nature plants

ARTICLES

https://doi.org/10.1038/s41477-018-0254-2

NPR1 mediates a novel regulatory pathway in cold acclimation by interacting with HSFA1 factors

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NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1) is a master regulator of plant response to pathogens that confers immunity through a transcriptional cascade mediated by salicylic acid 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 the fact that Arabidopsis NPR1 plays an essential role in cold acclimation by regulating cold-induced gene expression independently of salicylic acid 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 factor 1 (HSFA1) to promote the induction of HSFA1-regulated genes and cold acclimation. 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 signalling 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 non-freezing 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 3,000 genes[3]. Unfortunately, however, the role that the vast majority of these genes play in this adaptive process and the signalling pathways through which they operate remain to be explained.

In plants, accumulating evidence indicates that low temperature interacts with other environmental cues. Interestingly, several studies support that cold signals are closely associated with defence 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[4,5]. It is worth mentioning that these proteins, in addition to having a role in freezing tolerance, function in pathogen resistance[6]. 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 bind to the promoter regions of *PR* genes, thus inducing *PR* expression and enhancing resistance to pathogen infection[7,8]. Other cold-related proteins from *Arabidopsis*, such as the vascular plant one-zinc-finger proteins (VOZ), the mediator subunit SFR6/MED16 and the DREB and EAR motif protein 1 (DEAR1), also control *PR* expression and promote tolerance to pathogens[9–11]. Nonetheless, the molecular mechanisms whereby these cold-related proteins control the expression of *PR* genes to induce defence responses remains to be uncovered. It has been proposed that the accumulation of PR proteins under low-temperature conditions ensures an adequate strategy of defence against the pathogens that frequently propagate during cold seasons[6]. All these data indicate the existence of a wide range of signalling crosstalk between cold and pathogen responses.

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[12]. In unchallenged *Arabidopsis*, NPR1 is sequestered in the cytoplasm as an oligomer maintained by redox-sensitive intermolecular disulfide bonds. The oligomerisation of NPR1 is preserved by S-nitrosylation through *S*-NITROSOGLUTATHIONE (GSNO)[13]. On pathogen challenge, the levels of salicylic acid increase, inducing the expression of *NPR1* gene and the accumulation of the NPR1 protein[12]. In addition, the increase in salicylic acid levels generates changes in the cellular redox state, which, in turn, leads 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[14]. The salicylic-acid-induced NPR1 oligomer-to-monomer reaction is catalysed by THIOREDOXINS H3 and H5 (TRXH3, TRXH5)[13], while the nuclear import of NPR1 monomers is mediated by the SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8)[15]. The NPR1 protein holds at least two domains involved in protein–protein interactions, the BTB/POZ (broad-complex, tramtrack, and bric-a-brac/Pox virus and zinc finger) and the ankyrin-repeat domains, and also a nuclear localization sequence, but it does not contain a canonical DNA-binding domain[12]. Consistent with this structure, monomeric NPR1 acts as a transcriptional coactivator interacting with bZIP transcription factors of the TGA family. These factors have been shown to directly bind to TGACG elements in the promoters of *PR* genes, thus inducing their expression and the ensuing defence response[12]. In addition to interacting with TGA factors, NPR1 also interacts with NONINDUCIBLE-IMMUNITY-INTERACTIN (NIMIN) proteins to attenuate *PR* gene expression[16].

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Monomeric NPR1 is specifically targeted for degradation by the CUL3 E3 ligase and its adaptors, the NPR1 paralogs NPR3 and NPR4[17].

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 salicylic acid 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. 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 activate cold acclimation. NPR1, therefore, represents an integration node for pathogen and cold signalling, allowing plants to better respond and adapt to a fluctuating environment.

Results

NPR1 accumulates in response to low temperature and promotes cold acclimation. Given the close relationship that exists between cold and pathogen signalling in plants, we examined whether the master regulator of pathogen response, NPR1, could also play a role in cold response. 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[18]. Quantitative polymerase chain reaction (qPCR) experiments confirmed that *NPR1* mRNAs accumulated transiently in 2-week-old Col-0 (WT) plants exposed to 4 °C, reaching a peak after 6 h of treatment (Fig. 1a). This accumulation was mainly observed 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 drought (300 mM sorbitol) or high salt (150 mM NaCl) (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 *β-GLUCURONIDASE* (*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 analysed. In all cases, the levels of *GUS* mRNAs increased significantly when exposed to 4 °C, 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 4 °C strong GUS staining was detected in the leaves of all lines (Fig. 1d). These data showed that the accumulation of *NPR1* mRNAs by low temperature is regulated at the transcriptional level.

Because the expression of *NPR1* is induced by salicylic acid[12], whose levels have been described to increase under cold conditions[19], we tested the possibility that the accumulation of *NPR1* mRNAs by low temperature could be mediated by salicylic acid. We analysed the content of *NPR1* transcripts in WT plants and *sid2-1* and *sid2-2*, two *Arabidopsis* mutants deficient in salicylic acid[20], which were exposed to 4 °C for 6 h. We also analysed the content of *NPR1* transcripts in cold-treated transgenic *Arabidopsis* expressing *NahG*, a bacterial gene encoding a salicylate hydroxylase that converts salicylic acid to catechol[21]. No significant differences were found between WT, *sid2* and *NahG* plants (Supplementary Fig. 1c), showing that messengers corresponding to *NPR1* accumulate in response to low temperature independently of salicylic acid. In addition, we investigated whether the cold induction of *NPR1* was dependent on the C-REPEAT BINDING FACTORS (CBF) transcription factors and/or on abscisic acid (ABA), which mediate the two main signalling pathways controlling cold-induced gene expression[22]. Expression analyses in cold-treated CBF- and ABA-deficient *Arabidopsis* mutants (*cbf123-1* (ref. [3]) and *aba2-11* (ref. [23]) revealed that the increase of *NPR1* transcripts under low-temperature conditions was also independent of CBF and ABA (Supplementary Fig. 1c). Next, we assessed if the cold accumulation of *NPR1* mRNA was followed by an increase in the corresponding protein. Western blot (WB) experiments using *Arabidopsis* plants containing a single copy of a functional genomic fusion $NPR1_{PRO}$-*NPR1-MYELOCYTOMATOSIS ONCOGENE (MYC)* (*c-npr1a*; see later) showed that the levels of NPR1-MYC protein were more abundant after 3 h of cold treatment, correlating with those of *NPR1* transcripts (Fig. 1e). These results indicated that the levels of NPR1 augment under low-temperature conditions independently of salicylic acid, ABA and CBF.

These results suggest 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* (ref. [24]) and *npr1-2* (ref. [25]), to cold acclimate. Two-week-old mutant plants were cold acclimated (7 d, 4 °C) and subsequently exposed for 6 h to different freezing temperatures. Survival was scored after 10 d of recovery under control conditions. 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 were −9.4 °C and −10.1 °C, respectively (Fig. 1f). *npr1-1* mutants transformed with the $NPR1_{PRO}$-*NPR1-MYC* fusion were also analysed for their ability to cold acclimate. Three independent transgenic lines (*c-npr1a*, *c-npr1b*, *c-npr1c*) containing a single copy of the fusion in homozygosity were tested. All of them displayed a freezing tolerance similar to WT plants (Fig. 1g), 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 function. Furthermore, we evaluated the capacity to cold acclimate of an *Arabidopsis npr1-1* mutant containing a *35S:NPR1-GREEN FLUORESCENT PROTEIN (GFP)* construct[14]. The overexpression of *NPR1* significantly increased the freezing tolerance of cold-acclimated *Arabidopsis* (Fig. 1g). The LT_{50} value of *35S:NPR1-GFP* plants was estimated to be about −10.5 °C.

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 assayed in 5-day-old *npr1-1* and *npr1-2* seedlings, 1 week after being transferred to plates containing 300 mM sorbitol or 200 mM NaCl. In both cases, mutants showed similar tolerance to WT seedlings, as revealed by the quantification of their fresh weights and main root lengths (Supplementary Fig. 2a–c). These data provide 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 earlier, in response to pathogens, cytoplasmic NPR1 oligomers release monomers by the action of TRXH3 and TRXH5[13]. Subsequently, these monomers translocate to the nucleus through SnRK2.8-mediated phophorylation[15] where they activate *PR* gene expression. We therefore investigated the possibility that low temperature could also trigger the monomerisation 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-npr1a* plants, and the NPR1-MYC fusion protein was detected immunologically in each fraction. In plants

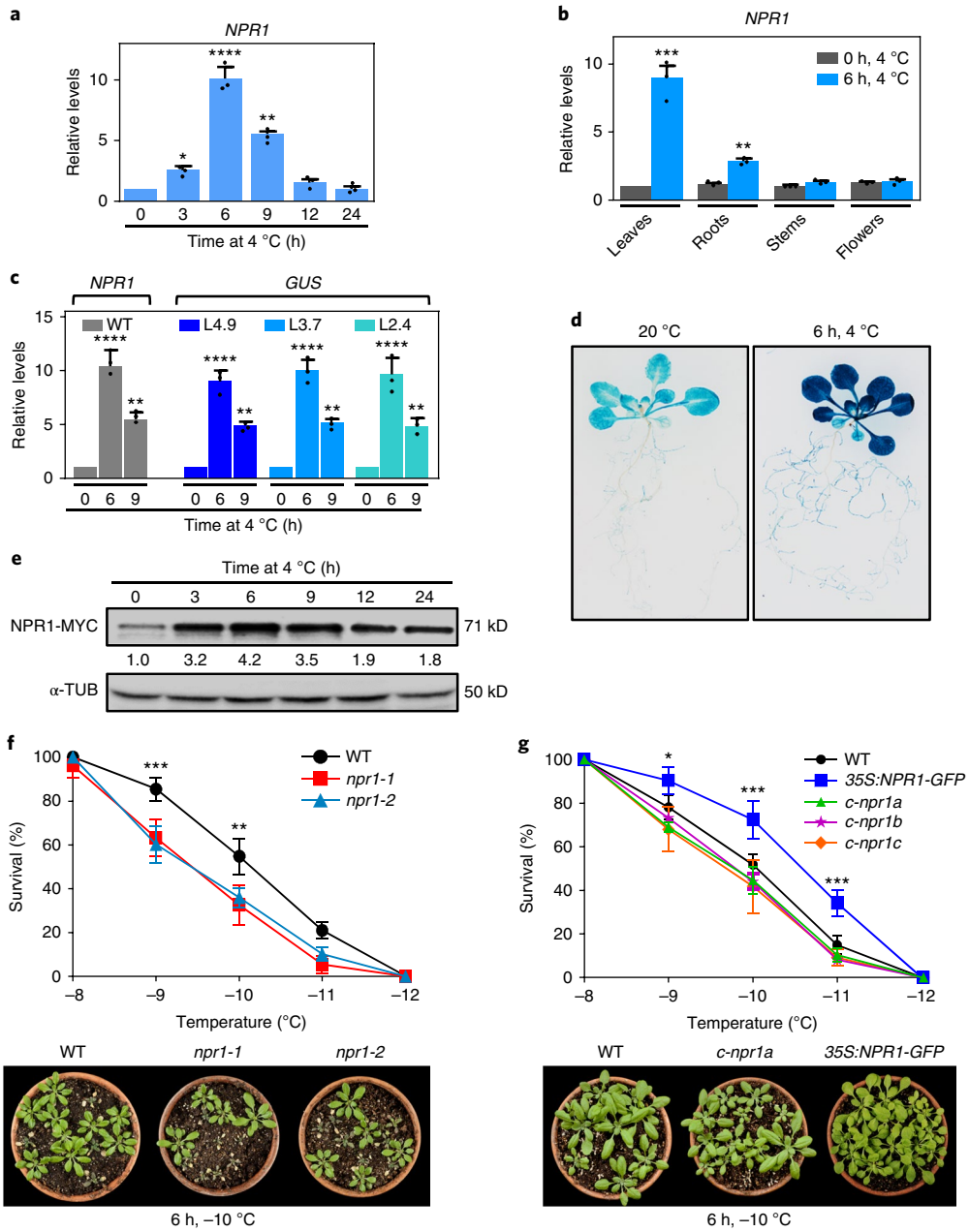

Fig. 1 | NPR1 accumulates in response to low temperature and positively regulates cold acclimation in *Arabidopsis*. **a**,**b**, Expression of *NPR1* in leaves from 2-week-old Col-0 plants (**a**) and in leaves, roots, stems and flowers from 6-week-old Col-0 plants (**b**), exposed to 4 °C for the indicated hours (h). **c**, Expression of *NPR1* and *GUS* in leaves from 2-week-old Col-0 (WT) plants and $NPR1_{PRO}$-*GUS* lines, respectively, exposed to 4 °C for the indicated hours. **d**, Histochemical analysis of GUS activity in 3-week-old plants from the $NPR1_{PRO}$-*GUS* line L4.9 grown under control conditions (20 °C) or exposed to 4 °C for 6 h. **e**, Levels of NPR1-MYC fusion protein in 2-week-old *c-npr1a* plants exposed to 4 °C for the indicated hours. α-Tubulin (α-TUB) was used as a loading control. Numbers below the blot indicate the quantification of the NPR1-MYC signals in each lane represented as the NPR1-MYC/α-TUB ratio relative to this ratio at time 0. **f**,**g**, Freezing tolerance of 2-week-old Col-0 (WT), *npr1-1* and *npr1-2* plants (**f**) and WT, *35 S:NPR1-GFP*, *c-npr1a*, *c-npr1b* and *c-npr1c* plants (**g**) exposed to the indicated freezing temperatures for 6 h after being acclimated for 7 d at 4 °C (upper panels). Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 10 d of recovery under control conditions. Lower panels show the freezing tolerance of representative cold-acclimated plants. In **a**–**c**, transcript levels, determined by qPCR, are represented as relative to the values of leaves at 0 h. Data represent the mean of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) between cold-treated and control (0 h) plants, as determined by one-sided *t*-test. In **d** and **e**, results are representative of three independent experiments. In **f** and **g**, data represent the mean of six independent experiments. Asterisks indicate significant differences (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$) to WT plants, as determined by one-sided *t*-test. In all cases, error bars show the standard deviation (s.d.).

grown under standard conditions, NPR1 was primarily localized in the cytoplasm. After cold exposure, however, NPR1 was clearly detected in the nuclear fraction (Fig. 2a). The cold-induced nuclear accumulation of NPR1 was practically disrupted in *trxh3trxh5* (see Methods) and *snrk2.8-1*[15] mutants expressing the $NPR1_{PRO}$-*NPR1-MYC* construct (Fig. 2b), evidencing that TRXH3, TRXH5 and SnRK2.8 are critical for its nuclear import in response to low temperature. Second, we performed WB assays with total protein

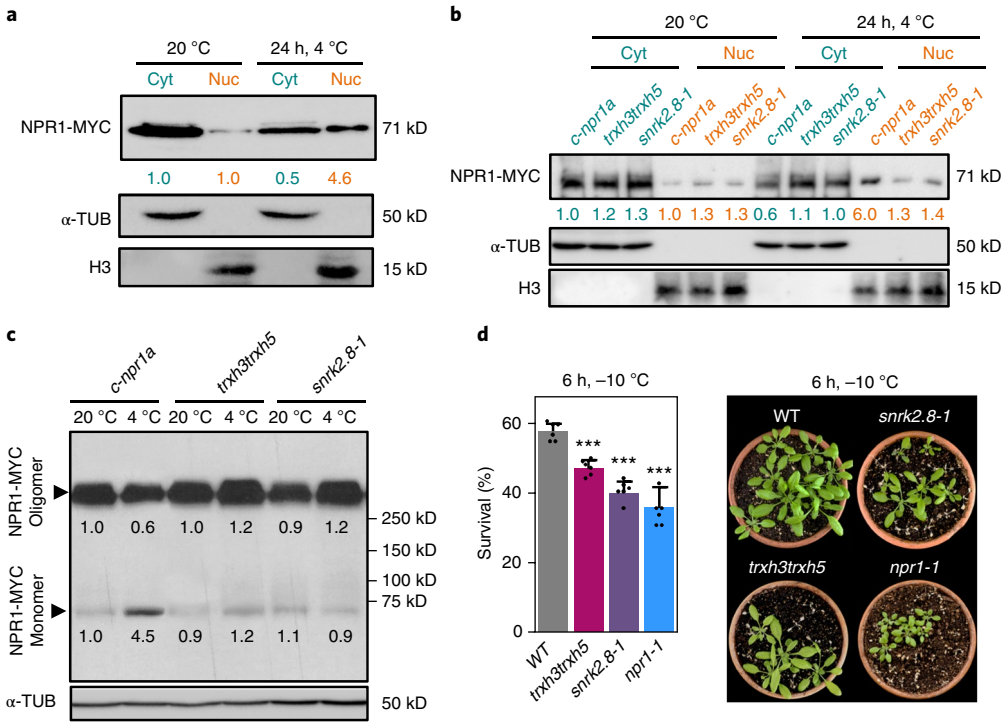

Fig. 2 | Monomerisation and nuclear localization of NPR1 depends on TRXH3, TRXH5 and SnRK2.8, and are required for cold acclimation. a,**b**, Levels of NPR1-MYC fusion protein in cytoplasmic (Cyt) and nuclear (Nuc) fractions from 2-week-old *c-npr1a* plants (**a**) and from 2-week-old *c-npr1a* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the *NPR1*$_{PRO}$*-NPR1-MYC* fusion (**b**) grown under control conditions (20 °C) or exposed to 4 °C for 24 h. α -TUB and Histone H3 (H3) were used for control of fractionations. Numbers below the blots indicate the quantification of the NPR1-MYC signals in each lane represented as the NPR1-MYC/α-TUB or NPR1-MYC/H3 ratios for the Cyt and Nuc fractions, respectively, relative to the ratios in the corresponding fractions at 20 °C (**a**) or in the corresponding *c-npr1a* samples at 20 °C (**b**). **c**, Levels of oligomeric and monomeric NPR1-MYC fusion protein in 2-week-old *c-npr1a* plants and *trxh3trxh5* and *snrk2.8-1* mutants containing the *NPR1*$_{PRO}$*-NPR1-MYC* fusion grown under control conditions (20 °C) or exposed to 4 °C for 24 h. α-TUB was used as a loading control. Numbers below the bands indicate the quantification of the NPR1-MYC signals in each lane represented as the NPR1-MYC/α-TUB ratio relative to this ratio in *c-npr1a* at 20 °C. **d**, Freezing tolerance of 2-week-old Col-0 (WT), *trxh3trxh5, snrk2.8-1 and npr1-1* plants exposed to −10 °C for 6 h after being acclimated at 4 °C for 7 d (left panel). Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 10 d of recovery under control conditions. Data represent the mean of six independent experiments and error bars show the s.d. Asterisks indicate significant differences (***$P < 0.001$) from WT plants, as determined by one-sided *t*-test. The right panel shows the freezing tolerance of representative cold-acclimated plants. In **a–c**, results are representative of three independent experiments.

extracts from control and cold-treated *c-npr1a* plants under non-reducing conditions to determine if low temperature promoted accumulation of monomeric NPR1. After 24 h of exposure to 4 °C, the levels of monomeric NPR1 were notably higher than at 20 °C (Fig. 2c). 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. 2c), therefore manifesting that it was mediated by TRXH3, TRXH5 and SnRK2.8.

The results described earlier suggest that the monomerisation and nuclear import of NPR1 may be necessary for proper development of the cold acclimation response. To test this hypothesis, we analysed the freezing tolerance of 2-week-old cold-acclimated (7 d, 4 °C) WT plants and *npr1-1*, *trxh3trxh5* and *snrk2.8-1* mutants. After 6 h at −10 °C, *trxh3trxh5* and *snrk2.8-1* mutants exhibited a survival rate significantly lower than WT plants, similar to that shown by *npr1-1* mutants (Fig. 2d). 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 monomerisation and nuclear import of NPR1 are needed for its function as a positive regulator of the adaptive response.

We also examined whether, as described in response to pathogens[26,27], NPR1 needs to be phosphorylated at its Ser11 and Ser15 residues and sumoylated at its SIM3 motif to function in cold response. The ability to cold acclimate of *npr1-2* mutant plants expressing phospho-mimic (*35S:npr1*$^{S11/15D}$*-GFP*), phospho-null (*35S:npr1*$^{S11/15A}$*-GFP*) or SIM3 (*35S:npr1*^{sim3}*-GFP*) versions of NPR1[26,27] was, in all cases, similar to that of *npr1-2* mutants expressing *35S:NPR1-GFP* (Supplementary Fig. 3), indicating that those post-translational modifications are not essential for NPR1 to promote cold acclimation.

NPR1 activates the cold-induction of HSFA1-regulated genes independently of the TGA transcription factors. The question was how NPR1 positively regulated cold acclimation. Since cold acclimation involves an extensive transcriptome reprogramming[28] and NPR1 has been implicated in regulating gene expression[29], 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 to 4 °C for 24 h. To this, we sequenced cDNA libraries prepared from cold-treated *npr1-1* and WT plants. The resulting reads (2.6 Gb per 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

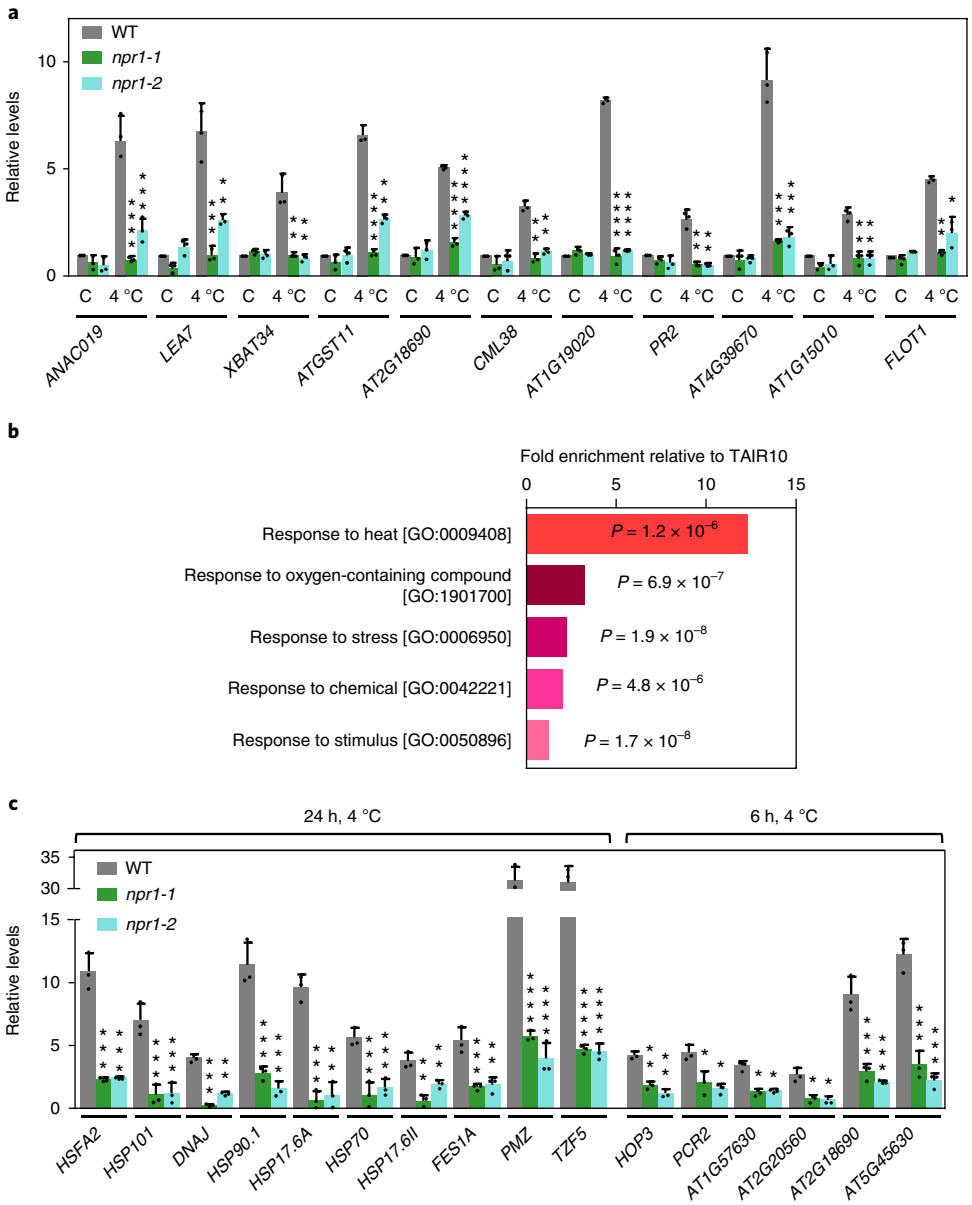

Fig. 3 | NPR1 activates the cold-induction of HSFA1-regulated genes. a, Expression of different cold-inducible genes in 2-week-old Col-0 (WT), *npr1-1* and *npr1-2* plants grown under control conditions (C) or exposed to 4 °C for 24 h. **b**, First five gene ontology (GO) terms enriched in the 71 cold-inducible genes downregulated in *npr1-1* mutant exposed to 4 °C for 24 h. Significantly enriched GO terms ($P \leq 2 \times 10^{-6}$) were established using the Benjamini–Hochberg corrected hypergeometric test. **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 4 °C for 24 h or 6 h. 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 three independent experiments and error bars show the s.d. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) between *npr1* mutants and WT exposed to 4 °C, as determined by one-sided *t*-test.

decreased at least two-fold compared with the WT (Supplementary Table 1). Remarkably, 71 out of the 200 downregulated genes (35.5%) had been reported to be induced (≥ two-fold) in response to cold[18] (Supplementary Table 2) and, therefore, could account for the impaired capacity of *npr1-1* to cold acclimate. Among these genes, neither the *CBF* nor genes involved in ABA biosynthesis or signalling were found (Supplementary Table 2). RNAseq results were validated analysing the expression of several downregulated cold-inducible genes in independent RNA samples from WT, *npr1-1* and *npr1-2* mutant plants grown at 20 °C or subjected to 4 °C for 24 h by means of qPCR experiments (Fig. 3a). 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[12]. To determine whether the role of NPR1 in promoting cold-induced gene expression was also mediated by the TGA factors, we evaluated the cold induction of the genes whose downregulated expression in *npr1* we had validated by qPCR assays (Fig. 3a) in *tga2–1tga5-1tga6-1* (*tga2/5/6*) triple mutants[30]. Results showed that the cold induction of all genes, including *PR2* whose expression by pathogens is mediated by NPR1 through the TGA factors[31], was not significantly affected in the triple mutant (Supplementary Fig. 4a). These observations indicated that NPR1 activates cold-induced gene expression independently of the class II TGA transcription factors.

GO analysis revealed that a significant number of the 71 cold-inducible genes whose expression was downregulated in *npr1* mutants were related to the response to heat stress. Out of the first five enriched GO categories, 'response to heat' (GO: 0009408) had the highest fold enrichment (fold change = 12.2; $P = 1.2 \times 10^{-6}$) (Fig. 3b). 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 as being regulated by the class A1 heat shock factors (HSFA1s)[32], a family of four partially redundant transcriptional activators in *Arabidopsis* (HSFA1a, HSFA1b, HSFA1d, HSFA1e) that work as master regulators of the heat shock response[33]. A detailed analysis of the 71 cold-inducible genes downregulated in *npr1* mutants also unveiled that 16 (22.5%) (Supplementary Table 3) belonged to the HSFA1 regulon[32]. That these genes were indeed downregulated in *npr1* mutants in response to low temperature, as indicated by the RNAseq data, was confirmed by analysing their expression in independent RNA samples from WT, *npr1-1* and *npr1-2* mutant plants grown at 20 °C or exposed to 4 °C for 24 h through qPCR assays (Fig. 3c). Furthermore, the cold-induction of these genes was independent of the TGA transcription factors because it was not affected in any case in *tga2/5/6* triple mutants (Supplementary Fig. 4b). These results provide 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[33]. Given the very close relationship existing between these responses and that to low temperature, and the results described earlier, 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*)[33] and WT plants (Wassilewskija (Ws) for *hsfa1a* and *hsfa1b* mutants and Col-0 for *hsfa1d* and *hsfa1e*) subjected to 4 °C for 24 h. The cold induction of all genes was significantly lower in the *QK* mutant than in WT plants, indicating that the HSFA1 factors mediated the induction of heat stress-responsive genes during cold acclimation (Fig. 4a). The cold induction of *NPR1* and *CBF* genes, however, was not mediated by these factors (Supplementary Fig. 5a, b).

The implication of the HSFA1 factors in cold acclimation 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 2-week-old *QK* mutants[33], we used the four triple mutants, *hsfa1b/d/e* (*aTK*), *hsfa1a/d/e* (*bTK*), *hsfa1a/b/e* (*dTK*) and *hsfa1a/b/d* (*eTK*), which do not show significant morphological differences with WT plants[33]. 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 at 4 °C for 7 d and subsequently exposed to −10 °C for 6 h (Fig. 4b). The impaired ability to cold acclimate exhibited by all triple mutants was consistent with the proposed partial functional redundancy for the HSFA1 factors[33]. Nonetheless, the different mutants showed different abilities, the most affected was the *eTK* mutant (Fig. 4b). 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 considerable part of the NPR1-mediated cold acclimation appears to be due to the activation of the HSFA1 regulon.

Our data demonstrated that the HSFA1 factors would act as positive regulators of cold acclimation by inducing the expression of heat stress-responsive genes. The heat stress-responsive gene expression regulated by these factors was involved in cold acclimation, which was determined by analysing the ability to acclimate of two null mutant alleles for *HSFA2*, *hsfa2-1* and *hsfa2-2* (ref. [34]). *HSFA2* is one of the 16 cold-inducible genes downregulated in cold-treated *npr1* mutants (Fig. 3c), a target of HSFA1 factors[32], and encodes a secondary regulator of the heat shock response[33]. Compared to WT, cold-acclimated (7 d, 4 °C) *hsfa2-1* and *hsfa2-2* mutants showed a significantly lower percentage of survival (≈20%) after being subjected to −10 °C for 6 h (Fig. 4c), showing that 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. Considering that NPR1 functions as a coactivator of gene expression[12] and the capacity of the HSFA1 factors to activate transcription and interact with other proteins[35], we hypothesized that NPR1 could activate cold-induced heat stress-responsive gene expression, and consequently the cold acclimation process, by interacting with HSFA1 factors. This interaction was first studied by means of pull-down assays using recombinant purified HIS-HSFA1 fusion proteins and extracts from *c-npr1a* plants grown under control conditions or subjected to 4 °C for 24 h. Results revealed that NPR1 was clearly pulled down by all HIS-HSFA1 proteins, and the efficiency was higher when using extracts from cold-treated *c-npr1a* plants (Supplementary Fig. 6a). An in vivo interaction between NPR1 and HSFA1 transcription factors was verified through bimolecular fluorescence complementation (BiFC) analysis in *Nicotiana benthamiana* leaves exposed to 4 °C for 24 h. As shown in Fig. 5a, a significant proportion of cells co-transformed with nYELLOW FLUORESCENT PROTEIN (nYFP)-NPR1 and cYFP-MYC-HSFA1a, cYFP-MYC-HSFA1b, cYFP-MYC-HSFA1d or cYFP-MYC-HSFA1e and displayed intense yellow fluorescence, denoting interaction between these proteins. Consistent with the subcellular localization of NPR1 in response to low temperature (Fig. 2a, b), NPR1-HSFA1s interactions were mainly observed in the nucleus of cold-treated *N. benthamiana* cells (Fig. 5a). These interactions were also detected under control conditions but, as observed in the pull-down experiments, they were less evident than those noticed in the cold (Fig. 5a). However, no interaction was detected between NPR1 and LSM8, a nuclear protein[36] used as a negative control in the experiments (Supplementary Fig. 6b). Similar expression of NPR1, HSFA1 and LSM8 proteins with or without cold treatment was established by WB experiments (Supplementary Fig. 6c). In consonance with the observations from the BiFC analyses, we found that, similar to NPR1, the HSFA1 factors localized preferentially in the nucleus when transiently expressed in leaves of *N. benthamiana* under low-temperature conditions (Supplementary Fig. 7a). Additional evidence that NPR1 interacts with HSFA1 factors in vivo was obtained by co-immunoprecipitation (co-IP) experiments in *N. benthamiana* plants infiltrated with MYC-NPR1 and HA-HSFA1 fusion proteins and exposed, or not, to 4 °C for 24 h. Confirming the results from the BiFC assays, we observed a physical interaction between NPR1 and all HSFA1 factors, and this interaction was more pronounced in plants subjected to 4 °C (Fig. 5b). All these data show that NPR1 interacts in planta with HSFA1 factors and that this interaction is favoured in the presence of low temperature. Consistent with this interaction and with the presumption that it is required for cold acclimation completion, we found that the overexpression of HSFA1 factors in *npr1-1* mutants

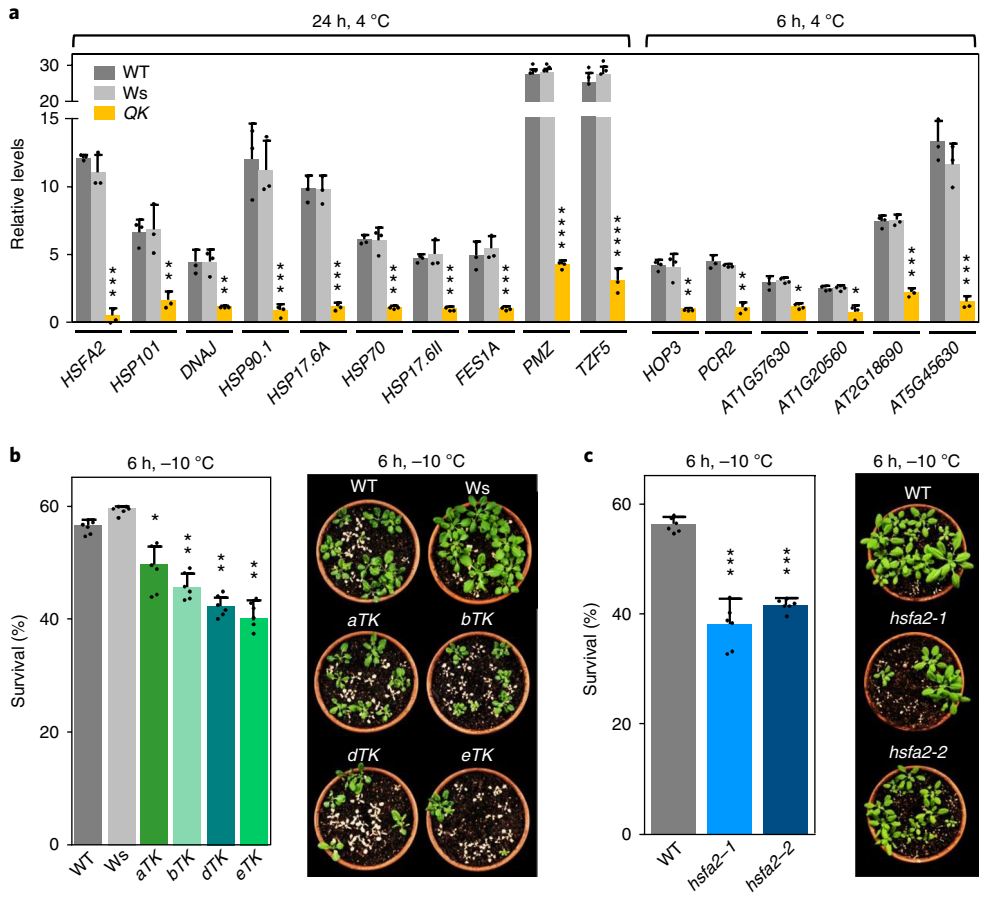

Fig. 4 | HSFA1 factors promote cold acclimation by inducing heat stress-responsive gene expression under low-temperature conditions. a, Expression of cold-inducible genes belonging to the HSFA1 regulon in 2-week-old Col-0 (WT), Wassilewskija (Ws) and *QK* plants grown under control conditions or exposed to 4 °C for 24 h or 6 h. Transcript levels, determined by qPCR, are represented as relative to their corresponding values under control conditions. Data represent the mean of three independent experiments and error bars show the s.d. 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 4 °C, as determined by one-sided analysis of variance (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 to −10 °C for 6 h after being acclimated at 4 °C for 7 d (left panels). Freezing tolerance was estimated as the percentage of plants surviving −10 °C after 10 d of recovery under control conditions. Data represent the mean of six independent experiments and error bars show the s.d. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) between *TK* mutants and WT and Ws, as determined by one-sided ANOVA (Bonferroni's post hoc test) (**b**), and between *hsfa2* mutants and WT (***$P < 0.001$), as determined by one-sided *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.

(Supplementary Fig. 7b) did not rescue their impaired ability to cold acclimate (Supplementary Fig. 7c).

Our results strongly suggest that the NPR1/HSFA1s interaction is essential for the cold induction of heat stress-responsive gene expression mediated by the HSFA1 transcription factors and, therefore, for full development of cold acclimation. To provide further support for this interpretation, the expression levels of the 16 cold-inducible genes downregulated in cold-treated *npr1* mutants that belonged to the HSFA1 regulon were analysed in *trxh3trxh5* and *snrk2.8-1* plants, which were deficient in NPR1 oligomer-to-monomer transition and nuclear translocation (Fig. 2b,c), exposed to 4 °C for 24 h. In all cases, the cold induction of these genes was significantly lower in *trxh3trxh5* and *snrk2.8-1* than in WT plants (Fig. 5c), indicating that the nuclear localization of NPR1 and, therefore, its interaction with HSFA1 factors is necessary to activate cold-induced heat stress-responsive gene expression. Overall, these findings demonstrated that NPR1 interacts with HSFA1 transcription factors to promote cold-induced heat stress-responsive gene expression and the cold acclimation response.

Discussion

Until now, 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 because *NPR1* transcripts do not accumulate by other cold-related stresses such as drought or high salt. In contrast to the response to pathogens, salicylic acid 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). This region, however, does not contain any described low-temperature responsive element.

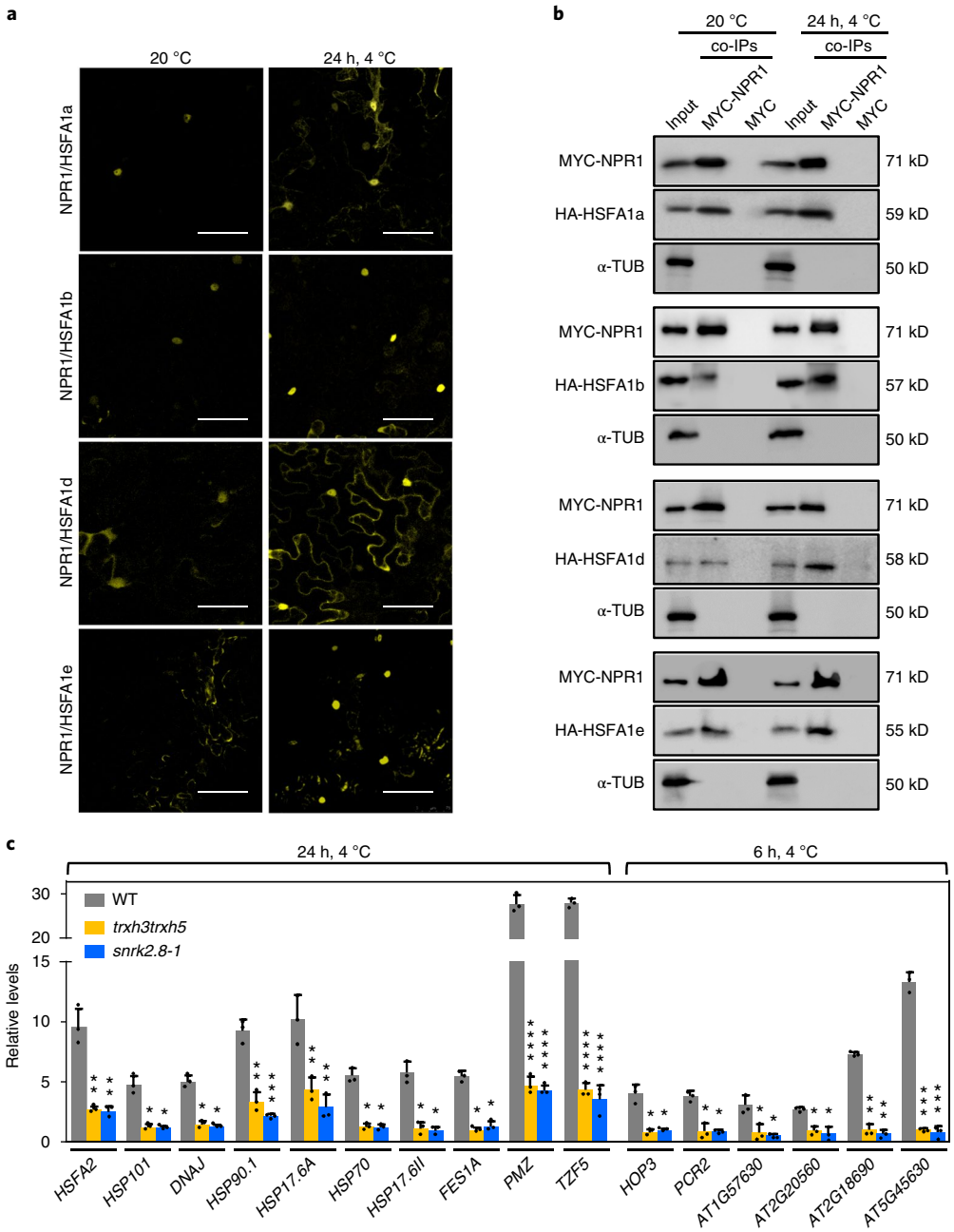

Fig. 5 | NPR1 interacts with HSFA1 factors to activate cold-induced heat stress-responsive gene expression. a, In vivo interactions between NPR1 and HSFA1 proteins by BiFC assays in *N. benthamiana* leaf cells under control (20 °C) or cold conditions (4 °C, 24 h). Reconstitution of YFP is shown. Scale bars, 75 µm. **b**, In vivo interactions between NPR1 and HSFA1 proteins by co-IP experiments in *N. benthamiana*. Input lanes contain protein extracts from plants grown at 20 °C or exposed to 4 °C for 24 h, expressing individual HA-HSFA1s fusion proteins and the MYC-NPR1 protein. co-IP lanes contain the same extracts used for inputs after being incubated with MYC antibody-conjugated Dynabeads (MYC-NPR1 lane), or extracts from plants expressing individual HSFA1s-HA fusion proteins and the MYC epitope alone (MYC lane). MYC-NPR1 and HA-HSFA1 fusion proteins were detected by immunoblotting with anti-MYC and anti-HA antibodies, respectively. Anti-α-TUB antibody was employed to verify equal amounts of protein extracts in inputs lanes. **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 4 °C for 24 h or 6 h. Levels, determined by qPCR, are represented as relative to their corresponding values in WT plants under control conditions. Data represent the mean of three independent experiments and error bars show the s.d. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) between mutants and WT exposed to 4 °C, as determined by one-sided *t*-test. In **a** and **b**, results are representative of three independent experiments.

NPR1 expression is positively regulated by pathogen-induced WRKY transcription factors during the activation of the plant defence response[37]. The *Arabidopsis* genome contains 29 *WRKY* genes whose expression is cold-induced[18]. The expression of two of these genes, *WRKY33* and *WRKY46*, in response to low temperature is mediated by NPR1 (Supplementary Table 2), suggesting that they could also mediate the cold induction of *NPR1*. Still, *wrky33-1* (ref. [38]) and *wrky46-3* (ref. [39]) mutants are not affected in cold induction of *NPR1* (Supplementary Fig. 8a), demonstrating that this is not the case. Understanding the

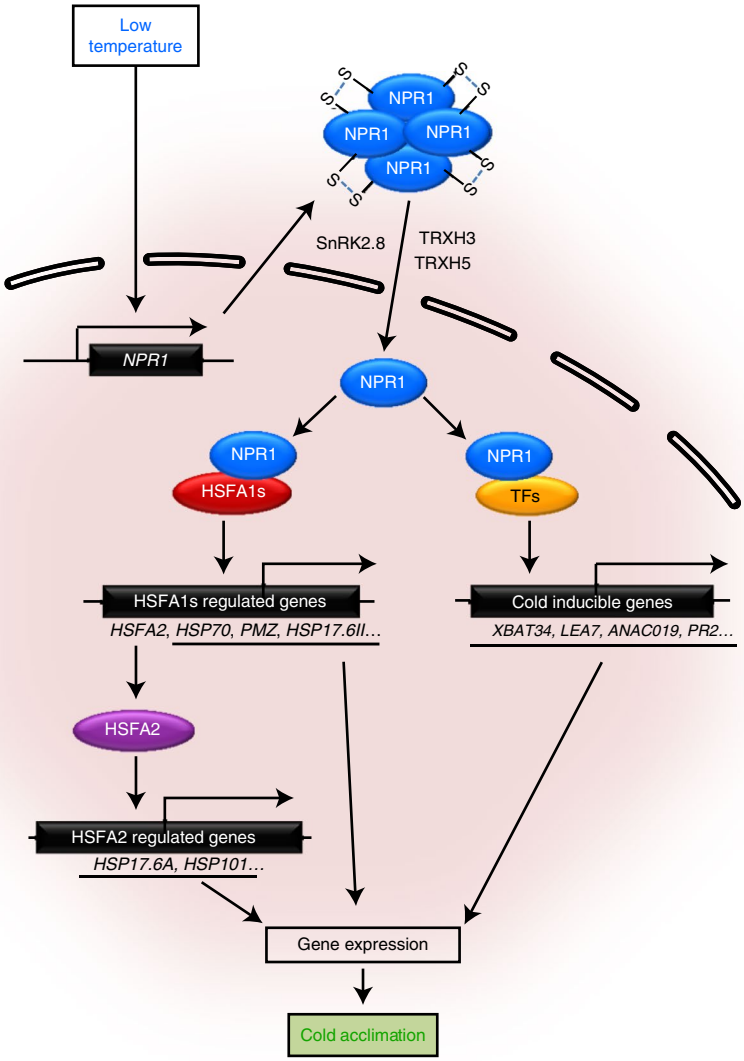

Fig. 6 | Proposed model for the function of NPR1 in cold acclimation response. The expression of NPR1 is induced in response to low temperature and is followed by an increase of the NPR1 protein, which accumulates in the nucleous in its monomeric form through a TRXH3/TRXH5–SnRK2.8-dependent pathway. In the nucleolus, NPR1 interacts with different transcription factors, including HSFA1s, to promote cold-induced gene expression and cold acclimation. The interaction of NPR1 with HSFA1 factors induces the expression of heat stress-responsive genes encoding chaperones that would minimize the impact of low temperatures and are essential for full development of the cold acclimation process.

molecular mechanisms underlying the induction of *NPR1* transcripts by low temperature, including the implication of some WRKY factors, 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[13], we found that at 20 °C NPR1 preferentially localizes to the cytoplasm of *Arabidopsis* cells in its oligomeric form. Furthermore, because it happens after pathogen infection[15], when plants are exposed to low temperature, it accumulates chiefly in the nucleus as a monomer. In this case, however, both redox modifications and phosphorylation of NPR1 by thioredoxins TRXH3/TRXH5 and SnRK2.8 kinase, respectively, are necessary for the oligomer-to monomer transition and its subsequent nuclear translocation. The implication of thioredoxins in the conformation of NPR1 seems to be related to the redox state of the cells[13]. Indeed, *trxh3trxh5* double mutants exhibit higher levels of reactive oxygen species (ROS) than WT plants under both control and cold conditions (Supplementary Fig. 8b), suggesting that TRXH3/TRXH5 contribute to controlling the cellular redox status and to attenuating the oxidative stress generated by low temperature, allowing the reduction of NPR1 oligomers and the release of NPR1 monomers.

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. Loss-of-function *npr1* mutants show a significantly lower capacity to cold acclimate than WT plants. In line with these results, *Arabidopsis* plants with increased levels of NPR1 display increased capacity to cold acclimate. Our preliminary data suggest that NPR1 also positively regulates *Arabidopsis* constitutive freezing tolerance (Supplementary Fig. 8c), indicating that it would be involved in both intrinsic and cold-induced tolerance to freezing temperatures. Deciphering the signalling pathways through which NPR1 promotes constitutive freezing tolerance represents an engaging challenge for future research. 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. It therefore does not play a general role in *Arabidopsis* tolerance to abiotic stresses but seems to

have a specific function in freezing tolerance. The cold induction of *NPR1* is independent of salicylic acid, 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 salicylic acid levels do not increase during cold acclimation in *Arabidopsis*, and that *Arabidopsis* mutants deficient in salicylic acid are not affected in their capacity to cold acclimate (Supplementary Fig. 9a,b). Still, the role of NPR1 in cold acclimation requires its monomerisation and subsequent nuclear translocation because *Arabidopsis* mutants deficient in TRXH3/TRXH5 and SnRK2.8 activities show impaired cold acclimation ability, similar to that of *npr1* mutants.

Global transcriptome profiles indicate that NPR1 positively regulates cold acclimation in *Arabidopsis* by promoting cold-induced gene expression. After 24 h of exposure to 4 °C, 71 cold-inducible genes display lower induction (≥two-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-quarter (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, HSFA1b, HSFA1d, HSFA1e) that function as the master regulators of the heat shock response[33]. The corresponding genes are constitutively expressed[32], and it has been estimated that more than 65% of the heat-stress-induced genes are HSFA1-dependent[33]. 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 which encode a secondary regulator of the heat shock response[32,33]. In addition to activating the heat shock response, HSFA1 and HSFA2 factors have been described as enhancing the plant response to other adverse environmental conditions, including anoxia, salt and osmotic stresses[33]; however, the implication of these transcription factors in plant response to low temperature has still not been documented. Here, we show that *Arabidopsis* plants deficient in HSFA1 or HSFA2 are unable to cold acclimate properly, demonstrating 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 mainly operate as molecular chaperones, facilitating the synthesis and ensuring the structural stability of other intracellular proteins under harsh environments[40]. Accordingly, during cold acclimation they would act as buffers to limit misfolding and resolve aggregates, minimizing the impact of the low temperatures on the proteome. Heat shock proteins in plants have also been linked to signalling, protein targeting and degradation[41–44], and they could carry out any of these functions in response to cold. 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 enhance gene expression[12]. To date, NPR1 has only been described as interacting with transcription factors from the TGA family, principally with TGA2, TGA5 and TGA6, after pathogen attack to induce *PR* gene expression and the subsequent defence response[12]. The data obtained in this work reveal that the NPR1 function in cold response promoting 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, tomato HSFA1 factors have also been reported as being able to interact with different proteins to induce transcription[45]. Our findings demonstrate that NPR1 interacts with the four HSFA1 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 promoting 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 acclimation through different regulatory pathways. A detailed study of the transcriptomic data does not offer any clue for identifying these roles and pathways. Deciphering their nature and the corresponding underlying molecular mechanisms requires additional investigation.

Based on the data described here, a hypothetical model for NPR1 function in *Arabidopsis* cold acclimation is proposed in Fig. 6. In response to low temperature, the expression of *NPR1* would be induced independently of salicylic acid, ABA and CBF. Concomitantly with this induction, there would be an increase of NPR1 protein that would translocate to the nucleus in its monomeric form in a TRXH3/TRXH5-SnRK2.8-dependent pathway. In the nucleus, monomeric NPR1 would operate, promoting cold-induced gene expression and cold acclimation by interacting with different transcription factors, including the HSFA1s. The interaction of NPR1 with the HSFA1 factors would induce the expression of numerous heat stress-responsive genes, mainly encoding chaperones that would act as powerful buffers to minimize the impact of low temperatures, and would be essential for the full development of the cold acclimation process. Interestingly, several of the 71 genes whose cold induction is mediated by NPR1, such as *PR2*, *WRKY46*, *DMR6*, *ANAC019* or *CRK6*, (Supplemental Table 2), have been implicated in pathogen response[46–49]. Considering that plant pathogens often propagate during cold seasons[6], it is tempting to speculate that in response to low temperature NPR1 would also promote increasing levels of PR proteins to prepare plants from possible pathogen attacks, in addition to promoting the accumulation of heat shock proteins. Hence, NPR1 seems to serve as a regulatory hub where pathways mediating biotic and abiotic stress responses converge and integrate to guarantee the precise development of *Arabidopsis* tolerance to adverse conditions. Identifying the complete repertoire of clients through which NPR1 mediates cold acclimation, and the molecular mechanisms that determine NPR1 involvement in pathogen and/or cold signalling, constitutes a remarkable goal for future studies that will provide new insights on how plants respond and adapt to fluctuating, and often adverse, natural environments.

Methods

Plant materials. *Arabidopsis thaliana* Col-0 and Ws ecotypes, and mutants *npr1-1* (ref. [25]), *sid2-2* (ref. [20]), *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). Plants containing the fusion *35 S:NPR1–GFP* and the *35 S:NPR1–GFP*, *35 S:NPR1^{sim3}-GFP*, *35 S:NPR1$^{S11/15A}$-GFP*, *35 S:NPR1$^{S11/15D}$-GFP* fusions in *npr1-1* (ref. [14]) and *npr1-2* (refs [26,27]) backgrounds, respectively, as well as the *npr1-1* (ref. [24]) *and cpr5* (ref. [50]) mutants were provided by X. Dong. The *sid2-1* mutant[51] was procured by R. Solano. The *aba2–11* mutant[23] was received from P. Rodriguez. The *tga2–1tga5–1tga6-1* triple mutant[30] was obtained from X. Li. The *aTK*, *bTK*, *dTK*, *eTK* and *QK* mutants[33] were supplied by Y.-Y. Charng. *NahG* transgenic plants[21] were furnished by M.E. Alvarez. The *cbf123-1* mutant[3] was obtained from J.-K. Zhu. The *wrky33-1* (ref. [38]) and *wrky46-3* (ref. [39]) insertion mutants were provided by I. Somssich. 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* 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[52]. The *NPR1*$_{PRO}$*-GUS* fusion was then introduced to WT plants, and the *NPR1*$_{PRO}$*-NPR1-MYC* fusion to *npr1-1*, *trxh3trxh5* and *snrk2.8-1* mutants via *Agrobacterium tumefaciens* (GV3101 strain) using the floral dip method[53]. For *NPR1*$_{PRO}$*-NPR1-MYC* fusion in *npr1-1* mutant, three independent transgenic lines (*c-npr1a*, *c-npr1b*, *c-npr1c*) were used for analyses. All transgenic lines were genetically determined to have the fusions integrated at a single locus in homozygosis. Full-length cDNAs corresponding to *NPR1*, *HSFA1a*, *HSFA1b*, *HSFA1d*, *HSFA1e* and *LSM8* genes were amplified with suitable primers (Supplementary Table 4) and the resulting PCR products cloned into the *pDONR207* vector (Invitrogen) to be subsequently tranferred to different destiny vectors. For BiFC assays, they were transferred to the *pYFN43* vector[54] to generate the *nYFP-NPR1* fusion or to a modified *pYFC43* vector[54], to which we added an MYC epitope (*pYFC43-MYC*) to generate the *cYFP-MYC-HSFA1s* and *cYFP-MYC-LSM8* fusions. For subcellular localization, the *HSFA1* cDNAs cloned in *pDONR207* were transferred to the *pMDC43* vector (Invitrogen) to obtain *GFP-HSFA1* fusions. For co-IP and pull-down assays (see later), the *HSFA1* cDNAs in *pDONR207* were transferred to the *pGWB15*[52] or *pDEST17* (Invitrogen) vectors to generate *HA-HSFA1* or *HIS-HSFA1* constructs, respectively. The *NPR1* cDNA cloned in *pDONR207* were transferred to the *pGWB618*[52] vector to generate the *35 S:MYC–NPR1* construct used in co-IP experiments. Plasmids containing the *HSFA1s* cDNAs fused to *YFP*, *GFP* and *HA*, the *LSM8* cDNA fused to *YFP*[16], and the *35 S:MYC–NPR1* construct were introduced into *Agrobacterium* strain GV3101 for agroinfiltration in 3-week-old *N. benthamiana* leaves (see later). Furthermore, the *pGWB15*[52] binary vectors containing the *HSFA1* cDNAs were used to transform *npr1-1* mutants via *Agrobacterium* (see earlier) and to obtain *35 S:HSFA1 Arabidopsis* plants. *pGWB* vectors were provided by T. Nakagawa. All constructs used in this work were made using Gateway technology and were validated by sequencing.

Growth conditions and treatments. Seeds were surface-sterilized, germinated and grown under standard conditions (20 °C under long-day photoperiods [16 h 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 at 4 °C for different times under a long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 40 μmol 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 300 mM sorbitol or 150 mM 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 at 4 °C for one additional day. For freezing tolerance assays, seeds from the different genotypes, each one coming from the same parent and collected at the same time, were sown in soil-containing pots and allowed to develop for 2 weeks under control conditions. Afterwards, several plants from each pot were removed in order to have a similar number (≈25) of individuals uniformly developed and distributed in all pots. Constitutive freezing tolerance was assessed by exposing plants to 4 °C for 30 min in darkness and subsequently decreasing the temperature at a rate of −1 °C per 30 min until reaching −6 °C. Six hours later, temperature was increased to 4 °C at the same rate and thawing was allowed for 12 h before returning plants to control conditions under long-day light regime for recovering. To determine freezing tolerance after cold acclimation, plants were acclimated at 4 °C for 7 d under long-day photoperiod and subsequently subjected to freezing in the dark by progressively decreasing the temperature (−1 °C per 30 min) until attaining the desired freezing temperature. After exposing plants to the appropriate freezing temperature for 6 h, temperature was gradually increased to 4 °C (+1 °C per 30 min). Twelve hours later, plants were transferred to 20 °C under long-day light regime for recovering. In all cases, survival rate was evaluated after 10 d of recovering. 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 300 mM sorbitol or 200 mM NaCl for 1 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. In each experiment, pots or plates were randomly positioned in the growth or treatment chambers.

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. In all cases, the relative expression values were calculated using the *At4g24610* gene as a reference[55]. Primers used are listed in Supplementary Table 4. All reactions were carried out 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 4 °C for 24 h 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 (Spain). RNAseq reads were aligned to the TAIR10 WT reference genome using TopHat2[56] 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[57]. This package internally estimates size factors for each sample, calculates dispersion for each gene and then fits a negative binomial generalized linear model 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 were cold induced and also downregulated in the *npr1-1* mutant. Selected settings were 'AtGenExpress-stress series' as data set and 'cold stress' as research area[18]. 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 two-fold. GO categorization was done with the ThaleMine data mining tool from Araport (www.araport.org). Significantly enriched GO terms ($P \leq 2 \times 10^{-6}$) were established using the Benjamini–Hochberg corrected hypergeometric test.

BiFC assays and microscopy analysis. Transient expression of fusion proteins for BiFC assays and for subcellular localization of HSFA1 factors was analysed by confocal microscopy, 3 days after agroinfiltration in leaves of 3-week-old *N. benthamiana* plants exposed to 20 °C or 4 °C for 24 h, as reported[58]. Similar expression of fusion proteins in BiFC assays was confirmed by WB experiments. 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 independent samples.

Immunoblot analysis and subcellular fractionation. Total proteins were extracted from 2-week-old *c-npr1a* plants grown under control conditions or exposed to 4 °C for different periods of time. Plants were ground in extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonident P-40) with inhibitors (40 μM MG132, protease inhibitor cocktail EDTA-free (Roche), 0.6 mM phenylmethyl sulfonyl fluoride, PMSF), and cell debris were pelleted by centrifugation (16,000*g*, 4 °C, 20 min) to obtain clear protein extracts. Protein concentration was determined by Bradford, using the Bio-Rad Protein Assay (Bio-Rad). Loading buffer containing 6 mM beta-mercaptoethanol was added to protein extracts for visualizing total NPR1 protein (reducing conditions). To visualize both monomeric and oligomeric NPR1 forms, we used loading buffer without beta-mercaptoethanol (non-reducing conditions). Proteins (50 μg) were resolved by electrophoresis on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to Hybond P 0.45 polyvinylidene difluoride membranes (Amersham), according to the manufacturer's protocol. To detect NPR1-MYC, cYFP-MYC-HSFA1s and cYFP-MYC-LSM8 fusion proteins, we used anti-c-MYC monoclonal antibody (sc-40; Santa Cruz Biotechnology). HA-HSFA1s and nYFP-NPR1 fusion proteins was detected by anti-HA monoclonal antibody (sc-7392; Santa Cruz Biotechnology) and anti-GFP antibody (ab190584; Abcam), respectively. α-TUB, employed as a protein loading control, was detected using anti-α-tubulin monoclonal antibody (T60T4; Sigma). Western blots were quantified using the ImageJ software (https://imagej.nih.gov/ij/). The intensities of the band signals were corrected with respect to the corresponding loading controls and represented as relative to a given control lane.

Subcellular fractionation was performed as reported previously[59] using extracts from 2-week-old *c-npr1a* plants and transgenic *trxh3trxh5* and *snrk2.8-1* plants containing the *NPR1*$_{PRO}$*-NPR1-MYC* fusion grown under control conditions or exposed to 4 °C for 24 h. Isolated proteins were fractionated by electrophoresis, transferred to membranes as described earlier and analysed by immunoblotting using anti-α-tubulin monoclonal (see earlier) and anti-Histone H3 polyclonal (Ab1791, Abcam) antibodies for control of the cytoplasmic and nuclear fractions, respectively.

In all cases, horseradish peroxidase-conjugated secondary antibodies were used for primary antibody detection. Signals were detected with the ECL Western Blotting Detection Kit (Amersham) and assays were performed in triplicate using three independent protein samples.

co-IP assays. co-IP assays were conducted as previously described[60]. Briefly, plasmids containing the *35S-HA-HSFA1a*, *35S-HA-HSFA1b*, *35S-HA-HSFA1d*, *35S-HA-HSFA1e*, *35S-MYC-NPR1* and 35S-*MYC* constructs (see earlier) were introduced into *A. tumefaciens*. Three-week-old *N. benthamiana* plants were then co-infiltrated with two *Agrobacterium* cultures, one expressing a *HSFA1* construct and the other the *35S-MYC-NPR1* fusion. The 35S-*MYC* construct was co-infiltrated in negative controls. For agroinfiltration, cultures were at an optical density of 0.2 in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl and 200 µM acetosyringone). Three days after agroinfiltration, tobacco leaves were collected under control conditions or after being exposed to 4 °C for the last 24 h and total proteins extracted with co-IP buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 2 mM Na_3VO_4, and 2 mM NaF, 1 mM PMSF, protease inhibitor cocktail EDTA-free (Roche) and 50 µM proteasome inhibitor MG132). Debris were removed by centrifugation at 12,000*g* for 10 min, and 3 mg of total protein extracts were mixed with anti-MYC (SC-40, Santa Cruz Biotechnology) antibody-conjugated Dynabeads® Protein G (10003D, Invitrogen), following the manufacturer's instructions, and gently shaken overnight at 4 °C. Afterwards, beads with immunoprecipitates were extensively washed three times with co-IP buffer and resuspended in Laemmli buffer. Proteins were resolved in 12% SDS-PAGE gels. As an input, 6% of the total protein extracts was loaded. Immunodetections were performed with monoclonal antibodies anti-MYC and anti-HA (see earlier). We used anti-α-tubulin monoclonal antibody (see earlier) to confirm equal protein loading from control and cold-treated plants. In all cases, co-IP assays were performed in triplicate using three independent protein samples.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. All primers used in this study are described in Supplementary Table 4. 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.

Received: 9 October 2017; Accepted: 16 August 2018;
Published online: 24 September 2018

References

1. Levitt, J. *Chilling, Freezing, and High Temperature Stresses* 2nd ed, (Academic Press, New York, 1980).
2. Ruelland, E., Vaultier, M. N., Zachowski, A. & 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 Ttanscription factors in cold acclimation in *Arabidopsis*. *Plant Physiol.* **171**, 2744–2759 (2016).
4. Hon, W. C., Griffith, M., Mlynarz, A., Kwok, Y. C. & Yang, D. S. Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiol.* **109**, 879–889 (1995).
5. 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).
6. Griffith, M. & Yaish, M. W. F. Antifreeze proteins in overwintering plants: a tale of two activities. *Trends Plant Sci.* **9**, 399–405 (2004).
7. 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).
8. 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).
9. 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).
10. 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).
11. 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).
12. Fu, Z. Q. & Dong, X. Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* **64**, 839–863 (2013).
13. Tada, Y. et al. Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**, 952–956 (2008).
14. Kinkema, M., Fan, W. & Dong, X. Nuclear localization of NPR1 is required for activation of *PR* gene expression. *Plant Cell* **12**, 2339–2350 (2000).
15. Lee, H.-J. et al. Systemic immunity requires SnRK2.8-mediated nuclear import of NPR1 in *Arabidopsis*. *Plant Cell* **27**, 3425–3438 (2015).
16. Hermann, M. et al. The *Arabidopsis* NIMIN proteins affect NPR1 differentially. *Front. Plant Sci.* **4**, 1–15 (2013).
17. Fu, Z. Q. et al. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486**, 228–232 (2012).
18. 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).
19. Kim, Y., Park, S., Gilmour, S. J. & 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).
20. Wildermuth, M. C., Dewdney, J., Wu, G. & Ausubel, F. M. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562–565 (2001).
21. Lawton, K. et al. Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant Microbe. Interact.* **8**, 863–870 (1995).
22. 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).
23. 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).
24. Cao, H., Bowling, S. A., Gordon, A. S. & Dong, X. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**, 1583–1592 (1994).
25. Shah, J., Tsui, F. & 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).
26. Spoel, S. H. et al. Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* **137**, 860–872 (2009).
27. Saleh, A. et al. Post-translational modifications of the master transcriptional regulator NPR1 enable dynamic but tight control of plant immune responses. *Cell Host Microbe* **18**, 169–182 (2015).
28. Chinnusamy, V., Zhu, J. & Zhu, J.-K. Cold stress regulation of gene expression in plants. *Trends Plant Sci* **12**, 444–451 (2007).
29. Blanco, F. et al. Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol. Biol.* **70**, 79–102 (2009).
30. Zhang, Y., Tessaro, M. J., Lassner, M. & 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).
31. Fan, W. & 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).
32. 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).
33. Liu, H. C., Liao, H. T. & 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).
34. 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).
35. Scharf, K.-D., Berberich, T., Ebersberger, I. & Nover, L. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochim. Biophys. Acta* **1819**, 104–119 (2012).
36. Perea-Resa, C., Hernandez-Verdeja, T., Lopez-Cobollo, R., Castellano, Md. M. & Salinas, J. LSM proteins provide accurate splicing and decay of selected transcripts to ensure normal *Arabidopsis* development. *Plant Cell* **24**, 4930–4947 (2012).
37. Yu, D., Chen, C. & Chen, Z. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* **13**, 1527–1540 (2001).
38. Birkenbihl, R. P., Diezel, C. & Somssich, I. E. *Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. *Plant Physiol.* **159**, 266–285 (2012).
39. Moreau, M. et al. EDS1 contributes to nonhost resistance of *Arabidopsis* thaliana against Erwinia amylovora. *Mol. Plant Microbe. Interact.* **25**, 421–430 (2012).
40. Carey, C. C., Gorman, K. F. & Rutherford, S. Modularity and intrinsic evolvability of Hsp90-buffered change. *PLoS ONE* **1**, e76 (2006).
41. Huang, S. et al. HSP90s are required for NLR immune receptor accumulation in *Arabidopsis*. *Plant J.* **79**, 427–439 (2014).
42. Kadota, Y. & Shirasu, K. The HSP90 complex of plants. *Biochim. Biophys. Acta* **1823**, 689–697 (2012).

43. Kriechbaumer, V., von Löffelholz, O. & Abell, B. M. Chaperone receptors: guiding proteins to intracellular compartments. *Protoplasma* **249**, 21–30 (2012).
44. Lee, S. et al. Heat shock protein cognate 70-4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in *Arabidopsis*. *Plant Cell* **21**, 3984–4001 (2009).
45. Bharti, K. et al. Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. *Plant Cell* **16**, 1521–1535 (2004).
46. Hu, Y., Dong, Q. & Yu, D. *Arabidopsis* WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. *Plant Sci.* **185-186**, 288–297 (2012).
47. Zeilmaker, T. et al. DOWNY MILDEW RESISTANT 6 and DMR6-LIKE OXYGENASE 1 are partially redundant but distinct suppressors of immunity in *Arabidopsis*. *Plant J.* **81**, 210–222 (2015).
48. Bu, Q. et al. Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Res.* **18**, 756–767 (2008).
49. Yeh, Y. H. et al. Enhanced *Arabidopsis* pattern-triggered immunity by overexpression of cysteine-rich receptor-like kinases. *Front. Plant Sci.* **6**, 322 (2015).
50. Bowling, Sa, Clarke, J. D., Liu, Y., Klessig, D. F. & Dong, X. Thecpr5 mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* **9**, 1573–1584 (1997).
51. Adie, B. A. T. et al. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* **19**, 1665–1681 (2007).
52. Nakagawa, T. et al. Improved gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol. Biochem.* **71**, 2095–2100 (2007).
53. Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W. & Chua, N.-H. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* **1**, 641–646 (2006).
54. Belda-Palazón, B. et al. Aminopropyltransferases involved in polyamine biosynthesis localize preferentially in the nucleus of plant cells. *PLoS ONE* **7**, e46907 (2012).
55. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. & Scheible, W.-R. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139**, 5–17 (2005).
56. Kim, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **14**, R36 (2013).
57. Love, M. I. & Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNAseq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
58. English, J., Davenport, G., Elmayan, T., Vaucheret, H. & Baulcombe, D. Requirement of sense transcription for homology-dependent virus resistance and *trans*-inactivation. *Plant J.* **12**, 597–603 (1997).
59. Locascio, A., Blázquez, M. A. & Alabadí, D. Dynamic regulation of cortical microtubule organization through prefoldin-DELLA interaction. *Curr. Biol.* **23**, 804–809 (2013).
60. Lazaro, A., Mouriz, A., Piñeiro, M. & Jarillo, J. A. Red light-mediated degradation of CONSTANS by the E3 ubiquitin ligase HOS1 regulates photoperiodic flowering in *Arabidopsis*. *Plant Cell* **9**, 2437–2454 (2015).

Acknowledgements

We thank all our colleagues who 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 the 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. analysed the data. J.S. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41477-018-0254-2.

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Software and code

Policy information about availability of computer code

Data collection	TAIR (https://www.arabidopsis.org) ThaleMine (https://apps.araport.org/thalemine) BAR (bar.utoronto.ca)

Data analysis	Prism 6.0 (GraphPad Software Inc., http;//www.graphpad.com)
	ImageJ (https://imagej.nih.gov/ij/)

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The data that support the findings of this study are available from the corresponding author upon request. All primers used in this study are described in Supplementary Table 4. 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.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We chose the sample size according to previous studies (Catala et al., Plant Cell 26: 3326, 2014; Perea-Resa et al., Mol. Plant 10: 791, 2017).
Data exclusions	No data were excluded from the analyses.
Replication	All experiments were repeated at least three times and similar results were obtained.
Randomization	Plants were always randomly distributed in the growth/treatment chambers.
Blinding	Investigators were not blinded since this is not relevant to our study. Data were always collected according to the genotype of plants.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	All unique materials used are available from the authors of from standard commercial sources.

Antibodies

Antibodies used	α -MYC (9E10), cat. SC-40, Santa Cruz Biotechnology #I1415, dilution 1:500. α -TUB (B512), cat. T6074, Sigma , dilution 1:10,000. α –HIS, cat. H1029, Sigma, dilution 1:1,000. α-HA (F-7), cat. SC7392, Santa Cruz Biotechnology #I1115, dilution 1:500.

α-H3, cat. Ab1791, Abcam #GR3185000-1, dilution 1:1000.
α-GFP-HRP (E385), cat. Ab190584, Abcam #GR3193339-2, dilution 1:5000.
Goat-α-Rabbit-HRP, cat. SC-2004, Santa Cruz Biotechnology #F2116, dilution 1:5,000.
Goat-α-Mouse-HRP, cat. SC-2005, Santa Cruz Biotechnology #L0312, dilution 1:5,000.

Validation

All antibodies were tested with controls