Plant Science xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions

Carine Gery[a], Ellen Zuther[b], Elisa Schulz[b], Julie Legoupi[a], Aurélie Chauveau[c], Heather McKhann[c,d], Dirk K. Hincha[b], Evelyne Téoulé[a,e,*]

[a] *Institut Jean-Pierre Bourgin (IJPB), UMR1318 INRA-AgroParisTech, Bât. 7, INRA Centre de Versailles-Grignon, Route de Saint Cyr, 78026 Versailles Cedex, France*
[b] *Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Potsdam, Germany*
[c] *Etude de Polymorphisme des Genomes Végétaux (EPGV), INRA, CNG, 2, rue Gaston Crémieux, 91057 Evry Cedex, France*
[d] *DARESE Affaires Européennes, INRA, 147 rue de l'Université, 75338 Paris Cedex 07, France*
[e] *UPMC, 4 Place Jussieu, 75252 Paris Cedex 05, France*

ARTICLE INFO

Article history:
Received 30 March 2010
Received in revised form 13 July 2010
Accepted 17 July 2010
Available online xxx

Keywords:
Arabidopsis
Cold acclimation
CBF
RNAi
QTL mapping

ABSTRACT

Plants from temperate regions are able to withstand freezing temperatures and to increase their freezing tolerance during exposure to low, but non-freezing, temperatures through a process known as cold acclimation. Key regulatory proteins in this process are the cold-induced CBF1, 2 and 3 transcription factors which control many cold regulated genes. Although much work has focused on this signal transduction pathway, the details of its regulation and of its quantitative contribution to cold acclimation are still unclear. Here, we have used the large natural variation present in the 48 accessions of the Versailles core collection of *Arabidopsis thaliana* to further elucidate the function of the CBF transcription factors. *CBF* gene expression studies showed that the freezing sensitive accessions had mostly low expression levels 2 h after transfer of plants to 5 °C, while the most tolerant accessions showed a wide range of *CBF* expression levels. To investigate the quantitative contribution of *CBF* expression to plant freezing tolerance and low temperature growth performance, RNAi lines targeting all three *CBF* genes were produced in eight different accessions. We observed striking differences between different accessions in the effects that reduced *CBF* expression had on freezing tolerance, while effects on growth were generally too small to draw firm conclusions. Analysis of *CBF* expression indicated a tight co-regulation between *CBF1* and *CBF3*, while the relationship between the expression levels of *CBF2* and *CBF1* or *CBF3* strongly depended on the genetic background of the RNAi lines. In agreement with the observed differences between the different accessions, QTL analyses with two different RIL populations indicated that QTL localisation varies strongly between populations. Collectively, these results show that both the regulation of the *CBF* genes and their relative contribution to freezing tolerance strongly depend on the accession studied. In addition, natural variation is suggested to be an interesting source of novel regulatory pathways and genes that may be useful in the future for improving plant freezing tolerance.

1. Introduction

Low temperature is among the most important abiotic environmental factors affecting the geographical distribution of plant species, as well as growth and yield of crop plants. Therefore, understanding the networks and the molecular mechanisms underlying the cold responses of plants is of major importance to understand the ecology and physiology of wild species and to increase the yield potential of species of agronomic interest.

The ability of plants to survive freezing temperatures depends to a large extent on their capacity to cold acclimate, i.e. to increase their freezing tolerance during exposure to low, but non-freezing, temperatures [1]. For example, the acclimation process allows winter wheat to survive temperatures down to −20 °C while it is killed at around −5 °C in the nonacclimated state [8]. During this cold acclimation period, extensive modifications take place in the plant, such as changes in the lipid composition of membranes and increases of soluble proteins, sugars and proline, molecules that may serve as cryoprotectants (see [2] for review). These physiological changes are due, at least in part, to large-scale modifications of gene expression [3–7].

* Corresponding author at: Institut Jean-Pierre Bourgin, UMR1318 INRA-AgroParisTech, Bât. 7, INRA, Centre de Versailles-Grignon, Route de St Cyr, 78026 Versailles Cedex, France. Tel.: +33 1 30 83 33 06, fax: +33 1 30 83 33 19.
E-mail address: Evelyne.Teoule@versailles.inra.fr (E. Téoulé).

0168-9452/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2010.07.010

Recently many studies have been undertaken on model plants such as *Arabidopsis thaliana*. In this species, mechanisms underlying cold acclimation have been extensively studied and excellent reviews on this subject are available [9–11]. Most of these studies involved the characterisation of mutants selected by forward and reverse genetic screens in a few accessions such as Columbia-0 (Col-0) or Wassilewskija (WS). Although these strategies have been highly successful, the study of natural genetic variation has been shown in the past two decades to be an interesting alternative means to elucidate the functional role of candidate genes [12,13] and to identify novel genes involved in complex traits *via* quantitative trait locus (QTL) mapping [14–17].

The CBF pathway is one of the major signalling pathways involved in plant cold acclimation. In this pathway, the *CBF/DREB1* (*CRT*-binding factor/*DRE*-binding factor 1) genes act as main molecular switches. Numerous studies deal with *CBF1*, *2* and *3* in *Arabidopsis* and homologues have been identified in many other species such as barley [18–20], wheat [21] and eucalyptus [22], showing that this pathway is widely distributed in higher plants. In most species, at least some of the *CBF* genes are rapidly induced in response to low temperatures [1,5,23,24]. They encode proteins that are transcriptional activators containing the AP2/ERF DNA-binding domain, which is able to recognize the CRT/DRE element present in the promoters of a large number of cold regulated genes. Transcriptomic analysis in *A. thaliana* has shown that 12–20% of all cold-induced transcriptional changes are regulated by CBF1-3 [25]. Collectively, these genes have been termed the "CBF regulon". Moreover, over expression of individual *CBF* genes in transgenic *Arabidopsis* plants results in constitutive expression of target genes and increased freezing tolerance without an acclimation period [26]. In addition to these data, major QTL for freezing tolerance have been identified in both *Arabidopsis* [27] and wheat [21] that localise to the region of the *CBF* genes. These data all suggest that the *CBF* genes play a critical role in the increased freezing tolerance observed during cold acclimation. It is, however, still unclear whether the different members of the *CBF* gene family have redundant roles. In *Arabidopsis*, there is evidence that CBF2 is a negative regulator of *CBF1* and *CBF3* expression, through the study of the only known *CBF* mutant and of *CBF* anti-sense and RNAi lines [28,29]. However, the lack of additional mutants has precluded the analysis of loss-of-function phenotypes for *CBF1* and *CBF3*.

The natural genetic variation present in plant species is a powerful tool to elucidate the functional role of candidate genes in complex traits [30,31]. With respect to freezing tolerance, the core collection of 48 *A. thaliana* accessions generated in Versailles [32] has been characterised previously by evaluating damage to whole plants after freezing and by sequencing the *CBF1-3* genes [33]. In addition, a subset of accessions has also been analyzed for the cold-induced expression of *CBF1-3* and some of their *COR* target genes, and for freezing tolerance using electrolyte leakage assays [33]. Other studies have established a linear correlation between acclimated freezing tolerance of *Arabidopsis* accessions and minimum habitat temperature [34,35] indicating strong evolutionary pressure on this trait.

In the present study, we have measured *CBF* gene expression in all 48 accessions of the Versailles core collection to determine whether there is a general correlation between the degree of cold induction of these genes and freezing tolerance. To gain insights into the importance of the *CBF* pathway in the freezing tolerance and growth performance of natural accessions, we examined the effect of RNAi-induced silencing of the *CBF* genes on these traits. In parallel, we performed QTL mapping for freezing tolerance after acclimation in two RIL populations generated in the Biological Resource Center in Versailles. The data confirm the large variation in *CBF* gene expression in natural populations and indicate that the importance of the CBF signal transduction pathway varies among accessions. Similarly, QTL mapping led to different positions in the two populations tested, suggesting the presence of additional pathways important for freezing tolerance in *Arabidopsis*.

2. Materials and methods

2.1. Plant material and experimental conditions

All accessions are from the Versailles nested core collection [32]. Passport data for these accessions are available at [36]. For the analysis of *CBF* expression in the accessions after a short (2 h) cold treatment, plants were grown as described in McKhann et al. [33]. Briefly, the plants were grown in a greenhouse and watered to keep the substrate humid and shortly before they were transferred to 5 °C for cold acclimation. During cold acclimation, relative air humidity was about 50% and plants were not watered during acclimation or freezing. For the test of freezing tolerance of cold acclimated whole plants, conditions were as described by Bouchabke-Coussa et al. [37] except that only a freezing temperature of −5 °C was used. After freezing, the plants were transferred back into the greenhouse and watered the day after thawing.

For the growth experiments, seeds were germinated on soil and grown for 5 weeks at 4 °C with a 16 h day length at 90–100 μmol photons m^{-2} s^{-1}. Plants were then transferred to short day conditions (8 h light at 180 μmol photons m^{-2} s^{-1} at a temperature of 20 °C during the day and 16 °C during the night) for an additional two weeks. These plants were then divided into three groups of 8–10 plants each that were transferred into small growth chambers where they were exposed to 16 h day length at 150 μmol photons m^{-2} s^{-1} at a temperature regime of either 20 °C/18 °C, 15 °C/13 °C, or 10 °C/8 °C (day/night). Plants were randomized within each chamber to avoid positioning effects. Growth was quantified by counting the rosette leaves and measuring the rosette diameter at regular intervals. Experiments were terminated and rosettes from 4 to 5 plants were harvested by plunging into liquid nitrogen when the inflorescences of at least 50% of the plants of an accession and its respective RNAi lines had reached 4–6 cm in length.

For QTL detection, subsets of 164 Can-0 × Col-0 and Bur-0 × Col-0 RILs optimized for QTL mapping (http://dbsgap.versailles.inra.fr/vnat/) were grown in the greenhouse and phenotyped according to McKhann et al. [33] to map QTLs affecting freezing tolerance. RILs still segregating for a limited region around a QTL position were used to generate HIFs [38], which enabled the comparison of lines containing one of the parental alleles at the locus of interest in an otherwise identical background.

2.2. Generation of transgenic plants

For the RNAi gene construct targeting *CBF1*, *2* and *3*, a 200 bp partial coding region of *A. thaliana CBF2* (At4g25470) was cloned in pHannibal [39]. The primers used were CBF2r 448U24 (xxxxxxGACATGGAGGAGACCTT) and CBF2r 614L24 (xxxxxxGTCATCATCTCCCTCGA) with the appropriate restriction sites (xxxxxx). The recombined pHannibal (pCG102) was digested with Not1 and cloned into the binary vector pEC2 [40] leading to the plasmid pCG1001. This plasmid was introduced into target plants using *Agrobacterium*-mediated transformation by floral dipping [41]. T1 plants were screened for transformants on Basta in the greenhouse. T2 seeds were collected and screened *in vitro* for 3:1 segregation to select lines with a single insertion, then T3 homozygous lines were derived from this material.

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

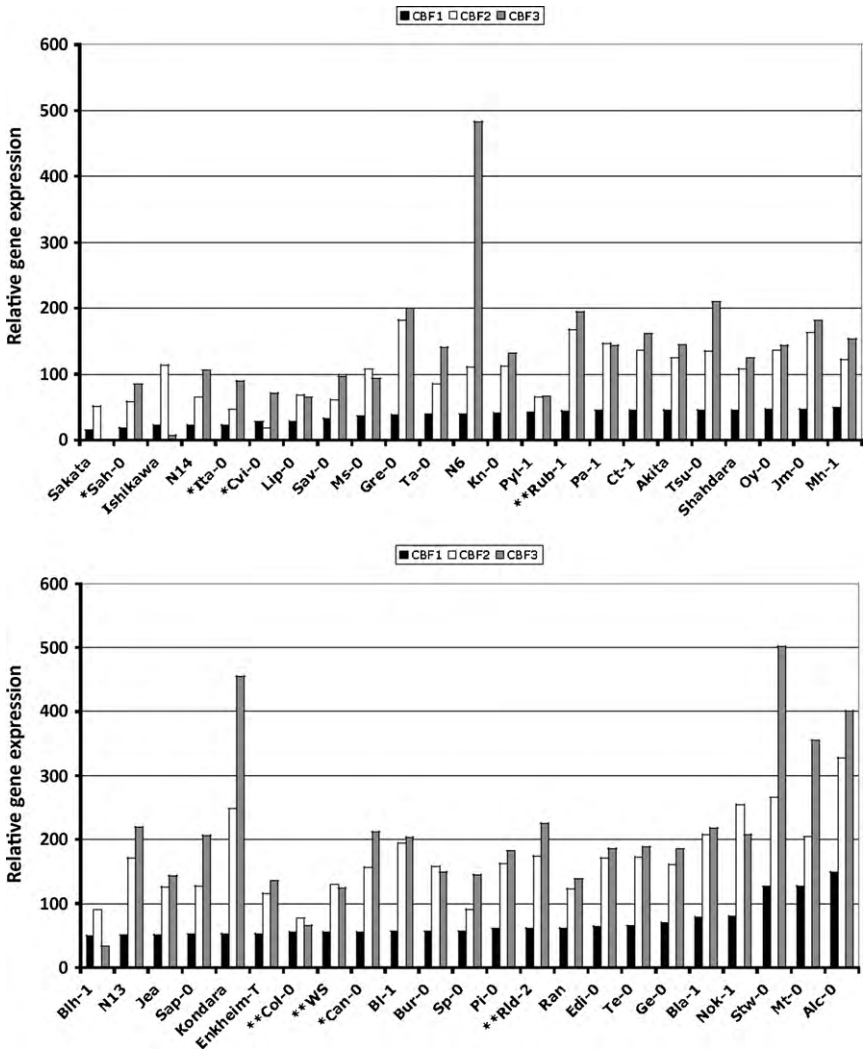

Fig. 1. *CBF* gene expression in 44 accessions from the Versailles core collection. In addition, WS and Col-0 were investigated as reference accessions. Expression levels were measured after 2 h at 5 °C in two biological replicates. Accessions are ordered as a function of *CBF1* expression level. Expression levels under control conditions are not presented because they were very low and not significantly different between accessions. A set of eight accessions with contrasting freezing tolerance that we characterised previously [33] is indicated by asterisks (*for sensitive and **for tolerant accessions).

2.3. RNA extraction, cDNA preparation and quantitative RT-PCR

For the analysis of *CBF* expression in the accessions after a short (2 h) cold treatment, RNA extraction, cDNA preparation and quantitative RT-PCR were performed according to protocol 1 in McKhann et al. [33]. For the analysis of *CBF* expression at the end of the growth experiments, the protocol of McKhann et al. [33] was used with some modifications. RNA was isolated from pools of 4 to 5 rosettes. cDNA was synthesized using Superscript III (Invitrogen) following the manufacturer's instructions and expression of the *CBF* genes was normalized to the mean of the expression levels of the housekeeping genes ubiquitin 10 (At4g05320) and actin 2 (At3g18780).

2.4. Freezing tolerance measurements

To measure viability after freezing at −5 °C, plants were transferred back into the greenhouse and survival was scored after 6 days. Four rows of the RNAi lines and two rows of the respective wild type plants from an accession were put in each square pot to optimize viability comparisons by reducing undesirable environmental variation. At least 400 plants were tested per line.

For QTL detection, the experimental design described in McKhann et al. [33] was used. The score for freezing damage is calculated on a mean of five replicates.

2.5. Statistical analysis

Differences in rosette diameters between the wild type and RNAi plants in the different accessions were tested for significance using an unpaired Student's *t*-test. Linear correlation analysis was done by least squares regression and the significance of the correlations was tested using a paired Student's *t*-test.

2.6. QTL analysis

QTL analyses were performed using QTL Cartographer [42], with standard methods for interval mapping as described by Loudet et al. [43]. Then, HIFs were used to confirm initial QTL positions [38].

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

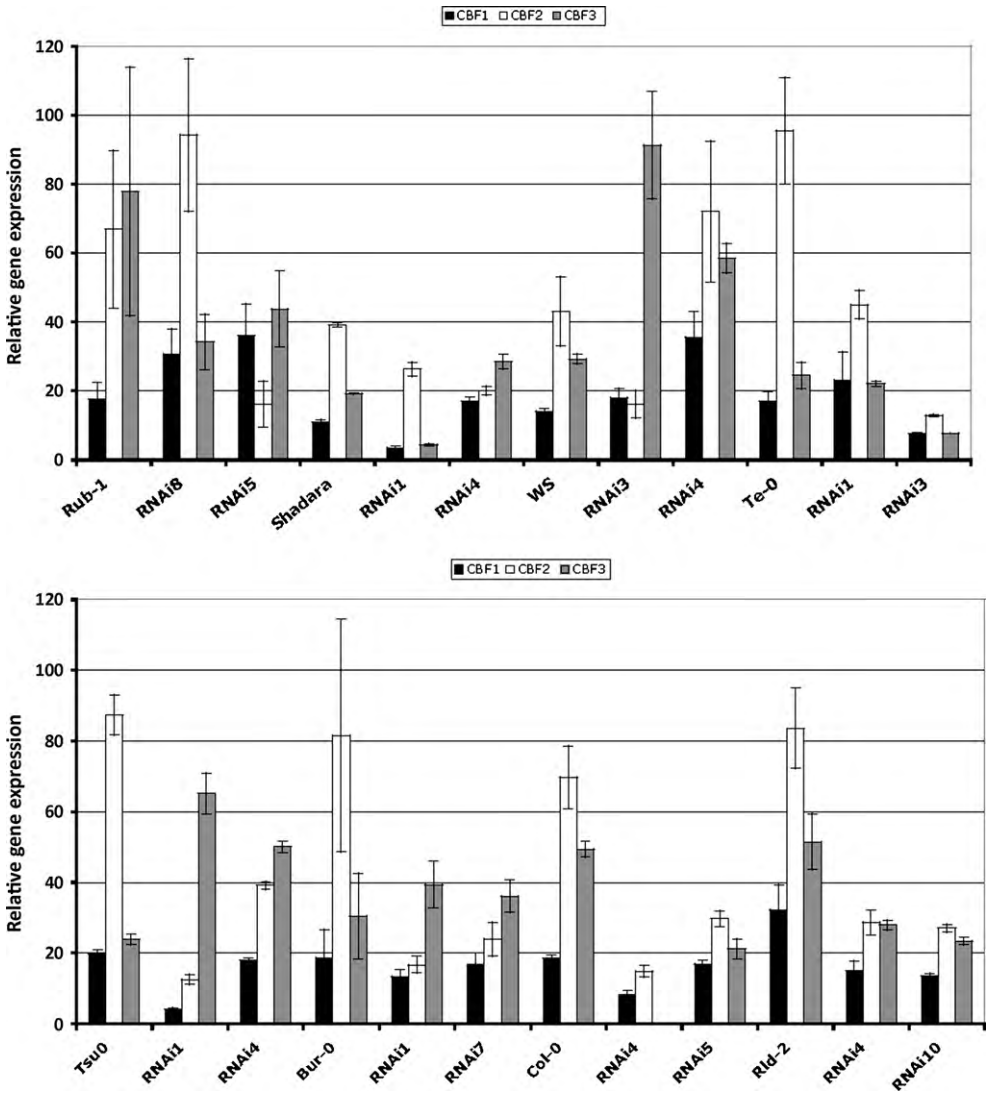

Fig. 2. Expression of *CBF* genes in RNAi lines in comparison to the wild type control. The expression level of each *CBF* gene has been measured after 2 h at 5 °C.

3. Results

3.1. Cold-induced CBF expression in natural accessions

In order to survey the overall range of *CBF* gene expression in the core collection of 48 accessions, a qRT-PCR study was carried out on the entire collection. Our previous studies had shown that maximum induction is achieved after 2 h at 5 °C for nearly all accessions [33]. Plants were thus exposed to 2 h at 5 °C. The experiment was repeated twice under identical conditions. The results (Fig. 1) are in accord with those previously observed on eight accessions (Col-0, Ita-0, Sah-0, Cvi-0, Can-0, Rld-2, Rub-1, WS; indicated by asterisks in Fig. 1). We note that Cvi-0 had the expected low level of *CBF2* expression (compare [28,23]). The freezing sensitive acces-

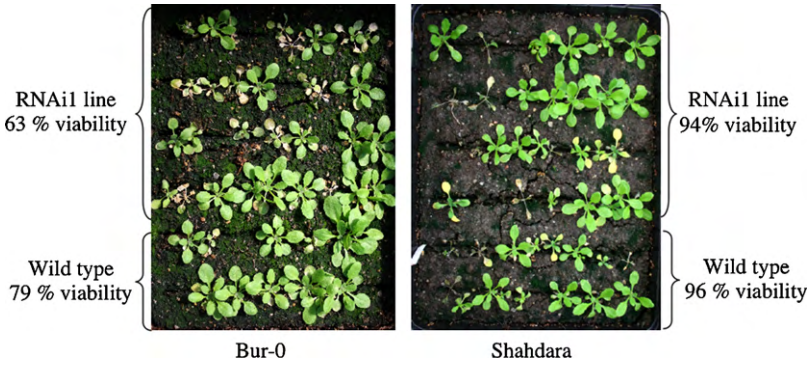

Fig. 3. Phenotyping of RNAi lines. Freezing tolerance after acclimation was determined as % survival after six days of recovery at 22 °C. A reduction in *CBF* expression in the RNAi lines resulted in reduced freezing tolerance in Bur-0 (left), but not in Shahdara (right). A quantitative analysis for all eight investigated accessions is shown in Table 1.

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

Table 1
Viability of the wild type accessions and their respective RNAi lines after freezing.

RNAi line	Accession	% viability of RNAi line	% viability of wild type	Significance
RNAi1	Te-0	95	100	S**
RNAi3	Te-0	75	84	S**
RNAi1	Tsu-0	87	93	S**
RNAi4	Tsu-0	72	89	S***
RNAi1	Bur-0	63	79	S**
RNAi7	Bur-0	84	96	S***
RNAi4	Col-0	93	76	S***
RNAi5	Col-0	58	31	S***
RNAi4	Rld-2	86	95	S**
RNAi10	Rld-2	1	80	S***
RNAi8	Rub-1	47	51	NS
RNAi5	Rub-1	97	98	NS
RNAi1	Shahdara	94	96	NS
RNAi4	Shahdara	90	89	NS
RNAi3	WS	81	89	S*
RNAi4	WS	98	99	NS

Plants were acclimated for 7 days at 4 °C and then exposed to −5 °C for 48 h. % viability was scored as described in Section 2. S*: significant with $0.05 > p > 0.01$; S**: significant with $0.01 > p > 0.001$; S***: significant with $p < 0.001$; NS: not significant.

sions Sah-0, Can-0 and Ita-0 showed overall low levels of expression of all three *CBF* genes. The two accessions we previously showed having high *CBF* expression levels, WS and Rub-1 were again at the high end of the range of expression levels. Also, the freezing sensitive accessions were mostly grouped together in the distribution, while the most tolerant ones such as Rld2, Rub-1, Kn-0 or Shahdara showed a wide range of *CBF* expression levels. Interestingly, the two Japanese accessions Ishikawa and Sakata had almost undetectable levels of *CBF3*, while maintaining low levels of *CBF1* and *CBF2* expression.

Among the accessions used for RNAi gene silencing of the *CBF* genes, all showed high levels of *CBF* expression except Shahdara.

3.2. Suppression of CBF gene expression has a variable impact on freezing tolerance among accessions

To inactivate the *CBF* pathway in different genetic backgrounds, a construct designed based on the *CBF2* sequence of Col-0 was generated and introduced into eight different accessions exhibiting good freezing tolerance after acclimation according to previous studies [33]. Two independent homozygous lines were selected for all further experiments. The transgenic plants all showed a phenotype similar to wild type plants at the selectable stage (two fully expanded leaves).

The *CBF2* sequence used in the RNAi construct exhibits 87.4% identity with *CBF1* and 83% with *CBF3* in Col-0. It is thus expected that the expression of the construct could inactivate not only *CBF2* but also *CBF1* and *CBF3*. We examined the induction of the three *CBF* genes at their cold induction peak after 2 h at 5 °C by qRT-PCR. This sampling time has been chosen after examining *CBF* expression data gathered over 14 days at 4 °C on a set of accessions with contrasting freezing tolerance (Fig. S1). None of the accessions showed any secondary expression peak during this period of cold acclimation. A significant reduction of expression was observed in almost all cases for *CBF2* and in many cases also for *CBF1* and *CBF3* (Fig. 2). For *CBF2*, the expression level was reduced at least by half in most RNAi lines compared to the wild type: the most significant reduction was observed for RNAi3 in Te-0 background and the least reduction was found in RNAi1 in Shahdara background. The only exceptions were observed in WS background for RNAi4 where the expression level of *CBF2* was increased by around 50% and in line RNAi8 in Rub-1 that showed a slight increase. For *CBF1*, the reduction in expression was generally lower, with the largest reduction in Tsu-0 background in RNAi1. Some RNAi lines exhibited *CBF1* expression levels similar to the corresponding wild type: RNAi1 in Te-0 background, RNAi4 in Tsu-0, RNAi1 and RNAi7 in Bur-0 background. As for *CBF2*, overexpression of *CBF1* was observed in the RNAi4 line in WS background. The expression level of *CBF3* was much more variable: severe reduction, as in RNAi1 (Sha-0), was rare. In some lines there was no apparent reduction, while there was a clear increase for example in RNAi1 (Tsu-0) or RNAi3 (WS). Lastly, the degree of reduction varied among lines and accessions, but complete inactivation of any of the three *CBF* genes was never observed.

The RNAi transgenic lines were then submitted to a freezing test together with the respective wild type accessions to evaluate the effect of variation in *CBF* gene expression on freezing tolerance. Plants were cold acclimated for 7 days at 5 °C, then exposed to a freezing treatment at −5 °C and viability was determined after 6 days of recovery in the greenhouse. To avoid positional effects, the wild type and the corresponding RNAi lines were sown in the same pots (Fig. 3) and each unit was replicated five times. The effect of *CBF* depletion varied significantly among accessions (Fig. 3 and Table 1). RNAi lines derived from Rub-1 and Shahdara showed no difference in freezing tolerance compared to the wild type plants, in spite of a clear reduction in *CBF* expression. The two lines derived from WS exhibited contrasting behavior, with a reduction in freezing tolerance in RNAi3, but no effect in RNAi4. It is important to point out that the *CBF* gene expression profiles also differed strongly between these lines. Particularly, in the unaffected line *CBF1*, *2* and *3* were overexpressed compared to the wild type WS background. RNAi lines derived from Te-0, Tsu-0, Bur-0 showed a significant reduction in freezing tolerance. In contrast, both lines derived from Col-0 seemed more tolerant than the wild type. Finally, the two lines derived from Rld2 were less tolerant than the wild type but the degree of reduction was different. While line RNAi10 showed almost 0% viability after freezing, line RNAi4 behaved similar to the lines derived from Te-0, Tsu-0 or Bur-0. Sister lines from RNAi10 also showed a very low level of survival after freezing (data not shown), indicating that this low level of freezing tolerance could be an additive effect of both a reduction in *CBF* expression and insertion position effects.

3.3. Growth and CBF gene expression are differentially affected by temperature in different accessions

The constitutive overexpression of *CBF* genes in *Arabidopsis* can lead to severe growth retardation [1] and a reduction in seed yield [2]. We were therefore interested to see whether a down-regulation of *CBF* expression may have the opposite effect, i.e. an increase in growth, especially under mild cold conditions. We performed growth experiments under three different temperature regimes

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

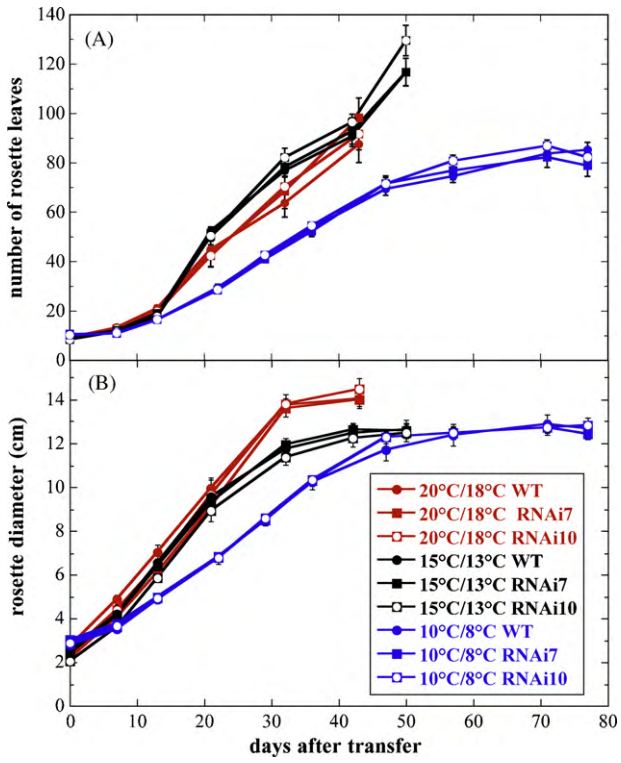

Fig. 4. Growth kinetics of the accession Bur-0 wild type (WT) plants and of two *CBF* RNAi lines (RNAi7 and RNAi10) at three different growth temperature regimes (20 °C/18 °C; 15 °C/13 °C; 10 °C/8 °C day/night). Plants were transferred to the different growth conditions 49 days after sowing (day 0). Growth was quantified as the number of rosette leaves (A) and as rosette diameter (B). Error bars denote the means ± SE for 8–10 plants. Experiments were terminated and plants harvested for analysis of *CBF* gene expression when the inflorescences had reached 4–6 cm in 50% of the plants.

(20 °C/18 °C; 15 °C/13 °C; 10 °C/8 °C day/night) under long day (16 h light) conditions, where growth was measured as either the number of rosette leaves or as rosette diameter until the plants entered the reproductive state. Fig. 4 shows a typical set of growth curves, obtained from the accession Bur-0. It can be seen that a shift of growth temperature from 20 °C/18 °C to 15 °C/13 °C only had a minor influence on the growth kinetics, but delayed inflorescence development by about one week. A further reduction of growth temperature significantly reduced growth rate compared to the higher growth temperatures and delayed inflorescence development even further.

For all following analyses, only the endpoints of the growth curves are compared, which represent the same developmental stage in all cases. The timing of inflorescence development, however, differed not only between the different growth conditions (Fig. 4), but also between the eight accessions that were investigated (Table 2).

Table 2
Harvest dates in days after transfer to the different growth conditions for the different accessions.

Accession	20 °C/18 °C	15 °C/13 °C	10 °C/8 °C
Rub-1	20	22	40
Te-0	25	34	57
Col-0	25	34	57
Rld-2	20	25	49
Sha-0	20	25	43
Tsu-0	31	39	62
Bur-0	43	50	77
Edi-0	25	25	46

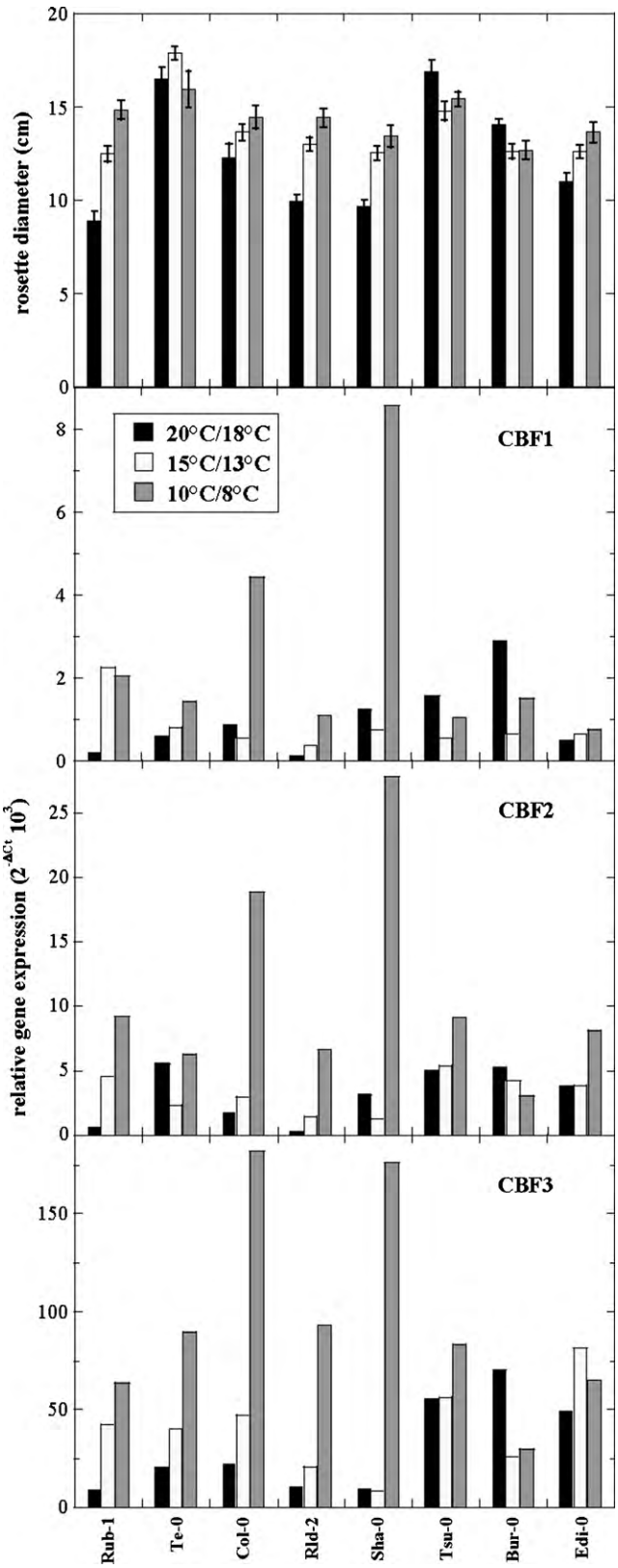

Fig. 5. Plant size, measured as rosette diameter, and expression of the *CBF1*, *2* and *3* genes in eight *Arabidopsis* accessions under three different growth conditions. Plants were harvested at the end of the growth experiments (compare Fig. 4). Data for rosette diameter show the means ± SE for 8–10 plants. Gene expression data are the means from two technical replicates measured on RNA isolated from pools of 4–5 whole rosettes. The harvest dates as days after transfer for the different accessions and temperature regimes are shown in Table 2. The accessions are ordered from the most freezing tolerant (Rub-1) on the left to the most sensitive (Edi-0) on the right.

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

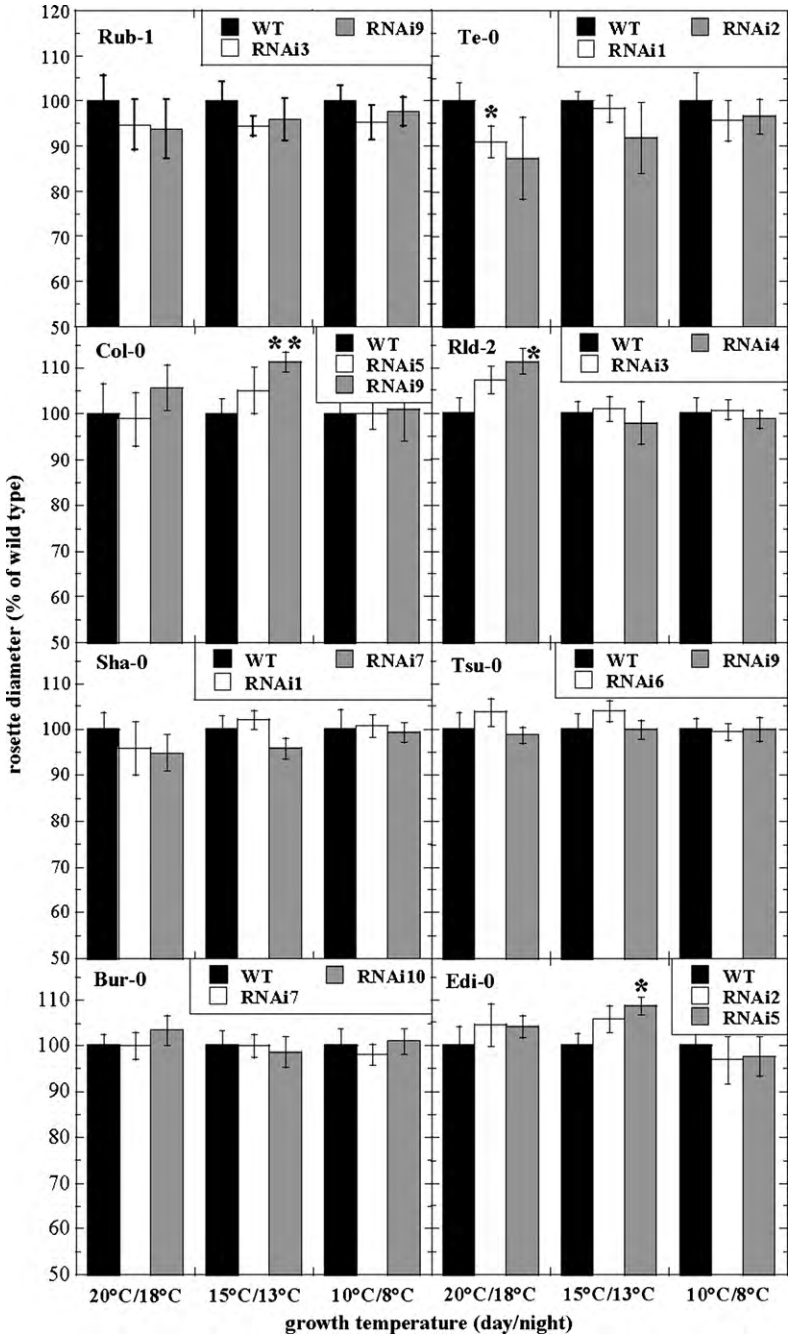

Fig. 6. Plant size at the end of the growth experiments (compare Fig. 4), measured as rosette diameter, in eight *Arabidopsis* accessions and their corresponding *CBF* RNAi lines under three different growth conditions. For each accession and growth condition, the rosette diameter of the wild type plants was set as 100%. Data show the means ± SE for 8–10 plants. Significant differences between wild type and RNAi plants were tested by Student's *t*-test and are indicated by asterisks (*$p < 0.05$; **$p < 0.01$).

The final plant size, measured as rosette diameter, varied both between the accessions and between the different growth conditions within the accessions (Fig. 5). In most accessions, the final size increased with decreasing growth temperature, emphasizing the fact that even 10 °C/8 °C was not a prohibitive temperature for *Arabidopsis* growth. It should of course be kept in mind that this increased size was also a function of the longer developmental time span. The expression of the *CBF* genes also varied between accessions and growth conditions (Fig. 5). However, in all accessions expression was highest in *CBF3* and lowest in *CBF1*, with *CBF2* showing a medium level of expression, similar to our earlier data [33] and the short term induction data reported in Fig. 1. Also, the expression of all three genes generally increased with decreasing temperature, as would be expected for cold-induced genes. Interestingly, this was more pronounced in the more freezing tolerant than in the three most sensitive accessions. However, there was no obvious relationship between the level of *CBF* expression and plant size at the different temperatures or between the accessions.

To test the influence of *CBF* gene expression on plant size more directly, we performed the same growth experiments also with two independent RNAi lines for each accession and calculated the relative size compared to the respective wild type under each temperature regime (Fig. 6). It is apparent that the differences between the RNAi lines and the respective wild type plants were rather small and were only statistically significant in four out of 48 cases. Nevertheless some trends could be observed. A small reduction in rosette size in the RNAi lines compared to the wild type was only observed in the two most freezing tolerant accessions Rub-1 and

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

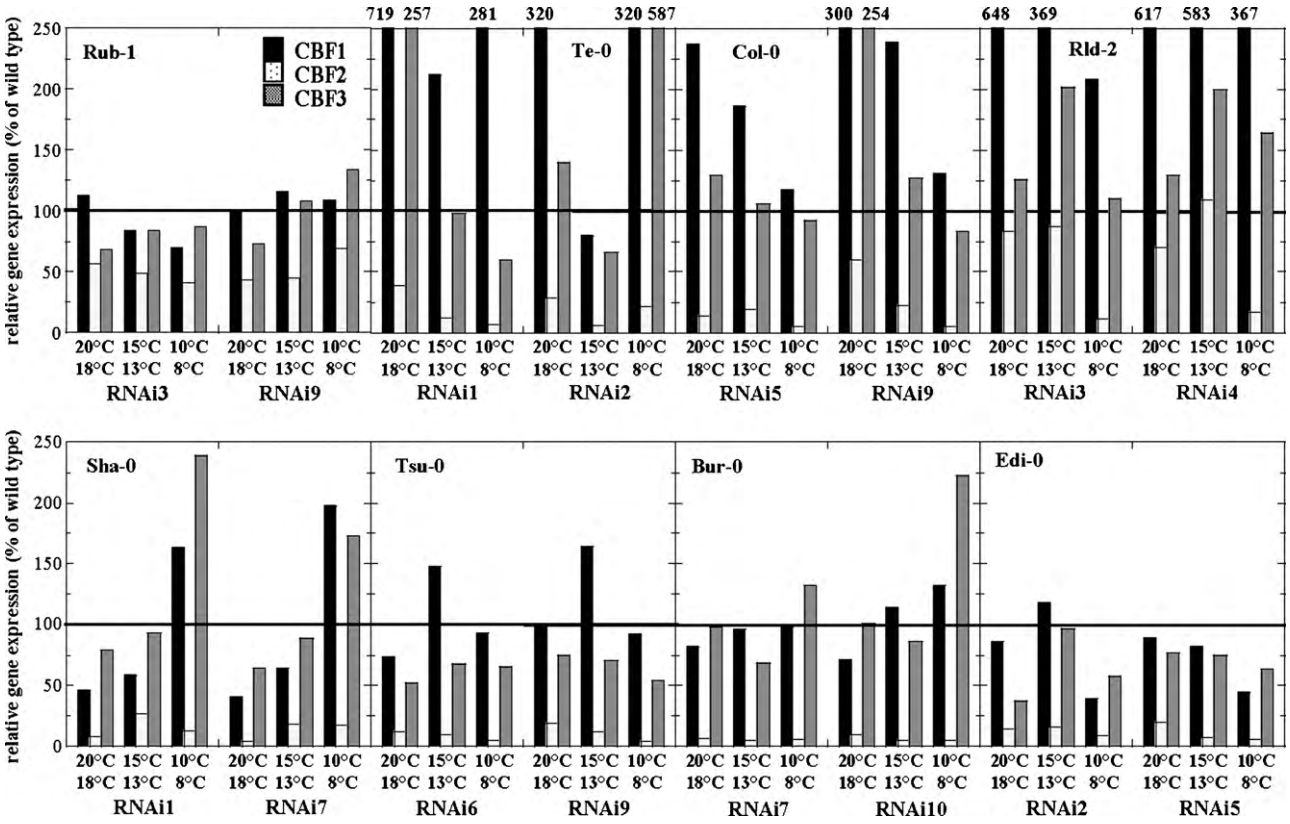

Fig. 7. Expression of the *CBF1*, *2* and *3* genes in eight *Arabidopsis* accessions and their corresponding *CBF* RNAi lines under three different growth conditions. Plants were harvested at the end of the growth experiments (Table 2). For each gene, accession and growth condition, gene expression of the wild type plants was set as 100%. Data are the means from two technical replicates measured on RNA isolated from pools of 4–5 whole rosettes. The numbers on top of the panels denote relative gene expression in those cases where it exceeded the chosen limit of the figures (>250%).

Te-0. All others showed either no differences (Sha-0, Tsu-0, Bur-0), or a minor increase in size at the higher growth temperatures (Col-0, Rld-2, Edi-0). Of course, these trends should be viewed with caution, as most of the differences were not statistically significant.

The expression of the *CBF* genes, measured in the same plants, showed some interesting patterns (Fig. 7). As mentioned earlier, the RNAi construct showed the greatest homology to the *CBF2* gene. Consequently, that is also the gene where we observed the strongest reduction in expression relative to the wild type in all accessions and under all growth conditions. In some cases, most prominently in the accessions Te-0, Col-0 and Rld-2, we found a compensatory increase in the expression of *CBF1* and/or *CBF3*. A similar observation has been made in a *CBF2* knock-out mutant in the Col-0 background [29]. However, this was not true in all accessions. In Edi-0, Bur-0 and Tsu-0 the suppression of *CBF2* expression was at least as strong as in Te-0, Col-0 and Rld-2, but *CBF1* and *CBF3* were mostly either not influenced or even showed a decrease in their expression. This indicates a strong influence of the genetic background on the regulation of *CBF* gene expression.

To obtain a more general picture of the relationship between the expression of the different *CBF* genes, we analyzed all three possible pairwise correlations (Fig. 8). A close correlation was only observed between the expression of *CBF1* and *CBF3*, while the correlation of the expression of either gene with *CBF2* expression was rather

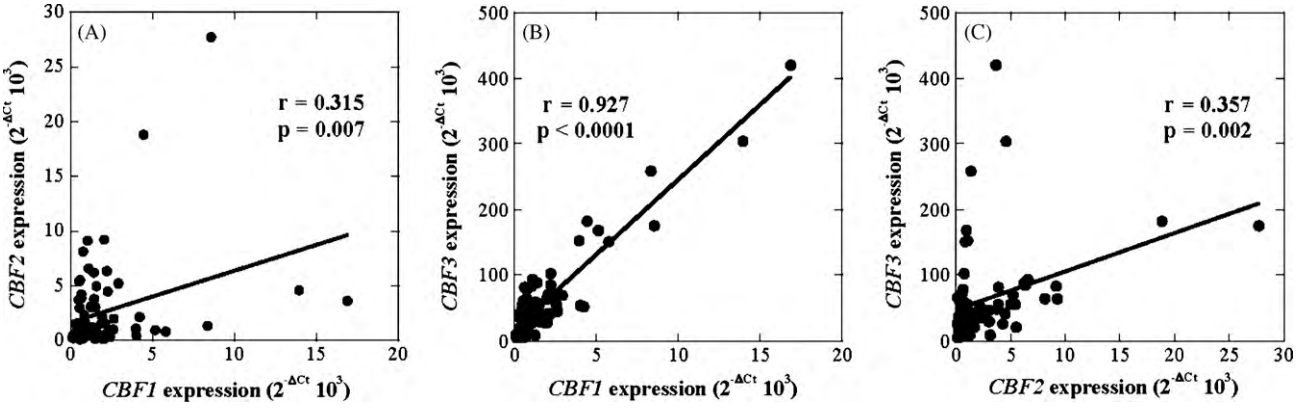

Fig. 8. Analysis of all three possible pairwise linear correlations (*CBF1* vs. *CBF2* (A), *CBF1* vs. *CBF3* (B), *CBF2* vs. *CBF3* (C)) of expression levels of the three *CBF* genes. Data were compiled from all accessions and the respective RNAi lines (compare Fig. 7). The lines were fitted to the data by least squares regression analysis. Correlation coefficients and *p*-values are shown in the respective panels.

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

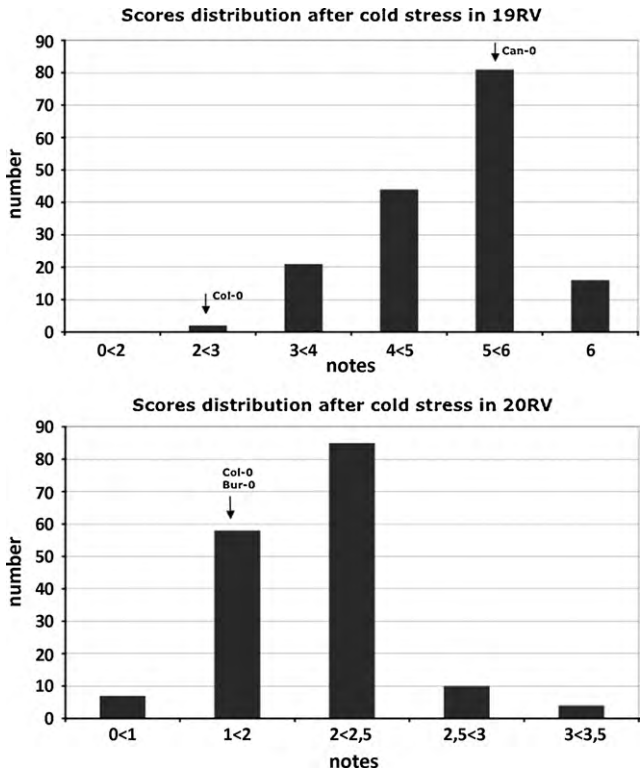

Fig. 9. Damage score distribution after freezing in the population 19RV (Can-0 × Col-0) and 20RV (Bur-0 × Col-0). The scale has been extended for the 20RV population for clarity.

weak. Significantly, there was no negative correlation between the expression of *CBF2* and the other two genes, indicating that *CBF2* is not generally a negative regulator of *CBF1* and *CBF3* gene expression in *Arabidopsis*, as proposed previously [29]. Also, there was no correlation between the expression of any of the *CBF* genes and plant growth at any growth temperature (data not shown). This indicates that within a physiological range, *CBF* expression has no strong influence on growth, in contrast to the results obtained with strong constitutive overexpression lines [1].

3.4. QTL analysis in two RIL populations

Genome-wide quantitative genetics has been used to investigate natural genetic variation for freezing tolerance after cold acclimation using subsets of 164 Recombinant Inbred Lines (RILs) optimized for QTL mapping that were grown and phenotyped under our standard conditions described in [33]. These experiments were performed on two RIL populations derived from Can-0 × Col-0 (19RV) and Bur-0 × Col-0 (20RV) crosses. The transgressive segregation of the damage score observed among RILs (Fig. 9) indicates that the genetic potential for the study of freezing tolerance after acclimation exists in these populations. Indeed, significant QTLs with LOD scores greater than 3.0 were mapped (Fig. 10) in each population. The experiment has been repeated at a different period of the year for the RIL population derived from the Can-0 × Col-0 cross to explore an eventual seasonal effect during the growth period in the greenhouse. Mapping of QTLs was completely congruent with the first experiment (data not shown).

In the Can-0 × Col-0 RIL population, several QTLs were predicted: one on the top of chromosome 3 explaining 9.4% of the phenotypic variance and several additional QTLs spanning most of chromosome 4, explaining from 27% to 47% of the phenotypic variance. As expected, considering previous data on the *CBF* pathway, one QTL on chromosome 4 maps in the region of the *CBF* gene cluster, but two others were detected on top of chromosome 4 and near the CBF region, respectively (Fig. 10). All additive effects showed negative values, indicating that Col-0 always contributed to increased freezing tolerance. To confirm QTL localisation at the different positions we used NILs (Near Isogenic Lines) obtained by producing Heterogeneous Inbred Families (HIFs). This material was generated taking advantage of the residual heterozygosity still segregating in F6 RILs. Initially, nine candidate RILs (343, 334, 290, 197, 133, 58, 46, 25 and 5), that were heterozygous around the predicted QTL positions on chromosome 4, were used. In four HIFs (334, 133, 46 and 25), the comparison between lines that were fixed for either parental allele (Col-0 or Can-0) at the QTL region revealed a significant difference in survival after freezing (Fig. 11). To reduce the size of the initial heterozygous region, which is generally large in F6 material (several Mb), progenies of HIFs have been genotyped to recover recombinants with a smaller heterozygous region, at least reduced by half. This material has been submitted to the same phenotyping experiment. Some lines exhibited differences when fixed for one parental allele or the other, confirming the localisation of the QTL in the corresponding region (Fig. 11).

In the RIL population derived from the cross of Bur-0 × Col-0, two QTLs were predicted, one at the bottom of chromosome 1 explaining 7.6% of the phenotypic variance and one at the top of the chromosome 4 explaining 15.3% of the phenotypic variance. Unlike in the previous population, the QTL in the region of the *CBF* gene cluster exhibited a low LOD score that is scarcely significant (Fig. 10). Additive effects showed positive and negative values, indicating that both parents contributed to the expression of the trait. To confirm the QTL localisation, nine candidate RILs (479, 451, 440, 356, 341, 277, 151, 60 and 13) that were heterozygous around the predicted QTL position on chromosome 4 were used. In two HIFs (440 and 341) the comparison between plants that were fixed for each parental allele (Col-0 or Bur-0) at the QTL region revealed a significant difference in survival after freezing, thus confirming this QTL position.

4. Discussion

As already indicated in our previous study with a much smaller group of accessions [33], natural accessions of the *Arabidopsis* Versailles nested core collection exhibited strong variability in *CBF* gene expression after a short period (2 h) of cold exposure. The most striking result of this large survey was the difference of distribution along the range of *CBF* expression levels between the most sensitive and tolerant accessions. The most sensitive accessions were grouped together among accessions with a low level of maximum cold-induced *CBF* expression. However, the most tolerant accessions were spread out across the whole range of cold-induced *CBF* expression, suggesting that the importance of the CBF cold signalling pathway in freezing tolerance could vary between accessions and that some populations could be interesting sources of new genes involved in the regulation of freezing tolerance. Overall, there was no simple correlation between *CBF* expression level and the degree of freezing tolerance under the conditions of the present experiments. While one possible explanation for this finding could be that *CBF* expression may not be equally important in all accessions, it should also be pointed out that we measured *CBF* expression at its peak, after 2 h in the cold, while freezing tolerance was measured after 7 days of cold acclimation. A correlation between *CBF* expression and freezing tolerance has previously been found in a small collection of nine accessions when gene expression was measured by microarray hybridization after 14 days at 4 °C [34], indicating that the timing of the expression measurements may also play a role. A set of eight accessions with contrasting freez-

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

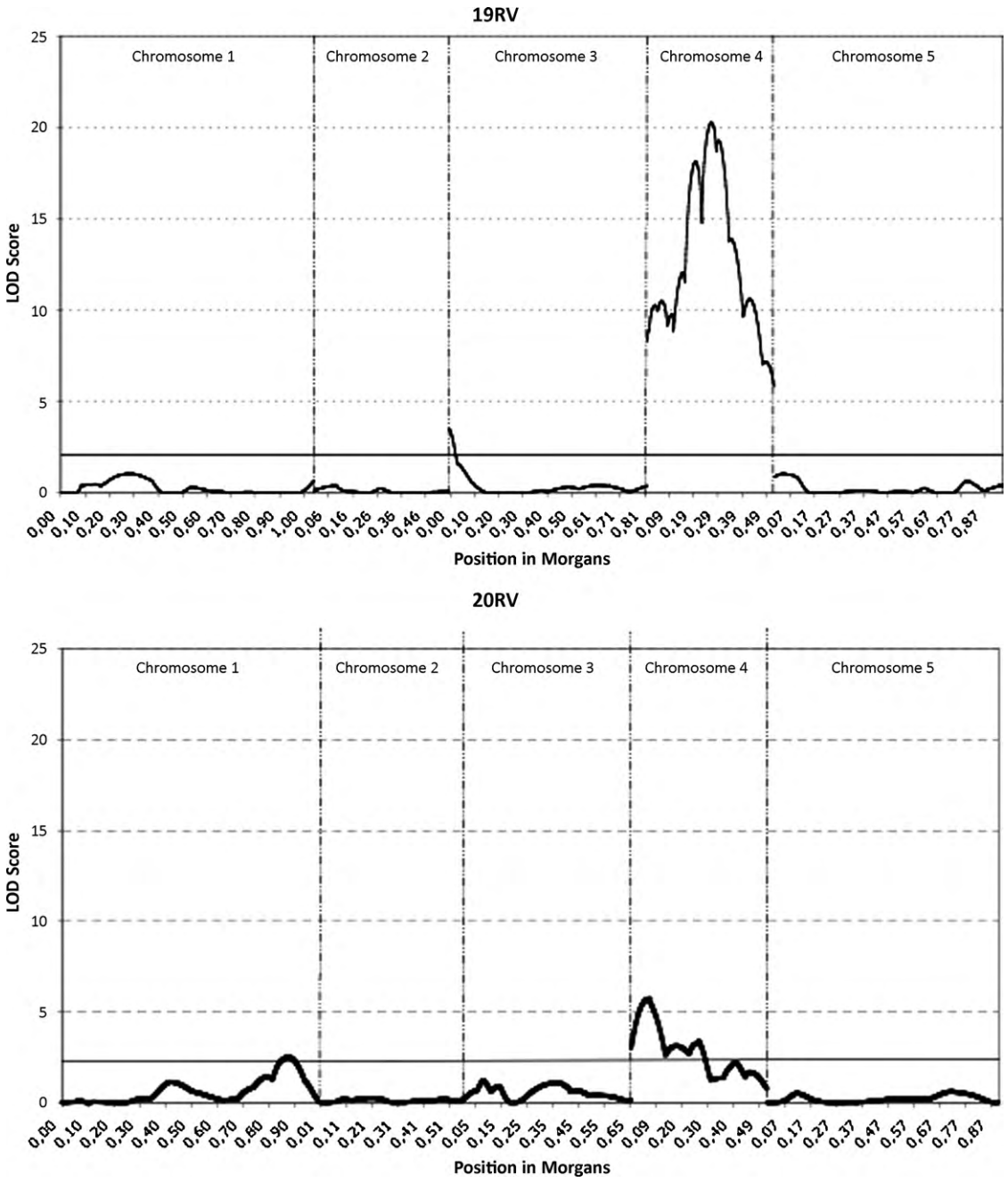

Fig. 10. QTL detection with QTL cartographer in the 19RV and 20RV populations. Positions are given in Morgans, the significant threshold for LOD score is indicated as a continuous black line.

ing tolerance has been studied over six weeks at 5 °C with weekly sampling to observe an eventual secondary *CBF* peak in the most tolerant accessions, as was shown in different lines of winter wheat [44]. However, no secondary peak was observed in *Arabidopsis* (data not shown), indicating that high acclimated freezing tolerance is sustained with low levels of *CBF* expression.

To address the question of the effect of differences in *CBF* expression on freezing tolerance more directly, we generated RNAi lines targeted against all three *CBF* genes in eight different accessions. While numerous previous studies have demonstrated an increase in freezing tolerance due to constitutive overexpression of *CBF* genes in different plant species from *Arabidopsis* to poplar [24,45–47], only one study reported the effect of inactivation of a *CBF* gene on freezing tolerance [29]. Interestingly, the conclusion from this study that investigated a T-DNA insertion knock-out mutant in the *CBF2* gene in Col-0 was that *CBF2* is a negative regulator of the expression of *CBF1* and *CBF3*. Consequently, the mutant line showed a higher freezing tolerance than the Col-0 wild type plants. This increase in freezing tolerance was also observed in the RNAi lines in Col-0 background investigated here. An increase in *CBF1* and *CBF3* expression together with a down-regulation of *CBF2* expression was only evident at the end of the growth experiments, but not after 2 h at 5 °C. Further detailed investigations into the kinetics of the cold acclimation process will be necessary to completely resolve this issue. A general role of CBF2 as a negative regulator of *CBF1* and *CBF3* expression, however, seems unlikely on the basis of our data, which show no negative correlation between the expression of *CBF2* and either of the other *CBF* genes. In addition, we observed strong repression of *CBF2* expression (up to 95%) in the RNAi lines of several accessions without any increase in the

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010

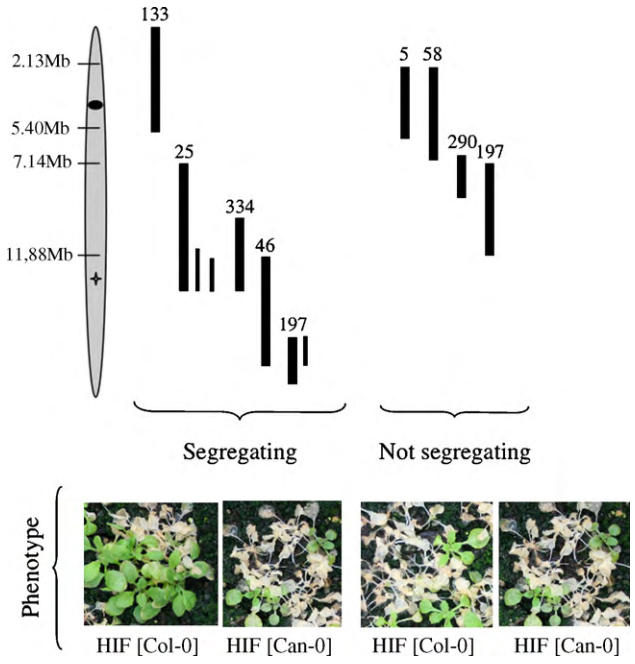

Fig. 11. QTL validation with HIFs in the 19RV population for QTL detected on chromosome 4. Bold lines represent heterozygous regions in the F6 generation and thinner lines represent recombinants. The importance of segregation is shown on an example of photos.

expression of the other *CBF* genes or an increase in freezing tolerance. These effects seem to be limited to a few accessions, but the reasons for these differences between different accessions are currently completely unclear. Interestingly, our correlation analyses indicate that the expression levels of *CBF1* and *CBF3* are tightly linked, while the expression of *CBF2* seems to be regulated independently of the other two genes. The molecular basis of these differences in regulation is also unclear at present.

Until now only a single knock-out mutant in a *CBF* gene in *Arabidopsis* has been reported for *CBF2* and the position of the three *CBF* genes in a cluster excludes the generation of double and triple mutants by classical genetic approaches. We therefore used a RNAi strategy to inactivate one or more members of this gene family. The absence of a complete knock-out of any gene in all our transgenic lines may be a significant observation. Two main reasons could explain this result. First, the construct may not be efficient enough to completely suppress the expression of the target genes, although the construct was clearly effective to some degree. Alternatively, it is possible that the total inactivation of *CBF* gene(s) could be deleterious and that the knock-out transgenic lines were lost during the selection process. The impaired development of plants overexpressing *CBF* coding sequences described in the literature [24,47] is congruent with the hypothesis that *CBF* genes have major roles in pathways other than cold acclimation. On the other hand, the *CBF2* knock-out line in Col-0 was clearly viable [28] and in the investigated RNAi lines no clear effects on growth could be detected. Screening of further RNAi or artificial microRNA lines generated with different *CBF* sequences will be necessary to resolve this question.

The freezing tolerance analysis of the RNAi lines showed obvious variation in the effect of down-regulation of *CBF* expression among natural accessions. Independent evolution of natural populations, allowing selection of different networks involved in stress response in different accessions, could partly explain this variability. In agreement with this hypothesis, we found that freezing tolerance QTLs mapped to different positions in the two tested populations.

Another possible explanation is that the effects of the RNAi construct are not straightforward because of interactions between the three *CBF* genes, as has been reported in the Col-0 background by Salinas et al. [28,29]. In agreement with this hypothesis, increased expression has been observed in some of our transgenic lines, suggesting complex regulation that depends in addition on the genetic background. To approach this question more comprehensively, artificial microRNA lines designed to target only one gene each have been generated in different genetic backgrounds and are currently under investigation.

Freezing tolerance is a complex trait of great agronomic interest appropriate for QTL analysis. Consequently, this approach has been successfully used in different species such as wheat, barley, sunflower, rice or pea [48–57]. QTL identification and mapping proved to be of great help in plant breeding to identify interesting regions in the genome and perform marker assisted selection. Different traits related to cold tolerance, such as germination capacity in cold and dark conditions [58] or freezing resistance under long or short day conditions [27] have been studied in *Arabidopsis* using a QTL approach. Here we have performed QTL analysis in two different RIL populations using freezing tolerance after acclimation as the phenotyped trait. The major result of these analyses is that the identified QTLs differ between two populations. The validation of QTL positions by analysing HIFs and the absence of known genes involved in freezing tolerance in the detected areas, except in the *CBF* region, indicates that novel genes could be identified using this approach. We are currently in the process of cloning candidate genes that are responsible for the identified QTLs.

Acknowledgements

The authors thank Michel Burtin (UMR1318, INRA) for technical assistance, Sylvie Lévêque (EPGV, INRA) for gene expression studies and Christine Camilleri (UMR1318, INRA) for great help in QTL analysis.

This work was realised in the context of the cooperation between Genoplante and GABI and received a financial support by the European project ANR-06-ERAPG-008 “Cold tolerance for the future: the CBF genes and beyond (FROSTY)”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.07.010.

References

[1] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two signal transduction pathways in drought and low-temperature-responsive gene expression, respectively, in Arabidopsis, Plant Cell 10 (1998) 1391–1406.
[2] M.W. Jackson, J.R. Stinchcombe, T.M. Korves, J. Schmitt, Costs and benefits of cold tolerance in transgenic *Arabidopsis thaliana*, Mol. Ecol. 13 (2004) 3609–3615.
[3] M. Smallwood, D.J. Bowles, Plants in a cold climate, Philos. Trans. R. Soc. Lond. B: Biol. Sci. 357 (1423) (2002) 831–847.
[4] M. Seki, M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, et al., Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray, Plant J. 31 (3) (2002) 279–292.
[5] M.A. Hannah, A.G. Heyer, D.K. Hincha, A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*, PLoS Genet. 1 (2005) e26.
[6] S. Fowler, M.F. Thomashow, *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway, Plant Cell 14 (8) (2002) 1675–1690.
[7] B.H. Lee, D.A. Henderson, J.K. Zhu, The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1, Plant Cell 17 (11) (2005) 3155–3175.
[8] M.F. Thomashow, Plant cold acclimation: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 571–599.

[9] V. Chinnusamy, J. Zhu, J.K. Zhu, Cold stress regulation of gene expression in plants, Trends Plant Sci. 12 (2007) 444–451.
[10] J. Zhu, C.H. Dongand, J.K. Zhu, Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation, Curr. Opin. Plant Biol. (2007) 290–295.
[11] C. Guy, F. Kaplan, J. Kopka, J. Selbig, D.K. Hincha, Metabolomics of temperature stress, Physiol. Plant 132 (2008) 220–235.
[12] C. Shindo, M.J. Aranzana, C. Lister, C. Baxter, C. Nicholls, M. Nordborg, C. Dean, Role of FRIGIDA and FLOWERING LOCUS C in determining variation in flowering time of *Arabidopsis*, Plant Physiol. 138 (2005) 1163–1173.
[13] J.D. Werner, J.O. Borevitz, N.H. Uhlenhaut, J.R. Ecker, J. Chory, D. Weigel, FRIGIDA-independent variation in flowering time of natural *Arabidopsis thaliana* accessions, Genetics 170 (2005) 1197–1207.
[14] M.C. DeVicente, S.D. Tanksley, QTL analysis of transgressive segregation in an interspecific tomato cross, Genetics 134 (1993) 585–596.
[15] I. Paran, D. Zamir, Quantitative traits in plants: beyond the QTL, Trends Genet. 19 (2003) 303–306.
[16] M. Simon, O. Loudet, S. Durand, A. Bérard, D. Brunel, F.X. Sennesal, M. Durand-Tardif, G. Pelletier, C. Camilleri, Quantitative trait loci mapping in five new large recombinant inbred line populations of *Arabidopsis thaliana* genotyped with consensus single-nucleotide polymorphism markers, Genetics 178 (2008) 2253–2264.
[17] O. Loudet, T.P. Michael, B.T. Burger, C. Le Metté, T.C. Mockler, D. Weigel, J. Chory, A zinc knuckle protein that negatively controls morning-specific growth in *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 17193–17198.
[18] E.J. Stockinger, J.S. Skinner, K.G. Gardner, E. Francia, N. Pecchioni, Expression levels of barley Cbf genes at the Frost resistance-H2 locus are independent upon alleles at Fr-H1 and Fr-H2, Plant J. 51 (2007) 308–321.
[19] A. Fricano, F. Rizza, P. Faccioli, D. Pagani, P. Pavan, A. Stella, L. Rossini, P. Piffanelli, L. Cattivelli, Genetic variants of HvCbf14 are statistically associated with frost tolerance in a European germplasm collection of *Hordeum vulgare*, Theor. Appl. Genet. 119 (2009) 1335–1348.
[20] C. Campoli, M.A. Matus-Cadiz, C.J. Pozniak, L. Cattivelli, B. Fowler, Comparative expression of *Cbf* genes in the *Triticeae* under different acclimation induction temperatures, Mol. Genet. Genomics 282 (2009) 141–152.
[21] A.K. Miller, G. Galiba, J. Dubcovsky, A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-Am2 in *Triticum monococcum*, Mol. Genet. Genomics 275 (2006) 193–203.
[22] M. Navarro, G. Marque, C. Ayax, G. Keller, J.P. Borges, C. Marque, C. Teulières, Complementary regulation of four Eucalyptus CBF genes under various cold conditions, J. Exp. Bot. 60 (2009) 2713–2724.
[23] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, *Arabidopsis thaliana* CBF1 encode an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1035–1040.
[24] K.R. Jaglo-Ottosen, S.J. Gilmour, D.G. Zarka, O. Schabenberger, M.F. Thomashow, *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance, Science 280 (1998) 104–106.
[25] H.A. Van Buskirk, M.F. Thomashow, *Arabidopsis* transcription factors regulating cold acclimation, Physiol. Plant 126 (2006) 72–80.
[26] S.J. Gilmour, S.G. Fowler, M.F. Thomashow, Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities, Plant Mol. Biol. 54 (2004) 767–781.
[27] C. Alonso-Blanco, C. Gomez-Mena, F. Llorente, M. Koorneef, J. Salinas, J.M. Martinez-Zapater, Genetic and molecular analysis of natural variation indicate CBF2 as a candidate gene for underlying a freezing tolerance quantitative trait locus in *Arabidopsis*, Plant Physiol. 139 (2005) 1304–1312.
[28] F. Novillo, J.M. Alonso, J.R. Ecker, J. Salinas, CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 3985–3990.
[29] F. Novillo, J. Medina, J. Salinas, *Arabidopsis* CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 21002–21007.
[30] C. Alonso-Blanco, M. Koorneef, Natually occurring variation in *Arabidopsis*: an underexploited resource for plant genetics, Trends Plant Sci. 5 (2000) 22–29.
[31] T. Mitchell-Olds, J. Schmitt, Genetic mechanisms and evolutionary significance of natural variation in *Arabidopsis*, Nature 441 (2006) 947–952.
[32] H.I. McKhann, C. Camilleri, A. Berard, T. Bataillon, J.L. David, X. Reboud, V. Le Corre, C. Caloustian, I. Gut, D. Brunel, Nested core collections maximising genetic diversity in *Arabidopsis thaliana*, Plant J. 38 (2004) 193–202.
[33] H.I. McKhann, C. Géry, A. Bérard, S. Lévêque, E. Zuther, D.K. Hincha, S. De Mita, D. Brunel, E. Téoulé, Natural variation in CBF gene sequence, gene expression and freezing tolerance in the Versailles core collection of *Arabidopsis thaliana*, BMC Plant Biol. 8 (2008) 105–122.
[34] M.A. Hannah, D. Wiese, S. Freund, O. Fiehn, A.G. Heyer, D.K. Hincha, Natural genetic variation of freezing tolerance in *Arabidopsis*, Plant Physiol. 142 (2006) 98–112.
[35] Y. Zhen, M.C. Ungerer, Relaxed selection on the CBF/DREB1 regulatory genes and reduced freezing tolerance in the southern range of *Arabidopsis thaliana*, Mol. Biol. Evol. 25 (2008) 2547–2555.
[36] VNAT, http://dbsgap.versailles.inra.fr/vnat/.
[37] O. Bouchabke-Coussa, M.L. Quashie, J. Seoane-Redondo, M.N. Fortabat, C. Géry, D. Linderme, J. Trouverie, F. Granier, E. Téoulé, M. Durand-Tardif, ESKIMO1 is a key gene involved in water economy as well as cold acclimation and salt tolerance, BMC Plant Biol. 8 (2008) 125.
[38] M.R. Tuinstra, G. Ejeta, P.B. Goldsbrough, Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative trait loci, Theor. Appl. Genet. 95 (1997) 1005–1011.
[39] V.S. Wesley, C. Helliwell, N.A. Smith, M.B. Wang, D. Rouse, Q. Liu, P.S. Gooding, S.R. Singh, D. Abbott, A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, P.M. Waterhouse, Construct design for efficient, effective and high-throughput gene silencing in plants, Plant J. 27 (2001) 581–590.
[40] M.E. Cartea, M. Migdal, A.-M. Galle, G. Pelletier, P. Guerche, Comparison of sens and antisens methodologies for modifying the fatty acid composition of *Arabidopsis thaliana* oil seed, Plant Sci. 136 (1998) 181–194.
[41] N. Bechtold, G. Pelletier, *In planta Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration, Methods Mol. Biol. 82 (1998) 259–266.
[42] C.J. Basten, B.S. Weir, Z.B. Zeng, QTL Cartographer, Version 1.14, North Carolina State University, Raleigh, 2000.
[43] O. Loudet, S. Chaillou, P. Merigout, J. Talbotec, F. Daniel-Vedele, Quantitative Trait Loci analysis of nitrogen use efficiency in *Arabidopsis*, Plant Physiol. 131 (1) (2003) 345–358.
[44] S. Kume, F. Kobayashi, M. Ishibashi, R. Ohno, C. Nakamura, S. Takumi, Differential and coordinated expression of Cbf and Cor/Lea genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance, Genes Genet. Syst. 80 (3) (2005) 185–197.
[45] T.J. Hsieh, J.T. Lee, Y.Y. Chang, M.T. Chan, Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced resistance to water deficit stress, Plant Physiol. 130 (2002) 618–626.
[46] S.C. Lee, K.W. Huh, K. An, G. An, S.R. Kim, Ectopic expression of a cold-inducible transcription factor CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.), Mol. Cells 18 (2004) 107–114.
[47] C. Benedict, J.S. Skinner, R. Meng, Y. Chang, R. Bhalerao, N.P.A. Huner, C.E. Finn, T.H.H. Chen, V. Hurry, The CBF1-dependant low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp., Plant Cell Environ. 29 (2006) 1259–1272.
[48] A.K. Knox, C. Li, A. Vágújfalvi, G. Galiba, E.J. Stockinger, J. Dubcovsky, Identification of candidate CBF genes for the frost tolerance locus Fr-Am2 in *Triticum monococcum*, Plant Mol Biol. 67 (3) (2008) 257–270.
[49] A.K. Miller, G. Galiba, J. Dubcovsky, A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-Am2 in *Triticum monococcum*, Mol. Genet. Genomics 275 (2) (2006) 193–203.
[50] A. Vágújfalvi, A. Aprile, A. Miller, J. Dubcovsky, G. Delugu, G. Galiba, L. Cattivelli, The expression of several Cbf genes at the Fr-A2 locus is linked to frost resistance in wheat, Mol. Genet. Genomics 274 (5) (2005) 506–514.
[51] E. Francia, D. Barabaschi, A. Tondelli, G. Laidò, F. Rizza, A.M. Stanca, M. Busconi, C. Fogher, E.J. Stockinger, N. Pecchioni, Fine mapping of a HvCBF gene cluster at the frost resistance locus Fr-H2 in barley, Theor. Appl. Genet. 115 (8) (2007) 1083–1091.
[52] A. Chen, J. Reinheimer, A. Brûlé-Babel, U. Baumann, M. Pallotta, G.B. Fincher, N.C. Collins, Genes and traits associated with chromosome 2H and 5H regions controlling sensitivity of reproductive tissues to frost in barley, Theor. Appl. Genet. 118 (8) (2009) 1465–1476.
[53] C. Allinne, P. Maury, A. Sarrafi, P. Grieu, Genetic control of physiological traits associated to low temperature growth in sunflower under early sowing conditions, Plant Sci. 177 (2009) 349–359.
[54] K. Fujino, H. Sekiguchi, Y. Matsuda, K. Sugimoto, K. Ono, M. Yano, Molecular identification of a major quantitative trait locus, qLTG3-1, controlling low-temperature germinability in rice, Proc. Natl. Acad. Sci. U.S.A. 105 (34) (2008) 12623–12628.
[55] J.P. Suh, J.U. Jeung, J.I. Lee, Y.H. Choi, J.D. Yea, P.S. Virk, D.J. Mackill, K.K. Jena, Identification and analysis of QTLs controlling cold tolerance at the reproductive stage and validation of effective QTLs in cold-tolerant genotypes of rice (*Oryza sativa* L.), Theor. Appl. Genet. 120 (5) (2010) 985–995.
[56] E. Dumont, V. Fontaine, C. Vuylsteker, H. Sellier, S. Bodèle, N. Voedts, R. Devaux, M. Frise, K. Avia, J.L. Hilbert, N. Bahrman, E. Hanocq, I. Lejeune-Hénaut, B. Delbreil, Association of sugar content QTL and PQL with physiological traits relevant to frost damage resistance in pea under field and controlled conditions, Theor. Appl. Genet. 118 (8) (2009) 1561–1571.
[57] I. Lejeune-Hénaut, E. Hanocq, L. Béthencourt, V. Fontaine, B. Delbreil, J. Morin, A. Petit, R. Devaux, M. Boilleau, J.J. Stempniak, M. Thomas, A.L. Lainé, F. Foucher, A. Baranger, J. Burstin, C. Rameau, C. Giauffret, The flowering locus Hr colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum L.*, Theor. Appl. Genet. 116 (8) (2008) 1105–1116.
[58] P.H. Meng, A. Macquet, O. Loudet, A. Marion-Poll, H.M. North, Analysis of natural allelic variation controlling *Arabidopsis thaliana* seed germinability in response to cold and dark: identification of three major quantitative trait loci, Mol. Plant 1 (1) (2008) 145–154.

Please cite this article in press as: C. Gery, et al., Natural variation in the freezing tolerance of *Arabidopsis thaliana*: Effects of RNAi-induced CBF depletion and QTL localisation vary among accessions, Plant Sci. (2010), doi:10.1016/j.plantsci.2010.07.010