NPR1 MEDIATES A NOVEL REGULATORY PATHWAY IN COLD ACCLIMATION BY INTERACTING WITH HSFA1 FACTORS

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ABSTRACT

NPR1 is a master regulator of plant response to pathogens that confers immunity through a transcriptional cascade mediated by salicylic acid (SA) and TGA transcription factors. Little is known, however, about its implication in plant response to abiotic stress. Here, we provide genetic and molecular evidence supporting that Arabidopsis NPR1 plays an essential role in cold acclimation by regulating cold-induced gene expression independently of SA and TGA factors. Our results demonstrate that, in response to low temperature, cytoplasmic NPR1 oligomers release monomers that translocate to the nucleus where they interact with heat shock transcription factors HSFA1 to promote the induction of HSFA1-regulated genes and cold acclimation. Accordingly, Arabidopsis mutants deficient in HSFA1 factors display reduced capacity to cold acclimate, and cold induction of heat stress-responsive genes is required for correct development of cold acclimation. All these findings unveil an unexpected function for NPR1 in plant response to low temperature, reveal a new regulatory pathway for cold acclimation mediated by NPR1 and HSFA1 factors, and place NPR1 as a central hub integrating cold and pathogen signaling for a better adaptation of plants to an ever-changing environment.

Low temperature is a major environmental stress that adversely affects growth and development of plants, determines their geographic distribution and limits crop productivity. Many plants from temperate regions, including Arabidopsis, have evolved an adaptive process whereby their constitutive freezing tolerance increases after being exposed to low nonfreezing temperatures[1]. This process, named cold acclimation, is complex and involves many physiological and biochemical changes[2]. Research over the last decades has shown that most of these changes are controlled by low temperature through changes in gene expression. Arabidopsis, for instance, reprograms its transcriptome during cold acclimation involving more than 3000 genes[3]. Unfortunately, however, the role that the vast majority of these genes play in this adaptive process and the signaling pathways through which they operate remain to be elucidated. The best characterized pathway is mediated by a family of three transcription factors, named CBF1 to CBF3, that are estimated to control the induction of around 12% of the Arabidopsis cold-responsive genes[4]. Plant hormones also have a significant function in cold signaling. Low temperature induces changes in the levels of abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and gibberellins (GA), and it has been shown that ABA, ET, JA, GA and brassinosteroids (BRs) signaling contribute to the regulation of cold acclimation[5]. Salicylic acid (SA) accumulates in response to cold in several species[6] but its function in the process still needs further investigation.

In plants, accumulating evidence indicate that low temperature interacts with other environmental cues. Interestingly, several studies support that cold signals are closely associated with defense responses. Thus, a number of pathogenesis-related (PR) proteins, such as β-1,3-glucanases, endochitinases, and thaumatin-like proteins, accumulate in winter rye during cold acclimation[7,8]. It is worth mentioning that these proteins, in addition of having a role in freezing tolerance, function in pathogen resistance[9]. How low temperature induces their accumulation and triggers pathogen resistance is still largely unknown. In Arabidopsis, some cold-regulated transcription factors, including the plasma membrane-bound NAC transcription factor NTL6 and the C2H2-type Zinc finger transcription factor AtZAT6, have been reported to directly binding to the promoter regions

of *PR* genes thus inducing *PR* expression and enhancing resistance to pathogen infection[10,11]. Other cold-related proteins from Arabidopsis, such as the vascular plant one-zinc-finger proteins (VOZs), the Mediator subunit SFR6/MED16, and the DREB and EAR motif protein 1 (DEAR1), also control *PR* expression and promote tolerance to pathogens[12-14]. Nonetheless, the molecular mechanisms whereby these cold-related proteins control the expression of *PR* genes to induce defense responses remains to be uncovered. It has been proposed that the accumulation of PR proteins under low temperature conditions ensures an adequate strategy of defense against the pathogens that frequently propagate during cold seasons[9]. All these data indicate the existence of a wide range of signaling crosstalk between cold and pathogen responses.

NONEXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1) is a master regulator of basal and systemic acquired resistance in plants, which confers immunity through a transcriptional cascade leading to massive induction of antimicrobial genes[15]. In unchallenged Arabidopsis, NPR1 is sequestered in the cytoplasm as an oligomer maintained by redox-sensitive intermolecular disulfide bonds. The oligomerization of NPR1 is preserved by S-nitrosylation through S-NITROSOGLUTATHIONE (GSNO)[16]. Upon pathogen challenge, the levels of SA increase inducing the expression of *NPR1* gene and the accumulation of the NPR1 protein[15]. In addition, the increase in SA levels generates changes in the cellular redox state, which, in turn, lead to the reduction of the disulfide bonds in NPR1 oligomers. The released NPR1 monomers subsequently translocate to the nucleus where they activate *PR* gene expression[17]. The SA-induced NPR1 oligomer-to-monomer reaction is catalyzed by THIOREDOXINS H3 and H5 (TRXH3, TRXH5), as well as by the SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8)[16,18]. The NPR1 protein holds, at least, two domains involved in protein-protein interactions, the BTB/POZ and the ankyrin-repeat domains, and a nuclear localization sequence, but it does not contain a canonical DNA-binding domain[15]. Consistent with this structure, monomeric NPR1 acts as a transcriptional coactivator interacting with bZIP transcription factors of the TGAs family. These factors have been shown to directly bind to *as-1* elements in the promoters of *PR* genes thus inducing their expression and the

ensuing defense response[15]. In addition to interacting with TGA factors, NPR1 also interacts with NIMIN proteins to attenuate *PR* gene expression[19]. Monomeric NPR1 is specifically targeted for degradation by the CUL3 E3 ligase and its adaptors, the NPR1 paralogs NPR3 and NPR4[20].

Intriguingly, despite the tight connections existing between cold and pathogen responses in plants, any implication of NPR1 in plant response to low temperature has been overlooked. In this study, we show that Arabidopsis NPR1 positively regulates cold acclimation by promoting cold-induced gene expression independently of SA and TGA factors. Our results demonstrate that the expression of *NPR1* is induced in response to low temperature and this induction is followed by an increase of NPR1 protein that accumulates in the nucleus in its monomeric form. There, NPR1 interacts with HSFA1 transcription factors, the master regulators of heat shock response, to activate the expression of HSFA1-regulated genes and, as a consequence, cold acclimation. Indeed, we further demonstrate that the HSFA1 factors also function as positive regulators of cold acclimation, and that the cold induction of heat stress-responsive genes is crucial for full development of this adaptive process in Arabidopsis. Collectively, the data reported here uncover an unanticipated function for NPR1 in cold response, triggering a new transcriptional cascade through its interaction with the HSFA1 factors to promote cold acclimation. NPR1, therefore, represents an integration node for pathogen and cold signaling, allowing plants to better respond and adapt to a fluctuating environment.

RESULTS

NPR1 accumulates in response to low temperature

Given the close relationship that exists between cold and pathogen signaling in plants, we decided to examine whether the master regulator of pathogen response, NPR1, could also play a role in cold response. Encouragingly, results from the eFP Browser database (bar.toronto.ca) indicated that the expression levels of *NPR1* gene from Arabidopsis (At1G64280) increase in response to low temperature[21]. Quantitative PCR (qPCR) experiments confirmed that, in fact, *NPR1* mRNAs accumulated transiently in 2-week-old Col-0 (WT) plants subjected to 4ºC, reaching a peak after 6 hours of treatment (Fig. 1a). This accumulation was mainly detected in the leaves of adult Arabidopsis plants (Fig. 1b). *NPR1* transcripts, however, did not increase in plants exposed to other related abiotic stresses, such as high salt (150mM NaCl) or drought (300mM sorbitol) (Supplementary Fig. 1a, b).

To further investigate the accumulation of *NPR1* transcripts in response to low temperature, we generated Arabidopsis transgenic lines containing a fusion between a *NPR1* promoter fragment (*NPR1*$_{PRO}$; -1986 to +3) and the *UidA* (*GUS*) reporter gene (*NPR1*$_{PRO}$-*GUS*). Three independent transgenic lines (L2.4, L3.7, L4.9) containing a single copy of the fusion in homozygosity were analyzed. In all cases, the levels of *GUS* mRNAs increased significantly when exposed to 4ºC, mirroring the expression pattern of the endogenous *NPR1* gene (Fig. 1c). As expected, transgenic lines showed weak GUS activity under control conditions, but after 6 h of exposure to 4ºC strong GUS staining was detected in the leaves of all lines (Fig. 1d). These data pointed out that the accumulation of *NPR1* mRNAs by low temperature is regulated at the transcriptional level.

Since the expression of *NPR1* is induced by SA[15], whose levels, in turn, have been described to increase under cold conditions[22], we tested the possibility that the accumulation of *NPR1* mRNAs by low temperature could be mediated by SA. This hormone is mainly synthesized from chorismate through the isochorismate synthase (ICS) pathway[23]. In Arabidopsis, ICS is encoded by two genes, *ICS1* and *ICS2*, with *ICS1* having the primary role in cold-induced SA

biosynthesis[22]. We, therefore, analyzed the content of *NPR1* transcripts in WT plants and mutants *sid2-1* and *sid2-2*, two loss-of-function alleles of *ICS1*[23], exposed 6h to 4ºC. We also analyzed the content of *NPR1* transcripts in cold-treated transgenic Arabidopsis expressing *NahG*, a bacterial gene encoding a salicylate hydroxylase that converts SA to catechol[24]. No significant differences were found between WT, *sid2* and *NahG* plants (Supplementary Fig. 1c), evidencing that messengers corresponding to *NPR1* accumulate in response to low temperature independently of SA. In addition, we investigated whether the cold induction of *NPR1* was dependent on the CBF transcription factors and/or on ABA, which mediate the two main signaling pathways controlling cold-induced gene expression[25]. Expression analyses in cold-treated CBF- and ABA-deficient Arabidopsis mutants (*cbf123-1*[3] and *aba2-11*[26]) revealed that the increase of *NPR1* transcripts under low temperature conditions was also independent of CBFs and ABA (Supplementary Fig. 1c).

Next, we assessed if the cold accumulation of *NPR1* mRNA was followed by an increase of the corresponding protein. Western blot (WB) experiments using Arabidopsis plants containing a single copy of a functional genomic fusion *NPR1*$_{PRO}$*-NPR1-MYC* (see below) showed that the levels of NPR1-MYC protein were also more abundant after some hours of cold treatment, correlating with those of *NPR1* transcripts (Fig. 1e). All in all, these results indicated that the levels of NPR1 augment under low temperature conditions independently of SA, ABA and the CBFs.

NPR1 positively regulates cold acclimation

The results described above suggested that NPR1 could be involved in the response of Arabidopsis to low temperature. To test this assumption, we examined the capacity of two *NPR1* loss-of-function mutant alleles, *npr1-1*[27] and *npr1-2*[28], to cold acclimate. Two-week-old mutant plants were cold-acclimated (7d, 4ºC) and subsequently exposed for 6h to different freezing temperatures. Survival was scored after 7 days of recovery under controlled growth conditions. Interestingly, cold acclimated *npr1* mutants exhibited a significantly lower freezing tolerance than cold acclimated WT plants, the LT_{50} (temperature

that causes 50% of lethality) values being -9.4ºC and -10.1ºC, respectively (Fig. 2a, c). *npr1-1* mutants transformed with the *NPR1*$_{PRO}$*-NPR1-MYC* fusion (*c-npr1*) recovered the wild-type capacity to cold acclimate (Fig. 2b, d), validating the fusion and establishing that the decreased capacity of *npr1-1* and *npr1-2* mutants to cold acclimate was a direct consequence of the absence of NPR1. Furthermore, we evaluated the capacity to cold acclimate of an Arabidopsis line containing a *35S-NPR1-GFP* construct[17]. The overexpression of *NPR1* significantly increased the freezing tolerance of cold-acclimated Arabidopsis (Fig. 2b, d). The LT_{50} value of *35S-NPR1-GFP* plants was estimated to be about -10.5ºC.

Although *NPR1* transcripts did not accumulate in Arabidopsis plants exposed to drought or high salt, we also explored a possible role of NPR1 in Arabidopsis tolerance to these cold-related abiotic stresses. Drought and high salt tolerance was examined in 5-day-old *npr1-1* and *npr1-2* seedlings, one week after being transferred to plates containing 300mM sorbitol or 200mM NaCl. In both cases, mutants exhibited similar tolerance as WT seedlings as revealed by the quantification of their fresh weights and main root lengths (Supplementary Fig. 2a, b). Together, all these data provided genetic evidence that NPR1 functions specifically in cold acclimation by positively regulating this adaptive response in Arabidopsis.

Low temperature-induced monomerization and nuclear import of NPR1 are required for full development of cold acclimation

As mentioned above, in response to pathogens, cytoplasmic NPR1 oligomers release monomers by the action of TRXH3, TRXH5 and SnRK2.8 that translocate to the nucleus where they activate *PR* gene expression[16,18]. Thus, we investigated the possibility that low temperature could also trigger the monomerization and nuclear translocation of NPR1. First, we studied the subcellular distribution of NPR1 in Arabidopsis plants exposed to control or low temperature conditions by cell fractionation followed of WB experiments. Cytoplasmic and nuclear protein extracts were obtained from control and cold-treated *c-npr1* plants, and the NPR1-MYC fusion protein was detected immunologically in each

fraction. In plants grown under standard conditions, NPR1 was primarily localized in the cytoplasm. After cold exposure, however, NPR1 was clearly detected in the nuclear fraction (Fig. 3a). The cold-induced nuclear accumulation of NPR1 was practically disrupted in *trxh3trxh5* (see Methods) and *snrk2.8-1*[18] mutants transformed with the *NPR1*$_{PRO}$*-NPR1-MYC* construct (Fig. 3b), evidencing that TRXH3, TRXH5 and SnRK2.8 are critical for its nuclear import in response to low temperature. Then, we performed WB assays with total protein extracts from control and cold-treated *c-npr1* plants under non-reducing conditions to determine if low temperature promoted accumulation of monomeric NPR1. Indeed, after 24h of exposure to 4ºC, the levels of monomeric NPR1 were notably higher than at 20ºC (Fig. 3c). When these assays were carried out with extracts from *trxh3trxh5* and *snrk2.8-1* plants containing the *NPR1*$_{PRO}$*-NPR1-MYC* fusion, the cold-induced accumulation of monomeric NPR1 was not detected (Fig. 3c), therefore indicating that it was mediated by TRXH3, TRXH5 and SnRK2.8.

The results described above suggested that the monomerization and nuclear import of NPR1 could be necessary for proper development of the cold acclimation response. To test this hypothesis, we analyzed the freezing tolerance of 2-week-old cold acclimated (7d, 4ºC) WT plants and *npr1*, *trxh3trxh5* and *snrk2.8-1* mutants. After 6h at -10ºC, *trxh3trxh5* and *snrk2.8-1* mutants exhibited a survival rate significantly lower than WT plants, similar to that shown by *npr1* mutants (Fig. 3d). These data demonstrated that TRXH3, TRXH5 and SnRK2.8 are required to ensure full development of cold acclimation and, therefore, that the low temperature-induced monomerization and nuclear import of NPR1 are needed for its function as a positive regulator of the adaptive response.

NPR1 activates the cold-induction of HSFA1-regulated genes independently of the TGA transcription factors

Now, the arising question was how NPR1 positively regulated cold acclimation. Since cold acclimation involves an extensive transcriptome reprogramming[29] and NPR1 has been implicated in regulating gene expression[30], we considered the possibility that it could

activate the adaptive response by promoting cold-induced gene expression. High-throughput RNA sequencing (RNAseq) was used to estimate the impact of the *npr1-1* mutation on the transcriptome of Arabidopsis plants exposed 24h to 4ºC. To this, we sequenced cDNA libraries prepared from cold-treated *npr1-1* and WT plants. The resulting reads (2.6 Gb/sample) were mapped to the Arabidopsis genome (TAIR10 version) and gene expression changes in the mutant were evaluated. The top 200 downregulated genes in *npr1-1*, based on fold change ratios with respect to their corresponding controls, were considered for analysis. The expression levels of these genes in mutant plants were decreased at least 2-fold compared with the WT (Supplementary Table 1). Remarkably, 71 out of the 200 downregulated genes (35.5%) had been reported to be induced (≥2-fold) in response to cold[21] (Supplementary Table 2) and, therefore, could account for the impaired capacity of *npr1-1* to cold acclimate. These findings were validated analyzing the expression of several downregulated cold-inducible genes in independent RNA samples from WT, *npr1-1* and *npr1-2* mutant plants grown at 20ºC or subjected 24h to 4ºC by means of qPCR experiments (Fig. 4a). We concluded that NPR1 is required for cold-induced gene expression.

In response to pathogens, NPR1 interacts with class II redundant TGA transcription factors (TGA2, TGA5 and TGA6) to foster *PR* gene expression[15]. To determine whether the role of NPR1 in promoting cold-induced gene expression was also mediated by the TGA transcription factors, we evaluated the cold induction of the genes whose downregulated expression in *npr1* we had validated by qPCR assays (Fig. 4a) in *tga2-1tga5-1tga6-1* (*tga2/5/6*) triple mutants[31]. Results uncovered that the cold induction of all genes, including *PR2*, whose expression by pathogens is mediated by NPR1 through the TGA factors[32], was not significantly affected in the triple mutant (Supplementary Fig. 3a). These observations indicated that NPR1 activates cold-induced gene expression independently of the class II TGA transcription factors.

Intriguingly, gene ontology (GO) analysis revealed that a significant number of the 71 cold-inducible genes whose expression was downregulated in *npr1* mutants, were related with the response to heat stress. In fact, out of the first five enriched GO categories, "response to heat" (GO: 0009408) had the highest fold enrichment (fold change=12.2; P=1.2E-6) (Fig. 4b). This category consisted of nine heat stress-inducible genes, including *HSFA2*, *HSP101*, *DNAJ*, *HSP90.1*, *HSP17.6A*, *HSP70*, *HSP17.6II*, *FES1A* and *WRKY33*. It is worth noting that the expression of all these genes, except that of *WRKY33*, had been described to be regulated by the class A1 heat shock factors (HSFA1s)[33], a family of four partially redundant transcriptional activators in Arabidopsis (HSFA1a, HSFA1b, HSFA1d, HSFA1e), that work as master regulators of the heat shock response[34]. More interesting, a detailed analysis of the 71 cold-inducible genes downregulated in *npr1* mutants unveiled that 16 (22.5%) (Supplementary Table 3) belonged to the HSFA1 regulon[33]. That these genes were indeed downregulated in *npr1* mutants in response to low temperature, as indicated by the RNAseq data, was confirmed by analyzing their expression in independent RNA samples from WT, *npr1-1* and *npr1-2* mutant plants grown at 20ºC or exposed 24h to 4ºC through qPCR assays (Fig. 4c). Furthermore, as expected, the cold-induction of these genes was independent of the TGA transcription factors since it was not affected, in any case, in *tga2/5/6* triple mutants (Supplementary Fig. 3b). Therefore, all these results provided evidence that NPR1 promotes the cold induction of HSFA1-regulated genes independently of the class II TGA factors.

HSFA1 transcription factors positively regulate cold acclimation by inducing heat stress-responsive gene expression under low temperature conditions

HSFA1 factors have been reported to play essential roles in other abiotic stress responses than heat shock, such as water and salt stress responses, by mediating the induction of heat stress-responsive genes[34]. Given the very close relationship existing between these responses and that to low temperature, and the results described above, we considered the possibility that the HSFA1 factors could be involved in cold acclimation by promoting the cold-induced expression of heat stress-responsive genes. This assumption was first assessed

by comparing the expression levels of the 16 cold-inducible genes that were downregulated in cold-treated *npr1* mutants and belonged to the HSFA1 regulon in a *hsfa1a/b/d/e* quadruple knockout mutant (*QK*)[34] and WT plants [Wassilewskija (Ws) for *hsfa1a* and *hsfa1b* mutants and Col-0 for *hsfa1d* and *hsfa1e*] subjected to 4ºC for 24h. The cold induction of all genes was significantly lower in the *QK* mutant than in WT plants, indicating that, in fact, the HSFA1 factors mediated the induction of heat stress-responsive genes during cold acclimation (Fig. 5a).

The implication of the HSFA1 factors in the adaptive process was ultimately established by examining the capacity of different *hsfa1* mutant plants to cold acclimate. Because of the very small size and pleiotropic phenotype of 3-week-old *QK* mutants[34], for these experiments we used the four triple mutants, *hsfa1a/b/d* (*eTK*), *hsfa1b/d/e* (*aTK*), *hsfa1a/b/e* (*dTK*) and *hsfa1a/d/e* (*bTK*), which do not show significant morphological differences with WT plants[34]. The prefixed letters in the triples represent the remaining functional *HSFA1* gene. All mutants displayed significantly reduced freezing tolerance compared to WT plants after being acclimated 7d at 4ºC and subsequently exposed 6h to -10ºC (Fig. 5b). The impaired ability to cold acclimate exhibited by all triple mutants was consistent with the proposed partial functional redundancy for the HSFA1 factors[34]. Nonetheless, the different mutants showed different abilities, the most affected being the *eTK* mutant (Fig. 5b) whose survival percentage (≈30%) was similar to that of *npr1* mutants under the same freezing conditions (Fig. 2a). The low ability of the *eTK* mutant to cold acclimate suggested that factors HSFA1a, HSFA1b and HSFA1d should play a prominent role in the adaptive process. Moreover, a large part of the NRP1-mediated cold acclimation appears to be due to the activation of the HSFA1 regulon.

Our data, therefore, pointed out that the HSFA1 factors would act as positive regulators of cold acclimation by inducing the expression of heat stress-responsive genes. That the heat stress-responsive gene expression regulated by these factors was indeed involved in cold acclimation was determined by analyzing the ability to acclimate of two null mutant alleles

for *HSFA2*, *hsfa2-1* and *hsfa2-2*[35]. *HSFA2* is one of the 16 cold-inducible genes downregulated in cold-treated *npr1* mutants (Fig. 4c), a target of HSFA1 factors[33] and encodes a secondary regulator of the heat shock response[34]. Compared to WT, cold-acclimated (7d, 4ºC) *hsfa2-1* and *hsfa2-2* mutants showed a low percentage of survival (≈40%) after being subjected to -10ºC for 6h (Fig. 5c), evidencing that, indeed, the cold-induced heat stress-responsive gene expression mediated by the HSFA1 factors is essential for full development of cold acclimation. Taken together, these results demonstrated that HSFA1 factors positively regulate cold acclimation in Arabidopsis by promoting heat stress-responsive gene expression under low temperature conditions.

NPR1 interacts with HSFA1 transcription factors to activate cold-induced heat stress-responsive gene expression and cold acclimation

Taking into account that NPR1 functions as a coactivator of gene expression[15] and the capacity of the HSFA1 factors to activate transcription and interact with other proteins[36], we hypothesized that NPR1 could activate cold-induced heat stress-responsive gene expression, and consequently the cold acclimation process, by interacting with HSFA1 factors. The interaction between NPR1 and HSFA1a, HSFA1b and HSFA1d, the factors with a more relevant role in the process, was first studied by means of bimolecular fluorescence complementation (BiFC) analysis in *Nicotiana benthamiana* leaves exposed 24h to 4ºC. Results revealed that a significant proportion of cells transformed with nYFP-NPR1 and cYFP-HSFA1a, cYFP-HSFA1b or cYFP-HSFA1d displayed intense yellow fluorescence (Fig. 6a), denoting interaction between these proteins. Consistent with the subcellular localization of NPR1 in response to low temperature (Fig. 3a, b), NPR1-HSFA1s interactions were mainly observed in the nucleus of cold-treated *N. benthamiana* cells (Fig. 6a). These interactions were also detected under control conditions, but, as expected given the low levels of NPR1 at 20ºC (Fig. 1e) and its main cytoplasmic localization (Fig. 3a, b), they were much less evident than those observed in the cold (Fig. 6a). No interaction, however, was noticed between NPR1 and LSM8, a nuclear protein[37] used as a negative control in the experiments (Fig. 6a). In consonance with these observations, we found that, like NPR1, the HSFA1

351 factors also localized preferentially in the nucleus when transiently expressed in leaves of
352 *N. benthamiana* under low temperature conditions (Supplementary Fig. 4). The interaction
353 between NPR1 and HSFA1s was confirmed by *in vivo* pull-down assays using recombinant
354 purified HIS-HSFA1a, HIS-HSFA1b and HIS-HSFA1d fusion proteins and extracts from *c-npr1*
355 plants grown under control conditions or subjected to 4ºC for 24h. As observed in the BiFC
356 experiments, NPR1 was clearly pulled down by all His-HSFA1 proteins, the efficiency being
357 higher when using extracts from cold-treated *c-npr1* plants (Fig. 6b).
358
359 Our results suggested that the NPR1/HSFA1s interaction is essential for the cold induction
360 of heat stress-responsive gene expression mediated by the HSFA1 transcription factors and,
361 therefore, for full development of cold acclimation. To provide further support to this
362 assertion, the expression levels of the 16 cold-inducible genes downregulated in cold
363 treated *npr1* mutants that belonged to the HSFA1 regulon were analyzed in *trxh3trxh5* and
364 *snrk2.8-1* plants, which were deficient in NPR1 oligomer-to-monomer transition and
365 nuclear translocation (Fig. 3b, c), exposed 24h to 4ºC. In all cases, the cold induction of these
366 genes was significantly lower in *trxh3trxh5* and *snrk2.8-1* than in WT plants (Fig. 6c),
367 indicating that the nuclear localization of NPR1 and, therefore, its interaction with HSFA1
368 factors is necessary to activate cold-induced heat stress-responsive gene expression.
369 Overall, these data demonstrate that NPR1 acts as a coactivator together with HSFA1
370 transcription factors to promote cold-induced heat stress-responsive gene expression and
371 the cold acclimation response.
372

DISCUSSION

Heretofore, the expression of *NPR1* has been considered to be exclusively induced in response to pathogen infection. Expression analyses presented in this work revealed that in Arabidopsis, *NPR1* transcripts also accumulate in response to low temperature. This accumulation is transient and seems to be stress specific, since *NPR1* transcripts do not accumulate by other cold-related stresses such as drought or high salt. In contrast to the response to pathogens, SA does not mediate the increase of *NPR1* mRNAs by low temperature. Furthermore, the levels of *NPR1* transcripts in CBF- and ABA-deficient mutants exposed to 4°C are identical to those in WT plants, denoting that they increase in response to low temperature through a CBF- and ABA-independent pathway. We show that the cold accumulation of *NPR1* mRNAs is regulated at the transcriptional level and that the cis-acting element(s) implicated are contained within its proximal promoter region (<2 kb). Intriguingly, however, this region does not contain any described low-temperature responsive element. Understanding the molecular mechanisms underlying the induction of *NPR1* transcripts by low temperature awaits further investigation. As expected from the expression data, the levels of NPR1 protein also increase after cold treatment, mirroring those of *NPR1* transcripts. In agreement with previous reports[16], we found that under control conditions NPR1 preferentially localizes to the cytoplasm of Arabidopsis cells in its oligomeric form. Remarkably, in response to low temperature it accumulates chiefly in the nucleus as monomer. Phosphorylation and redox modifications of NPR1 by SnRK2.8 kinase and thioredoxins TRXH3/TRXH5, respectively, have been shown to be necessary for the oligomer to monomer transition and its subsequent nuclear translocation that occurs after pathogen infection[16,18]. Our data demonstrate that SnRK2.8 and TRXH3/TRXH5 also mediate the cold-induced monomerization and nuclear localization of NPR1.

Consistent with the accumulation of NPR1 in response to low temperature, our genetic analyses provide evidence that it acts as a positive regulator for cold acclimation. In fact, loss-of-function *npr1* mutants show a significantly lower capacity to cold acclimate compared to WT plants. In line with these results, Arabidopsis plants with increased levels

of NPR1 display increased capacity to cold acclimate. NPR1, however, does not seem to be implicated in the ability of Arabidopsis to tolerate other important abiotic stresses such as drought and high salt. Thus, it does not play a general role in Arabidopsis tolerance to abiotic stresses but seems to have a specific function in cold acclimation. The cold induction of *NPR1* is independent of SA, which strongly suggests that this phytohormone does not mediate the role of NPR1 in cold acclimation. This assumption is further supported by the fact that SA levels do not increase during cold acclimation in Arabidopsis, and that Arabidopsis mutants deficient in SA are not affected in their capacity to cold acclimate (Supplementary Fig. 5). Still, the role of NPR1 in cold acclimation requires its monomerization and subsequent nuclear translocation since Arabidopsis mutants deficient in SnRK2.8 and TRXH3/TRXH5 activities show impaired cold acclimation ability, similar to that of *npr1* mutants.

The global transcriptome profiles indicate that NPR1 positively regulates cold acclimation in Arabidopsis by promoting cold-induced gene expression. After 24h of exposure to 4°C, 71 cold-inducible genes display lower induction (≥2.0-fold) in *npr1* than in WT plants. The reduced levels of the corresponding transcripts should account for the reduced capacity of the *npr1* mutants to cold acclimate. Unexpectedly, almost one fourth (16) of the 71 cold-inducible genes whose induction was mediated by NPR1 corresponded to heat stress-responsive genes belonging to the HSFA1 regulon. In Arabidopsis, there are four partially redundant HSFA1 transcription factors (HSFA1a, b, d, e) that function as the master regulators of the heat shock response[34]. The corresponding genes are constitutively expressed[33], and it has been estimated that more than 65% of the heat stress-induced genes are HSFA1 dependent[34]. It is worth noting that one of the 16 NPR1-mediated cold-inducible genes that belong to the HSFA1 regulon is *HSFA2*, a direct target of the HSFA1 factors that encodes a secondary regulator of the heat shock response[33,34]. In addition to activate the heat shock response, HSFA1 and HSFA2 factors have been described to enhance plant response to other adverse environmental conditions, including anoxia, salt and osmotic stresses[34]. The implication of these transcription factors in plant response to low

temperature, however, has not been still documented. Here, we show that Arabidopsis plants deficient in HSFA1 or HSFA2 are unable to cold acclimate properly, evidencing that they also play a positive role in regulating cold acclimation and that heat stress-responsive gene expression mediated by the HSFA1 factors is required for full development of this adaptive process. In this regard, it has been proposed that the heat shock proteins operate as buffers against environmental stresses[38]. HSFA1 and HSFA2, therefore, represent molecular integrators of plant responses to extreme temperatures. In the case of HSFA1 factors, consistent with their functional redundancy, all of them work in promoting cold acclimation although their contribution to the process is not the same. Our results suggest that HSFA1a, HSFA1b and HSFA1d have a more relevant role than HSFA1e.

As already mentioned, Arabidopsis NPR1 does not contain a canonical DNA binding domain and must interact with other transcription factors to act as coactivator to enhance gene expression[15]. To date, NPR1 has only been described to interact with transcription factors from the TGA family, principally with TGA2, TGA5 and TGA6, after pathogen attack to induce *PR* gene expression and the subsequent defense response[15]. The data obtained in this work reveal that the NPR1 function in cold response as coactivator of cold-induced gene expression is fully independent of class II TGA factors. Indeed, the cold-induced gene expression that is promoted by NPR1 during cold acclimation is not affected in *tga2/5/6* triple mutants, indicating that NPR1 must interact with factor(s) different from TGAs to operate in this process. We present compelling evidence that the HSFA1 factors constitute novel clients of Arabidopsis NPR1 through which cold acclimation is established. In line with these results, the tomato HSFA1 factors have also been reported to be able of interacting with coactivator proteins to induce transcription[39]. Our findings demonstrate that NPR1 interacts with HSFA1a, HSFA1b and HSFA1d transcription factors in the nucleus in response to low temperature to promote cold-induced heat-stress responsive gene expression and cold acclimation in Arabidopsis. Nevertheless, in addition to promote the induction of HSFA1-regulated genes under low temperature conditions, NPR1 also fosters the induction of other genes related to cold response, indicating that it must have additional roles in cold

460 acclimation through different regulatory pathways. The nature of these roles and the
461 corresponding underlying molecular mechanisms remain to be elucidated.
462
463 Based on the data described here, a hypothetical model for NPR1 function in Arabidopsis
464 cold acclimation is proposed in Figure 7. In response to low temperature, the expression of
465 *NPR1* would be induced independently of SA, ABA and the CBFs. Concomitantly with this
466 induction there would be an increase of NPR1 protein that would translocate to the nucleus
467 in its monomeric form in a TRXH3/TRXH5-SnRK2.8-dependent way. In the nucleus,
468 monomeric NPR1 would operate as a coactivator promoting cold-induced gene expression
469 and, therefore, cold acclimation by interacting with different transcription factors, including
470 the HSFA1s. The interaction of NPR1 with the HSFA1 factors would induce the expression
471 of numerous heat stress-responsive genes encoding chaperones that would act as powerful
472 buffers to minimize the impact of low temperatures, and would be essential for the full
473 development of the cold acclimation process. Hence, NPR1 seems to serve as a regulatory
474 hub where pathways mediating biotic and abiotic stress responses converge and integrate
475 to guarantee the precise development of Arabidopsis tolerance to adverse conditions.
476 Identifying the complete repertoire of clients through which NPR1 mediates cold
477 acclimation, and the molecular mechanisms that determine NPR1 involvement in pathogen
478 and/or cold signaling constitutes a remarkable goal for future studies that will provide new
479 insights on how plants respond and adapt to fluctuating, and often adverse, natural
480 environments.
481

METHODS

Plant materials

Arabidopsis thaliana Col-0 and Ws ecotypes, and mutants *npr1-2*[28], *sid2-2*[23], *trxh3* (SALK_111160), *trxh5* (SALK_144259), *snrk2.8-1* (SALK_073395), *hsfa2-1* (SALK_008978) and *hsfa2-2* (GK-650B06) were obtained from the Nottingham Arabidopsis Stock Centre. The *trxh3trxh5* double mutant was generated by crossing *trxh3* and *trxh5* single mutants, and homozygous lines were confirmed by PCR amplification with suitable primers (Supplementary Table 4). WT transgenic plants containing the fusion *35S-NPR1-GFP*[17] as well as the *npr1-1 and cpr5* mutants[40] were provided by Xinnian Dong. The *sid2-1* mutant[41] was procured by Roberto Solano. The *aba2-11* mutant[26] was received from Pedro Rodriguez. The *tga2-1tga5-1tga6-1* triple mutant[31] was obtained from Xin Li. The *aTK*, *bTK*, *dTK*, *eTK* and *QK* mutants[34] were supplied by Yee-Yung Charng. *NahG* transgenic plants[24] were furnished by Maria Elena Alvarez. The *cbf123-1* mutant[3] was obtained from Jian-Kang Zhu. To generate the $NPR1_{PRO}$*-GUS* fusion, a 1989-bp (−1986 to +3) promoter fragment from *NPR1* was amplified with appropriate primers (Supplementary Table 4) and cloned into the *pMDC162* Gateway™ binary vector (Invitrogen). The $NPR1_{PRO}$*-NPR1-MYC* fusion was obtained by amplifying the *NPR1* genomic region, including the $NPR1_{PRO}$ fragment, with pertinent primers (Supplementary Table 4) and cloning the resulting PCR product into the *pGWB616* binary vector[42]. The $NPR1_{PRO}$*-GUS* fusion was then introduced in WT, and the $NPR1_{PRO}$*-NPR1-MYC* fusion in *npr1-1* (*c-npr1*), *trxh3trxh5* and *snrk2.8-1* mutants via *Agrobacterium tumefaciens* (GV3101 strain), using the floral dip method[43]. All transgenic lines were genetically determined to have the fusions integrated at a single locus in homozygosis. For BiFC assays, full-length cDNAs corresponding to *NPR1*, *HSFA1a*, *HSFA1b*, *HSFA1d* and *LSM8* genes were amplified with suitable primers (Supplementary Table 4) and the resulting PCR products cloned into the *pDONR207* Gateway™ binary vector (Invitrogen)[42]. Subsequently, they were transferred to *pYFN43* and *pYFC43* binary vectors[44] using the Gateway™ cloning system to generate the *nYFP-NPR1* and the *cYFP-HSFA1a*, *cYFP-HSFA1b*, *cYFP-HSFA1d* and *cYFP-LSM8* fusions, respectively. For the subcellular localization of *HSFA1a*, *HSFA1b* and *HSFA1d* factors, the corresponding cDNAs cloned in *pDONR207*

were transferred to the *pMDC43* Gateway™ binary vector to obtain *GFP-HSFA1* fusions. Plasmids containing the YFP and GFP fusions were introduced into *Agrobacterium* strain GV3101 for agroinfiltration in 3-week-old *N. benthamiana* leaves (see below). For *in vivo* pull-down assays (see below), the *HSFA1a*, *HSFA1b* and *HSFA1d* cDNAs cloned in *pDONR207* were transferred to the *pDEST17* Gateway™ vector, to generate *HIS-HSFA1* fusions. All constructs used in this work were validated by sequencing.

Growth conditions and treatments

Seeds were surface-sterilized, germinated, and grown under standard conditions [20ºC under long-day photoperiods (16h light, of cool-white fluorescent light, photon flux of 90 µmol m^{-2} s^{-1})] in pots containing a mixture of organic substrate and vermiculite (3:1, v/v) or in Petri dishes containing Murashige and Skoog medium supplemented with 1% sucrose (GM) and solidified with 0.9% (w/v) plant agar. Low-temperature treatments for gene expression and immunoblot analyses were performed by transferring plants growing in pots or Petri dishes under standard conditions to a growth chamber set to 4°C for different times under a long-day photoperiod (16h of cool-white fluorescent light, photon flux of 40µmol m^{-2} s^{-1}). Water and salt stress treatments for gene expression assays were accomplished by transferring plants growing in Petri dishes under standard conditions to plates containing GM medium supplemented with 300mM sorbitol or 150mM NaCl, respectively, for different periods of time. In all cases, tissue samples were frozen in liquid nitrogen after treatment and stored at -80°C until use. For histochemical analysis of GUS activity, cold treatments were performed on 2-week-old plants expressing the *NPR1*$_{PRO}$*-GUS* fusion grown under standard conditions and subsequently transferred to a growth chamber set to 4°C for one additional day. Tolerance to freezing temperatures was determined on 2-week-old plants grown on soil under standard conditions and subsequently exposed to 4ºC for 7d (cold acclimated) as described[45]. Tolerance to water and salt stresses was assessed on 5-day-old seedlings grown on GM medium under standard conditions and then transferred to new plates containing GM medium supplemented with 300mM sorbitol or 200mM NaCl for one week. In both cases, tolerance was estimated as the percentage of the main root length and

fresh weight of the plants after treatments. All data reported about tolerances are expressed as standard deviations of the means of at least three independent experiments with 50 plants each.

Gene expression analysis and RNAseq experiments

For gene expression, total RNA was obtained using Purezol™ reagent (Bio-Rad) according to the manufacturer's instructions. RNA samples were treated with DNase I (Roche) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). cDNA was synthesized from each sample with the iScript™ cDNA synthesis kit (Bio-Rad), and qPCRs were performed with SsoFast™ EvaGreen Supermix (Bio-Rad) in a Bio-Rad iQ2 thermocycler. The relative expression values were calculated using the *At4g24610* gene as a reference[46]. Primers used are listed in Supplementary Table 4. All reactions were realized in triplicate employing three independent RNA samples.

For RNAseq experiments, total RNA was obtained from 2-week-old WT and *npr1-1* plants exposed to 4ºC for 24h using TRIzol™ Reagent (Invitrogen) and cleaned with the RNeasy Plant Mini Kit (Qiagen). cDNA libraries were generated from three independent RNA preparations each. RNA quality, library preparation, and subsequent sequencing were performed by the staff of Life Sequencing (Valencia, Spain). RNAseq reads were aligned to the TAIR10 WT reference genome using TopHat2[47] with default parameters. Uniquely mapped reads (Supplementary Table 5) were counted per representative gene model (excluding introns) according to the TAIR10 annotation using custom R scripts. Only genes with reads per kilobase per million >1 in at least one sample were used for differential expression analysis between WT and *npr1-1* plants using DEseq2[48]. This package internally estimates size factors for each sample, calculates dispersion for each gene, and then fits a negative binomial GLM to detect differentially expressed genes taking into account the size factors and dispersion values.

The Expression Browser tool of The Bio-Analytic Resource for Plant Biology

(http://bar.utoronto.ca) was used to determine the genes from our RNAseq data that, in addition of being downregulated in the *npr1-1* mutant, were cold induced. Selected settings were "AtGenExpress-stress series" as data set and "cold stress" as research area[21]. All tissue types, growth stages, and time points were considered, output options were set to "Average of replicate treatments relative to average of appropriate control", and induction was only contemplated when fold change was equal to or higher than 2-fold. Gene ontology (GO) categorization was done with the ThaleMine data mining tool from Araport (www.araport.org). Significantly enriched GO terms (P-value ≤ 2E-6) were established using the Benjamini Hochberg corrected hypergeometric test.

SA measurements

For total SA measurements, leaves from 2-week-old WT and *cpr5* plants were frozen in liquid nitrogen, ground and extracted as previously described[22]. Instrumental set up, data acquisition and calculations were performed as reported[49].

Determination of GUS activity

GUS activity in Arabidopsis transgenic plants containing the fusion *NPR1$_{PRO}$-GUS* was detected and measured as described[50].

Microscopy analysis

Subcellular localization of the NPR1-GFP fusion protein was performed by confocal microscopy in roots from 6-day-old transgenic seedlings containing the *35S-NPR1-GFP* construct grown in petri dishes under control conditions or exposed 24h to 4ºC. Transient expression of fusion proteins for BiFC assays and for subcellular localization of HSFA1 factors was analyzed, also by confocal microscopy, 3d after agroinfiltration in leaves of 3-week-old *N. benthamiana* plants exposed to 20°C or 24h to 4ºC, as reported by English et al. (1997)[51]. Microscopy images were collected using a TCS SP2 confocal laser spectral microscope (Leica Microsystems). The excitation lines for imaging GFP and YFP fusions were 488 and 514 nm, respectively. All microscopy analyses were performed, at least, in triplicate with

598 independent samples.

599

600 **Immunoblot analysis and subcellular fractionation**

601 Total proteins were extracted from 2-week-old *c-npr1* plants grown under control
602 conditions or exposed to 4ºC for different periods of time. Plants were ground in extraction
603 buffer [50mM TRIS pH 7.5, 150mM NaCl, 5mM EDTA, 0.1% Triton X-100, 0.2% Nonident P-
604 40] with inhibitors [40µM MG132, protease inhibitor cocktail EDTA-free (Roche), 0.6mM
605 PMSF], and cell debris were pelleted by centrifugation (16000g, 4ºC, 20 min) to obtain clear
606 protein extracts. Protein concentration was determined by Bradford, using the BioRad
607 Protein Assay (Bio-Rad). Loading buffer containing 6mM beta-mercaptoethanol was added
608 to protein extracts for visualizing total NPR1 protein (reducing conditions). To visualize both
609 monomeric and oligomeric NPR1 forms, we employed loading buffer without beta-
610 mercaptoethanol (non-reducing conditions). Proteins (50µg) were resolved by
611 electrophoresis on 12% SDS-polyacrylamide gels and transferred to Hybond P 0.45 PVDF
612 membranes (Amersham), according to the manufacturer's protocol. To detect the NPR1-
613 MYC protein, we used anti-c-MYC monoclonal antibody (sc-40; Santa Cruz Biotechnology).
614 α-Tubulin, employed as a protein loading control, was detected using anti-α-tubulin
615 monoclonal antibody (T60T4; Sigma).

616

617 Subcellular fractionation was performed as reported previously[52] using extracts from 2
618 week-old *c-npr1* plants and transgenic *trxh3trxh5* and *snrk2.8-1* plants containing the
619 *NPR1*$_{PRO}$*-NPR1-MYC* fusion grown under control conditions or exposed 24h to 4ºC. Isolated
620 proteins were fractionated by electrophoresis, transferred to membranes as described
621 above, and analyzed by immunoblotting using anti-α-tubulin monoclonal (see above) and
622 anti-Histone H3 polyclonal (sc-10809, Santa Cruz Biotechnology) antibodies for control of
623 the cytoplasmic and nuclear fractions, respectively.

624

625 In all cases, horseradish peroxidase-conjugated secondary antibodies were used for primary
626 antibody detection. Signals were always detected with the ECL Western Blotting Detection

Kit (Amersham), and assays were performed in triplicate employing three independent protein samples.

Pull down assays

HIS-HSFA1a, HIS-HSFA1b and *HIS-HSFA1d* constructs were expressed in *Escherichia coli* BL21-CodonPlus (DE3). Cells were grown in 250 ml of Terrific Broth medium at 28^{o}C until reaching an OD600 0.4-0.5. The induction of fusion proteins was performed by addition of 0.1mM IPTG and incubation at 28^{o}C for 12 h. Cells were then centrifuged at 13000g for 30min at 4^{o}C and pellet was resuspended in 3 ml/gr of resuspension buffer [50 mM Tris-HCl pH 8.0, 300mM NaCl, 1mM PMSF, 10mM Imidazole and protease inhibitor cocktail EDTA-free (Roche)]. After lysis by French press and centrifugation (13000g, 4^{o}C, 30 min), supernatants (70mg of protein) were mixed with 400 µl of ProBond™ Nickel-Chelating Resin (Thermo Fisher Scientific) and gently shaken for 2h at 4^{o}C. Resins were finally washed three times with resuspension buffer containing 0.5% Nonidet P-40 before used. Two-week-old WT and *c-npr1* plants grown under control conditions or exposed 24h to 4^{o}C were ground in liquid nitrogen and homogenized in pull-down extraction buffer [50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM PMSF, 0.5% Nonidet P-40, 0.05% Triton X100, 10% Glycerol, 25 µM MG132 and protease inhibitor cocktail EDTA-free (Roche)]. Homogenates were centrifuged (13000g, 4^{o}C, 15 min) and supernatants collected. For *in vivo* pull-down assays, 40µl of resin-bound HSFA1-His fusion proteins were added to 3 mg of total protein extracts and incubated 1h at 4^{o}C with gentle agitation. Then, resins were washed 3 times in pull-down extraction buffer, loaded on 12% SDS-PAGE gels, transferred to Hybond membranes and incubated with anti-His monoclonal antibody (H1029, Sigma), to verify that equal amounts of HIS-fused proteins were used in each assay, or with anti-c-MYC monoclonal antibody (see above), to detect the NPR1-MYC protein recovered in those assays. We employed anti-α-tubulin monoclonal antibody (see above) to confirm equal protein loading from control and cold exposed plant extracts in the pull-downs. In all cases, horseradish peroxidase-conjugated secondary antibodies were used for primary antibody detection. Pull-down assays were always realized in triplicate employing three independent protein samples.

Statistical analyses

The statistical significance of the results was determined by using PRISM 6.0 (GraphPad Software Inc., USA: http://www.graphpad.com). Comparisons between two groups of data were realized employing Student's *t*-test. Comparisons between multiple groups of data were made by means of one-way ANOVA and Bonferroni's post hoc test, taking $P < 0.05$. The values of control conditions or WTs were considered as references.

Data availability

Sequence data from the genes mentioned in this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Supplementary Table 6. The full names of these genes are also included in Supplementary Table 6. The RNAseq data from this article have been submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) and assigned the identifier accession GSE101483.

REFERENCES

1. Levitt, J. Responses of Plants to Environmental Stress. Chilling, Freezing, and High Temperature Stresses, 2nd ed. (New York: Academic Press) (1980).

2. Ruelland, E., Vaultier, M.N., Zachowski, A., and Hurry, V. Chapter 2 Cold Signalling and Cold Acclimation in Plants. *Adv. Bot. Res.* **49**, 35–150 (2009).

3. Zhao, C. et al. Mutational Evidence for the Critical Role of CBF Transcription Factors in Cold Acclimation in Arabidopsis. *Plant Physiol.* **171**, 2744-2759 (2016).

4. Jia, Y. et al. The *cbfs* triple mutants reveal the essential functions of *CBFs* in cold acclimation and allow the definition of CBF regulons in *Arabidopsis*. *New Phytol.* **212**, 345–353 (2016).

5. Barrero-Gil, J. and Salinas, J. CBFs at the crossroads of plant hormone signaling in cold stress response. *Mol. Plant* **10**, 542–544 (2017).

6. Miura, K. and Tada, Y. Regulation of water, salinity, and cold stress responses by salicylic acid. *Front. Plant Sci.* 10.3389/fpls.2014.00004 (2014).

7. Hon, W.C., Griffith, M., Mlynarz, A., Kwok, Y.C., and Yang, D.S. Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiol.* **109**, 879–889 (1995).

8. Pihakaski-Maunsbach, K. et al. Genes encoding chitinase-antifreeze proteins are regulated by cold and expressed by all cell types in winter rye shoots. *Physiol. Plant.* **112**, 359–371 (2001).

9. Griffith, M. and Yaish, M.W.F. Antifreeze proteins in overwintering plants: A tale of two activities. *Trends Plant Sci.* **9**, 399–405 (2004).

10. Seo, P.J. et al. Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. *Plant J.* **61**, 661–671 (2010).

11. Shi, H. et al. The Cysteine2/Histidine2-Type transcription *factor ZINC FINGER OF ARABIDOPSIS THALIANA6* modulates biotic and abiotic stress responses by activating salicylic acid-related genes and *C-REPEAT-BINDING FACTOR* genes in Arabidopsis. *Plant Physiol.* **165**, 1367-1379 (2014).

12. Tsutsui, T. et al. DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in Arabidopsis. *J. Plant Res.* **122**, 633–643 (2009).

13. Wathugala, D.L. et al. The Mediator subunit SFR6/MED16 controls defence gene expression mediated by salicylic acid and jasmonate responsive pathways. *New Phytol.* **195**, 217–230 (2012).

14. Nakai, Y. et al. Vascular plant one-zinc-finger protein 1/2 transcription factors regulate abiotic and biotic stress responses in Arabidopsis. *Plant J.* **73**, 761–775 (2013).

15. Fu, Z.Q. and Dong, X. Systemic Acquired Resistance: Turning Local Infection into Global Defense. *Annu. Rev. Plant Biol.* **64**, 839–863 (2013).

16. Tada, Y. et al. Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**, 952–956 (2008).

17. Kinkema, M., Fan, W., and Dong, X. Nuclear Localization of NPR1 Is Required for Activation of *PR* Gene Expression. *Plant Cell* **12**, 2339-2350 (2000).

18. Lee, H.-J. et al. Systemic immunity requires SnRK2.8-mediated nuclear import of NPR1 in Arabidopsis. *Plant Cell* **27**, 3425–3438 (2015).

19. Hermann, M. et al. The *Arabidopsis* NIMIN proteins affect NPR1 differentially. *Front. Plant Sci.* **4**, 10.3389/fpls.2013.00088 (2013).

20. Fu, Z.Q. et al. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486**, 228–232 (2012).

21. Kilian, J. et al. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.* **50**, 347–363 (2007).

22. Kim, Y., Park, S., Gilmour, S.J., and Thomashow, M.F. Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of Arabidopsis. *Plant J.* **75**, 364–376 (2013).

23. Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562–565 (2001).

24. Lawton, K. et al. Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant. Microbe. Interact.* **8**, 863–870 (1995).

25. Xue-Xuan, X. et al. Biotechnological implications from abscisic acid (ABA) roles in cold stress and leaf senescence as an important signal for improving plant sustainable survival under abiotic-stressed conditions. *Crit. Rev. Biotechnol.* **30**, 222–230 (2010).

26. González-Guzmán, M. et al. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell* **14**, 1833–1846 (2002).

27. Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**, 1583–1592 (1994).

28. Shah, J., Tsui, F., and Klessig, D.F. Characterization of a Salicylic Acid–Insensitive Mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* Gene. *Mol. Plant. Microbe. Interact* **10**, 69–78 (1997).

29. Chinnusamy, V., Zhu, J., and Zhu, J.-K. Cold stress regulation of gene expression in plants. *Trends Plant Sci.* **12**, 444–451 (2007).

30. Blanco, F. et al. Early genomic responses to salicylic acid in Arabidopsis. *Plant Mol. Biol.* **70**, 79–102 (2009).

31. Zhang, Y., Tessaro, M.J., Lassner, M., and Li, X. Knockout analysis of Arabidopsis transcription factors *TGA2*, *TGA5*, and *TGA6* reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* **15**, 2647–2653 (2003).

32. Fan, W. and Dong, X. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. *Plant Cell* **14**, 1377–1389 (2002).

33. Yoshida, T. et al. Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol. Genet. Genomics* **286**, 321–332 (2011).

34. Liu, H.C., Liao, H.T., and Charng, Y.Y. The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in *Arabidopsis*. *Plant, Cell Environ.* **34**, 738–751 (2011).

35. Nishizawa, A. et al. Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant J.* **48**, 535–547 (2006).

36. Scharf, K.-D., Berberich, T., Ebersberger, I., and Nover, L. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochim. Biophys. Acta* **1819**, 104–119 (2012).

37. Perea-Resa, C., Hernandez-Verdeja, T., Lopez-Cobollo, R., Castellano, M. d. M., and Salinas, J. LSM proteins provide accurate splicing and decay of selected transcripts to ensure normal Arabidopsis development. *Plant Cell* **24**, 4930–4947 (2012).

38. Carey, C.C., Gorman, K.F., and Rutherford, S. Modularity and intrinsic evolvability of Hsp90-buffered change. *PLoS One* **1**, e76 (2006).

771 39. Bharti, K. et al. Tomato heat stress transcription factor HsfB1 represents a novel type
772 of general transcription coactivator with a histone-like motif interacting with the
773 plant CREB binding protein ortholog HAC1. *Plant Cell* **16**, 1521–1535 (2004).

774 40. Bowling, S. a, Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. The *cpr5* mutant of
775 Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance.
776 *Plant Cel*l **9**, 1573–1584 (1997).

777 41. Adie, B.A.T. et al. ABA Is an essential signal for plant resistance to pathogens
778 affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* **19**,
779 1665–1681 (2007).

780 42. Nakagawa, T. et al. Improved Gateway binary vectors: high-performance vectors for
781 creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol.*
782 *Biochem.* **71**, 2095–2100. (2007).

783 43. Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., and Chua, N.-H. *Agrobacterium*-
784 mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat.*
785 *Protoc.* **1**, 641–646 (2006).

786 44. Belda-Palazón, B. et al. Aminopropyltransferases involved in polyamine biosynthesis
787 localize preferentially in the nucleus of plant cells. PLoS One **7**, e46907 (2012).

788 45. Catalá, R., Medina, J., and Salinas, J. Integration of low temperature and light
789 signaling during cold acclimation response in Arabidopsis. *Proc. Natl. Acad. Sci. U. S.*
790 *A.* **108**, 16475–16480 (2011).

791 46. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.-R. Genome-
792 Wide Identification and Testing of Superior Reference Genes for Transcript
793 Normalization in Arabidopsis. *Plant Physiol.* **139**, 5-17 (2005).

794 47. Kim, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of
795 insertions, deletions and gene fusions. *Genome Biol.* **14**, R36 (2013).

796 48. Love, M.I, Huber, W., Anders, S.l. Moderated estimation of fold change and
797 dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

798 49. Pastor, V. et al. Detection, characterization and quantification of salicylic acid
799 conjugates in plant extracts by ESI tandem mass spectrometric techniques. *Plant*
800 *Physiol. Biochem.* **53**, 19–26 (2012).

801 50. Medina, J., Catala, R., Salinas, J. Developmental and stress regulation of *RCI2A* and
802 *RCI2B*, two cold-inducible genes of Arabidopsis encoding highly conserved
803 hydrophobic proteins. *Plant Physiol.* **125**, 1655–1666 (2001).

804 51. English, J., Davenport, G., Elmayan, T., Vaucheret, H., and Baulcombe, D.
805 Requirement of sense transcription for homology-dependent virus resistance and
806 *trans*-inactivation. *Plant J.* **12**, 597–603 (1997).

807 52. Locascio, A., Blázquez, M.A., and Alabadí, D. Dynamic regulation of cortical
808 microtubule organization through prefoldin-DELLA interaction. *Curr. Biol.* **23**, 804–
809 809 (2013).

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We thank all our colleagues that kindly provided us with the mutants and transgenic plants used in this work (see Methods section for details). Furthermore, we thank J.J. Sanchez-Serrano, R. Solano, J. Barrero-Gil and R. Catalá for helpful discussions and comments. This research was supported by grants BIO2013-47788-R from MINECO and BIO2016-79187-R from AEI/FEDER, UE to J.S. and grants 1141202 from FONDECYT and NC130030 from the Millennium Science Initiative to L.H. E.O. was recipient of a PhD fellowship from CONICYT and an I-COOP+ scholarship from the CSIC.

AUTHOR CONTRIBUTIONS

E.O., L.H. and J.S. conceived and designed the experiments. E.O. performed the experiments. E.O., J.M.M., and J.S. analyzed the data. J.S. wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

MATERIALS AND CORRESPONDENCE

Correspondence and requests for materials should be addressed to J.S.

FIGURE LEGENDS

Figure 1. Arabidopsis NPR1 accumulates in response to low temperature.

a, b, Expression of *NPR1* in leaves from 2-week-old Col-0 plants **(a)**, and in leaves, roots, stems and flowers from 6-week-old Col-0 plants **(b)**, exposed to 4ºC for the indicated hours (h). In all cases, transcript levels, determined by qPCR, are represented as relative to the values of leaves at 0h.

c, Expression of *NPR1* and *GUS* in leaves from 2-week-old Col-0 (WT) plants and *NPR1$_{PRO}$-GUS* lines, respectively, exposed to 4ºC for the indicated hours (h). Transcript levels, determined by qPCR, are represented as relative to the values at 0h.

d, Histochemical analysis of GUS activity in 3-week-old plants from the *NPR1$_{PRO}$-GUS* line L4.9 grown under control conditions (20ºC) or exposed 6h to 4ºC.

e, Levels of NPR1-MYC fusion protein in 2-week-old *c-npr1* plants exposed to 4ºC for the indicated hours (h). α-Tubulin (α-TUB) was used as a loading control.

In **a**, **b** and **c**, data represent the mean of three independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between cold-treated and control (0h) plants, as determined by *t*-test.

In **d** and **e**, results are representative of 3 independent experiments.

Figure 2. NPR1 positively regulates cold acclimation in Arabidopsis

a,b, Freezing tolerance of 2-week-old Col-0 (WT), *npr1-1* and *npr1-2* plants **(a)**, and WT, *35S-NPR1-GFP* and *c-npr1* plants **(b)** exposed 6h to the indicated freezing temperatures after being acclimated for 7d at 4°C. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001) from WT plants, as determined by *t*-test.

c,d, Freezing tolerance of representative cold-acclimated plants 7d after being exposed to -10ºC for 6h.

Figure 3. Monomerization and nuclear localization of NPR1 depends on TRXH3, TRXH5 and SnRK2.8, and are required for cold acclimation.

a, Levels of NPR1-MYC fusion protein in cytoplasmic (Cyt) and nuclear (Nuc) fractions from 2-week-old *c-npr1* plants grown under control conditions (20ºC) or exposed to 4ºC for 24h. α-Tubulin (α-TUB) and Histone H3 were used for control of fractionation.

b, Levels of NPR1-MYC fusion protein in cytoplasmic (Cyt) and nuclear (Nuc) fractions from 2-week-old *c-npr1* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the *NPR1PRO-NPR1-MYC* fusion grown under control conditions (20ºC) or exposed to 4ºC for 24h. α-TUB and H3 were used for control of fractionation.

c, Levels of oligomeric and monomeric NPR1-MYC fusion protein in 2-week-old *c-npr1* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the *NPR1PRO-NPR1-MYC* fusion grown under control conditions (20ºC) or exposed to 4ºC for 24h. α-TUB was used as a loading control.

d, Freezing tolerance of 2-week-old Col-0 (WT), *trxh3trxh5*, *snrk2.8-1* and *npr1-1* plants exposed 6h to -10ºC after being acclimated at 4°C for 7d (left panel). Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (***P ≤ 0.001) from WT plants, as determined by t-test. The right panel shows the freezing tolerance of representative cold-acclimated WT, *trxh3trxh5*, *snrk2.8-1* and *npr1-1* plants 7d after being exposed to -10ºC for 6h.

In **a**, **b** and **c**, results are representative of 3 independent experiments.

Figure 4. . NPR1 activates the cold-induction of HSFA1-regulated genes.

a, Expression of different cold-inducible genes in 2-week-old Col-0 (WT), *npr1-1* and *npr1-2* plants grown under control conditions (C) or exposed to 4ºC for 24h.

b, First five gene ontology (GO) terms enriched in cold-inducible genes downregulated in *npr1-1* mutant exposed 24h to 4ºC.

c, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old WT, *npr1-1* and *npr1-2* plants grown under control conditions or exposed to 4ºC for 24 or 6h. In **a** and **c**, transcript levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between *npr1* mutants and WT exposed to 4ºC, as determined by *t*-test.

Figure 5. HSFA1 factors promote cold acclimation by inducing heat stress-responsive gene expression under low temperature conditions.

a, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old Col-0 (WT), Wassilewskija (Ws) and *QK* plants grown under control conditions or exposed to 4ºC for 24 or 6h. Transcript levels, determined by qPCR, are represented as relative to their corresponding values under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between *QK* mutants and WT and Ws exposed to 4ºC, as determined by ANOVA (Bonferroni's post hoc test). No significant differences between WT and Ws plants were observed in any case.

b,c, Freezing tolerance of 2-week-old plants from WT, Ws, *aTK*, *bTK*, *dTK* and *eTK* **(b)**, and WT, *hsfa2-1* and *hsfa2-2* **(c)** exposed 6h to -10ºC after being acclimated at 4°C for 7d (left panels). Freezing tolerance was estimated as the percentage of plants surviving -10ºC after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001) between *TK* mutants and WT and Ws, as determined by ANOVA (Bonferroni's post hoc test) **(b)**, and between *hsfa2* mutants and WT, as determined by *t*-test **(c)**. No significant differences between WT and Ws plants were observed in any case. Right panels show the freezing tolerance of representative cold-acclimated plants from WT, Ws, *aTK*, *bTK*, *dTK* and *eTK* **(b)**, and WT, *hsfa2-1* and *hsfa2-2* **(c)** 7d after being exposed to -10ºC for 6h.

Figure 6. NPR1 interacts with HSFA1 factors to activate cold-induced heat stress-responsive gene expression.

a, *In vivo* interaction between NPR1 and HSFA1a, HSFA1b and HSFA1d proteins by BiFC assays in *N. benthamiana* leaf cells under control (20ºC) or cold conditions (4ºC, 24h). Reconstitution of YFP is shown. The interaction of NPR1 with LSM8 was also assayed as a negative control. Scale bars, 75mm.

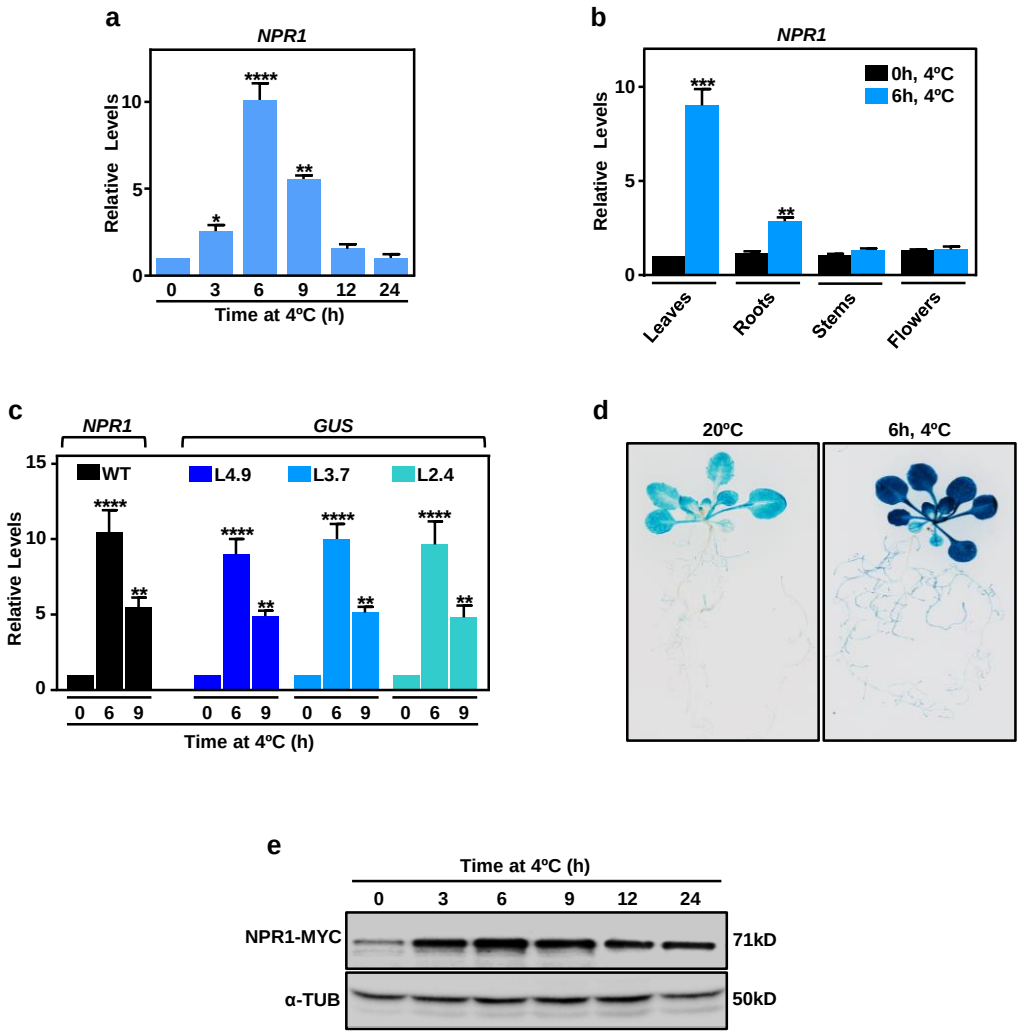
b, Interactions between NPR1 and HSFA1a, HSFA1b and HSFA1d proteins by *in vivo* pull down experiments. Input lanes contain protein extracts from *c-npr1* plants grown at 20ºC or exposed 24h to 4ºC. Pull down lanes contain pull down reactions using protein extracts from *c-npr1* plants grown at 20ºC or exposed 24h to 4ºC and resin-bound recombinant HIS-HSFA1 proteins (HSFA1s) or unbound resin (Resin). Levels of NPR1-MYC were detected by immunoblotting with anti-c-MYC antibody. Anti-α-Tubulin (α-TUB) and anti-HIS antibodies were employed to verify that equal amounts of protein extracts and HSFA1-HIS proteins were used in each reaction, respectively.

c, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old Col-0 (WT), *trxh3trxh5* and *snrk2.8-1* plants grown under control conditions or exposed to 4ºC for 24 or 6h. Levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between mutants and WT exposed to 4ºC, as determined by *t*-test.

In **a** and **b**, results are representative of 3 independent experiments.

Figure 7. Proposed model for the function of NPR1 in cold acclimation response.

FIGURE 1



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c, Expression of *NPR1* and *GUS* in leaves from 2-week-old Col-0 (WT) plants and *NPR1*$_{PRO}$*-GUS* lines, respectively, exposed to 4ºC for the indicated hours (h). Transcript levels, determined by qPCR, are represented as relative to the values at 0h.
d, Histochemical analysis of GUS activity in 3-week-old plants from the *NPR1*$_{PRO}$*-GUS* line L4.9 grown under control conditions (20ºC) or exposed 6h to 4ºC.
e, Levels of NPR1-MYC fusion protein in 2-week-old *c-npr1* plants exposed to 4ºC for the indicated hours (h). α-Tubulin (α-TUB) was used as a loading control.
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FIGURE 2

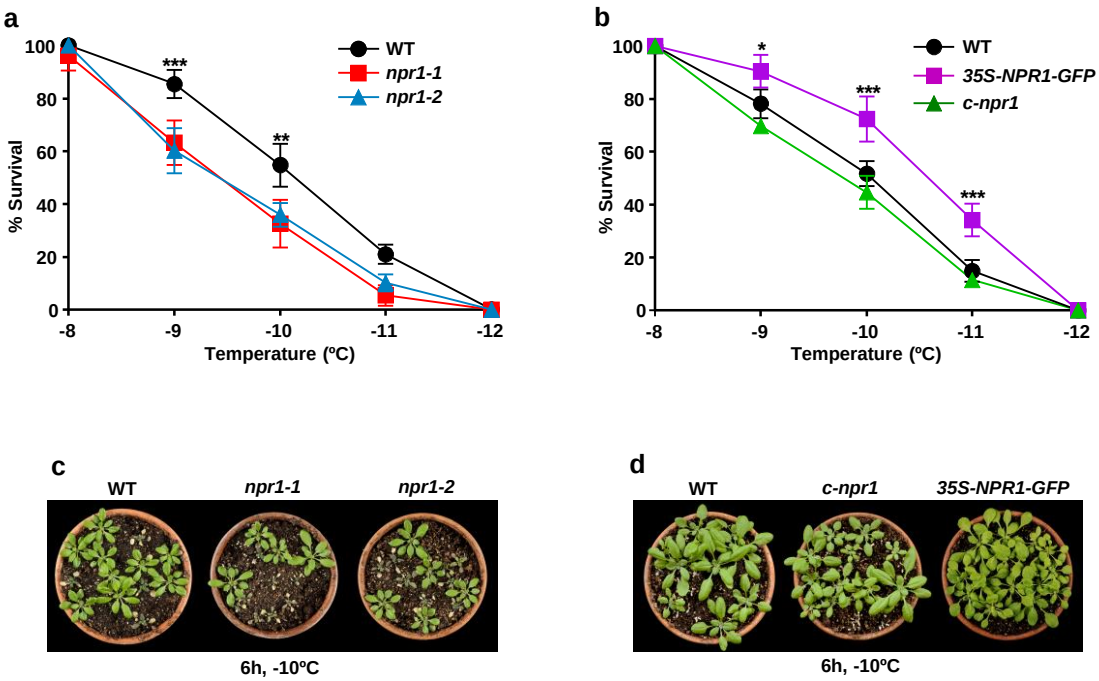

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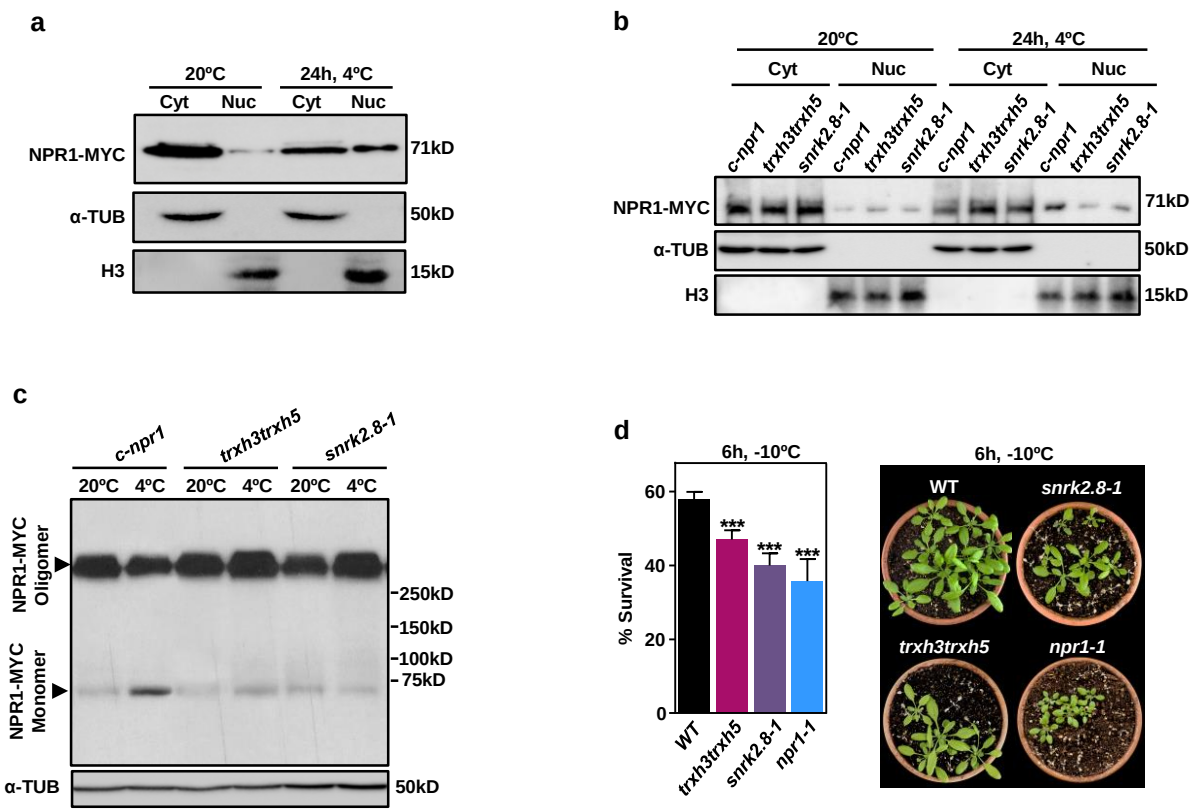
FIGURE 3



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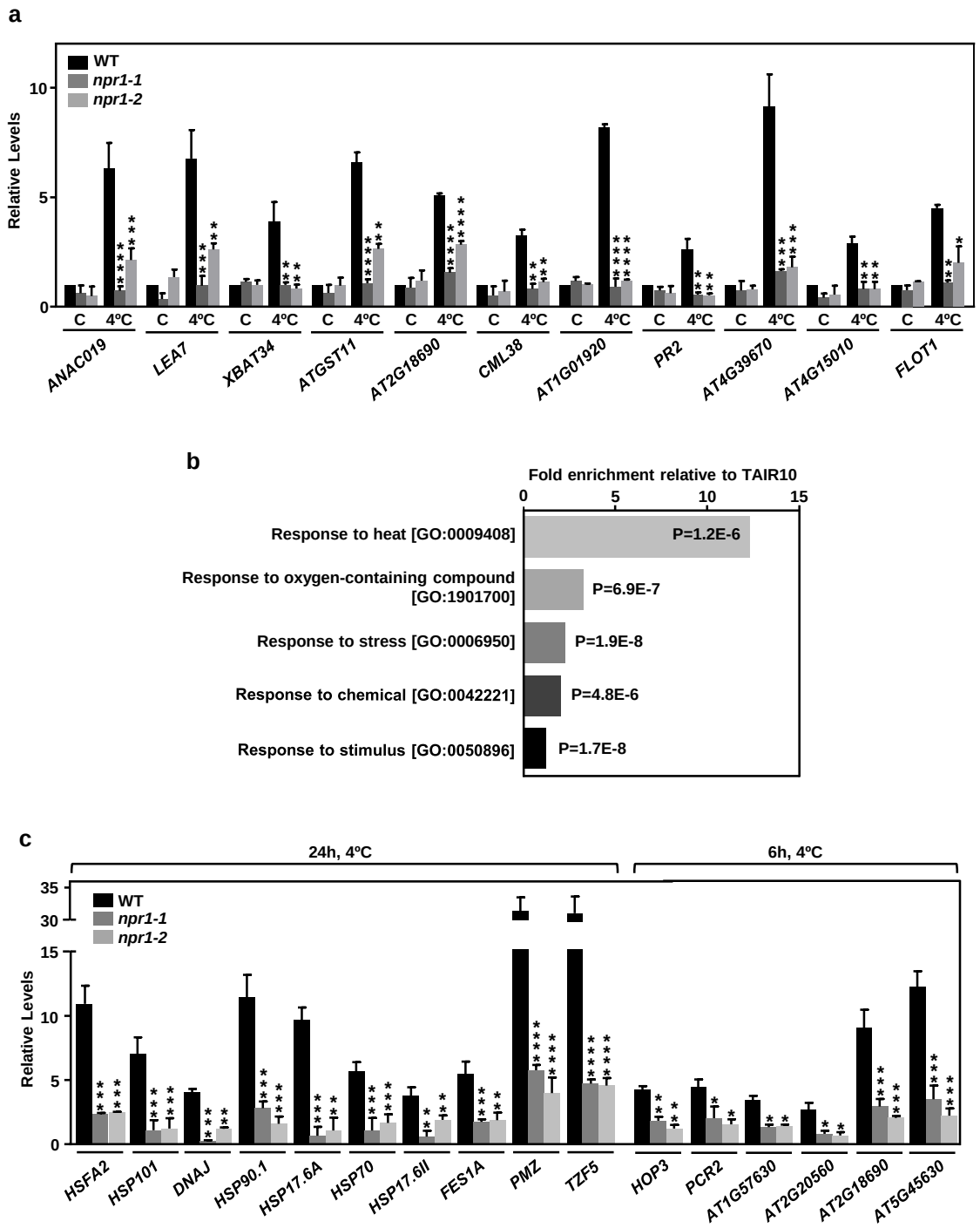
b, Levels of NPR1-MYC fusion protein in cytoplasmic (Cyt) and nuclear (Nuc) fractions from 2-week-old *c-npr1* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the $NPR1_{PRO}$-*NPR1-MYC* fusion grown under control conditions (20ºC) or exposed to 4ºC for 24h. α-TUB and H3 were used for control of fractionation.
c, Levels of oligomeric and monomeric NPR1-MYC fusion protein in 2-week-old *c-npr1* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the $NPR1_{PRO}$-*NPR1-MYC* fusion grown under control conditions (20ºC) or exposed to 4ºC for 24h. α-TUB was used as a loading control.
d, Freezing tolerance of 2-week-old Col-0 (WT), *trxh3trxh5, snrk2.8-1 and npr1-1* plants exposed 6h to -10ºC after being acclimated at 4°C for 7d (left panel). Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (***P ≤ 0.001) from WT plants, as determined by *t*-test. The right panel shows the freezing tolerance of representative cold-acclimated WT, *trxh3trxh5*, *snrk2.8-1* and *npr1-1* plants 7d after being exposed to -10ºC for 6h.
In **a**, **b** and **c**, results are representative of 3 independent experiments.

FIGURE 4



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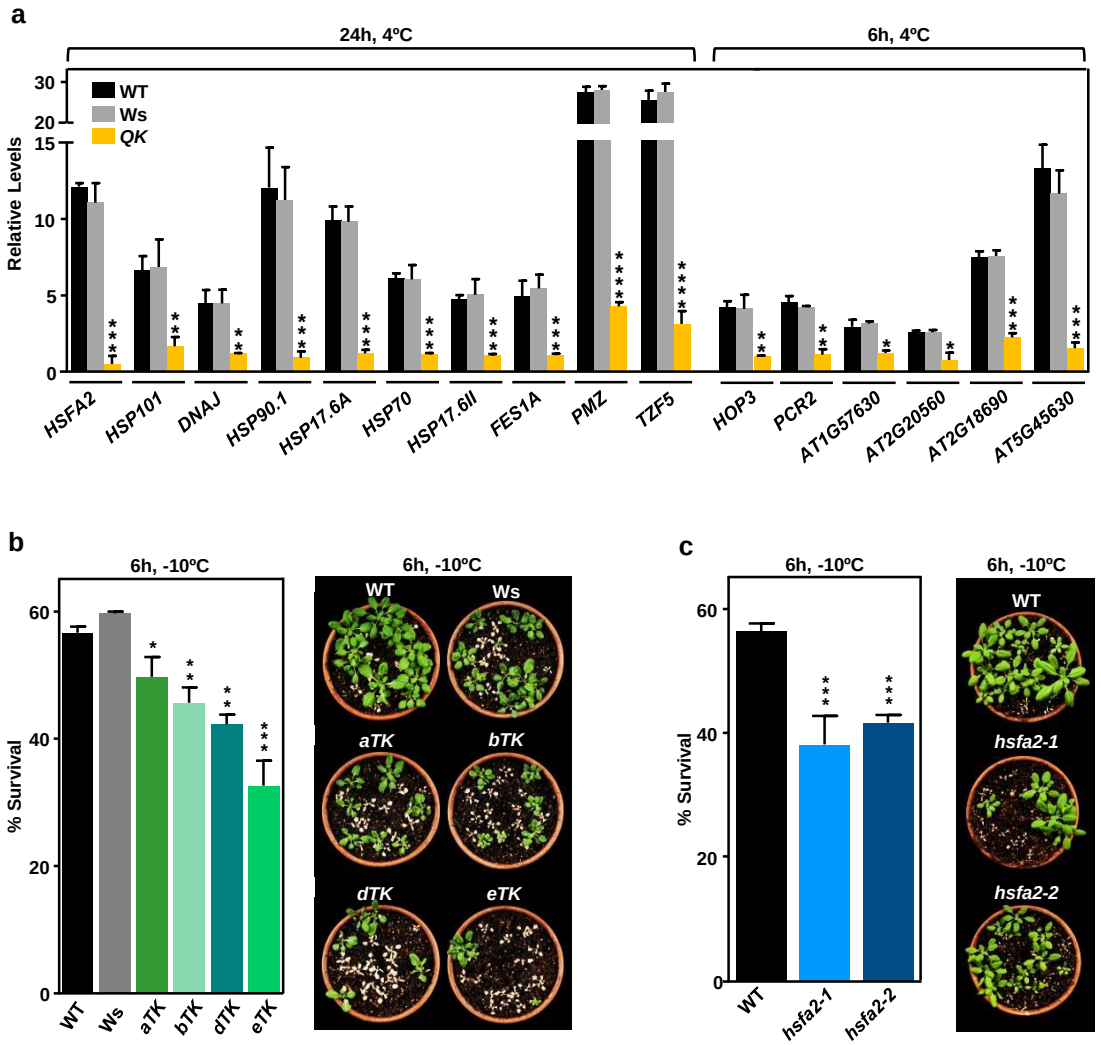
a, Expression of different cold-inducible genes in 2-week-old Col-0 (WT), *npr1-1* and *npr1-2* plants grown under control conditions (C) or exposed to 4ºC for 24h.
b, First five gene ontology (GO) terms enriched in cold-inducible genes downregulated in *npr1-1* mutant exposed 24h to 4ºC.
c, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old WT, *npr1-1* and *npr1-2* plants grown under control conditions or exposed to 4ºC for 24 or 6h.
In **a** and **c**, transcript levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between *npr1* mutants and WT exposed to 4ºC, as determined by *t*-test.

FIGURE 5



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b,c, Freezing tolerance of 2-week-old plants from WT, Ws, *aTK*, *bTK*, *dTK* and *eTK* **(b)**, and WT, *hsfa2-1* and *hsfa2-2* **(c)** exposed 6h to -10ºC after being acclimated at 4°C for 7d (left panels). Freezing tolerance was estimated as the percentage of plants surviving -10ºC after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001) between *TK* mutants and WT and Ws, as determined by ANOVA (Bonferroni's post hoc test) **(b)**, and between *hsfa2* mutants and WT, as determined by *t*-test **(c)**. No significant differences between WT and Ws plants were observed in any case. Right panels show the freezing tolerance of representative cold-acclimated plants from WT, Ws, *aTK*, *bTK*, *dTK* and *eTK* **(b)**, and WT, *hsfa2-1* and *hsfa2-2* **(c)** 7d after being exposed to -10ºC for 6h.

FIGURE 6

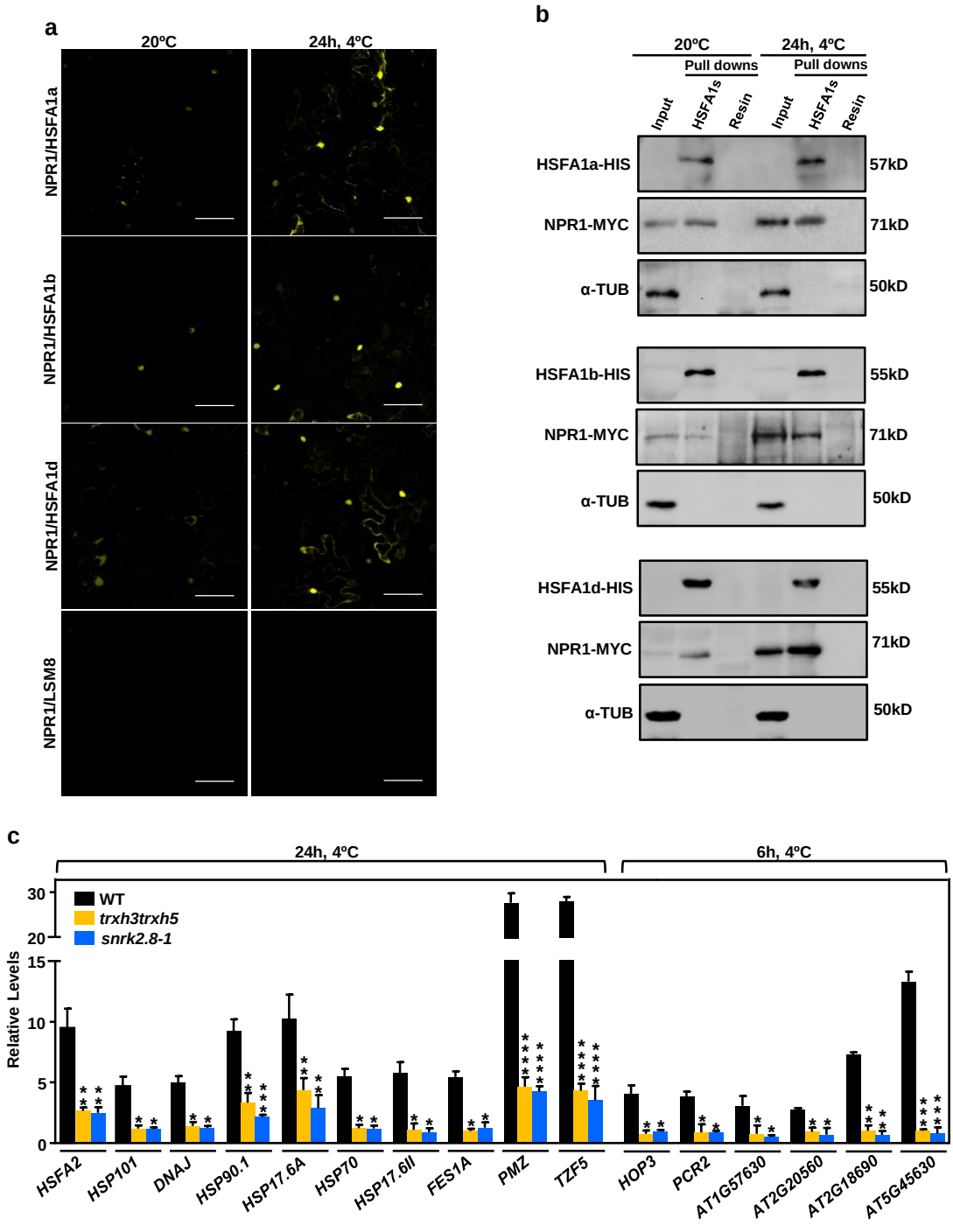


Figure 6. NPR1 interacts with HSFA1 factors to activate cold-induced heat stress-responsive gene expression.
a, *In vivo* interaction between NPR1 and HSFA1a, HSFA1b and HSFA1d proteins by BiFC assays in *N. benthamiana* leaf cells under control (20ºC) or cold conditions (4ºC, 24h). Reconstitution of YFP is shown. The interaction of NPR1 with LSM8 was also assayed as a negative control. Scale bars, 75mm.
b, Interactions between NPR1 and HSFA1a, HSFA1b and HSFA1d proteins by *in vivo* pull down experiments. Input lanes contain protein extracts from *c-npr1* plants grown at 20ºC or exposed 24h to 4ºC. Pull down lanes contain reactions using protein extracts from *c-npr1* plants grown at 20ºC or exposed 24h to 4ºC and resin-bound recombinant HIS-HSFA1 proteins (HSFA1s) or unbound resin (Resin). Levels of NPR1-MYC were detected by immunoblotting with anti-c-MYC antibody. Anti-α-Tubulin (α-TUB) and anti-HIS antibodies were employed to verify that equal amounts of protein extracts and HSFA1-HIS proteins were used in each reaction, respectively.
c, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old Col-0 (WT), *trxh3trxh5* and *snrk2.8-1* plants grown under control conditions or exposed to 4ºC for 24 or 6h. Levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between mutants and WT exposed to 4ºC, as determined by *t*-test. In **a** and **b**, results are representative of 3 independent experiments.

FIGURE 7

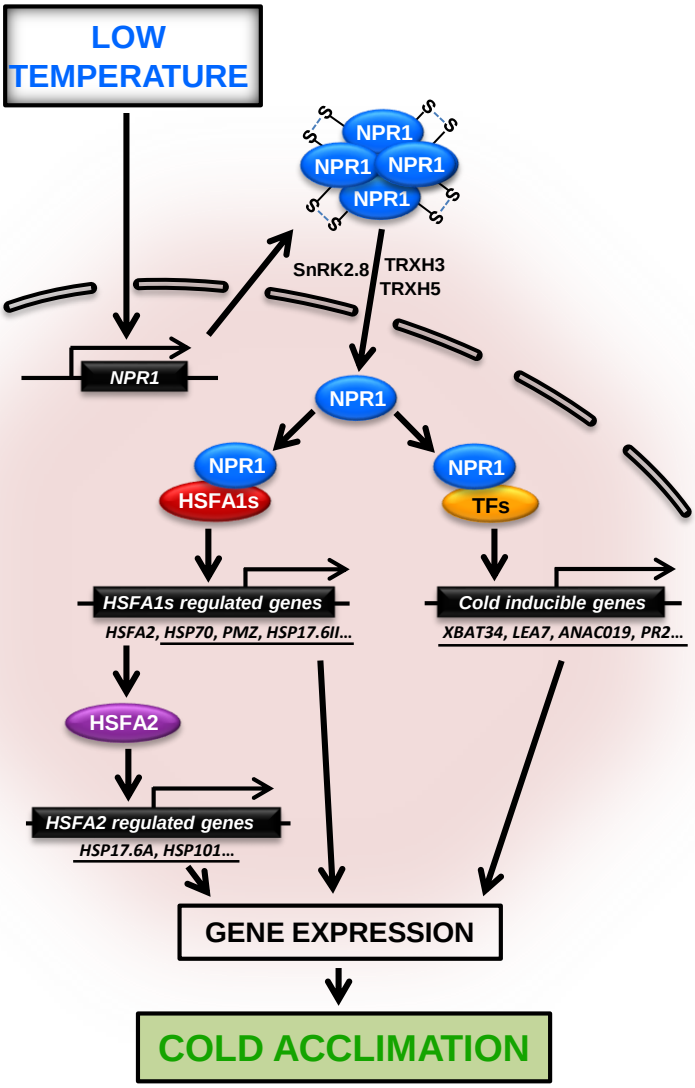
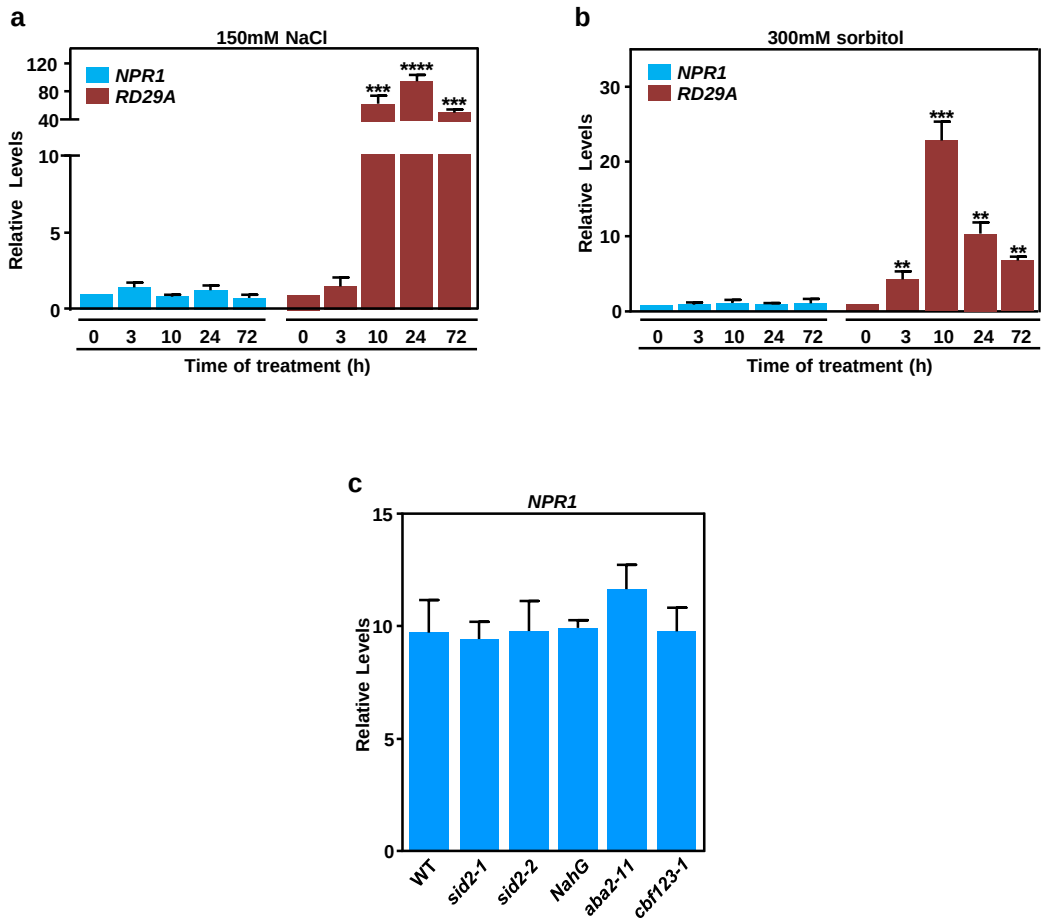


Figure 7. Proposed model for the function of NPR1 in cold acclimation response.

SUPPLEMENTARY FIGURE 1

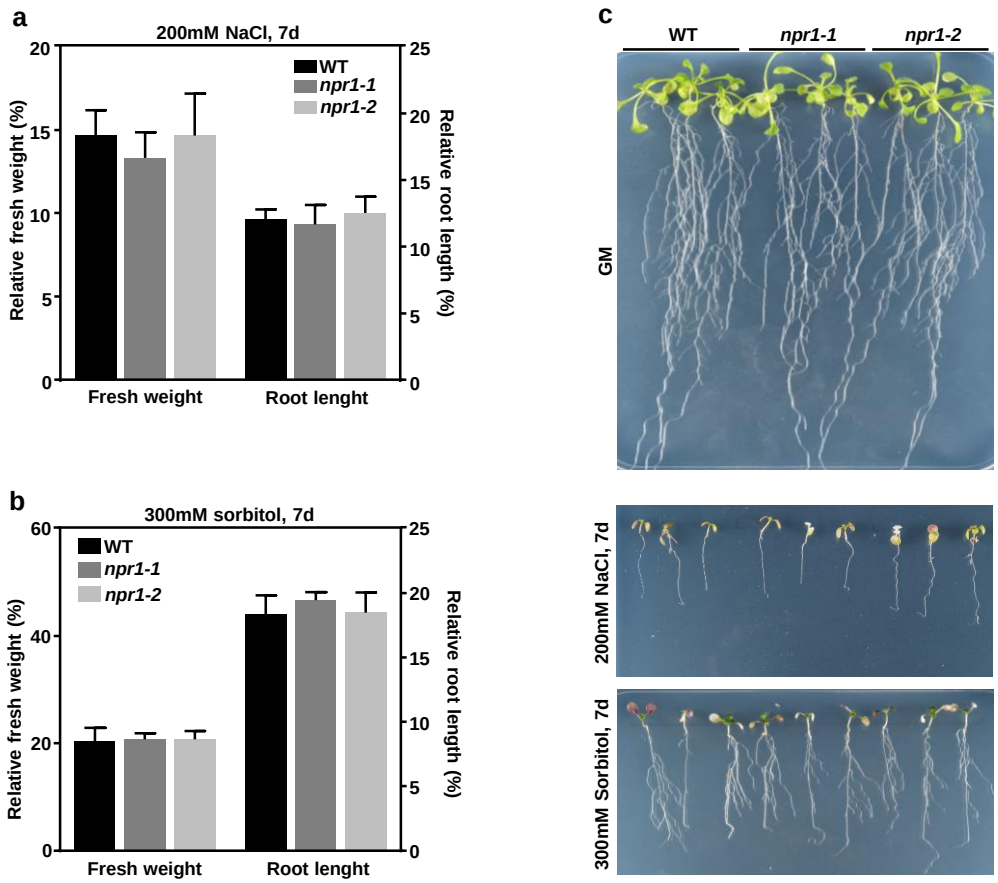


Supplementary Figure 1. *NPR1* expression is induced by low temperature independently of SA, ABA and CBFs but not by drought or high salt.

a,b, Expression of *NPR1* in leaves from 2-week-old Col-0 plants exposed to drought **(a)** or high salt conditions **(b)** for the indicated hours (h). The efficiency of drought and high salt treatments was controlled by analyzing the expression of *RD29A*. In all cases, expression levels, determined by qPCR, are represented as relative to the values at 0h. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (**P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001) between cold-treated and control (0h) plants for *RD29A* expression, as determined by *t*-test. No significant differences between cold-treated and control (0h) plants were observed in any case for *NPR1* expression.

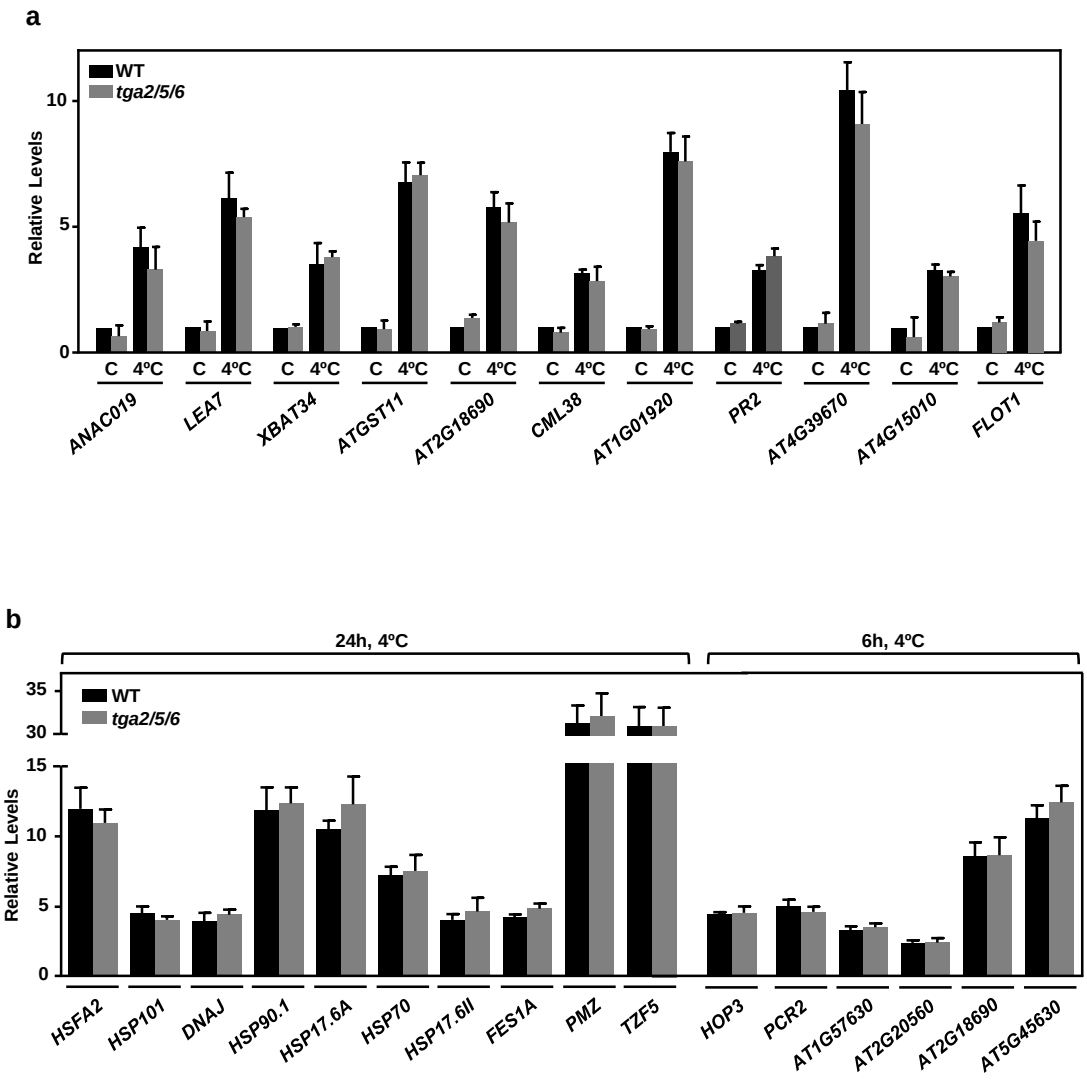
c, Expression of *NPR1* in leaves from 2-week-old Col-0 (WT), *sid2-1*, *sid2-2*, *NahG*, *aba2-11* and *cbf123-1* plants exposed 6h to 4ºC. Expression levels, determined by qPCR, are represented as relative to the value in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. No significant differences between mutants and WT were observed.

SUPPLEMENTARY FIGURE 2



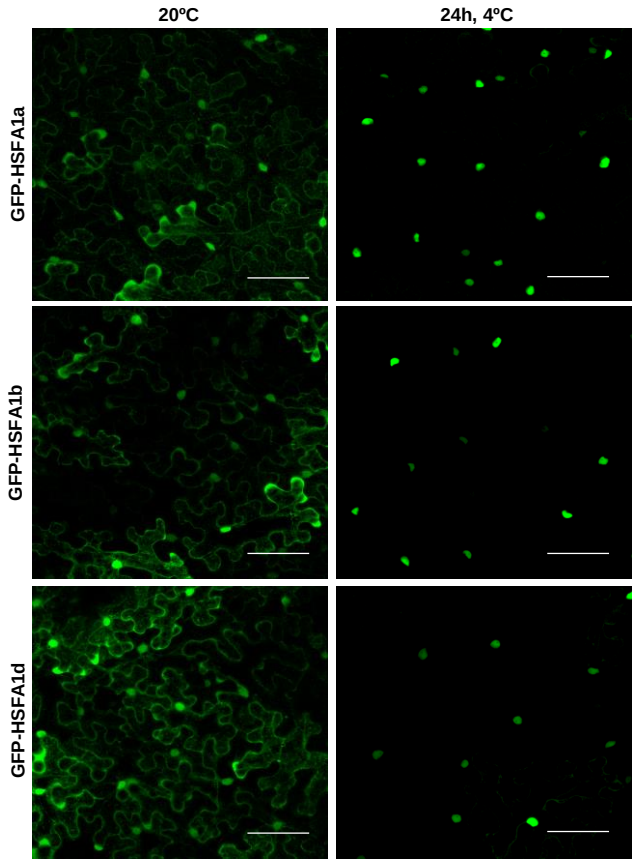
Supplementary Figure 2. NPR1 is not involved in drought or salt tolerance.
a,b, Drought **(a)** and salt **(b)** tolerance of 5-day-old Col-0 (WT), *npr1-1* and *npr1-2* seedlings. Tolerances were calculated as the relative fresh weights and main root lengths of seedlings exposed 7d to 300mM sorbitol or 200mM NaCl respect to seedlings grown under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. No significant differences between mutants and WT were observed in any case.
c, Representative seedlings grown on GM or exposed 7d to 300mM sorbitol or 200mM NaCl.

SUPPLEMENTARY FIGURE 3



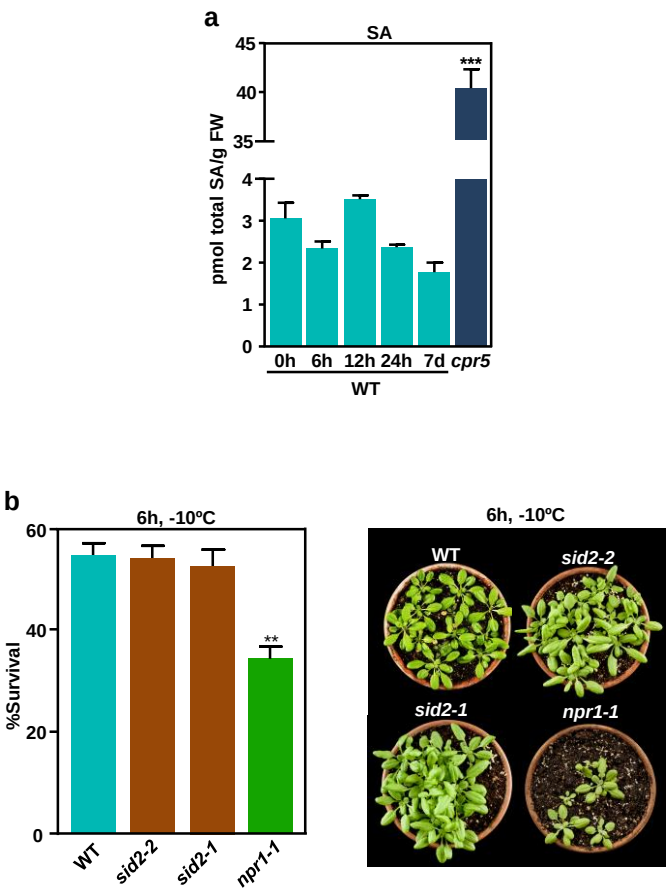
Supplementary Figure 3. NPR1 promotes cold-induced gene expression independently of class II TGA transcription factors.
a, Expression of different cold-inducible genes in 2-week-old Col-0 (WT) and *tga2/5/6* plants grown under control conditions (C) or exposed to 4ºC for 24h.
b, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old WT and *tga2/5/6* plants grown under control conditions or exposed to 4ºC for 24 or 6h.
In **a** and **b**, transcript levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. No significant differences between *tga2/5/6* mutants and WT under cold conditions were observed in any case.

SUPPLEMENTARY FIGURE 4



Supplementary Figure 4. HSFA1 factors localize in the nucleus under low temperature conditions.
Subcellular localization of GFP-HSFA1a, GFP-HSFA1b and GFP-HSFA1d fusion proteins in *N. benthamiana* leaf cells under control (20ºC) or cold conditions (4ºC, 24h). Scale bars, 75µm. Results are representative of 3 independent experiments.

SUPPLEMENTARY FIGURE 5



Supplementary Figure 5. The role of NPR1 in cold acclimation is not mediated by SA.
a, Levels of total SA in 2-week-old Col-0 (WT) plants exposed to 4ºC for the indicated hours (h) or days (d). As a positive control, SA levels were also measured in 2-week-old plants of *cpr5* mutant grown under control conditions. Data represent the mean of 3 independent experiments and error bars show the SD. Asterisks indicate significant differences (***P ≤ 0.001) between *cpr5* and WT plants, as determined by *t*-test. No significant differences between cold-treated and control (0h) WT plants were observed.
b, Freezing tolerance of 2-week-old WT, *sid2-1*, *sid2-2* and *npr1-1* plants exposed 6h to -10ºC after being acclimated at 4°C for 7d (left panel). Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7d of recovery under control conditions. Data represent the mean of 6 independent experiments and error bars show the SD. Asterisks indicate significant differences (**P ≤ 0.01) between *npr1-1* and the rest of plants analyzed, as determined by *t*-test). No significant differences between *sid2* mutants and WT plants were observed. The right panel shows the freezing tolerance of representative cold-acclimated plants from WT, *sid2-1*, *sid2-2* and *npr1-1* plants after being exposed to -10ºC for 6h.