Inducer of CBF Expression 1 (ICE1) Promotes Cold-enhanced Immunity by Directly Activating Salicylic Acid Signaling

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ABSTRACT

Cold stress affects plant immune responses, and this process may involve the salicylic acid (SA) signaling pathway. However, the underlying mechanism by which low temperature signals coordinate with SA signaling to regulate plant immunity remains poorly characterized. Here, we found that low temperatures enhanced the disease resistance of Arabidopsis against *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. This process required Inducer of CBF expression 1 (ICE1), the core transcription factor in cold-signal cascades. ICE1 physically interacted with Non-expresser of *PR* genes 1 (NPR1), the master regulator of the SA signaling pathway. Enrichment of ICE1 on the *PR1* promoter and its ability to transcriptionally activate *PR1* were enhanced by NPR1. Further analyses revealed that cold stress signals cooperate with SA signals to facilitate plant immunity against pathogen attack in an ICE1-dependent manner. Cold treatment promoted interactions of NPR1 and TGA3 with ICE1, and increased the ability of the ICE1–TGA3 complex to transcriptionally activate *PR1*. Together, our results characterize a previously unrecognized role of ICE1 as an indispensable regulatory node linking low temperature activated- and SA-regulated immunity. Discovery of a crucial role of ICE1 in coordinating multiple signals associated with immunity broadens our understanding of plant–pathogen interactions.

INTRODUCTION

In nature, plants can be attacked by a wide range of pathogens, including bacteria, fungi, oomycetes, viruses, and nematodes. In response, plants have developed a sophisticated innate immune system during co-evolution. The first layer of the defense system is governed by the plasma membrane (PM)-localized pattern recognition receptors (PRRs). The PRRs recognize the microorganism-derived pathogen-associated molecular patterns (PAMPs) and trigger the first layer of defense responses–this is known as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Dodds and Rathjen, 2010). PTI is generally believed to be sufficient to prevent non-pathogenic microbes from proliferating *in planta*. However, some successful pathogens can deliver effector proteins to evade or suppress host PTI, allowing them to multiply aggressively (Kinya Nomur and He, 2005; Boller and Felix, 2009). To counter pathogen inhibition of PTI, plants have evolved intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) that detect cellular effectors, directly or indirectly, and cause strong and robust immune responses. These responses, known as effector-triggered immunity (ETI), can stop virulent pathogens from causing severe disease symptoms (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Deslandes and Rivas, 2012). Along with the two branches of immune system, the defense hormone salicylic acid (SA) plays a central role in these responses (Spoel and Dong, 2012). For example, the accumulation of SA at infection sites subsequently leads to systemic acquired resistance (SAR) against *Pseudomonas syringae* throughout the plant to limit pathogen propagation (Durrant and Dong, 2004; Vlot et al., 2009).

In the SA signaling transduction pathway, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) has been well documented as one of the SA receptors that perceives and transduces SA signals (Wang et al., 2020; Kumar et al., 2022). In the resting state, NPR1 exists in the cytoplasm as an oligomer. After induction, NPR1 monomers are released from oligomers and translocated to the nucleus, where they act as cofactors of transcription factors, such as the TGACG motif-binding transcription factors (TGAs), which regulate global transcriptional reprogramming and resistance to a broad spectrum of pathogens (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Johnson et al., 2003; Kumar et al., 2022). Recently, NPR1 has attracted

attention because of its central roles in plant growth and development, as well as in plant immune responses. For instance, it was reported that NPR1 interferes with the binding of ETHYLENE-INSENSITIVE 3 (EIN3), a key transcription factor involved in ethylene (ET) signaling, to the promoter regions of its target genes to mediate antagonism between SA and ET in apical hook formation (Huang et al., 2020). The transcriptional activation activity of EIN3 is enhanced by NPR1 in a process coordinated by SA and ET during the modulation of leaf senescence (Wang et al., 2021; Yu et al., 2021). Moreover, NPR1 promotes the polyubiquitination and degradation of GID1, the gibberellin receptor, to balance growth and defense (Yu et al., 2022). NPR1 is also involved in facilitating cell survival during ETI by ubiquitinating key proteins involved in immune responses, such as ENHANCED DISEASE SUSCEPTIBILITY 1 and WRKY transcription factors (Zavaliev et al., 2020). NPR1 inhibits the transcriptional activation activity of MYC2, the master regulator of jasmonic acid signaling pathway, to suppress pathogen virulence (Nomoto et al., 2021).

The TGA transcription factors are members of the basic leucine zipper (bZIP) family of proteins, which specifically bind to the TGACG-core activation sequence 1 (*as-1*) to regulate the transcriptional levels of their target genes (Després et al., 2000; Zhou et al., 2000; Johnson et al., 2003). Ten TGA transcription factors are encoded in the Arabidopsis genome, of which TGA1 to TGA7 have been characterized in terms of their ability to interact with NPR1 (Zhang et al., 1999; Després et al., 2000). Among them, TGA3 is mainly required for basal resistance and *PR* gene expression (Kesarwani et al., 2007; Kumar et al., 2022), and also integrates with other signals. For instance, TGA3 interacts with ARR2, the cytokinin-activated transcription factor, to promote plant immunity through NPR1/TGA3-dependent SA signaling (Choi et al., 2010). A protein complex of TGA3 and WRKY53 acts on the *Cestrum yellow leaf curling virus* promoter to achieve synergistic up-regulation of SA-associated gene expression (Sarkar et al., 2018). BRASSINOSTEROID INSENSITIVE2 (BIN2) balances SA-induced immunity and brassinosteroid-mediated plant growth through phosphorylating TGA3 to activate gene expression (Han et al., 2022).

Low temperature has significant effects on plant growth and development

(Orvar et al., 2000; Wang et al., 2009). The ICE1/C-REPEAT BINDING FACTOR/DRE-BINDING FACTOR 1 (ICE1/CBF/DREB1) pathway plays an essential role in plants' tolerance to low temperature stress, and ICE1 acts as an important transcriptional regulator of cold-responsive gene expression and cold tolerance (Stockinger et al., 1997; Kim et al., 2015). ICE1, a basic helix-loop-helix (bHLH) protein in the MYC class, directly binds to the MYC site (CANNTG) in the promoter of *CBF3* to induce its expression under cold stress (Gilmour et al., 1998; Chinnusamy et al., 2003). ICE1 also binds to the promoters of other genes encoding regulatory factors such as cold-responsive (COR), calmodulin binding transcription activator 2, mitogen-activated protein kinase 3 (MPK3), MPK4, and high expression of osmotically responsive genes 1 (HOS1), all of which are critical for the cold response (Tang et al., 2020). As an essential component of the cold signaling network, ICE1 functions as a key cross-talk node between low temperature signals and other signals. For instance, HOS1, SUMO E3 ligase (SIZ1), Open Stomata 1 (OST1), MPK3/6, and BIN2 are involved in regulating responses to freezing by maintaining homeostasis of the ICE1 protein under cold stress (Dong et al., 2006; Miura et al., 2007; Ding et al., 2015; Li et al., 2017; Zhao et al., 2017; Ye et al., 2019). Moreover, ICE1 integrates endogenous and environmental signals to mediate diverse physiological processes, such as seed germination, flowering time, and stomatal development (Lee et al., 2015, 2017; Hu et al., 2019; An et al., 2021; An et al., 2022).

Plant–pathogen interactions are affected by the ambient temperature, and low temperature can promote plant immune responses. For example, under cold temperatures, winter rye (*Secale cereale*) accumulates abundant pathogenesis-related (PR) proteins that help protect against pathogen attack (Griffith and Yaish, 2004). Singh (2014) found that 7 days of repeated cold stress activated the PTI immune response of Arabidopsis in a histone acetyltransferase 1 (HAT1)-dependent manner, thereby enhancing resistance to the virulent pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Wu (2019) reported that low temperatures induced a transient defense response to *Pst* DC3000 in plants. Specifically, low temperatures induced H_2O_2 accumulation, callose deposition, and defense gene expression (Wu et al., 2019). Notably, there is a growing body of evidence showing that SA-mediated

defense responses are activated at low temperature in the interaction between Arabidopsis and *Pseudomonas syringae*. In particular, SA biosynthetic genes, including *ISOCHORISMATE SYNTHASE1* (*ICS1*), *ISOCHORISMATE SYNTHASE2* (*ICS2*), *CALMODULIN BINDING PROTEIN 60-LIKE.g* (*CBP60g*), *SAR DEFICIENT1* (*SARD1*), and *PHENYLALANINE AMMONIA LYASE1* (*PAL1*), are up-regulated by cold stress, activating the SA-mediated immune pathway (Kim et al., 2013; Kim et al., 2017; Wu et al., 2019; Li et al., 2020). Cold temperatures induce expression of the SA signaling-responsive genes *PR1*, *PR2,* and *PR5,* whose encoded products increase plant immunity (Seo et al., 2010). These results suggest that there are broad signaling interactions between low temperature signals and SA responses. Nonetheless, the underlying molecular mechanisms and signaling pathways are unknown.

In this study, we found that the enhancement of plant immune responses by low temperatures largely depends on ICE1, the core transcription factor in the cold signaling pathway. Mechanistic analyses revealed that the ICE1 protein physically interacts with the SA receptor NPR1 and is involved in SA-mediated resistance against *Pst* DC3000. Further analyses demonstrated that ICE1 directly activates the transcription of *PR1* and NPR1 promotes its activities. We also found that ICE1 interacts with TGA transcription factors to cooperatively stimulate *PR1* expression under low temperatures. Taken together, our results demonstrate that the NPR1-TGA3/ICE1 regulatory module acts as a crucial node integrating cold signals and SA signaling in the synergistic regulation of plant immunity.

RESULTS

ICE1 Is Involved in Low Temperature-enhanced Plant Immunity

Low temperature has complicated effects on plant immunity (Yang and Hua, 2004; Huang et al., 2010; Yang et al., 2010; Wigge, 2013). To investigate the underlying relationship between low temperature and plant immunity, we assessed effect of low temperature on plant immune responses against the hemibiotropic pathogenic bacteria, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. For these analyses, we monitored disease symptoms and populations of *Pst* DC3000 in Arabidopsis Columbia-0 (Col-0) plants that were grown at normal temperature (22°C) for 4 weeks and then exposed to a

205 long-term (3 weeks) or short-term (10 hours) cold treatment (4°C) before
206 infiltration with the pathogen. We observed that the pathogen-related
207 water-soaking symptom, one of the earliest and most common symptoms of
208 phyllosphere bacterial diseases, as well as bacterial proliferation in infected
209 leaves, were largely reduced in Col-0 plants exposed to a short or long cold
210 treatment, compared with those kept at 22°C (Fig. 1 A–D). These results
211 confirmed that both long- and short-term low temperature treatments promote
212 plant immunity against *Pst* DC3000, consistent with the results of previous
213 studies (Seo et al., 2010; Kim et al., 2017; Wu et al., 2019).
214 ICE1 is a transcriptional inducer of genes that encode important components
215 of chilling tolerance of Arabidopsis (Chinnusamy et al., 2003; Lee et al., 2005;
216 Benedict et al., 2006; Chinnusamy et al., 2007). At the early stages of cold
217 stress, ICE1 in its phosphorylated form activates the expression of *CBFs* to
218 enhance plant freezing tolerance (Ding et al., 2015; Ye et al., 2019). Given the
219 critical role of ICE1 at the early stage of the cold stress response, we
220 hypothesized that ICE1 may be involved in short-term low
221 temperature-enhanced immunity. To test this possibility, we investigated *Pst*
222 proliferation in Col-0, the loss-of-function *ice1-2* mutant, and an
223 *ICE1*-complemented mutant (*ice1-2/ICE1*) after short-term low temperature
224 treatment. As shown in Fig. 1E, F, the leaves of Col-0 and *ice1-2/ICE1*
225 exhibited clearly enhanced disease-resistance phenotypes, with less severe
226 water-soaking symptoms and lower pathogen populations after cold treatment,
227 compared with the control. However, in *ice1-2* mutants, the water-soaking
228 symptoms were severe and the pathogen population was large (Fig. 1E, F).
229 These results suggest that ICE1 may positively regulate cold stress-enhanced
230 plant immunity. To confirm the role of ICE1, we used two stable
231 *ICE1*-overexpressing transgenic lines, *35S::GFP-ICE1* (Chinnusamy et al.,
232 2003); hereinafter *ICE1-OE 1*) and *35S::3Myc-ICE1* (hereinafter *ICE1-OE 2*)
233 for bacterial propagation assays. As expected, compared with Col-0, the
234 *ICE1*-overexpressing plants showed enhanced resistance, including less
235 severe water soaking symptoms and lower pathogen proliferation after low
236 temperature stress (Fig. 1G, H). Together, these results indicate that ICE1 acts
237 as a positive regulator of cold stress-enhanced plant immunity.
238

ICE1 Physically Interacts with NPR1

ICE1 regulates diverse physiological processes through associating with other proteins (Ding et al., 2015; Lee et al., 2015; Hu et al., 2019; Ye et al., 2019; An et al., 2021; An et al., 2022). To explore the underlying molecular mechanism by which ICE1 regulates plant immune responses, we performed yeast two-hybrid screening analyses to identify its potential interacting protein partners. A potential candidate, NPR1, was identified and further characterized (Fig. 2A). To identify the functional region responsible for the interaction between ICE1 and NPR1, two truncated versions of the proteins were used in yeast two-hybrid assays. As shown in Fig. 2A, deletion of the N-terminal amino acid residues 1–260 of ICE1 (AD-ICE1$^{1-260}$) did not affect the ICE1–NPR1 interaction, whereas deletion of the C-terminal residues of ICE1 containing the bHLH domain (AD-ICE1$^{261-494}$) eliminated the interaction completely (Fig. 2A). Additionally, the C-terminal amino acid residues of NPR1 containing the BTB structural domain (BD-NPR1$^{1-194}$) strongly interacted with ICE1, while the N-terminal fragment (BD-NPR1$^{178-593}$) did not. These results demonstrate that the bHLH structural domain of ICE1 and the BTB structural domain of NPR1 are necessary for the ICE1–NPR1 interaction.

Bimolecular fluorescence complementation (BiFC) assays were conducted to further confirm the interaction between ICE1 and NPR1. For these assays, NPR1 fused to the C-terminal of YFP (NPR1-cYFP) and ICE1 fused to the N-terminal of YFP (ICE1-nYFP) were co-expressed in *Nicotiana benthamiana* leaves, and then fluorescence in the leaves was observed under a laser confocal microscope. Almost no YFP fluorescence was observed when NPR1-cYFP was co-expressed with ICE1-nYFP in *N. benthamiana* leaves at 22°C (Supplemental Fig. S1A), but the fluorescence signal became very strong after induction at 4°C for 4 h (Fig. 2B; Supplemental Fig. S2A), suggesting that the association between NPR1 and ICE1 may depend on low temperature *in vivo*. To further verify this interaction, we performed a split luciferase assay by fusing these proteins to the C-terminus (cLUC) or N-terminal (nLUC) of luciferase, with LUC fused with β-glucuronidase (GUS) as the negative control. Similarly, very faint LUC activity was detected when NPR1-cLUC was transiently co-expressed with ICE1-nLUC in *N. benthamiana*, but strong LUC signals were observed after low temperature induction (Fig. 2C; Supplemental

Fig. S1B).

Consistently, in a co-immunoprecipitation (Co-IP) assay, ICE1 was immunoprecipitated by anti-GFP agarose beads from Arabidopsis protoplasts co-expressing NPR1-YFP and ICE1-Myc, but not from those co-expressing YFP and ICE1-Myc, and more precipitation products were detected after low temperature induction (Fig. 2D). Taken together, these results indicate that ICE1 interacts with NPR1 both *in vitro* and *in vivo*, and that low temperature potentiates the association between ICE1 and NPR1 *in planta*.

ICE1 Is Required for SA-mediated Plant Immunity

Because ICE1 physically associates with the SA receptor NPR1 (Fig. 2), we hypothesized that ICE1 may participate in SA signal transduction. To test this hypothesis, we first detected changes in the transcript levels of *ICE1* in response to benzothiadiazole (BTH), a synthetic analog of SA that effectively induces SA responses and is extensively used as an alternative to SA signaling (Huot et al., 2017; Kim et al., 2022). Interestingly, BTH treatment did not affect the transcript level of *ICE1*, but led to the accumulation of the ICE1 protein to high levels (Supplemental Fig. S3). Next, we examined whether ICE1 is involved in SA-regulated immunity by conducting a BTH protection assay. For this assay, wild-type Col-0, *ice1-2,* and *ice1-2/ICE1* plants were pre-treated with BTH for 24 h before pathogen infiltration, and then their susceptibility to disease caused by *Pst* DC3000 was evaluated. Compared with the mock pretreated plants, the BTH-pretreated Col-0 and *ice1-2/ICE1* plants exhibited greatly reduced disease symptoms on the leaves after *Pst* DC3000 infection (Fig. 3A and 3B). In contrast, the leaves of *ice1-2* displayed severe disease symptoms, i.e., severe water soaking symptoms and strong pathogen growth, after *Pst* DC3000 infection (Fig. 3A, B). The transcript levels of *PR1* were also lower in BTH-pretreated *ice1-2* plants (Fig. 3C). These results suggest that ICE1 may be required for effective SA-regulated immunity. To further confirm the essential role of ICE1 in SA-regulated immunity, we analyzed the disease symptoms of pretreated Col-0 and *ICE1*-overexpressing plants (*ICE1-OE 1* and *ICE1-OE 2*). Compared with Col-0 plants, BTH-pretreated *ICE1*-overexpressing plants showed less severe disease symptoms after inoculation with *Pst* DC3000, accompanied by BTH-induced

307 high transcript levels of *PR1* (Fig. 3D–F).

308 *PR1*, whose expression is activated by the NPR1–TGA regulation module,
309 is a principal output gene of SA-associated immunity (Zhang et al., 1999;
310 Després et al., 2000; Zhou et al., 2000; Johnson et al., 2003). Given that ICE1
311 interacts with NPR1 and plays an essential role in the SA signaling pathway
312 (Figures 2 and 3), we wondered whether ICE1 directly regulates the
313 transcription of *PR1* in a similar manner as TGA transcription factors. ICE1 is a
314 MYC-like bHLH transcriptional activator that binds specifically to the MYC
315 recognition sequences (CANNTG, also known as the E-box) in the *CBF3*
316 promoter (Chinnusamy et al., 2003; Tang et al., 2020). We analyzed the
317 promoter of PR1 and found that some MYC recognition sequences exist in the
318 promoter of *PR1*, 1000 bp upstream of ATG (Supplemental Fig. S4). Hence,
319 we performed electrophoresis mobility shift assays (EMSAs) to determine
320 whether ICE1 could bind directly to the E-box *cis*-element in the promoter of
321 *PR1*. As shown in Fig. 3G, the ICE1 protein was able to bind to the E-box in the
322 *PR1* promoter (Fig. 3G). To verify these findings, we conducted chromatin
323 immunoprecipitation (ChIP) assays with *ICE1-Myc* (*ICE1-OE 2*) plants treated
324 or not with BTH. We observed that ICE1 was enriched in the *PR1* promoter
325 region, and BTH promoted its enrichment (Fig. 3H). Next, a dual-luciferase
326 reporter assay was conducted to determine the effects of ICE1 on *PR1*
327 transcription. *LUC* expression driven by the *PR1* promoter was significantly
328 activated by ICE1 (Fig. 3I). Taken together, these results indicate that ICE1 is
329 required for effective SA-associated immunity *via* direct activation of *PR1*
330 transcription.

331

332 **NPR1 Enhances the Ability of ICE1 to Transcriptionally Activate *PR1***

333 Having established that ICE1 interacts with NPR1 and positively regulates SA
334 responses through direct binding to the promoter region of *PR1*, we
335 investigated whether ICE1 functions in a NPR1-relevant manner. To test this
336 possibility, we generated *ice1-2 npr1-1* double mutant plants by crossing the
337 *ice1-2* mutant with *npr1-1*, a loss-of-function mutant allele of *NPR1* (Col-0
338 background;(Cao et al., 1997)). Similar to the *ice1-2* and *npr1-1* single mutants,
339 the *ice1-2 npr1-1* double mutant displayed a highly susceptible phenotype to
340 *Pst* DC3000 (Fig. 4A, B), implying that ICE1 may act in the same pathway as

341 NPR1 to mediate plant immunity.

342 NPR1 has long been believed to associate with downstream transcription
343 factors, such as TGAs, to trigger their transcriptional activation of *PR* genes
344 (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Johnson et al.,
345 2003; Kumar et al., 2022). Thus, we wondered whether NPR1 could promote
346 the ability of ICE1 to activate the transcription of *PR1*. To test this possibility,
347 ChIP assays were conducted with *npr1-1 ICE1-Myc* and *NPR1-HA ICE1-Myc*
348 plants treated or not with BTH. As shown in Fig. 4C, D, ICE1 was enriched on
349 the *PR1* promoter in *ICE1-Myc*, and BTH promoted its enrichment. However,
350 the high enrichment of ICE1 on the promoter of *PR1* was impaired in the
351 background of *npr1-1*, but enhanced in the *NPR1*-overexpressing line (Fig. 4C,
352 D). These results suggest that the enrichment of ICE1 on the promoter of *PR1*
353 is promoted by NPR1.

354 Next, dual-luciferase reporter assays using Arabidopsis mesophyll
355 protoplasts were conducted to validate the effect of NPR1 on the
356 transcriptional activation activity of ICE1. The effectors consisted of GFP,
357 NPR1, and ICE1 under the control of *Pro35S*, and the reporter consisted of the
358 *PR1* promoter fused to the *LUC* gene (Supplemental Fig. S5). Interestingly,
359 *LUC* expression driven by the *PR1* promoter was also induced when NPR1
360 and GFP were co-expressed, implying that basal TGA or other proteins were
361 involved. However, *LUC* expression was induced to a much higher level when
362 NPR1 and ICE1 were co-expressed than when GFP and ICE1 were
363 co-expressed (Fig. 4E). In contrast, *LUC* expression driven by the *PR1*
364 promoter was compromised by the loss-of-function of *NPR1* (Fig. 4F). These
365 results provide further evidence that the ability of ICE1 to activate the
366 transcription of *PR1* is stimulated by NPR1.

367

368 **ICE1 Integrates Low Temperature-enhanced and SA-regulated Immunity**

369 ICE1 is involved in low temperature-activated plant immunity (Fig. 1) and
370 plays a crucial role in the SA signaling pathway (Fig. 3), implying that it may
371 function as a central node linking these two signaling pathways. To test this
372 hypothesis, we assessed disease symptoms, including water-soaking
373 symptoms and pathogen proliferation, in Col-0, *ice1-2,* and *ice1-2*/*ICE1* plants
374 treated with BTH for 24 hours with or without a 10-hour low temperature

375 pretreatment. This allowed us to characterize the critical role of ICE1 in
376 integrating low temperature signals and SA signals to induce pathogen
377 resistance. As shown in Fig. 5A, B, in the leaves of Col-0 and *ice1-2/ICE1,* the
378 water soaking symptoms and pathogen proliferation were alleviated by either
379 BTH or 4°C pretreatment, and significantly diminished by BTH combined with
380 cold treatment, compared with the mock treatment. In contrast, the leaves of
381 *ice1-2* showed severe water soaking symptoms and high pathogen
382 proliferation under all the different treatments. The transcript levels of *PR1*
383 were also lower in *ice1-2* plants compared with Col-0 and *ice1-2/ICE1* plants
384 under different conditions (Fig. 5C). We tested the enrichment of ICE1 on the
385 promoter of *PR1* in the different treatments. As shown in Fig. 5D, accumulation
386 of ICE1 on the promoter of *PR1* was enhanced by the combined low
387 temperature and BTH treatment, compared with either of these treatments
388 alone. Collectively, these results indicate that ICE1 functions as a crucial node
389 by integrating low temperature signals and SA signals to promote plant
390 disease immunity synergistically.
391 To further explore the underlying molecular mechanism by which ICE1 is
392 synergistically regulated by these two important cues, we determined the
393 amount of ICE1 protein that accumulated in response to a combined low
394 temperature and BTH treatment. Consistent with previous results, ICE1 protein
395 remained stable within 2 hours of 4°C treatment (Ding et al., 2015). Strikingly,
396 ICE1 accumulated to a much higher level under 6 hours BTH treatment
397 combined with 2 hours of 4°C treatment than in either the low temperature or
398 BTH treatment alone (Fig. 5E-F).
399
400 **ICE1 Physically Interacts with TGA Transcription Factors**
401 TGA transcription factors were confirmed to be interacting proteins of NPR1 in
402 yeast two-hybrid screening assays (Zhang et al., 1999; Zhou et al., 2000), and
403 are believed to be the nuclear targets that bind to the *as-1* element required for
404 SA-induced *PR1* gene expression (Zhang et al., 1999; Zhou et al., 2000;
405 Johnson et al., 2003; Kesarwani et al., 2007; Ding et al., 2018). To explore the
406 regulatory role of ICE1 in SA signaling comprehensively, we investigated
407 whether ICE1 interacts with TGA transcription factors. The full-length coding
408 sequences (CDSs) of *TGA1–TGA7* were fused to pGBK-T7 to construct

409 pGBK-TGAs vectors, and then each one was transformed into the yeast strain
410 with pGAD-ICE1. Interestingly, ICE1 interacted with all tested TGA proteins in
411 yeast (Fig. 6A). Among the seven members of the TGA family in Arabidopsis,
412 TGA3 plays an important role to bind to the *as-1* element of *PR1* to induce its
413 expression (Kesarwani et al., 2007; Choi et al., 2010; Kumar et al., 2022).
414 Hence, we focused on TGA3 in subsequent experiments.

415 The results of the BiFC, split luciferase and Co-IP assays further confirmed
416 that ICE1 interacted with TGA3 in plant cells. For the BiFC assays, full-length
417 CDSs of *TGA1* (clade I), *TGA2* (clade II), *TGA3*, and *TGA7* (clade III) were
418 fused to the C-terminal yellow fluorescent protein (cYFP) fragment to generate
419 TGA1-cYFP TGA2-cYFP, TGA3-cYFP, and TGA7-cYFP, respectively, and the
420 full-length CDS of *ICE1* was fused to the N-terminal fragment of YFP (nYFP) to
421 produce ICE1-nYFP. Truncated TGA3$^{193\text{-}384}$-cYFP and ICE1$^{1\text{-}260}$-nYFP that
422 could not interact with ICE1 or TGA3 were used as negative controls
423 (Supplemental Fig. S6). When ICE1-nYFP was transiently co-expressed with
424 TGAs-cYFP in leaf cells of *N. benthamiana*, recombinant YFP fluorescence
425 was observed in the nucleus, whereas no fluorescence signals were detected
426 when either TGA3$^{193\text{-}384}$-nYFP and ICE1-nYFP or ICE1$^{1\text{-}260}$-nYFP and
427 TGA3-cYFP were co-expressed (Fig. 6B; Supplemental Fig. S2B).

428 For the split luciferase assay, TGA3 fused to the C-terminus (cLUC) and
429 ICE1 fused to N-terminal (nLUC) of luciferase were co-expressed in *N.*
430 *benthamiana* leaves. As shown in Fig. 6C, LUC activity could be detected
431 when TGA3 interacted with ICE1. In the Co-IP assay, ICE1 was
432 immunoprecipitated by anti-GFP agarose beads in Arabidopsis protoplasts
433 co-expressing TGA3-YFP and ICE1-Myc, but not in those co-expressing YFP
434 and ICE1-Myc. This result provided further evidence for the interaction
435 between ICE1 and TGA3 *in vivo* (Fig. 6D). Taken together, these results
436 demonstrate that ICE1 physically interacts with the TGA3 transcription factor *in*
437 *planta*.

438

439 **ICE1 and TGA3 Works Synergistically during Low Temperature-enhanced**
440 **Immunity**

441 Having demonstrated that ICE1 associates with TGA3, we wondered if these
442 two proteins act cooperatively to modulate disease resistance in plants

443 exposed to cold treatment. Hence, we constructed the *TGA3*-knockout mutant
444 *tga3*^{CR} (Supplemental Fig. S7), and then crossed *ice1-2* with *tga3*^{CR} to obtain
445 *ice1-2 tga3*^{CR} double mutant plants. The disease phenotypes of these plants
446 were then determined. As shown in Fig. 7A, B, compared with the *ice1-2* and
447 *tga3*^{CR} single mutants, the *ice1-2 tga3*^{CR} double mutant showed a more
448 susceptible phenotype, including severe water soaking symptoms and high
449 pathogen proliferation after inoculation with *Pst* DC3000 both at normal and
450 low temperatures, indicating a synergistic role of TGA3 and ICE1 in the
451 regulation of plant immunity.
452 ICE1 acts as an essential node integrating SA-regulated and low
453 temperature-enhanced immunity by direct enrichment on the promoter of *PR1*
454 (Fig. 5). To further delineate the regulatory relationship between ICE1 and
455 TGA3, we conducted ChIP assays to investigate if TGA3 promotes the
456 enrichment of ICE1 on the promoter of *PR1 in vivo*. For this purpose, *tag3*^{CR}
457 *ICE1-Myc* and *TGA3-HA ICE1-Myc* seedlings were treated at 4°C for 2 h.
458 Seedlings grown at 22°C served as the control. As shown in Fig. 7C, the
459 enrichment of ICE1 on the *PR1* promoter was decreased in the background of
460 *tag3*^{CR} compared with Col-0. In contrast, the accumulation of ICE1 on the *PR1*
461 promoter was increased in the background of *TGA3-HA-OE* plants compared
462 with Col-0, and was further enhanced under low temperature (Fig. 7D).
463 Dual-luciferase reporter assays using Arabidopsis mesophyll protoplasts were
464 conducted to validate the effect of TGA3 on the transcriptional activation
465 activity of ICE1. The effector consisted of GFP, TGA3, and ICE1 under the
466 control of *Pro35S*, and the reporter consisted of the *PR1* promoter fused to the
467 LUC gene (Supplemental Fig. S5). *LUC* expression driven by the *PR1*
468 promoter was induced to higher level when TGA3 and ICE1 were
469 co-expressed than when GFP and ICE1 were co-expressed (Fig. 7E). In
470 contrast, ICE1 induced *LUC* expression was compromised in the *tag3*^{CR}
471 mutant compared with Col-0 (Fig. 7F). These results indicate that the ability of
472 ICE1 to activate the transcription of *PR1* is promoted by TGA3. Noticeably, the
473 ability of TGA3 to increase the transcription of *PR1* was diminished in the
474 *ice1-2* mutant, suggesting that ICE1 also stimulates the transcriptional
475 activation activities of TGA3 (Fig. 7G). These results support the notion that
476 ICE1 and TGA3 act cooperatively to mediate cold-enhanced plant immunity

through increased enrichment on the *PR1* promoter to activate its expression in association with SA.

The NPR1-TGA3 complex has been well documented that plays an indispensable role in *PR1* expression. Given ICE1 interacts with both NPR1 and TGA3, we questioned whether ICE1 could enhance the transcriptional activities of NPR1-TGA3 module on *PR1* expression. To clarify the potential role of ICE1 during the complex, we conducted the dual-luciferase reporter assay. Noticeably, compared with NPR1 and TGA3 were co-expressed, *LUC* expression driven by the *PR1* promoter was induced higher when NPR1, TGA3 and ICE1 were co-expressed (Fig. 7H), implying that ICE1 promotes the function of NPR1-TGA3 in *PR1* expression regulating.

DISCUSSION

In this study, we demonstrate a previously uncharacterized function of ICE1 as an essential node bridging cold stimulus and plant immunity. We found that ICE1 is involved in cold-activated immunity as well as SA-regulated immunity, as demonstrated by the fact that the *ice1-2* mutant exhibited enhanced susceptibility to pathogen infection, including severe pathogen-related water soaking symptoms and high pathogen proliferation, and decreased expression of *PR1* (Fig. 1 and Fig. 3). Most importantly, the coordinated function of cold- and SA-enhanced immunity was diminished in *ice1-2* mutant plants, suggesting that ICE1 is the crucial regulator linking these two cues (Fig. 5). The major underlying mechanism is that NPR1 associates with ICE1 to promote its ability to activate *PR1* transcription (Fig. 2 and Fig. 4). Additionally, TAG3 works synergistically with ICE1 under low temperature (Fig. 6 and Fig. 7). The relationship between cold stress and immunity has not been elucidated in plants (Wigge, 2013; Kim et al., 2017; Wu et al., 2019). The identification of the NPR1-TGA3-ICE1 regulatory module in this study represents a significant step in understanding SA signaling during cold-activated resistance of plants to pathogen attack.

NPR1, the master regulator of SA signaling, is required for basal and systemically acquired resistance in plants (Cao et al., 1997; Kesarwani et al., 2007). Because it lacks a typical DNA-binding domain, NPR1 was long believed to function as a transcriptional coactivator that interacts with other

511 proteins, including TGAs, EIN3, and Heat shock transcription factor 1 (HSFA1),
512 to modulate physiological processes in plants (Zhang et al., 1999; Després et
513 al., 2000; Olate et al., 2018; Huang et al., 2020; Wang et al., 2021; Yu et al.,
514 2021). However, in terms of plant immune regulation, TGAs are so far the only
515 group of transcription factors that are known to be direct targets of NPR1 and
516 to activate *PR1* gene expression (Zhang et al., 1999; Després et al., 2000;
517 Zhou et al., 2000; Johnson et al., 2003). Interestingly, in the present study, we
518 demonstrate a novel function of ICE1 to activate SA signaling against
519 pathogen infection. ICE1 interacts with NPR1 (Fig. 2) and functions in the
520 same signal pathway with it (Fig. 4). As a MYC-like bHLH transcription factor,
521 ICE1 can bind directly to the MYC recognition sequences of the *PR1* gene
522 promoter to activate its expression (Supplemental Fig. S4; Fig. 3G-I), and its
523 transcriptional activation activity is enhanced by NPR1 and TGA3 (Fig. 4C–F
524 and Fig. 7C–F). In addition, ICE1 promotes the transcriptional activation of
525 NPR1-TGA3 complex on expression of *PR1* (Fig. 7H). These findings not only
526 provide evidence that ICE1 is an essential component of SA signal pathway at
527 low temperature (Supplemental Fig. S8), but also increase our understanding
528 of the role of the NPR1-ICE1-TGA3 regulatory module in integrating low
529 temperature signal in activating plant immunity.

530 Recently, increasing attention has been paid to the interactions between low
531 temperature stress and plant immunity. Previous studies have shown that the
532 SA content increases in Arabidopsis plants exposed to cold stress (4°C) for
533 more than 1 week, and that prolonged exposure (3 weeks) to low temperature
534 promotes immunity against the bacterial plant pathogen, *Pst* DC3000 (Kim et
535 al., 2013). Further study has clarified that low temperature can overcome the
536 calmodulin-binding transcriptional activator 3 (CAMTA3) mediated genes
537 inhibition of the SA pathway (Kim et al., 2017). Similarly, moderate low
538 temperature (16°C) also enhances immunity accompanied by up-regulation of
539 SA biosynthesis and signaling genes (Li et al., 2020). Wu et al. found that
540 exposure to short-term cold stress (4°C for 10 hours) activates SA-dependent
541 immunity as well as H_2O_2 and callose deposition (Wu et al., 2019). The results
542 of those studies imply that SA may play a crucial role in low
543 temperature-activated immunity, however, the underlying mechanism
544 remained poorly characterized. In this study, we discovered that ICE1 acts an

545 indispensable regulatory node bridging low temperature cues and the SA
546 signaling pathway. Our results show that low temperature treatment enhances
547 disease resistance against the bacterial pathogen, *Pst* DC3000 (Fig. 1A–C),
548 and that ICE1 is required for this modulation (Fig. 1E–H). Interestingly, we
549 found that cold stress coordinates SA signaling to promote immunity, and this
550 synergistic regulation occurs in an ICE1-dependent manner. This is highlighted
551 by the fact that the enhanced immunity caused by low temperature combined
552 with SA signaling was compromised in *ice1-2* mutant plants compared with
553 Col-0 plants (Fig. 5A, B). Low temperature has multifaceted influences on the
554 SA signaling pathway, which involves ICE1. For instance, in the Co-IP assays,
555 low temperature facilitated interactions between ICE1 and NPR1 (Fig. 2D).
556 Notably, in the BiFC and LUC assays, the NPR1 and ICE1 interaction only
557 occurred after cold treatment (Fig. 2B, C; Supplemental Fig. S1), consistent
558 with the results of previous studies showing that NPR1 accumulates in
559 response to low temperature and enters the nucleus as a monomer to exert its
560 function as a co-factor (Olate et al., 2018). The enrichment of ICE1–TGA3 on
561 the *PR1* promoter and the ability of this complex to transcriptionally activate
562 *PR1* were found to be significantly enhanced under low temperature (Fig.
563 7C–G). These results provide direct evidence for the co-operative regulation of
564 low temperature cues and SA signaling *via* ICE1, illuminating the relationship
565 between cold stress and SA-regulated immunity.

566 It has been proposed that NPR1 is present in both an inactive oligomer form
567 and active monomer form and that SA treatment and low temperature promote
568 the conformation change from oligomer to monomer (Mou et al., 2003; Tada et
569 al., 2008; Yu et al., 2022). Although, in normal temperature and absence of SA,
570 the majority of NPR1 proteins are in the oligomer form, some NPR1 proteins
571 are in the monomer form, which may explain why NPR1 can interact with other
572 proteins, including HSFA1, GID1 and EIN3 in normal temperature and absence
573 of SA, however, the interactions can be enhanced by SA and low temperature
574 (Olate et al., 2018; Huang et al., 2020; Yu et al., 2022). Consistent with these
575 findings, we found that NPR1 could interact with ICE1 in normal temperature,
576 and this process was enhanced by low temperature (Fig. 2B-D). Low
577 temperature may promote NPR1-ICE1 interaction through two mechanisms.
578 On one hand, the conformation of NPR1 may be changed by low temperature

579 and enhance its interaction with ICE1. On the other hand, low temperature
580 may promote the accumulation of NPR1 monomer, leading to more active
581 NPR1 proteins targeting ICE1.
582 Temperature is an essential environmental factor affecting plant growth and
583 development, as well as resistance against pathogens. Recent studies have
584 shown that elevated temperatures negatively affect pathogen resistance by
585 suppressing SA production in Arabidopsis (Huot et al., 2017; Kim et al., 2022).
586 Interestingly, the results of several studies, including ours, show that the SA
587 pathway also plays indispensable role in the defense against bacterial
588 pathogen attack under cold stress conditions (Fig. 5) (Seo et al., 2010; Kim et
589 al., 2013; Kim et al., 2017; Wu et al., 2019; Li et al., 2020). Climate change will
590 have a profound effect on plant immunity, so an in-depth understanding of the
591 regulatory role of low temperature on plant immunity is instrumental for
592 revealing the mechanisms of environmental adaptation in plants under cold
593 stress. The results of studies on the temperature regulation of pathogen
594 resistance may provide a theoretical basis for the development of new
595 strategies to improve plants' resistance to pathogens in future agricultural
596 production. In addition, because of their sessile lifestyle, plants are constantly
597 exposed to a variety of abiotic stresses, and endogenous hormone signals
598 often interact with multiple exogenous environmental cues to regulate their
599 resistance to pathogens. Dissecting the regulatory mechanism of the
600 integration of low temperature and SA signals in response to pathogen attack
601 also provides new possibilities for research on the cross-talk between SA
602 signaling and other environmental signals.
603
604 **MATERIALS AND METHODS**
605 **Plant Materials and Growth Conditions**
606 The wild-type and mutant Arabidopsis plants used in this study were in the
607 Columbia (Col-0) genetic background. All Arabidopsis plants were soil-grown
608 for four weeks under an artificial growth chamber at 22°C and 60% relative
609 humidity, and 10 h light/14 h dark photoperiod. The *ice1-2*
610 (SALK_003155;(Kanaoka et al., 2008)) mutant and *npr1-1* (CS3726;(Cao et al.,
611 1994)) mutant have been described. Seeds of *ice1-2/ICE1* were kindly
612 provided by Prof. Shuhua Yang (China Agricultural University). The

613 *35S::GFP-ICE1* (CS68099) line was obtained from the Arabidopsis Resource
614 Center at Ohio State University (http://abrc.osu.edu). To generate the
615 overexpression transgenic plants, the full-length cDNA of *ICE1, TGA3* were
616 cloned into the pOCA30 vector in the sense orientation behind the CaMV 35S
617 promoter. *tga3*^{CR} mutant was constructed by a CRISPR/ Cas9-mediated
618 method as described previously (Liang et al., 2016).
619
620 **Bacterial Disease Assays**
621 Syringe-infiltration was performed in this study. Briefly, *Pst* DC3000 was
622 cultured in modified Luria–Bertani medium (Xin et al., 2016) containing 100 mg
623 l^{-1} rifampicin at 28 °C to an OD_{600} of 0.8–1.0. Bacteria were collected by
624 centrifugation and re-suspended in 0.25 mM $MgCl_2$. Cell density was adjusted
625 to OD_{600} = 0.2 (approximately 1 × 10^8 cfu ml^{-1}) and further diluted to cell
626 densities of 1 × 10^5–1 × 10^6 cfu ml^{-1}. The infiltrated plants were first placed
627 under ambient humidity for 1–2 h to allow water to evaporate, and then placed
628 in high humidity (about 95%; by covering plants with a clear plastic dome) for
629 disease development after the leaves returned to the state before infiltration.
630 For quantification of *Pst* DC3000 bacterial populations, leaf disks were taken
631 using a disposable biopsy punch with 4 mm diameter (Integra LifeSciences)
632 and ground in 0.25 mM $MgCl_2$. Colony-forming units were determined by serial
633 dilutions and plating on Luria Marine plates containing 100 mg l^{-1} rifampicin.
634 One biological replicate consists of 12 leaf disks (that is, from 3 leaves of one
635 plant) and at least 3 biological replicates were included in each experiment.
636 Experiments were repeated at least three times.
637 For BTH protection assay, four-week-old *Arabidopsis thaliana* were sprayed
638 with benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH; Chem
639 Service, 0.1% DMSO, 0.05% Silwet L77) or Mock (0.1% DMSO, 0.05% Silwet
640 L77) 24 h before bacterial syringe-infiltration.
641
642 **RNA Extraction and qRT-PCR**
643 Total RNA was extracted by using Trizol reagent (Invitrogen). The RT-qPCR
644 analysis was conducted as described (He et al., 2023; Li et al., 2023). In brief,
645 1.0 μg DNase-treated RNA was reverse transcribed in a reaction volume of 20
646 μl containing oligo-(dT)19 primer and Moloney murine leukemia virus reverse

647 transcriptase (Thermo Fisher Scientific). RT-qPCR analysis was then
648 performed with 1.0 μl of 5-fold diluted cDNA using a SYBR premixed Ex Taq kit
649 (Takara), and a Light Cycler 480 Real-Time PCR System (Roche) for the
650 reaction. In each RT-qPCR experiment, at least three biological replicates
651 were used and three technical replicates were performed for each biological
652 replicate. The *ACTIN2* (*AT3G18780*) gene was used as an internal reference
653 gene.
654
655 **Electrophoresis Mobility Shift Assay (EMSA)**
656 To generate the ICE1 recombinant protein, the sequence encoding full-length
657 *ICE1* was cloned into pET28a and transformed into *Escherichia coli* strain
658 BL21 (DE3). The protein was induced with 0.5 mM isopropyl
659 β-D-1-thiogalactopyranoside (IPTG) for 18 h at 16°C, and purified with Ni-NTA
660 resin. EMSA was performed using biotin-labeled probes and
661 Chemiluminescent EMSA Kit (Beyotime; GS009) referring to the instructions.
662 The primers used for vector construction are listed in Supplemental Table S1.
663
664 **Yeast Two-Hybrid Screening and Confirmation**
665 The full-length coding sequences (CDS) of *NPR1* and *TGA* were fused to the
666 bait vector pGBKT7 (Clontech, Palo Alto, CA, USA) to generate BD-NPR1 and
667 BD-TGA, while the full-length coding sequence of *ICE1* was ligated to the prey
668 vector pGADT7 to produce AD-ICE1. To identify structural domains essential
669 for protein interactions, several truncated NPR1 and TGA3 were cloned into
670 pGBKT7 and truncated ICE1 into pGADT7. The yeast two hybrid (Y2H) assays
671 were performed as described previously (Li et al., 2023). The vector pairs were
672 co-transformed into the yeast strain AH109, protein interactions were indicated
673 by the ability of cells to grow on dropout medium lacking Leu, Trp, His, and Ade
674 for 2 days after plating. The primers used for vector construction are listed in
675 Supplemental Table S1.
676
677 **Split Luciferase Complementation Assay**
678 The split luciferase complementation assays were performed as described
679 previously (Fujikawa and Kato, 2007; Chen et al., 2008). The full-length CDS
680 of *ICE1* was cloned into the pCAMBIA1300-nLUC vector under the control of

the 35S promoter. Full-length CDS of NPR1 and TGA3 were cloned into the pCAMBIA1300-cLUC vectors. After co-expression in *Nicotiana benthamiana* leaves for 48 h，the leaves were sprayed with 1 mM D-luciferin (0.01% Triton X-100) solution and then incubated for 5 min in the dark before detecting. Luciferase intensity was visualized on Tanon-5200 Chemiluminescent Imaging System (Tanon Science&Technology). The primers used for vector construction are listed in Supplemental Table S1.

Bimolecular Fluorescence Complementation Assays

The sequences encoding the C-terminal (64 amino acids) YFP (cYFP) fragment and the N-terminal 173 amino acid-enhanced YFP (nYFP) fragment were amplified by PCR and cloned into the labeled pFGC5941 plasmid to generate pFGC-nYFP and pFGC-cYFP, respectively (Kim et al., 2008). The full-length or truncated CDS sequences of *ICE1* were cloned into pFGC-nYFP to generate the N-terminal in-frame fusions with n-YFP (ICE1-nYFP, ICE1$^{1-260}$-nYFP and ICE1$^{261-494}$-nYFP), whereas full-length or truncated *NPR1* and *TGA* coding sequences were inserted into pFGC-cYFP to form a C-terminal intraframe fusion (NPR1-cYFP, NPR1$^{1-194}$- cYFP, NPR1$^{178-593}$- cYFP and TGA3- cYFP). The plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 and injected into tobacco (*N. benthamiana*) leaves. As described previously (He et al., 2023; Li et al., 2023), YFP and 4,6-diamidino-2-phenylindole (DAPI) fluorescence of infected leaves were observed under a confocal laser scanning microscope (Olympus) after 48 h of co-expression. Primers used for cloning are listed in Supplemental Table S1.

Co-immunoprecipitation Assays

To generate proteins fused with YFP or Myc tags, we amplified the full-length CDS of *NPR1* and *TGA3* by PCR and then cloned into the pEarleyGate 101 vector with YFP-tagged sequences under the control of *Pro35S* using the gateway method, whereas the full-length CDS of *ICE1* was inserted into the Myc-tagged pGWB517 vector. Arabidopsis protoplasts were transformed with different combinations of plasmids, including 35S::YFP and 35S:: ICE1-Myc, 35S::NPR1-YFP and 35S:ICE1-Myc, 35S::TGA3-YFP and 35S:ICE1-Myc at

714 22°C or 4°C for 16 h. Total protein was extracted with an IP buffer containing
715 50 mM MOPs, 5 mM EDTA, 0.2% v/v Triton X-100, 5 mM dithiothreitol, 1 mM
716 PMSF and 1 x complete protease inhibitor cocktail (Roche).
717 Immunoprecipitation experiments with GFP-trap beads were performed
718 according to the manufacturer's protocol. Briefly, cell lysates were incubated
719 with GFP-trap beads (ChromoTek) at 4°C overnight. After incubation, the
720 beads were washed three to five times with IP buffer, and then
721 co-immunoprecipitated proteins were detected by immunoblotting with an
722 anti-Myc antibody (Abmart; 1:10,000). Primers used for vector construction are
723 listed in Supplementary Table S1.
724
725 **ChIP Analyses**
726 ChIP Assays were performed substantially as described earlier
727 (Mukhopadhyay et al., 2008; Jiang et al., 2014). In brief, Col-0, *ICE1-Myc*,
728 *npr1-1 ICE1-Myc*, *NPR1-HA ICE1-Myc*, *TGA3-HA ICE1-Myc*, and *tga3*^{CR}
729 *ICE1-Myc* plants were treated with 1% formaldehyde (cross-linking treatment)
730 to isolate their chromatin. Proteins were immunoprecipitated using anti-Myc
731 antibody (1:1,000). The precipitated DNA was purified using a PCR purification
732 kit (Qiagen). To quantitatively determine the ICE1–DNA (target promoter)
733 binding, RT-qPCR analysis was performed with the *ACTIN2* 3' untranslated
734 region sequence as an endogenous control. Relative enrichment was
735 calculated based on DNA binding ratio.
736
737 **Transient Transcriptional Activation Assays**
738 The full-length CDSs of *ICE1*, *NPR1*, *TGA3*, and *GFP* were cloned into the
739 pGreenII 62-SK vector as effectors, respectively. The promoter sequence of
740 *PR1* was amplified by PCR and inserted into the pGreenII 0800-LUC vector
741 (Biovector Science Lab) as the reporter (Hellens et al., 2005). Combinations of
742 plasmids were transformed into the Col-0 and/or mutant leaf mesophyll
743 protoplasts according to the Sheen laboratory protocol (Sheen, 2001).
744 Transfected cells were cultured for 16-18 h followed by analysis of relative
745 luciferase (LUC) activity using the Dual- Luciferase Reporter Assay System
746 (Promega), which detects the activities of firefly LUC and the internal control
747 Renilla reniformis (REN). Primers used for vector construction are listed in

Supplemental Table S1.

STATISTICAL ANALYSIS

Statistical analysis was performed by analysis of variance. The results of statistical analyses are shown in Supplemental Data Set S1.

ACCESSION NUMBERS

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *ICE1*, AT3G26744; *NPR1*, AT1G64280; *TGA1*, AT5G65210; *TGA2*, AT5G06950; *TGA3*, AT1G22070; *TGA4*, AT5G10030; *TGA*5, AT5G06960; *TGA6*, AT3G12250; *TGA7*, AT1G77920; *PR1*, AT2G14610; *ACTIN2*, AT3G18780.

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AUTHOR CONTRIBUTIONS

S.Q.L. and Y.J.J. designed and performed experiments; S.Q.L., L.H., Y.P.Y. and X.H. and Y.R.H analyzed data; S.Q.L. and Y.J.J. wrote the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTAL DATA

The following materials are available in the online version of this article.

Supplemental Figure S1. No interacting signal of ICE1 and NPR1 was detected at normal temperature.

Supplemental Figure S2. Percentage of transformed cells with YFP fluorescence in the BiFC assays presented in Figures 2B and 6B.

Supplemental Figure S3. Transcriptional and protein levels of ICE1 responding to BTH treatment.

Supplemental Figure S4. The promoter sequence of *PR1*.

782 **Supplemental Figure S5.** Schematic of effectors and reporters used in
783 transient transactivation assays.
784 **Supplemental Figure S6.** The bHLH motif and bZIP motif was required for
785 interaction of ICE1 and TGA3.
786 **Supplemental Figure S7.** Generation of *tga3*^{CR} knockout by CRISPR/Cas9
787 technology.
788 **Supplemental Figure S8.** Working model of NPR1-TGA3/ICE1 regulating
789 module against *Pst* DC3000 after cold stress.
790 **Supplemental Figure S9.** Uncropped images for protein gels.
791 **Supplemental Table S1.** Primers used for generating various clones.
792 **Supplemental Data Set S1.** The results of statistical analyses performed by
793 ANOVA.
794
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799
800 *Conflict of interest statement.* All authors state that they have no conflict of
801 interest in relation to this research.
802
803 **DATA AVAILABILITY**
804 All data supporting the findings of this study are available within the article and
805 its supplemental materials.
806
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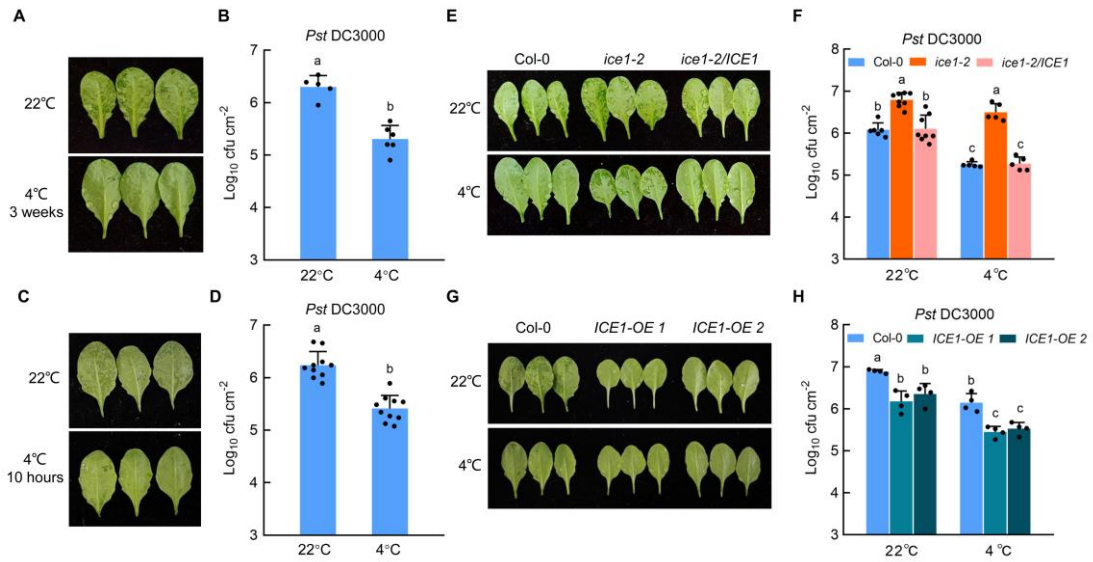

Figure 1. ICE1 involves in low temperature-enhanced plant immunity. (A) Col-0 leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 3 weeks of 4°C treatments (down), 22°C as control (up). (B) Bacterial populations in leaves described in (A). Values are displayed as mean ± SEM (n ≥ 5 biological replicates). (C) Col-0 leaves infiltrated with *Pst* DC3000 (OD600 = 0.0001) after 10 hours of 4°C treatments (down), 22°C as control (up). (D) Bacterial populations on leaves described in (C). Values are displayed as mean ± SEM (n = 10 biological replicates). (E) Col-0, *ice1-2*, *ice1-2/ICE1* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 10 hours of 4°C treatments (down), 22°C as control (up). (F) Bacterial populations on leaves described in (E). Values are displayed as mean ± SEM (n ≥ 5 biological replicates). (G) Col-0, *ICE1-OE 1* and *ICE1-OE 2* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 10 hours of 4°C treatment (down), 22°C as control (up). (H) Bacterial populations of *Pst* DC3000 on plants described in (G). Values are displayed as mean ± SEM (n = 4 biological replicates). Photos were taken after 48 h pathogen inoculation. Different letters indicate statistically significant differences (two-way ANOVA, $P < 0.05$). Experiments were repeated at least three times with similar trends.

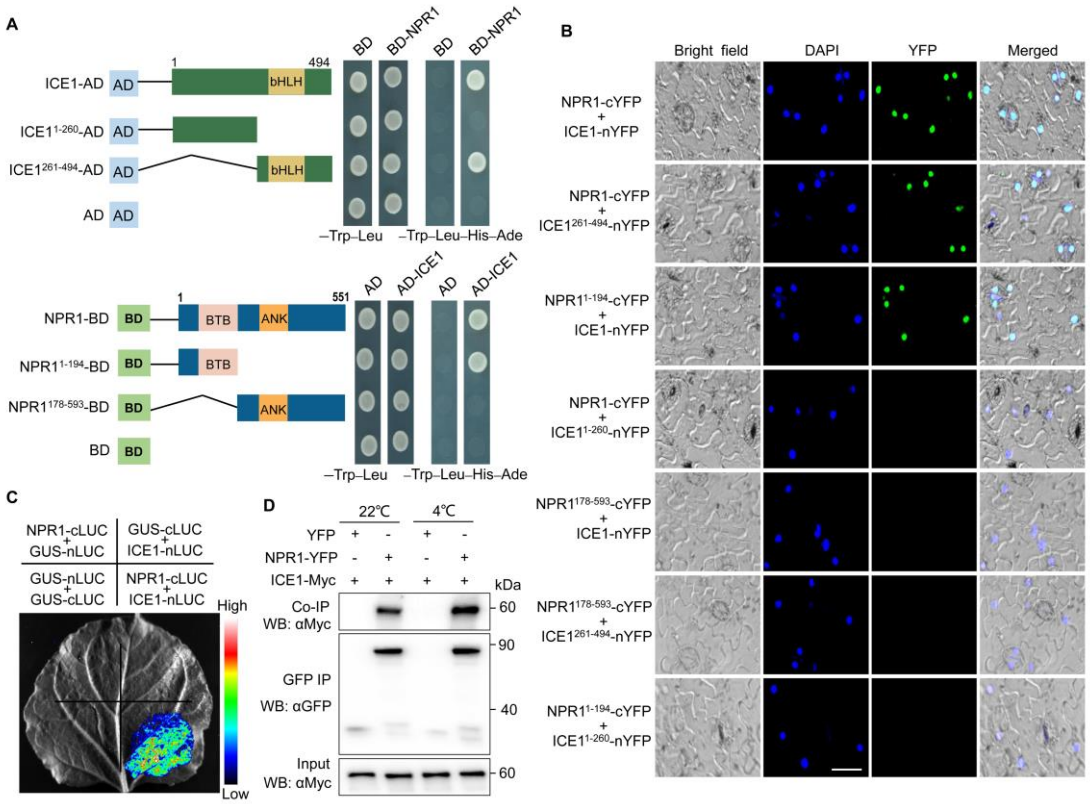

Figure 2. ICE1 interacts with NPR1. (A) Yeast two hybrid assays showing the interactions between ICE1, NPR1, and their truncated versions. Protein interactions were indicated by the growth of yeast cells after 2 days of incubation in dropout medium lacking Leu, Trp, His and Ade. The numbers indicate the positions of amino acids, pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. (B) BiFC assay. Fluorescence was observed in the nucleus of transformed *N. benthamiana* cells co-expressing ICE1-nYFP (or ICE1$^{261-494}$-nYFP) with NPR1-cYFP or ICE1-nYFP with NPR1$^{1-191}$-cYFP at 4°C for 4 h. No signal was obtained in the negative controls which NPR1-cYFP (or NPR1$^{1-194}$-cYFP) with ICE1$^{1-260}$-nYFP or ICE1-nYFP (or ICE1$^{261-494}$-nYFP) with NPR1$^{370-593}$-cYFP were co-expressed. Nuclei are indicated by DAPI staining. (C) Interaction between ICE1 and NPR1 in split luciferase assay. ICE1-cLUC and NPR1-nLUC were co-expressed in *N. benthamiana* leaves, the luminescence intensity was detected at 4°C for 4 h after 48 h of incubation by an imaging system. GUS-nLUC and GUS-cLUC were set as a negative control. (D) Co-IP assay. Arabidopsis protoplasts expressing NPR1-YFP with ICE1-Myc, YFP with ICE1-Myc were incubated for 16 h. For low-temperature induction, protoplasts were processed at 4°C for 2 h before total protein was extracted. Then immunoprecipitated with GFP-trap agarose beads. Co-immunoprecipitated proteins were detected with anti-Myc antibody. Experiments were repeated at least three times with similar trends.

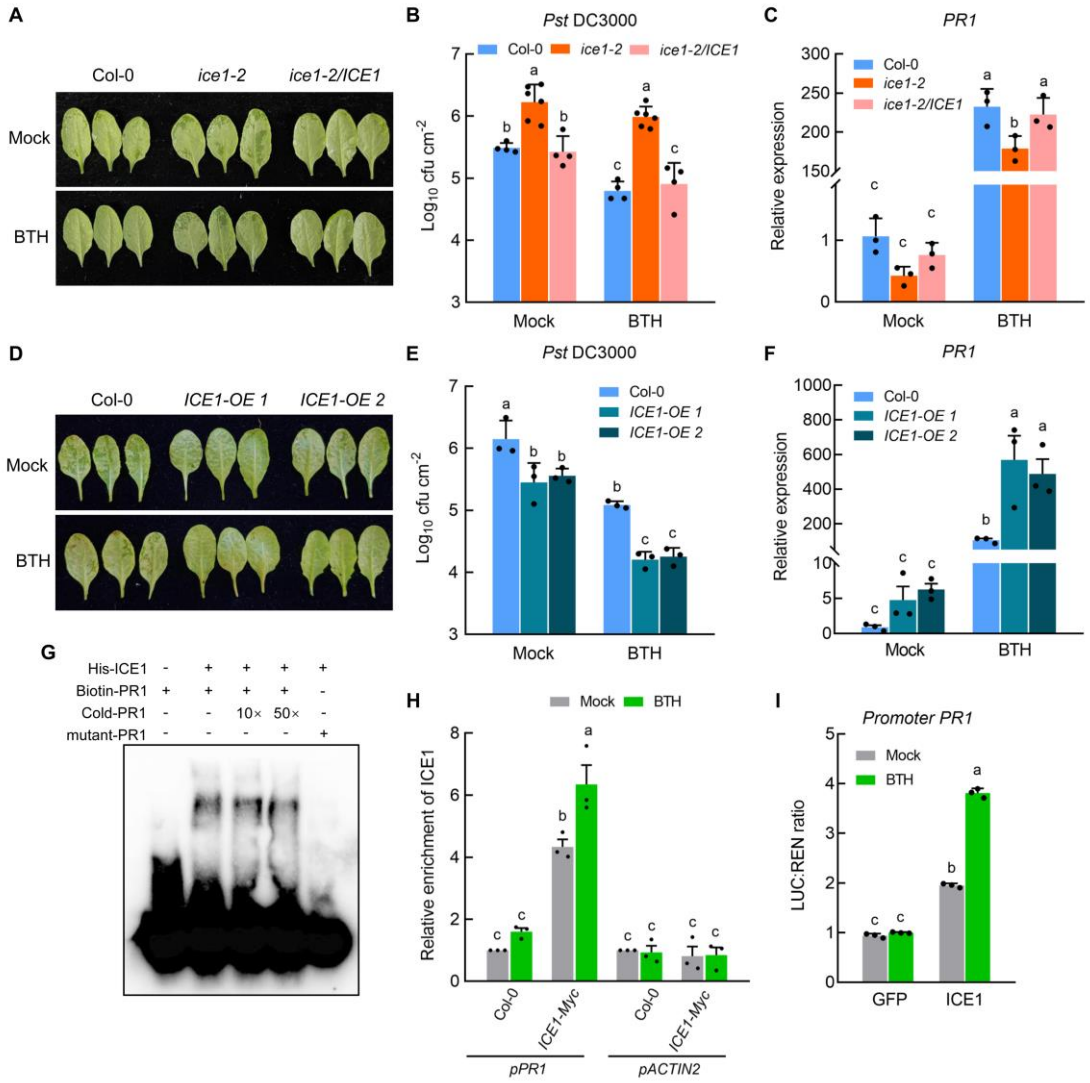

Figure 3. ICE1 involves in SA-regulated immunity. (A) Col-0, *ice1-2*, *ice1-2/ICE1* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 24 hours pretreating with 100 μM BTH (down), mock as control (up). (B) Bacterial populations in leaves described in (A). Values are displayed as mean ± SEM (n ≥ 4 biological replicates). (C) qRT-PCR analysis of *PR1* expression in leaves described in (A). Values are displayed as mean ± SEM (n = 3 biological replicates). (D) Col-0, *ICE1-OE 1* and *ICE1-OE 2* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 24 hours pretreating with 100 μM BTH (down), mock as control (up). (E) Bacterial populations in leaves described in (D). Values are displayed as mean ± SEM (n = 3 biological replicates). (F) qRT-PCR analysis of *PR1* expression in leaves described in (C). Values are displayed as mean ± SEM (n = 3 biological replicates). (G) EMSA assay examines ability of ICE1 binding to the E-box (CANNTG) of *PR1* promoter. Biotin-labeled probes were incubated with ICE1-His proteins, and the free and bound DNAs were separated on an acrylamide gel. As indicated, unlabeled probe (cold-PR1) was used as competitor and biotin labeled mutant probe (mutant-PR1) was used as negative control. Mutated probe in which the

CATTTG motif was replaced with AATTTC. (H) ChIP-qPCR analysis of the relative enrichment of ICE1 on the promoter of *PR1*. Col-0 and *ICE1*-overexpressing (*ICE1-Myc*) plants were treated with mock or 100 μM BTH for 6 h and pooled for ChIP assays using anti-Myc antibody, the *ACTIN2* untranslated region sequence (*pACTIN2*) as a negative control. Values are displayed as mean ± SEM (n ≥ 3 biological replicates). (I) Transient dual-LUC reporter assays showing that ICE1 activates the expression of *PR1*. Values are displayed as mean ± SEM (n ≥ 3 biological replicates). Each biological replicate was from different leaves of more than 60 Col-0 plants. Photos were taken after 48 h pathogen inoculation in A and D. Different letters indicate statistically significant differences (two-way ANOVA, $P < 0.05$). Experiments were repeated at least three times with similar trends.

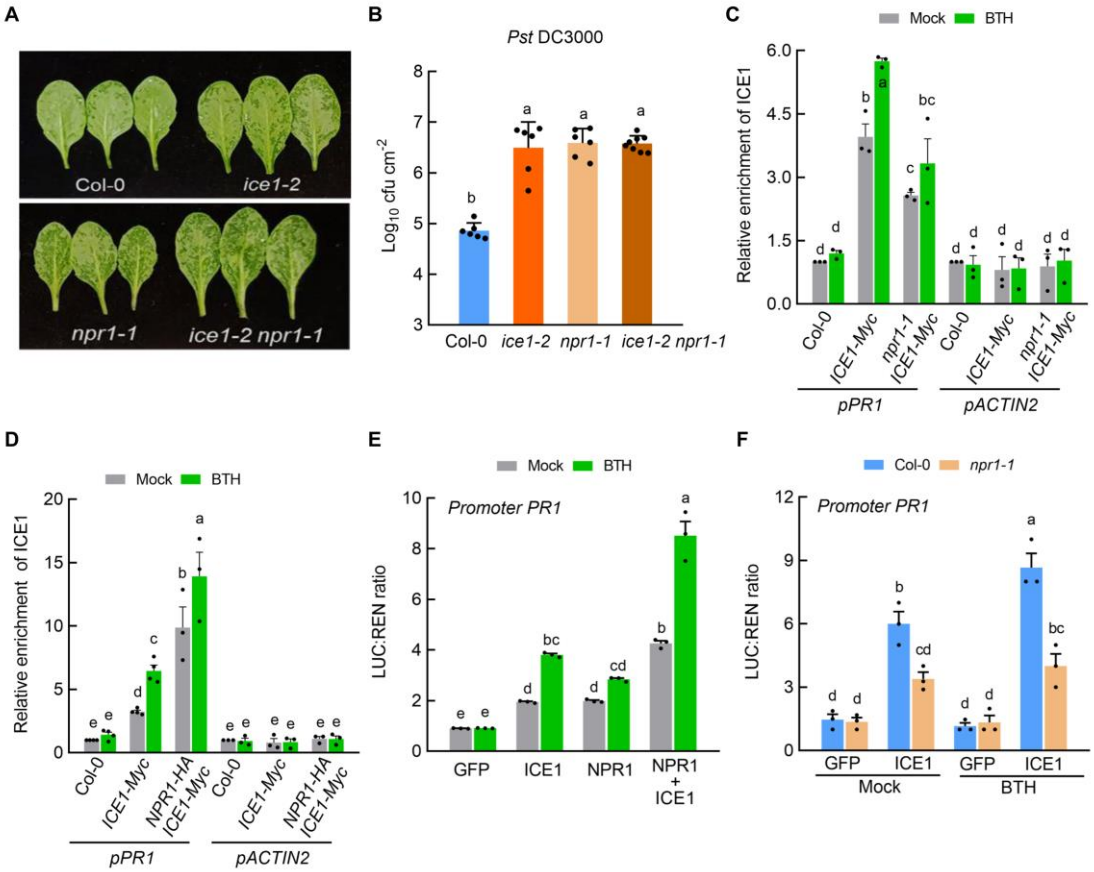

Figure 4. NPR1 promotes the binding and transcriptional activation of ICE1 to *PR1*. (A) Col-0, *ice1-2*, *npr1-1* and *ice1-2 npr1-1* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001). Photos were taken after 48 h pathogen inoculation. (B) Bacterial populations in leaves described in (A). Values are displayed as mean ± SEM (n ≥ 6 biological replicates). (C and D) ChIP-qPCR analysis of the relative enrichment of ICE1 on the promoter regions of *PR1*. Col-0, *ICE1*-overexpressing (*ICE1-Myc*), *npr1-1 ICE1-Myc* and *NPR1-HA ICE1-Myc* plants were treated with mock or 100 μM BTH for 6 h and pooled for ChIP assays using anti-Myc antibody, the *ACTIN2* untranslated region sequence (*pACTIN2*) as a negative control. Values are displayed as mean ± SEM (n = 3 biological replicates). (E) Transient transcriptional activity assays showing that NPR1 enhances the transcriptional activation of ICE1 to *PR1*. Values are displayed as mean ± SEM (n = 3 biological replicates). (F) Transient transcriptional activity assays showing that activation of the *PR1* promoter by ICE1 is compromised in the *npr1-1* mutant. Values are displayed as mean ± SEM (n = 3 biological replicates). Each biological replicate was from different leaves of more than 60 plants in (E) and (F). Different letters indicate statistically significant differences (two-way ANOVA, $P < 0.05$). Experiments were repeated at least three times with similar results.

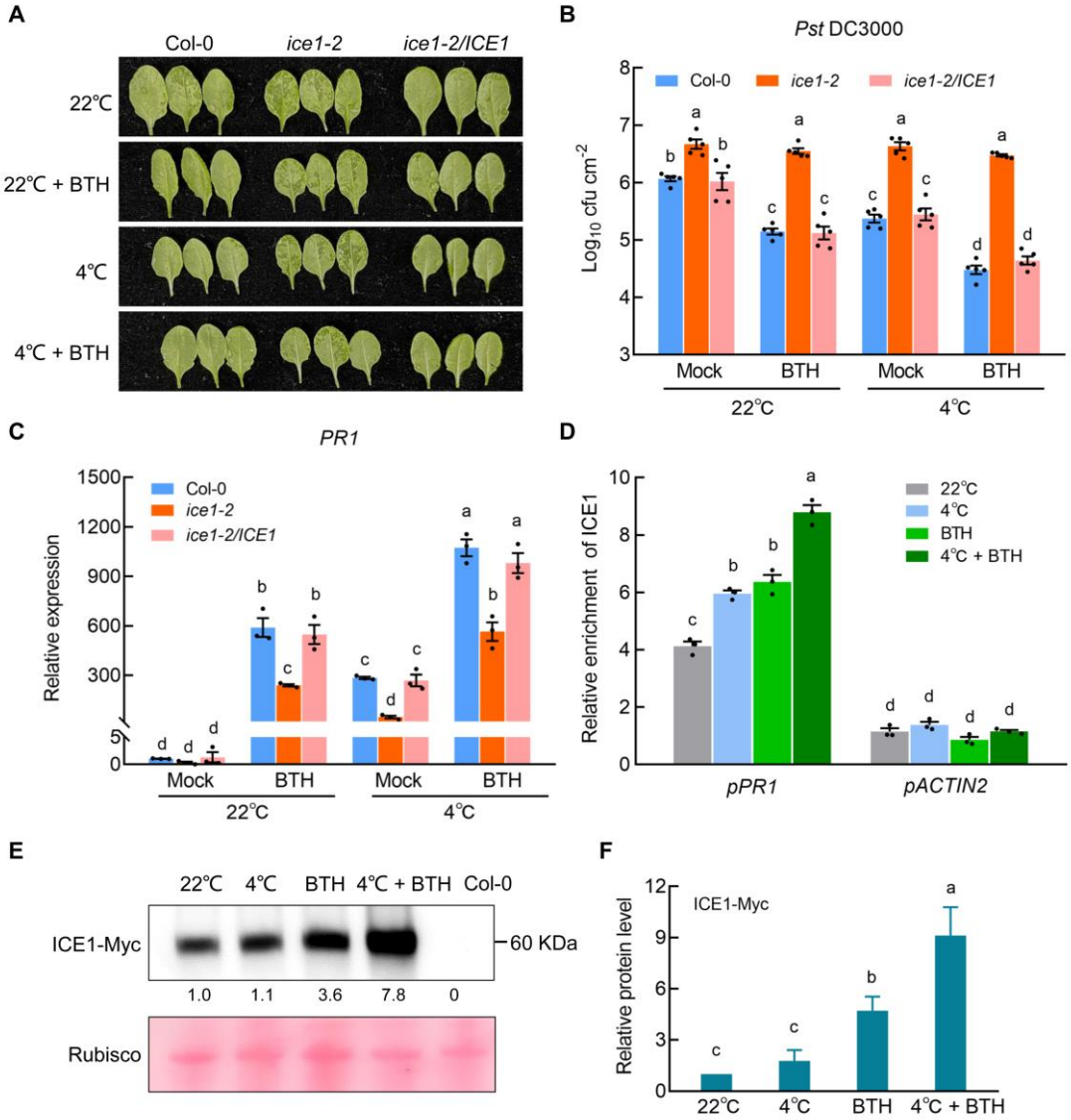

Figure 5. ICE1 links low temperature signals and SA pathway to promote plant immunity. (A) Col-0, *ice1-2* and *ice1-2/ICE1* leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after BTH with low temperature pretreating (briefly, 14 hours BTH treatment at 22°C and then move to 4°C growth chamber for 10 hours cold treatment). Photos were taken after 48 h pathogen inoculation. (B) Bacterial populations in leaves described in (A). Values are displayed as mean ± SEM (n = 5 biological replicates). (C) qRT-PCR analysis of *PR1* expression in leaves described in (A). Values are displayed as mean ± SEM (n = 3 biological replicates). (D) ChIP-qPCR analysis of the relative enrichment of ICE1 on the promoter regions of *PR1*. *ICE1*-overexpressing plants (*ICE1-Myc*) were treated with 100 μM BTH for 6 hours and 4°C for 2 hours and pooled for ChIP assays using anti-Myc antibody, the *ACTIN2* untranslated region sequence (*pACTIN2*) as a negative control. Values are displayed as mean ± SEM (n = 3 biological replicates). (E) Immunoblot analyzing the accumulation of ICE1 protein. Ten-day-old *ICE1*-overexpressing seedlings (*ICE1-Myc*) were treated with 100 μM BTH for 6 h and/or 4°C for 2 hours before protein extraction. The accumulation of ICE1 protein was detected with anti-Myc antibody. (F) Relative protein levels of ICE1-Myc in (E), which were quantified

by Image J. Values are displayed as mean ± SEM (n = 3 biological replicates). Different letters indicate statistically significant differences (two-way ANOVA, $P < 0.05$). Experiments were repeated at least three times with similar trends.

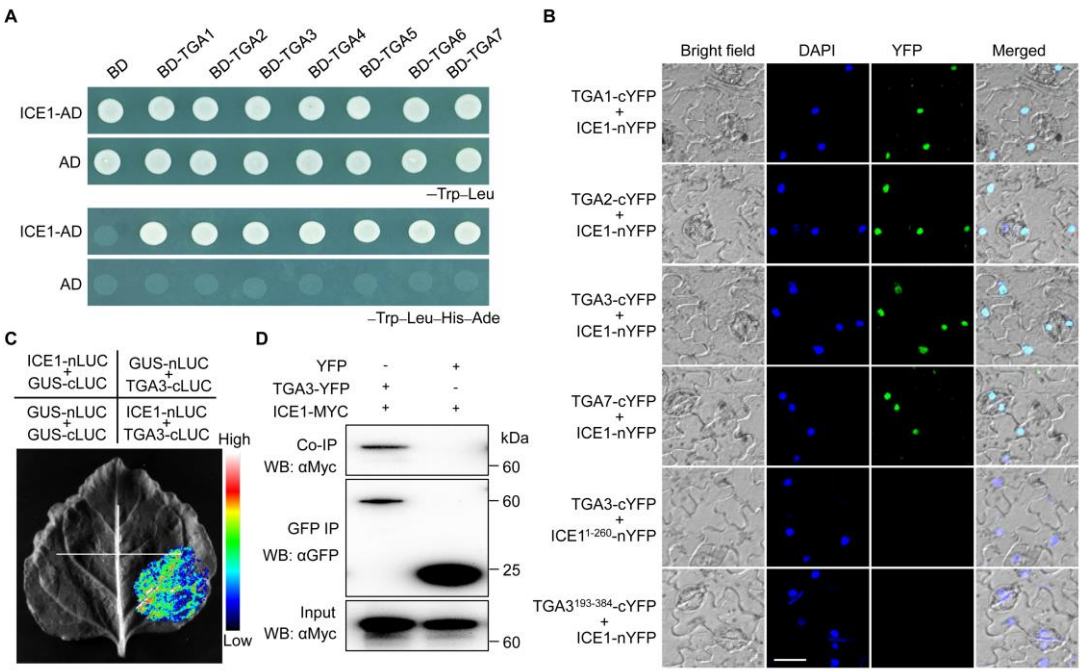

Figure 6. ICE1 interacts with TGA transcription factors. (A) Yeast two hybrid assays. Interactions of ICE1 with TGAs were indicated by the ability of yeast cells to grow on dropout medium lacking Leu, Trp, His, and Ade for 2 days after plating. BD and AD were used as negative controls. (B) BiFC assay. Fluorescence was observed in the nuclear compartment of transformed *N. benthamiana* cells, resulting from the complementation of ICE1-nYFP with TGA1/2/3/7-cYFP. No signal was obtained for the negative controls in which ICE1$^{1-260}$-nYFP with TGA3-cYFP or ICE1-nYFP with TGA3$^{193-384}$-cYFP were coexpressed. Nuclei are indicated by DAPI staining. (C) Split luciferase assay. nLUC-ICE1 and cLUC-TGA3 constructs were co-transformed into *N. benthamiana*. Luminescence intensity was measured after 48 h of incubation by an imaging system. GUS-nLUC and GUS-cLUC were set as a negative control. (D) Co-IP assay. Arabidopsis protoplasts expressing TGA3-YFP with ICE1-Myc, YFP with ICE1-Myc were incubated for 16 h, and total protein was extracted. Then immunoprecipitated with GFP-trap agarose beads. Co-immunoprecipitated proteins were detected with anti-Myc antibody. Experiments were repeated at least three times with similar trends.

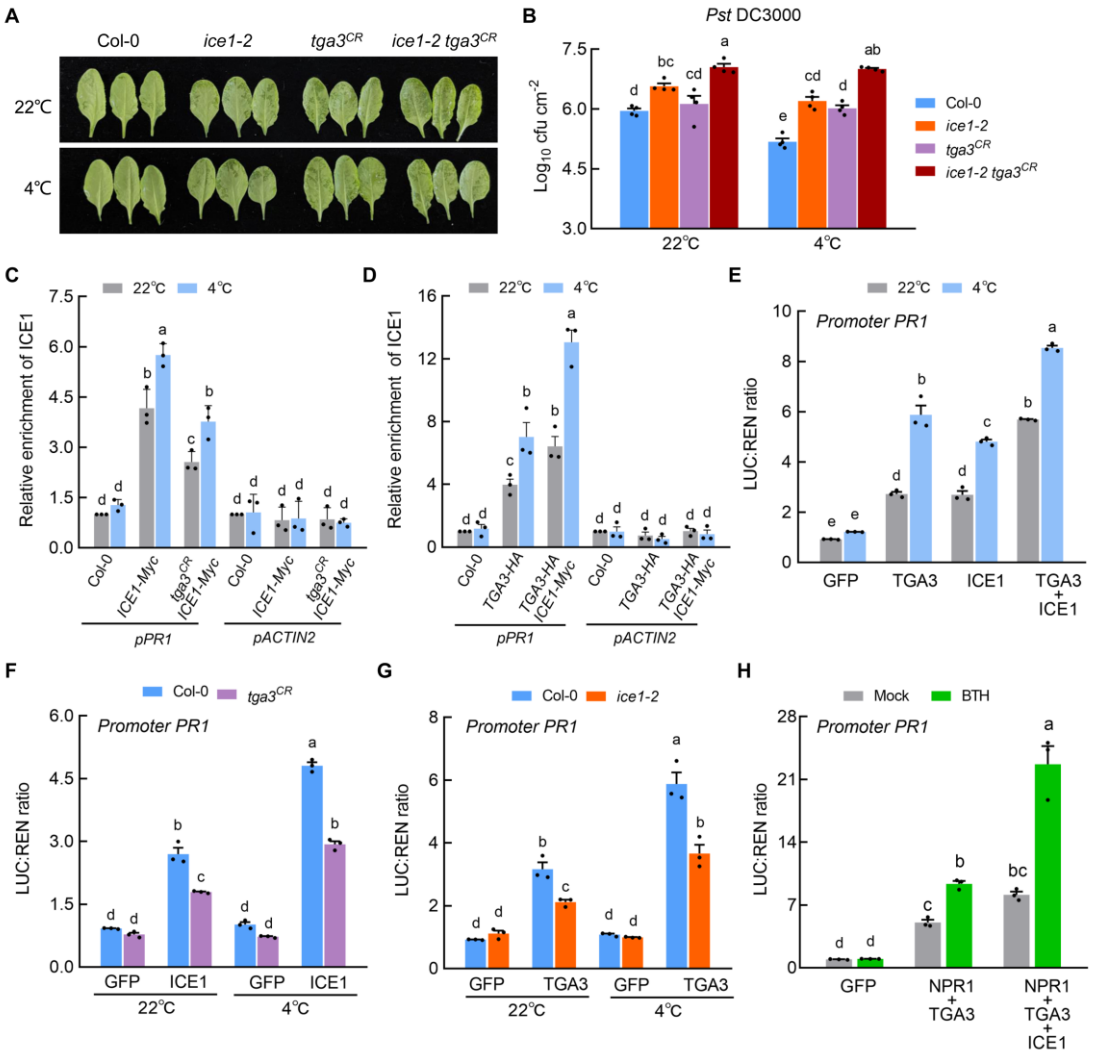

Figure 7. ICE1 and TGA3 works synergistically to activate expression of *PR1* during low temperature stress. (A) Col-0, *ice1-2*, *tga3*^{CR} and *ice1-2 tga3*^{CR} leaves infiltrated with *Pst* DC3000 (OD_{600} = 0.0001) after 10 hours of 4°C treatment (down), 22°C as control (up). Photos were taken after 48 h pathogen inoculation. (B) Bacterial populations in leaves described in (A). Values are displayed as mean ± SEM (n = 4 biological replicates). (C) ChIP-qPCR analysis of the *tga3*^{CR} mutation impaired the relative enrichment of ICE1 to the *PR1* promoter. Col-0, *ICE1*-overexpressing (*ICE1-Myc*) and *tga3*^{CR} *ICE1-Myc* plants were treated for 10 h of 4°C, 22°C as control. (D) ChIP-qPCR analyses of the relative enrichment of ICE1 to the *PR1* promoter enhanced by TGA3. Col-0, *ICE1*-overexpressing plants (*ICE1-Myc*) and *TGA3-HA ICE1-Myc* (hybridized F1 generation) were treated for 10 h of 4°C, 22°C as control. Treated leaves were pooled for ChIP assays using anti-Myc antibody, the *ACTIN2* untranslated region sequence (*pACTIN2*) as a negative control in (C) and (D). (E) Transient transcriptional activity assays showing that TGA3 enhances the transcriptional activation of ICE1 to *PR1*. (F) Transient transcriptional activity assays showing that activation of the *PR1* promoter by ICE1 is compromised in the *tga3*^{CR} mutant. (G) Transient transcriptional activity assays showing that activation of the *PR1* promoter by ICE1 is diminished in the *ice1-2* mutant. Each biological replicate was from different leaves of more than 60 plants in (E-G). (H) Transient transcriptional activity

assays showing that ICE1 enhances the transcriptional activation of NPR1-ICE1 complex to *PR1* expression. Values are displayed as mean ± SEM (n = 3 biological replicates) for (C-H). Different letters indicate statistically significant differences (two-way ANOVA, $P < 0.05$). Experiments were repeated at least three times with similar trends.

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