The ICE1 transcription factor, also known as SCREAM (SCRM), is thought to be a master regulator of genes that impart freezing tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Knight and Knight, 2012; Guo et al., 2018; Shi et al., 2018). In this issue of The Plant Cell, Kidokoro et al. (2020) present results that challenge this thinking. In this commentary, invited by the journal editors, we first provide background information establishing a context for the Kidokoro et al. study and then summarize their findings and present our thoughts on fundamental questions raised by the results.

Plants from cold environments increase in freezing tolerance in response to low nonfreezing temperatures, a phenomenon known as cold acclimation (Xin and Browse, 2000). The CBF/DREB1 transcription factors, which are highly conserved in higher plants, have a major role in regulating cold acclimation. Arabidopsis has three CBF/DREB1 genes—CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A—that are located in tandem array and rapidly induced upon exposing plants to low temperature (~4°C) (Thomashow, 2010). Induction of the CBF/DREB1 genes is followed by induction of CBF/DREB1-targetted COR (cold-regulated) genes, some of which contribute to an increase in freezing tolerance. Arabidopsis mutants in which all three CBF/DREB1 genes are inactivated are severely impaired in freezing tolerance (Jia et al., 2016; Zhao et al., 2016; Park et al., 2018).

Given the importance of the CBF/DREB1 genes in cold acclimation, it is not surprising that considerable effort has been directed at determining how they are induced in response to low temperature. A major breakthrough was reported by Chinnusamy et al. (2003) who identified ICE1 (Inducer of CBF Expression 1) as a positive regulator of CBF3/DREB1A expression and freezing tolerance. In particular, the investigators mutagenized a transgenic line of Arabidopsis that carried a luciferase (LUC) reporter under control of the CBF3/DREB1A promoter and screened for mutants that showed reduced expression of the CBF3/DREB1A::LUC reporter gene in response to low temperature. This led to identification of the ice1-1 mutation, now designated ice1-1, a dominant mutation that caused reduced cold induction of both the CBF3/DREB1A::LUC reporter gene and the endogenous CBF3/DREB1A gene and caused a reduction in freezing and chilling tolerance. Further analysis indicated that the ICE1 gene encoded a MYC-like bHLH transcription factor and that the ice1-1 allele having a dominant negative effect on CBF3/DREB1A expression (expression of the endogenous CBF3/DREB1A gene was not reported). Additionally, overexpression of wild-type ICE1 resulted in a transient up-regulation of CBF3/DREB1A and CBF2/DREB1C in response to low temperature and an increase in freezing tolerance. Taken together, these results provided a strong case that ICE1 had an important role in regulating the CBF/DREB1 genes and freezing tolerance.

In a subsequent unrelated study that is germane here, ICE1 was identified as a regulator of stomatal development (Kanaoka et al., 2008). The gene, named SCRM, and its paralog SCRM2/ICE2, were shown to promote stomatal differentiation via forming heterodimers with core stomatal transcription factors, including SPEECHLESS (SPCH) (Kanaoka et al., 2008). Consistently, the ice1-2 scrm2-1 double knockout mutant developed an epidermis devoid of stomata, identical to the spch mutant phenotype. By contrast, the gain-of-function mutant scrm-D elevated expression of the stomatal gene EPF1 and produced a 'screaming' epidermis solely composed of stomata (Pilletteri et al., 2011; Putarjunan et al., 2019). Of importance here is that the molecular lesion of scrm-D was found to be the same ICE1(R236H) substitution found in ice1-1. Thus, as would be expected, the ice1-1 mutant produces overwhelming stomata on the epidermis (Figure 1).

Now, Kidokoro et al. (2020) present results that challenge the thinking that ICE1 has a major role in regulating the CBF/DREB1 genes. This challenge comes via two approaches, the first involving a genetic analysis of the ice1-1 mutant. Kidokoro et al. crossed the ice1-1 mutant with a newly made transgenic line carrying the emerald luciferase (ELUC) reporter fused to a portion of the CBF3/DREB1A promoter; the construct was designated 1AR::ELUC. Analysis of the F2 population showed that the ice1-1/scrm-D phenotypes of increased numbers of stomata and the induction of EPF1 did not cosegregate with impaired cold induction of the 1AR::ELUC reporter or CBF3/DREB1A. Additionally, it was found that transformation of plants with the ice1-1 gene resulted in the induction of EPF1 but did not reduce cold induction of the endogenous CBF3/DREB1A gene. These results indicate that the ICE1(R236H) protein encoded by ice1-1/scrm-D is not responsible for the impaired cold induction of CBF3/DREB1A as initially reported for the ice1-1 mutation (Chinnusamy et al., 2003). If ice1-1/scrm-D was not responsible, what was? Additional analysis...
altered by transgene-induced DNA methylation: a CBF3/DREB1A::LUC transgene present in the ice1-1 mutant caused an increase in DNA methylation of the endogenous CBF3/DREB1A locus resulting in reduced cold induction of its expression.

The genetic analysis of Kidokoro et al. is thorough but includes a troublesome mystery. As the investigators note, their results indicate that the CBF3/DREB1A::LUC transgene responsible for DNA methylation of the endogenous CBF3/DREB1A gene in the ice1-1 mutant (ABRC stock: CS67843), as well as a second CBF3/DREB1A::LUC transgene located on another chromosome, are not present in the germline distributed as the progenitor of ice1-1 (ABRC stock: CS67845). The question thus raised is where did these two T-DNAs originate? At this point, there is no clear answer to this question. Thus, it is uncertain whether the ice1-1 mutant analyzed by Kidokoro et al. (ABRC stock: CS67843) is the same ice1-1 mutant isolated and analyzed by Chinnusamy et al. Consequently, it is unclear whether transgene-induced methylation was an issue in the Chinnusamy et al. analysis. Nevertheless, the genetic analysis presented by Kidokoro et al., in conjunction with their ice1-1 transformation experiment, provides a compelling case that the ICE1(R236H) protein encoded by ice1-1/scrm-D does not impair cold induction of CBF3/DREB1A.

But what about the wild-type ICE1 protein? Does it have a role in regulating the CBF/DREB1 genes? In a second approach, Kidokoro et al. addressed this question by testing the effects of up- and down-regulation of ICE1 activity on cold induction of the CBF/DREB1 genes. What they found is that ICE1 overexpression had no effect on cold induction of any of the three CBF/DREB1 genes. Additionally, the authors found that the null T-DNA insertion mutations ice1-2 and scrm2-1 had no effect on cold induction of any of the three CBF/DREB1 genes. Likewise, an ice1-2 scrm2-1 double mutation had no effect on cold induction of CBF1/DREB1B and CBF2/DREB1C and only a small transient effect on the induction of CBF3/DREB1A.

So, where do the Kidokoro et al. results leave us regarding the role of ICE1 in regulating the CBF/DREB1 genes? If their results were the only results available on the topic, it would be reasonable to conclude that ICE1 has little if any role in cold induction of the CBF/DREB1 genes. However, their results are not the only results on the topic and other studies lead to a different conclusion. For instance, whereas the results of Kidokoro et al. indicate that the ice1-2 mutation has no effect on cold-induction of the CBF/DREB1 genes, the results of Ding et al. (2015) and Kim et al. (2015) indicate that the ice1-2 mutation reduces peak cold induction of the CBF/DREB1 genes by 15 to 55% depending on the gene and study. Also, whereas Kidokoro et al. found that overexpression of ICE1 had no effect on cold induction of the CBF/DREB1 genes, Miura et al. (2007) reported that ICE1 overexpression increased peak expression of CBF1/DREB1B and CBF3/DREB1A by about 30% and CBF2/DREB1C by about 2.4-fold. Moreover, the same group (Miura et al., 2011) further found that an ICE1(S403A) mutation that resulted in increased ICE1 protein stability at cold temperature also resulted in about a 2-fold increase in cold induction of CBF3/DREB1A.

How might one resolve these apparent conflicting findings on the role of ICE1 in regulating the CBF/DREB1 genes? Perhaps there is a simple answer: the conflict is only apparent. That is, the differences may reflect the fact that regulation of the CBF/DREB1 genes is highly complex. Indeed, the CBF/DREB1 genes have been shown to be positively regulated by multiple transcription factors including CAMTA1,2,3,5, CESTA, BZR1/BE1 and CCA1/LHY and negatively regulated by multiple transcription factors including MYB15, PIF3,4,7, EIN3 and SOC1 (Shi et al., 2018). Additionally, the CBF/DREB1 genes are regulated by photoperiod (Lee and Thomashow, 2012); their induction is gated by the circadian clock (exposing Arabidopsis plants to low temperature in the early morning results in much greater induction of the CBF/DREB1 genes than when plants are exposure to low temperature at the end of the day) (Dong et al., 2011); and they are induced in response to touch (Gilmour et al., 1998). Given the complexity and diversity of the regulatory networks that contribute to CBF/DREB1 regulation—temperature, light, phytohormones, development, the clock, photoperiod, mechanical stress, calcium flux—it does not seem unreasonable to think that even small differences in growth conditions—age of plants, light quality and quantity, humidity, temperature, growth on soil or solid media, harvesting procedures—might affect the relative contributions that the various transcription factors have in regulating the CBF/DREB1 genes in a given experiment.

What about the role of ICE1 in freezing tolerance? Multiple studies have reported that overexpression of ICE1 increases the freezing tolerance of cold acclimated plants (Chinnusamy et al., 2003; Miura et al., 2007; Miura et al., 2011). Moreover, overexpression of ICE1 variants that stabilize the ICE1 protein at low temperature have been shown to increase the freezing tolerance of cold acclimated plants (Miura et al., 2011; Li et al., 2017). Do such results indicate that ICE1 acts as a positive regulator of the CBF/DREB1 pathway in all of these experiments? In short, the answer is “no.” This is because the CBF/DREB1 pathway is not the only regulatory pathway that contributes to

![Image](https://example.com/image1.png)

**Figure 1. The epidermal phenotype of ice1-1.** Shown are confocal microscopy images of abaxial cotyledon epidermis of Arabidopsis wild-type (A) and the original ice1-1 mutant (B). Images were taken by Dr. Masahiro Kanaoka.
freezing tolerance. This is evident from the finding that Arabidopsis plants carrying null mutations in all three CBF/DREB1, though severely impaired in freezing tolerance, still increase in freezing tolerance in response to low temperature (Jia et al., 2016; Zhao et al., 2016; Park et al., 2018). These results suggest that other transcription factors contribute to freezing tolerance independent of the CBF/DREB1 pathway. Indeed, there is direct evidence for this: for instance, overexpression of ZAT12 (Vogel et al., 2005) and HSFC1 (Park et al., 2015) has been shown to increase plant freezing tolerance and up-regulate expression of many COR genes including some that are co-regulated by CBF/DREB1. Thus, even if ICE1 does not have a major role in regulating the CBF/DREB1 pathway under a given set of conditions, it might contribute to freezing tolerance by affecting expression of other transcription factors that contribute to freezing tolerance.

One additional note, it is known that dysfunction of ICE1 confers a severe developmental phenotype (Kanaoka et al., 2008) which could impact cold tolerance indirectly. The surface of the ice1-1/scrm-D mutant plants are covered with stomata (Figure 1) (Kanaoka et al., 2008). These mutant plants lack a means to shield their bodies from the external environment. On the other hand, the ice1-2 scrm2-1 mutant produces an epidermis solely composed of pavement cells (Kanaoka et al., 2008) and thus unable to do gas exchange or transpiration via stomatal pores. With stomata serving a critical interface between a plant and the atmosphere, it is possible that such epidermal defects may impact cold tolerance in a given growth condition.

The discovery of ICE1 by Chinnusamy et al. some 17 years ago launched what has become a vibrant area of cold acclimation research. Indeed, a search of the Web of Science with the topic terms “ICE1 and freezing tolerance” yields over 170 publications which have been cited more than 9,000 times. The body of knowledge generated by these studies has resulted in the current wisdom which holds ICE1 as a major regulator of the CBF/DREB1A pathway—often referred to as the ICE1-CBF-COR pathway—and, as a consequence, serves a role in freezing tolerance. It is within this body of work that the results of Kidokoro et al. must be placed. One take home lesson is that conclusions from previous work using the ice1-1 mutant may have to be reinterpreted given the seemingly clear results of Kidokoro et al. indicating that the ice1-1/scrm-D mutation—encoding the ICE1(R236H) protein—does not impair cold induction of the CBF/DREB1 genes. A second lesson is that ICE1 may not be an indispensable master regulator of the CBF/DREB1 genes. Certainly, the results presented by Kidokoro et al. provide evidence that under a given set of experimental conditions overexpression of ICE1 or lack of ICE1 function (or even a lack of both ICE1/SCRM and SCRM2/ICE2 function) has little effect on cold induction of the CBF/DREB1 genes. Whether ICE1 contributes significantly to CBF/DREB1 expression under some conditions and not others and whether ICE1 contributes significantly to freezing independent of CBF/DREB1 are questions for future study. Indeed, it would seem prudent to think, as Kidokoro et al. propose, that “the current ICE1-DREB1 regulatory model should be reevaluated without the previous assumptions”.

AUTHOR CONTRIBUTIONS
Both authors contributed to writing this article.

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