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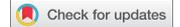Taylor & Francis Group

RESEARCH PAPER

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Responses of PYR/PYL/RCAR ABA Receptors to Contrasting stresses, Heat and Cold in Arabidopsis

Qian Zhang, Xiangge Kong, Qin Yu, Yongqiang Ding, Xiaoyi Li, and Yi Yang ●

Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, State Key Laboratory of Hydraulics and Mountain River Engineering, College of Life Sciences, Sichuan University, Chengdu, China

ABSTRACT

Plants growing in natural habitats have evolved a wide range of mechanisms to copy with environmental challenging, including biotic and abiotic stresses. Abiotic stresses-induced increases in Abscisic acid (ABA) levels in plants suffering from stresses, including drought, cold or heat stress. To explore the function of the core components in ABA signaling, we used the overexpression of *RCARs* transgenic plants to expose in heat or cold stress. In this study, overexpression of *RCAR12* or *RCAR13* (*R12*-OE or *R13*-OE) transgenic plants had higher germination and survival rate than the wild-type (WT) Arabidopsis, indicating that they are both positively responsive to the high temperature. And the heat shock genes *HSP18.2* and *HSP70* were significantly induced by RCAR12 or RCAR13. Further, the results inferred that the over-expression of *RCAR12* or *RCAR13* could tolerance the cold stress, through induction CBFs expressions, the cold-responsive genes when plants were challenged the cold tress. And when complementation of *RCAR12* to the 1124 mutant (R12:1124), the results indicated that RCAR12 could recover the insensitivity of 1124 to heat and cold stresses. Hence, we propose that RCAR12 and RCAR13, the ABA receptors, may play the positive roles in regulating the extreme temperature, including cold and high temperature in Arabidopsis.

1. Introduction

Plants as sessile organisms have evolved complex developmental and physiological strategies to adapt to the unfavorable and changing environments such as drought, high salinity, and temperature fluctuations.[1,2] When extreme temperature emerges, plants should face balancing adaptation of heat and cold stresses. Heat stress causes cellular damage of plants, ultimately leading to severe growth retardation and possible death.[3,4] Low temperatures can severely disrupt the metabolism and physiological homeostasis of plant cells, and also result in weakness of stem, root and leaf development.[5] Under natural conditions, vegetable crops eventually face episodes of heat and cold stresses that cause a loss of yield and agricultural quality. Therefore, understanding extreme temperature stresses, and how they damage plants are of practical importance.

Previous studies on Arabidopsis have suggested that phytohormones are involved in stress adaptation, especially for abscisic acid (ABA), which is generally considered a stress hormone.[6–8] ABA regulates many essential physiological and biochemical processes. Importantly, it mediates many stress responses, such as the inhibition of primary root growth, guard cell closure and stress gene expression.[9–11] Numerous reports have showed that ABA plays an essential role of dehydration stress.[12–14] However, it has also been suggested that the plant hormone ABA induces thermotolerance in maize,[15] or regulates heat stress transcription factors (HSFs) and heat shock proteins (HSPs) in *Festuca arundinacea* Schreb.[16]

ABA-deficient and -insensitive mutants are sensitive to heat stress, whereas ABRE/ABF-OE plants showed enhanced thermotolerance.[17–20] ABA as a signal molecule is not only important for adaption to high temperature, but also cold stress. The literature reports indicate that cold acclimation can be triggered by exposing plants to exogenous ABA.[5,21] Meanwhile, cold stress is accompanied with increased levels of endogenous ABA in many plant, such as maize, rice, cucumber, and pepper.[22–24]

ABA core signal pathway includes three components: pyrabactin resistance (PYR)/pyrabactin resistance-like (PYL)/regulation component of ABA receptors (RCAR) (hereafter referred as RCARs), protein phosphatase 2C (PP2Cs) and sucrose non-fermenting (SNF1)-related protein kinase 2 (SnRK2).[25,26] In the absence of ABA, SnRK2s physically interacts with PP2Cs, which inactivate SnRK2s to phosphorylate downstream transcription factors.[27] In the presence of ABA, RCARs would bind with ABA and subsequently tend to inhibit the activity of PP2Cs phosphatase.[7] This lead to the release of SnRK2s and then the kinases of SnRK2s could phosphorylate ABRE/ABF/ABI5 transcription factors or guard cell anion channel (SLAC1), all of which further activate ABA-induced gene expressions.[28–30] ABA receptors constitute a 14-member family, all of which are able to activate ABA-responsive gene expression in protoplast transfection assays.[28,31]

According to the alignment of amino acid sequences and expression patterns, substantial functional differences and redundancy among them should be expected.[32,33] For example, RCAR3

conferred greater ABA sensitivity to the PP2C regulation than RCAR1, whereas regulation of the protein phosphatase activity by RCAR1 and RCAR3 was more sensitive to ABA for ABI1 than for ABI2.[34] CARK1 interacts and phosphorylates RCAR3 and RCAR11 to involve in the ABA signal pathway, respectively.[35] Except for functions of regulation in the ABA signal pathway, ABA receptors also mediate abiotic stresses. The expression profile of ABA core signaling component genes shifts under heat and cold stress[36] ABA receptor genes, including *RCAR8* and *RCAR9*, were upregulated in *UBP1b*-ox plants.[37] Constitutive overexpression of rice landrace Nagina 22 *PYL3* showed improved cold stress tolerance in *Arabidopsis*.[24] However, it is not known how ABA-related genes both regulate heat and cold stress.

Here, we report that overexpressing of ABA receptors, including *RCAR12* and *RCAR13* response to heat and cold stress, respectively. The results show that overexpression of *RCAR12* or *RCAR13* increases thermotolerance in Arabidopsis through accumulation of heat short proteins (HSP), *HSP18.2* and *HSP70*. Interestingly, we also found that the gain-of-function of RCAR12 and RCAR13 plants could tolerance cold stress through the induction of C-repeat binding factor (CBF) genes.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis ecotype Col-0 was used as the wild-type (WT) control in this study. The quadruple 1124 mutant from the lab of Prof. Rodriguez is genetic impairment of *PYR1, PYL1 (RCAR12), PYL2* and *PYL4*.[25,38] The transgenic plants (*RCAR12 and RCAR13*-OE) were from our lab. The transgenic plants were identified.[39–41] For plate culture, seeds were surface sterilized by treatment with 75% ethanol for 5 min, followed by commercial bleach (20%) for 15 min, and finally, four washes with sterile-distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Then, seeds were sown on MS medium containing 2% sucrose and 0.8% agar, pH 5.7 with KOH. All plants were incubated in the growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 $\mu E\ m^{-2}\ s^{-1}$.

2.2. RNA extraction and quantitative real-time PCR (RT-PCR) analysis

Total RNA was extracted from 10-d-old Arabidopsis seedlings treated with heat or cold stress using TRIzol RNA reagent (TaKaRa, China). The cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, China), following the manufacturer's instruction. PR-PCR was carried out on a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA) with iQ™ SYBR Green Supermix and gene-specific primers (Table S1). All reactions were performed in triplicate with the following cycling conditions: 95°C for 5 min; 45 cycles each at 95°C for 20 s and 52°C for 20 s, and 72°C for 20 s. Actin2/8 was used as an internal standard.

2.3. Phenotype analysis

Seven or 10-d seedling on Murashige and Skoog agar medium (MS) were heated in the water incubator at 42°C for 1 h. The seed heat tolerance assay, seeds after stratification were heated in the water incubator at 50°C for 2 h. The freezing tolerance assay, 7 or 10-d seedlings were put at −20°C for 70 min, followed by 4°C for 24 h, and then 23°C in the chamber for 2 d.

3. Results

ABA receptors RCARs are well known to mediate plants response to ABA-dependent drought signal pathway and function as a major part of the ABA signal pathway. In this study, we tested whether RCARs are involved in response to other abiotic stress, including heat stress and cold stress.

3.1. RCARs involve in the heat stress response

The transcript expressions of *RCARs* were detected in Arabidopsis before and after heat stress. The expression of *RCAR12* had a 6.5-fold increase in WT plants exposed to the high temperature at 38°C for 2 h and increased 12.5-fold at 42°C for 2 h (Figure S1). The expression of *RCAR13* increased 17.9-fold at 52°C for 2 h, while it did not changed exposing to 38°C or 42°C. ABA receptors had a medium response to high temperature. Although ABA receptors are highly conserved in Arabidopsis,[32] the response to heat stress are different.

To explore the roles of signal transduction pathway involved in heat-stressed pathway, we tested the heat stress tolerance of over-expressing *RCARs* transgenic plants. The survival rates of *RCAR12*-OE and *RCAR13*-OE were significantly higher (~40%) than that of the wild-type plants (~15%) after the seedlings were exposed to the high temperature at 42°C for 2 h (Figure 1). To detect the heat tolerance during seed germination, seeds were exposed to the high temperature at 50°C for 2 h. The analysis demonstrated that of *RCAR12*-OE and *RCAR13*-OE plants presented higher germination rates than WT (Figure 2). Therefore, ABA receptors RCAR12 and RCAR13 had positive roles in heat stress response of plants.

The previous literature suggests a relationship between heat stress tolerance and heat shock proteins (HSPs) in plants.[16,42] Thus, the transcript abundance of *HSPs* was detected in different *RCARs* transgenic plants by qRT-PCR. Our results revealed that the transcription expression levels of *HSP18.2* and *HSP70* in *RCAR13*-OE plants had 40 and 37-fold increase, comparing with the control under the normal condition after heat treatment (Figure 3a). RCAR12 also could induce the expressions of *HSP18.2* and *HSP70* (Figure 3a). In addition, the complementation of *RCAR12* in the 1124 mutant showed higher germination rates than that of 1124 and WT (Figure 3b). Therefore, these results further demonstrated that ABA receptors RCAR12 and RCAR13 were involved in heat stress responses to plants by the induction of *HSPs* expressions.
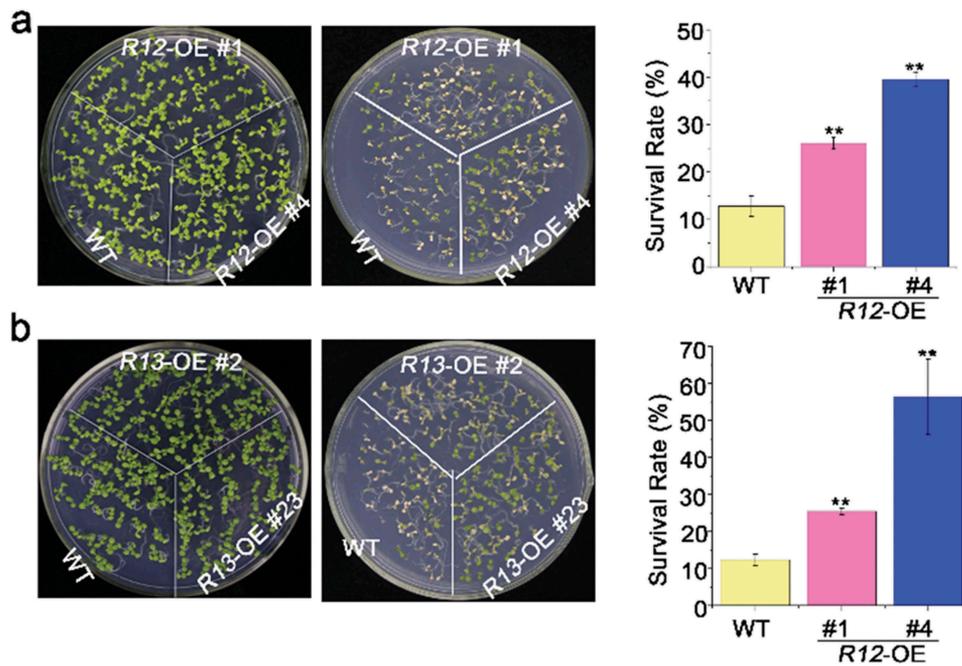
Figure 1. Heat tolerance of overexpressing of *RCAR12* and *RCAR13* transgenic plants in seedlings.
(a and b) 4-d-old seedlings in wild-type (WT), overexpression *RCAR12* plants (a), overexpression *RCAR13* plants on MS agar medium exposing to heat shock at 42°C for 2 h. Survival rates were cored at day 2 after heat stress treatment. Seedlings of plants on the control were 7-day-old plants. Data are means ± SD of the three independent biological experiments. ** Significant at *P* < .01 compared with the WT.
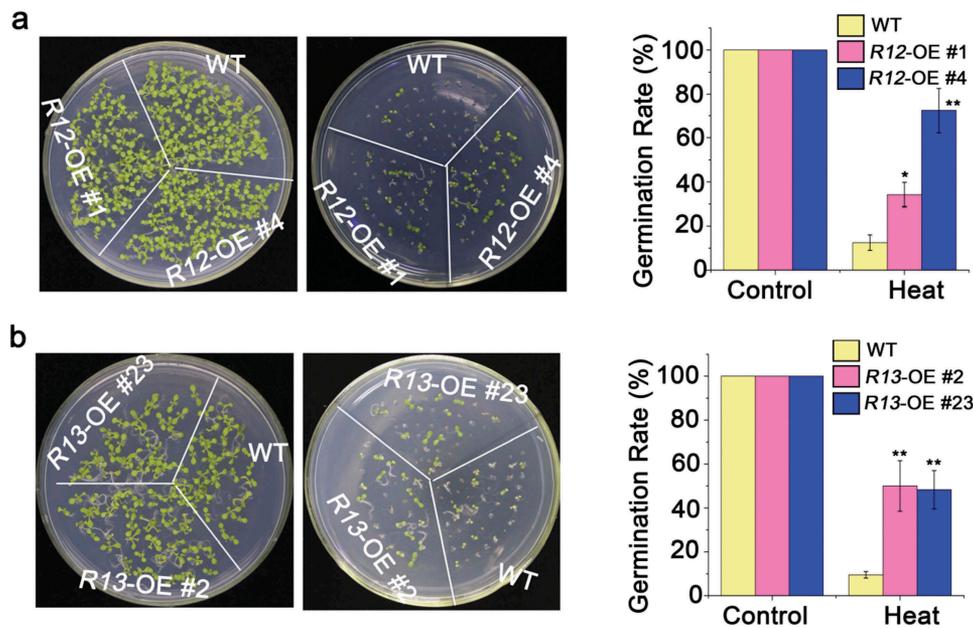

Figure 2. Heat tolerance of overexpressing of *RCAR12* and *RCAR13* transgenic plants.
(a and b) Seeds after stratification at 4°C for 3 days in the dark were sown on MS agar medium after 50°C for 2 h. The germination rates of plants (WT, *RCAR12*-OE (a) and *RCAR13*-OE (b)) were measured at day 10. Data are means ± SD of the three independent biological experiments. ** Significant at *P* < .01 compared with the WT, Student's *t*-test.

3.2. RCARs regulates CBFs during plant cold responses

Subsequently, we tested the ABA receptors response to the cold or freezing stress, including *RCAR12* and *RCAR13*. After the chilling or freezing treatment, the expression levels of *RCAR12* had about twofold increase, while *RCAR13* was significantly increased in the WT plants after −20°C for 60 min comparing with that in normal conditions (Figure S2). However, phenotypic analysis showed that *RCAR12/13*-OE after the freezing treatment had a higher level of survival rate than WT (Figure 4a,b and Figure S3). C-repeat/DREB binding factor (CBFs) as key transcription factors are critical for cold acclimation in high plants.[2,43] In this study, the qRT-PCR was employed to analyze the changes of expressions of *CBFs* in transgenic plants before and after cold treatment. The results indicated
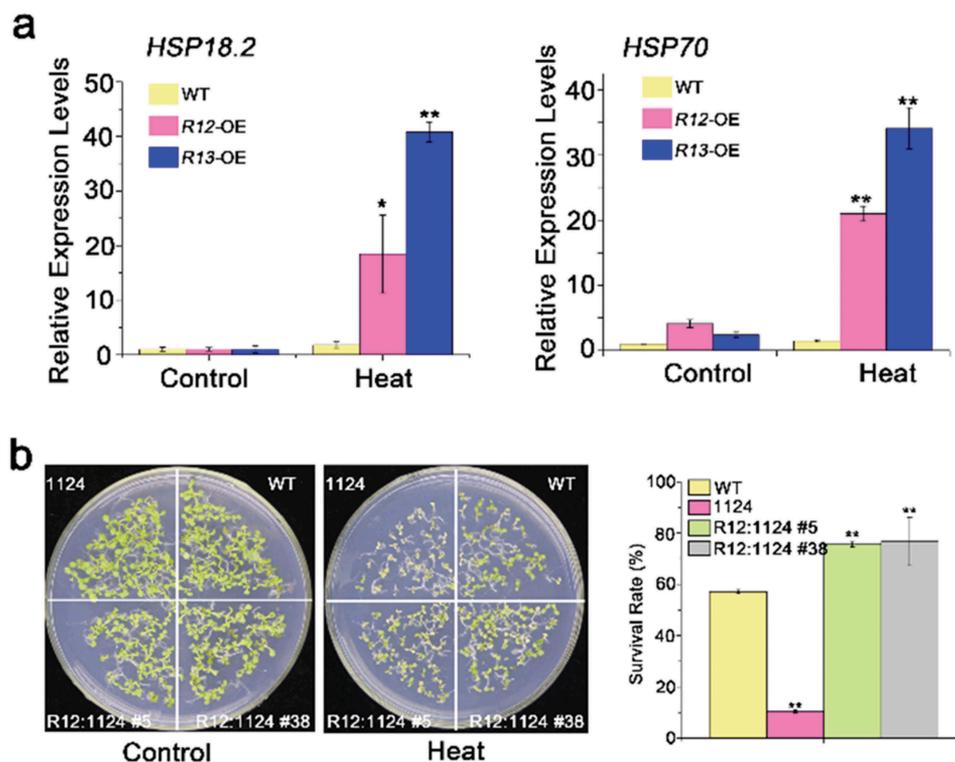
Figure 3. Heat-responsive genes expressions in overexpressing of *RCAR12* and *RCAR13* transgenic plants and RCAR12 recovers the heat-sensitive phenotype in 1124 mutant. (a) Relative expression levels of heat-responsive genes *HSP18.2* and *HSP70* in wild-type and *RCAR11*, *RCAR12* and *RCAR13* overexpression transgenic plants. 10-d-old seedlings in MS agar medium were treated with or without heat stress at 42℃ for 2 h. The transcriptional levels were determined by qRT-PCR analysis. Values are means ± SD (n = 3). *ACTIN2/8* was used as an internal control. The experiments were repeated three times (*P < .05, **P < .01, Student's *t*-test). (b) Seeds after stratification at 4℃ for 3 days in the dark were sown on MS agar medium after 50℃ for 2 h. The germination rates of plants were measured on the tenth day.
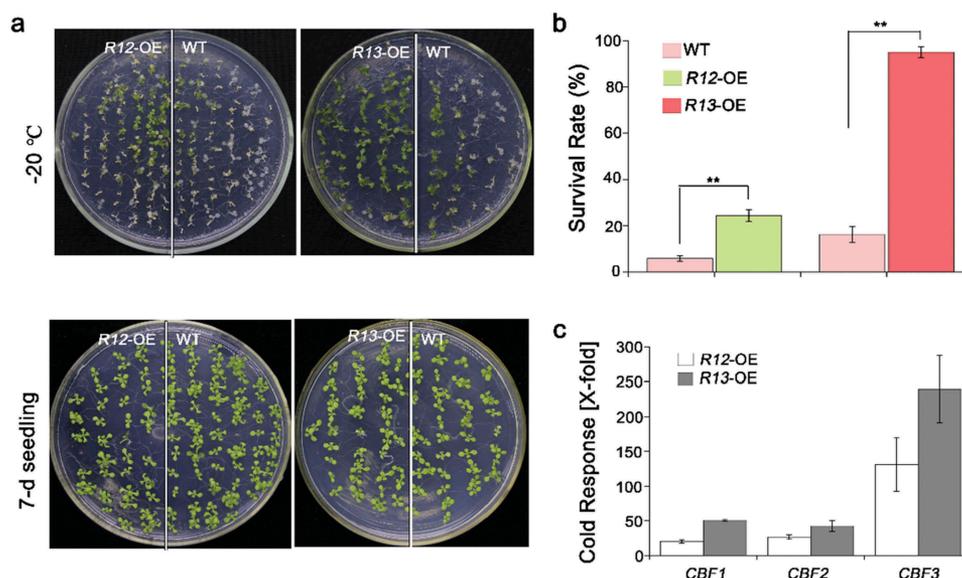

Figure 4. Freezing phenotypes of the plants. (a) 7-d-old plants including WT, *RCAR12*-OE (*R12*-OE #1) and *RCAR13*-OE (*R13*-OE #4) under freezing stress for 70 min and then were recovered in the plant chamber. (b) Survival rates were cored at day 2 after recovery. Data are means ± SD of the three independent biological experiments. ** Significant at P< .01 compared with the WT. Independent biological experiments were performed. (c) Relative expression levels of heat-responsive genes *ABF1*, *ABF2* and *ABF3* in wild-type, *RCAR12* and *RCAR13* overexpression transgenic plants. 10-d-old seedlings in MS agar medium were treated with or without heat stress at 4℃ for 12 h. The transcriptional levels were determined by qRT-PCR analysis. Values are means ± SD (n = 3). *ACTIN2/8* was used as an internal control. The experiments were repeated three times (*P < .05, **P < .01, Student's *t*-test).

that *CBF1/2/3* transcription levels in the overexpression *RCAR12* or *RCAR13* plants after cold stress had highly induced comparing with the control (Figure 4c). Furthermore, the transgenic plants with overexpressing *RCAR12* in the 1124 mutant were generated and were cooled at −20℃ for 70 min. The results showed that the R12:1124 transgenic plants had significantly higher survival rates

than that of 1124 mutant and the wild type plants (Figure 5). Taken together, RCAR12 and RCAR13 mediated the cold stress response of plants in the manner of inducing *CBFs* expressions.

4. Discussion

The phytohormone abscisic acid (ABA) would accumulate under stressed conditions, including drought, high and low temperature.[5] RCARs, as a key regulator, involve in the ABA signaling pathway and ABA-dependent drought-stress signal pathway.[41,44] Here, we reported the functions of ABA receptors in extreme temperature.

Drought stress usually accompanies with high temperature. Therefore, we hypothesis that RCARs also play an essential role in plant response to high temperature. The seeds and seedlings of *RCAR12/13*-OE showed heat tolerance through induction of *HSPs*. These results indicate that RCAR12 and RCAR13 play a positive role in regulation of heat-stress signal pathway. This conclusion is consistent with that HSP6a, participates in ABA-mediated resistance, as a positive regulator in heat-stress response.[20] In addition, ABA-responsive element binding protein 3 (AREB3), function as upstream trans-acting factors and regulate transcriptional activities of FaHSFA2c and the downstream FaHSPs leading to improve heat tolerance in *Festuca arundinacea* Schreb.[16] However, it is interesting that *RCAR1*-OE could not bear high temperature. Although ABA receptors are conserved, their functions also have divergence. For example, the PP2C AHG1 is regulated by RCAR1, RCAR2, and RCAR3, but not other members.[31] Therefore, it is reasonable

to propose that the components in the ABA signal pathway also involve in heat-stress responses through regulation of heat shock proteins.

Another common environmental stress, a low temperature, severely inhibits plant growth and development. A recent report suggests that the phosphorylation of ICE1 by the kinase SnRK2.6 could depress the degradation of ICE1, ultimately positively mediates the plant cold-stress response.[45] In plants, ABA accumulates under stress condition and ABA receptors bind to ABA, thus releasing the inhibition of SnRK2.6 by PP2Cs.[25,26] Our results indicate that *RCAR12*-OE and *RCAR13*-OE have higher levels of survival rates than other plants, and induce the expressions of CBF1/2/3. Therefore, we postulate that RCAR12 and RCAR13 binding to ABA inhibit the activities PP2Cs, resulting that SnRK2.6 enhances ICE1 stability through phosphorylation.[45] In polar, overexpression of *PtPYR1* or *PtPYL5* could increase the resistance to cold stress.[1]

5. Conclusion

In conclusion, RCAR12 and RCAR13 could positively regulate the plant responses to the extreme temperature through induction the expressions of downstream genes. In this study, we demonstrated that overexpression of *RCAR12* or *RCAR13* could enhance the plants in responses to the heat tolerance, through induction of the heat shock proteins, including *HSP18.2* and *HSP70*. In addition, RCAR12 or RCAR13 mediated the cold-stress signal pathway through induction of *CBFs* in Arabidopsis (Figure 6). However, more information is needed to explore how ABA receptors have an effect on cold resistance.
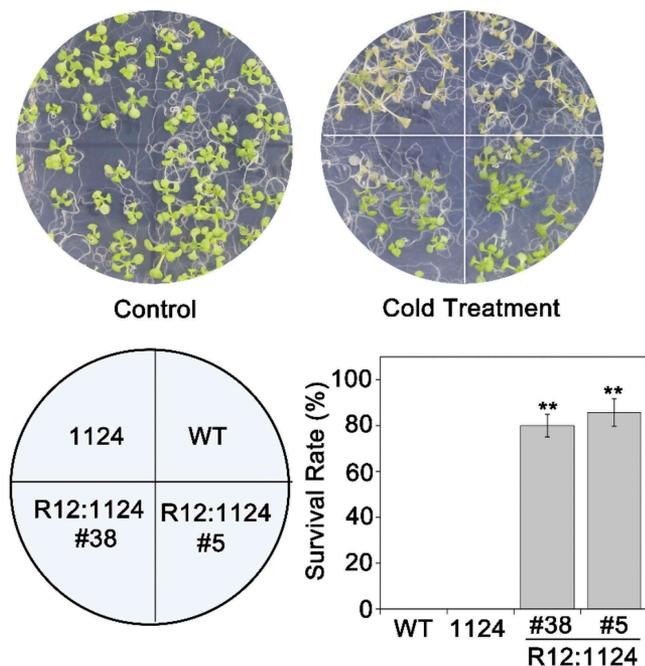

Figure 5. RCAR12 could recover the sensitivity of the 1124 mutant to cold stress. 7-d-old plants including WT, 1124 and *RCAR12*:1124 (R12:1124) under freezing stress for 70 min and then were recovered in the plant chamber and the survival rates were determined. Independent biological experiments were performed. ** Significant at *P* < .01.
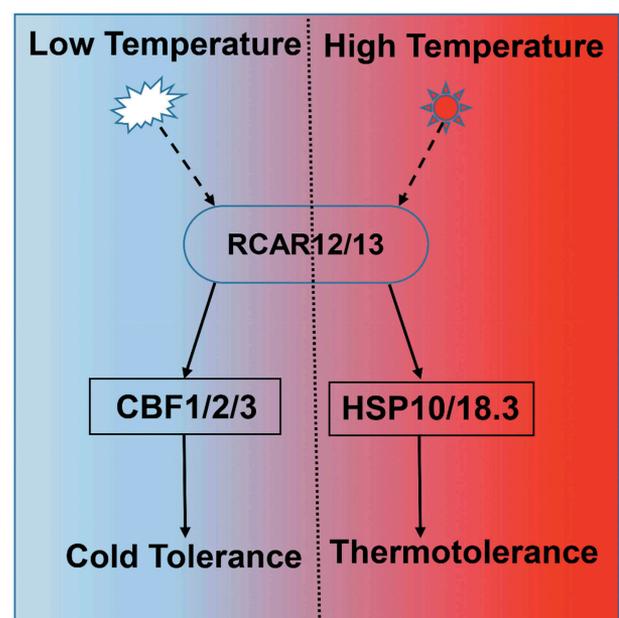

Figure 6. Model illustrating how RCARs response to heat and cold stresses in plants.

ORCID

Yi Yang ⓘD http://orcid.org/0000-0001-6051-4992

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