrated using the Chromeleon™ 7.1 chromatography data system. The flow rate was 0.5 ml min^{-1}, and the volume injected was 5–20 µl. Thiochrome derivatives were detected by fluorescence with excitation at 365 nm and emission at 435 nm. Detector response was calibrated by using thiochrome forms of thiamin, ThMP and ThDP standards.

Chloroplast isolation

Chloroplasts were isolated using the protocol described in Kley et al. (2010) with minor modifications. Chloroplast integrity was verified by both observation under a microscope and estimation of the ratio of major soluble stroma and thylakoid proteins by SDS–PAGE. Chloroplast extracts were free of mitochondrial contamination as determined by the absence of fumarase activity.

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Bacterial inoculation, enumeration of populations and assessment of disease

Pst strain DC3000 and its non-pathogenic derivative strain HrcC were cultured on solidified King's medium B for 2 d at 27°C, then suspended in 10 mM $MgCl_2$ to a population density of 2×10^7 CFU ml^{-1}. Potted plants were arranged with a complete randomized block design with five replicate blocks. At least six leaves per replicate (30 leaves per genotype) were inoculated with Pst DC3000 or the HrcC mutant by pressure infiltration on the underside of marked leaves with a needleless 3 ml syringe. Four days after inoculation, leaves were examined for bacterial speck symptoms. Four to five leaves per replicate were harvested, weighed, diced finely with a sterile razor blade and placed in 10 mM phosphate buffer. Samples were sonicated for 3 min in a bath-style sonicator and serially diluted; dilutions were spread on King's medium B amended with 50 µg ml^{-1} rifampicin and cycloheximide. Colonies were counted and converted to log_{10} (CFU per 0.1 g FW leaf tissue). Log_{10}-transformed population sizes were analyzed with analysis of variance (ANOVA), and mean population sizes of DC3000 or HrcC established in leaves of Columbia were compared with the population sizes in each genotype with a two-tailed *t*-test at $P < 0.05$.

Total antioxidant capacity and reactive oxygen species determinations

Arabidopsis leaf samples (100 mg) were ground in liquid nitrogen with a mortar and pestle. After addition of 1 ml of 1 × phosphate-buffered saline, samples were thoroughly vortexed and centrifuged for 10 min at 13,000 r.p.m. The supernatant was transferred to a new microfuge tube and kept on ice, and used for TAC and ROS determinations. TAC and ROS were determined with the OxiSelect™ Total Antioxidant Capacity Assay kit and the OxiSelect™ In vitro ROS/RNS Assay kit (Cell Biolabs, Inc.), respectively, according to the manufacturer's recommendations.

Determination of enzymatic activities

Methods for the determination of SOD, CAT, GR and APX activities were essentially as described before (Jiang and Zhang 2002). Frozen Arabidopsis leaf samples (100 mg) were ground in liquid nitrogen with a mortar and pestle. After addition of 2 ml of 50 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP), with the addition of 1 mM ascorbic acid in the case of the APX assay, samples were thoroughly vortexed and centrifuged for 10 min at 13,000 r.p.m. at 4°C. The supernatant was transferred to a new tube and kept on ice for enzymatic assays. Concentrations of proteins were determined by using the Bradford Protein Assay kit (Bio-rad) with bovine serum albumin as a standard.

Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of NBT as described by Jiang and Zhang (2002) with the following modifications. The reaction mixture (3 ml in 10 ml glass tubes) contained 50 mM potassium phosphate pH 7.0, 1 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 100 µl of protein extract. The reaction mixtures were illuminated for 15 min under 7,000 lux light intensity. One unit of SOD activity is defined as the amount of enzyme required to inhibit 50% of the reduction of NBT as monitored at 560 nm.

CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240 nm (extinction molar coefficient 43.1 $M^{-1} cm^{-1}$) for 45 s. The 1.5 ml reaction mixture contained 50 mM potassium phosphate pH 7.0, 10 mM H_2O_2 and 66 µl of protein extract. The reaction was started by adding H_2O_2.

GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction molar coefficient 6,220 $M^{-1} cm^{-1}$) for 3 min in a 1 ml reaction mixture containing 50 mM potassium phosphate pH 7.0, 2 mM EDTA, 0.15 mM NADPH, 0.5 mM GSSG and 100 µl of protein extract. The reaction was started by adding NADPH.

APX (EC 1.11.1.11) activity was determined by following the oxidation of ascorbic acid at 290 nm (extinction molar coefficient 2,800 $M^{-1} cm^{-1}$) for 30 s. The 1 ml reaction mixture contained 50 mM potassium phosphate pH 7.0, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µl of protein extract. The reaction was started by adding H_2O_2.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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