Unraveling the cold response in Draba

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Molecular and phylogenetic analysis of the cold regulated CBF and COR genes in the genus Draba

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Plants have evolved a wide array of responses to deal with cold temperatures. At the molecular level it is known from *Arabidopsis thaliana* that CBF (C-repeat binding factor) transcription factors play an important role in the cold response pathway and are involved in the activation of Cold Responsive (COR) genes. Here we report the molecular cloning and identification of CBF- and COR15-like cDNAs from the genus *Draba*. *Draba*, like *Arabidopsis*, belongs to the family of Brassicaceae. It is a genus with a worldwide distribution, and contains temperate, tropical-, and arctic-alpine species. *Draba*, therefore, allows us to study the evolution of cold related genes from closely related species adapted to very different habitats. This study includes two arctic-alpine species: *D. lactea* and *D. nivalis*, two temperate species: *D. muralis* and *D. verna*, and four tropical-alpine species: *D. alyssoides*, *D. aretioides*, *D. hookeri*, and *D. steyermarkii*.

We have identified three putative CBF homologs from *Draba*; CBFα, CBFβ, and CBFc. CBFα and CBFβ were isolated from the tropical-alpine species *D. hookeri* and *D. alyssoides*, CBFc from the temperate *D. verna* and *D. muralis*, whereas the arctic-alpine *D. nivalis* contained both CBFα and CBFc homologs. The *Draba* CBF genes have the typical structure found in *Arabidopsis* CBF genes; the nuclear localization signal (NLS), the AP2-binding domain, and the acidic region. Most of the variation in the cDNA nucleotide sequences between *Arabidopsis* and *Draba* occurred within the acidic region. Variation ‘hotspots’ within the acidic region differed between both genera and therefore do not appear to reflect a common functional effect.

We isolated one CBF-target gene from *Draba* with a high similarity (>81%) to *A. thaliana* COR15b, and labeled this gene *Draba* COR15a. In addition, one new COR15-like gene unique to *Draba* was identified. This new COR15c gene was characterized by a 76 amino acid insert. Both *Draba* COR15 genes were found to contain a potential chloroplast cleavage site and several irregularly repeated 13-amino acid sequence motifs. Thus, similar to *Arabidopsis* COR15a/b, COR15a/c identified in *Draba* are presumably targeted to the chloroplast.

This study showed that CBF-like proteins together with their CBF-targeted COR15 genes are conserved within different members of the genus *Draba*. With these molecular tools at hand we can start investigating the role of CBF and COR15 in the cold response in *Draba*.

Cold is a very common stress for plants growing in arctic or alpine regions. Plants have developed an array of responses at physiological, cellular and molecular levels that allow them to cope with cold stress. Additionally, many plants in temperate regions acquire an enhanced freezing tolerance via a process known as cold acclimation when exposed to low but non-freezing temperatures (Guy, 1990; Thomashow, 1998; Xin and Browse, 2000). Studies of *Arabidopsis* have shown that cold acclimation involves rapid cold-induced expression of the C-repeat binding factor (CBF) transcriptional activators followed by expression of CBF-target genes that increase freezing tolerance (Stockinger et al., 1997; Gilmour et al., 1998; Medina et al., 1999). In *Arabidopsis* CBF comprises a small family of three genes; CBF1-3, tandem repeated (CBF1 -> CBF3 -> CBF2) on chromosome 4, and all involved in cold tolerance (Gilmour et al., 1998; Haake et al., 2002). CBF proteins are characterized by the presence of a potential nuclear localization sequence, followed by an AP2 (APETALA2) domain, and an acidic region (Medina et al., 1999). The AP2 domain is thought to function as DNA binding domain, while the acidic region might act as activator domain (Stockinger et al., 1997). The DNA binding domain of the CBF transcription factors recognizes the cold- and dehydration responsive DNA regulatory element designated the C-repeat/dehydration responsive element (CRT/DRE; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). CRT/DRE elements are typical for the promoter regions of many cold-responsive (COR) genes of *Arabidopsis* (Thomashow, 1999). In transgenic
Arabidopsis plants constitutive CBF expression results in the induction of COR genes and enhances freezing tolerance (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). COR15 is thought to be involved in the freezing tolerance of plants as it has been identified as acting as a cryoprotectant in cold acclimated plants (Lin and Thomashow, 1992; Baker et al., 1994; Artus et al., 1996; Steponkus et al., 1998; Nakayama et al., 2007). This illustrates the importance of CBF and its target genes in the cold tolerance of plants.

Both CBF and COR genes have been increasingly studied in species other than Arabidopsis. With the aim of reducing yield losses due to freezing, various studies have been conducted into the cold tolerance of crops such as Brassica napus, tomato, rice, barley, grape, ryegrass, and Eucalyptus (Jaglo et al., 2001; Choi et al., 2002; Gao et al., 2002; Zhang et al., 2004; Ito et al., 2006; Xiao et al., 2006; Xiong and Fei, 2006). Studies to better understand the freezing tolerance mechanism of plants have also been conducted. Natural genetic variation of freezing tolerance has been studied intensively in accessions of Arabidopsis (Hannah et al., 2006) as well as the natural abilities of close relatives of Arabidopsis, such as Thlaspi, Capsella, and Thellungiella, to withstand freezing temperatures (Wang et al., 2004; Griffith et al., 2007; Zhou et al., 2007).

Draba, like Arabidopsis, belongs to the family of Brassicaceae. It is the largest genus within the family comprising more than 360 species. The genus Draba has a worldwide distribution, characterized by temperate, tropical-alpine, and arctic-alpine species. This study includes two arctic-alpine species: D. lactea and D. nivalis, two temperate species: D. muralis and D. verna, and four tropical-alpine species: D. alyssoides, D. aretioides, D. hookeri, and D. steyermarkii. The arctic- and tropical-alpine Draba species are perennials while both temperate species are winter annuals. All studied species, thus, experience winter freezing and must have evolved ways to cope with cold. This allows us to study the evolution and expression of cold related genes within a single clade. The first step is to identify CBF- and COR-like genes in Draba, using the existing sequence data from Arabidopsis. The expression of the genes can then be studied in plant material collected during laboratory or field experiments. Since no genomic sequences or ESTs were available from Draba we set out to investigate the cold tolerance using a CBF and COR15 candidate gene approach. Here we report the molecular cloning and identification of CBF- and COR15-like cDNAs from cold treated plants of the genus Draba.

We first cloned and sequenced putative CBF and COR genes from cold treated plant material of the different Draba species studied. Then, based on the obtained sequence information, we constructed gene trees to investigate the relationship between the different identified CBF and COR genes. This was done first for the Draba sequences, before expanding the analysis to a wider context of the Brassicaceae family later.

Materials and Methods

Plant material and cold treatment

Draba species included in this study originate from the arctic-alpine regions of Norway, temperate Europe and the tropical-alpine region of the Ecuadorian Andes. Arctic-alpine Draba included are D. lactea and D. nivalis (provided by Prof. Christian Brockmann, National Centre for Biosystematics, Norway), temperate D. muralis and D. verna (collected in the field by Dr. J.G.B. Oostermeijer and A.C. Ellis-Adam of the Universiteit van Amsterdam), and finally tropical-alpine D. alyssoides, D. aretioides, D. hookeri, and D. steyermarkii (collected in the field in Ecuador by N. von Meijenfeldt). All Draba species
included are tetraploid (flow cytometry measurements; see Chapter 1) except *D. nivalis* that is diploid, and *D. lactea* that is either tetra- or hexaploid (Grundt et al., 2005).

*Draba nivalis* seeds were scarified with very fine sandpaper prior to vapor-phase sterilization according to the method described by Clough and Brent (http://www.arabidopsis.org/portals/education/vapor.jsp). Seeds of all other *Draba* species were surface sterilized by washing them for 30sec with 70% EtOH prior to an additional washing of 15min with 4x diluted bleach and a drop of Tween 20. Subsequently the seeds were washed with 10mM HCl for 10min before rinsing 3x with miliQ. After scarification and sterilization all *Draba* seeds were imbibed on moist filter paper for 24-48h in a climate room with standard growing conditions of a 21/10°C or 21/15°C (day/night) temperature regime and a 12h photoperiod. Illumination was set at 150μEinstein. The seeds were subsequently vernalized at 4°C in the dark for two weeks prior to being transferred back to the climate room. After germination the seedlings were transferred to autoclaved sowing soil. At three to four weeks of age plants were transferred to a cold room set to 3-4°C with continuous light and leaves were harvested after 0, 1, 2, 6, 12, and 24h of cold treatment. Leaves were quickly frozen in liquid nitrogen after harvest and stored at −80°C until further analysis.

**RNA extraction and gDNA isolation**

Leaf material of cold treated plants of *D. alyssoides*, *D. hookeri*, *D. lactea*, *D. muralis*, *D. nivalis*, and *D. verna* was finely ground in liquid nitrogen with use of mortar and pestle. To maximize the presence of *CBF* or *COR* expression in *Draba*, plant material of the 1, 2, and 6h cold treated samples were pooled together. The same applied for the material from the 12 and 24h samples. Total RNA was extracted from 150mg plant material by using the hot phenol method adapted and optimized from Slater (1984). Extraction buffer (1% SDS; 10mM EDTA; 0.2M NaAc pH5) and hot, pure phenol were mixed in a 2:1 ratio and heated to 65°C to form a final extraction buffer. 5ml/g of this mixture was added to the finely ground leaf material and extracted for 10min in a 65°C water bath with two periods of vortexing. The aqueous phase was separated by centrifugation at 13.000rpm and 4°C for 20min. After an additional extraction with an equal volume of buffer-saturated phenol, the aqueous phase was again separated by centrifugation. Subsequently, half a volume of phenol:chloroform (1:1) was added and the centrifugation step repeated. The remaining phenol was washed away by addition of half a volume of chloroform followed by centrifugation at 4°C and 13.000rpm for 10min. RNA was precipitated overnight at 4°C with 1/3 volume 8M LiCl. After centrifugation at max rpm and 4°C for 30min, the RNA pellets were washed twice with 70% EtOH, before being dissolved in TE buffer (10mM Tris-CL, 1mM EDTA, ph 7.5).

cDNA was synthesized with an Oligo d(T)-adapter primer (APPENDIX 1, primer 28) from 5μg RNA using SuperscriptII (Invitrogen) in 25μl reaction volume. Prior to diluting the cDNA to 50μl, RNase was added to a final concentration of 50μg/ml and the solution was heated at 37°C for 30min to remove RNA. To verify the cDNA quality a test PCR was performed (APPENDIX 1, primers 33-36). Aliquots of 1μl cDNA were used in PCR experiments.

Total genomic DNA was isolated from non-cold treated *D. alyssoides*, *D. aretioides*, *D. hookeri*, *D. muralis*, *D. nivalis*, *D. steyermarkii*, *D. verna*, and *A. thaliana* Col-wt leaves. Prior to gDNA extraction leaf material was ground as described above for RNA isolation. gDNA was extracted by using the CTAB extraction method (200mg/ml 2% CTAB extraction buffer; 100mM Tris-HCl pH8, 20mM EDTA pH8, 1.42M NaCl, 2% PVP-40, 2% CTAB and 0.2% B-mercaptoethanol) with minor modifications from Doyle and Doyle (1987).
DNA was dissolved in 200ul TE. Subsequently, DNA stock solutions were diluted with miliQ water to a working solution of 5ng/ul. This was used as template for PCR further analysis.

**Isolation of CBF and COR genes from Draba**

We started our search for CBF and COR homologs in Draba from cDNA of *D. alyssoides* and *D. lactea*, and later expanded this to the other species. Primers were designed based on alignments of CBF and COR sequences available on NCBI Genbank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) from *Arabidopsis thaliana*, *Brassica napus*, *Brassica rapa*, *Capsella bursa-pastoris*, and *Thellungiella salugsinea*. PCR amplification of cDNA was performed with degenerate primers for the CBF genes and non-degenerate primers for the COR genes. Where required the products of this first PCR were re-amplified with nested PCR (Appendix 1, primers 1-7 and 18-21). PCR conditions were optimized for each template-primer system. PCR products were cleaned up from agarose gel with the QIAquick Gel Extraction kit (Qiagen, the Netherlands) or the Invisorb® Spin DNA Extraction Kit (Invitek, Germany). The different CBF and COR PCR fragments were cloned into pGEMT-easy (Promega, the Netherlands) and multiple clones were sequenced with use of M13 forward and reverse primers (Appendix 1, primers 44-45). Amplification from *D. lactea* did not yield clear PCR products, so after identification of two COR copies it was decided to exclude this species from further studies. Specific primers were then designed for rapid amplification of cDNA 5' end (5' RACE) according to the sequence information of the partial *D. alyssoides* cDNA fragment only, in order to obtain full length sequences of the genes. For CBF three antisense gene-specific primers (GSP1 CBF, GSP2-1 CBF and GSP2-2 CBF; Appendix 1, primers 37-39) were designed and for COR two (GSP1 COR and GSP2 COR; Appendix 1, primers 40-41). The RACE reactions were performed according to the manufacturer’s protocol (Fermentas, Germany). CBF and COR RACE products were cloned, sequenced and a single full-length cDNA sequence for both genes was obtained by combining the 5' RACE fragment with the cDNA fragment. Once full-length sequences were obtained for *D. alyssoides* CBF and COR and Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed our products had a high similarity to other Brassicaceae CBF and COR genes, we focused on the other *Draba* species.

New alignments were made based on the identified *D. alyssoides* CBF and COR genes and the Brassicaceae CBF and COR genes from NCBI Genbank used previously. Based on these alignments new degenerate primers were designed. PCR amplification of CBF and COR from gDNA and cDNA was performed with different combinations of degenerate and non-degenerate primers using high-fidelity polymerase (Phusion™ High-Fidelity Polymerase; Finnzymes) to ensure high quality sequences. PCR products were purified from agarose gel and cloned as described above. To identify potentially different clones, inserts of ten to twenty-four blue/white bacterial colonies were amplified (Appendix 1, primers 46-47) and used in a restriction analysis with Alu I en Dde I (Fermentas/NEB) according to the manufacture’s protocol. After screening the restrictions patterns clones with a unique pattern were cleaned and sequenced (BigDye® Terminator v1.1Cycle Sequencing Kit, Applied Biosystems; Appendix 1, primers 46-47).

In an attempt to obtain full-length coding sequences the 3'-UTR region was amplified. New cDNA was synthesized according to the procedure described under ‘RNA extraction and gDNA isolation’ but this time with use of an anchored polyT-adapter (Appendix 1,
PCR amplification was performed with species-specific forward primers in combination with the anchored polyT-adapter specific primer (Appendix 1, primers 8-10, 16-17, 22-26 and 32). For D. hookeri and D. nivalis nested primers had to be developed in order to amplify the 3'-UTR region (Appendix 1, primers 11-15, and 32). Subsequently, the procedure of purifying and cloning the PCR products and selecting and sequencing the clones was repeated.

All resulting sequence fragments were aligned per species in BioEdit (version 7.0.5, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) using default parameters. By comparing and aligning all the partial coding, 3'-UTR, and gDNA sequences full length CBF and COR sequences were compiled.

**Southern blot analysis**

Genomic DNA (7-11 μg) from D. hookeri, D. muralis, D. nivalis, D. verna, and A. thaliana was digested with restriction enzymes: HindIII, XmiI, and EcoRI. The resulting fragments were separated by electrophoresis on 0.8% agarose 1xTAE gel. gDNA was transferred to Hybond-XL nitrocellulose membrane (Amersham Biosciences, UK) by capillary transfer in 0.5M NaOH, 1.5M NaCl for at least 12 hours and cross-linked by UV illumination. The DalyssCBFb-specific probe consisting of a 580bp fragment was PCR-amplified from cDNA using a degenerate forward primer and the cDNA adapter-reverse primer (Appendix 1, primers 2 and 29). The AtCBF2-specific probe was PCR-amplified from gDNA with an AtCBF2 specific forward and reverse primer (Appendix 1, primers 42-43). The resulting fragment was 635bp long and covered the entire coding region except for 10bp. All DNA fragments to be used as probes were isolated from a 1.5% w/v agarose gel and purified with an Invisorb® Spin DNA Extraction kit (Invitek). DNA probes were labelled with [α^{32}P]-dATP using the DecaLabel kit (Fermentas) and purified with Sephadex-G50 columns. Prehybridization (30min) and hybridization (overnight) were performed in BLOTTO (0.14M NaH₂PO₄, 0.3M Na₂HPO₄, 10mM EDTA, 7% SDS, 1% BSA) at 62°C. After hybridization, membranes were washed at 62°C for 5min with 5x SSC, 0.1% SDS and 30min twice with 2x SSC, 0.1% SDS. Hybridization signals were visualized by autoradiography using phosphoimaging (Molecular Dynamics). After autoradiography the membranes were stripped by boiling for 10min in 0.1% SDS solution before briefly being rinsed in 2x SSC. The membranes were subsequently autoradiographed for three days to ensure they were no longer radioactive before allowing hybridization with a new probe.

**Sequence and phylogenetic analyses**

Sequence similarity searches and comparisons were performed using the Basic Local Alignment Search Tool (BLAST) of the NCBI server. Once confirmed, all Draba CBF and COR sequences were visualized and aligned using the BioEdit program (version 7.0.5, http://www.mbio.ncsu.edu/BioEdit/bioedit.html with default settings). CBF and COR sequences of other Brassicaceae present in the NCBI GenBank database (Appendix 2) were added to the Draba sequences and also aligned. CBF1-3 sequences of Arabidopsis lyrata were obtained by blasting AtCBF2 against the Arabidopsis lyrata v 1.0 assembly scaffolds database of JGI online (genome.jgi-psf.org). Multiple sequence alignments were generated with the online MULTIPLE SEQUENCE COMPARISON BY LOG-EXPECTATION method (Labarga et al., 2007). Alignments were made at the nucleotide as well as the inferred amino acid level. Nucleotide alignments were subsequently analyzed with FindModel (www.hiv.lanl.gov) to
test which substitution model best described our sequence data. The resulting model was used for tree building. Maximum likelihood trees for both the nucleotide and the amino acid data were generated using PHYML online (Guindon and Gascuel, 2003). Bootstrap analyses were performed with 1000 replicates.

DNA polymorphisms and ratios of synonymous to nonsynonymous substitutions in the AP2-domain and acidic region of the CBF genes from Draba, Arabidopsis thaliana, and A. lyrata were estimated and compared with use of DNAsp version 5 (Librado and Rozas, 2009).

To obtain a reference of the evolutionary relationships between the Draba species studied, trnL introns, trnL-F intergenic spacer, and ITS sequences from Draba were collected from the NCBI Genbank database (Appendix 2). Not all Draba species contained both trnL introns and trnL-F intergenic spacer sequences, so alignments of all trn sequences present were made with MAFFT version 6 (Katoh and Toh, 2008). Based on these alignments the overlapping region was selected and combined with the ITS sequences to form one compiled trn-ITS sequence per species. These sequences were further analyzed in a similar manner as described above for the CBF and COR sequences.

**RESULTS**

**Complexity of the CBF gene family in Draba**

To investigate the variation in CBF copies in the Draba genome, DNA from four different species was digested with HindIII and Eco88I, and separated by gelelectrophoresis. After blotting, the membrane was hybridized consecutively with a 635bp Arabidopsis thaliana CBF2 DNA fragment and a 490bp Draba alyssoides CBFb DNA fragment (FIGURE 1A and B). Hybridization of the AtCBF2 probe with Arabidopsis DNA showed the expected pattern based on the restriction map of the CBF region in Arabidopsis chromosome 4 (FIGURE 1D). The HindIII digestion rendered three bands as expected (FIGURE 1D): two short (parts of AtCBF2) and one long (AtCBF3+AtCBF1) fragment, while Eco88I rendered three fragments: a 3599bp band (containing parts of AtCBF1+AtCBF3) a 2866bp band (AtCBF3+AtCBF2), and a 1986bp band (AtCBF2). Weak bands were visible in the DNA of all Draba.

Hybridization with the DalCBFb probe showed that multiple CBF genes were present in Draba (FIGURE 1B). Although the exact number cannot be identified from these results, the number of CBF copies in Draba appears comparable to that in Arabidopsis. The hybridization patterns of HindIII and Eco88I are not identical yet they are consistent for all species. In D. hookeri, D. muralis and D. verna more than one CBF containing fragment was detected, while in D. nivalis hybridization resulted in a single band. Still, the longer bands may contain several CBF genes in tandem, similar to Arabidopsis. The hybridization patterns suggest the presence of several CBF copies in all Draba species. Arabidopsis CBF1 and CBF3 containing bands cross-hybridized with the DalCBFb probe. Pairwise DNA sequence comparison showed that the DalCBFb probe shared a 62% identity with the coding region of AtCBF1, 59% identity with AtCBF2, and 61% identity with AtCBF3 (Appendix 3A).

**Three classes of CBF genes exist; CBFa, CBFb, and CBFc**

With degenerate primers and RT-PCR a total of ten genes were cloned from cDNA and gDNA from the different Draba species. The cDNA was obtained from cold treated plants,
**Figure 1. CBF genes in the Arabidopsis and Draba genome.** DNA-gelblot hybridization of *A. thaliana* (At), *D. hookeri* (Dh), *D. muralis* (Dm), *D. nivalis* (Dn), and *D. verna* (Dv) genomic DNA (7-11µg) digested with *HindIII* and *Eco88I*. The probes used were the α³²P-labelled 635bp *A. thaliana* CBF2 DNA probe (A) and the 490bp *D. alyssoides* CBFb DNA probe (B). Both probes correspond to almost the entire coding region of the genes involved. DNA loading was visualized by means of ethidium bromide staining (C). (D) Schematic drawing of the *A. thaliana* chromosome 4 region containing the three CBF copies in tandem repeat. Horizontal arrows show the orientation of the genes. Digestion sites of restriction enzymes *HindIII* and *Eco88I* are depicted (vertical arrows) together with their resulting fragment lengths.
the gDNA from control plants. We started out with a total of 143 clones of which those with differing restriction patterns were sequenced in order to pick up as many putative CBF genes as possible. In the end sequences of 114 clones rendered the 10 different CBF copies as identified from the seven Draba species studied here. Fragments of which only a single copy was found were excluded from further analysis. We are, therefore, most likely dealing with an incomplete set of possible CBF genes in Draba. The different CBF copies isolated from different Draba species were labeled CBFa, CBFb, and CBFc, based on their clustering into three groups.

As different primer combinations were used to isolate the possible CBF homologs in Draba, sequences were not all of equal length. Therefore, we used the full length sequence from D. alyssoides, DalCBFa, as a prototype. This cDNA contained a 612bp open reading frame (ORF) encoding a 204aa protein. The other sequences of all Draba species were then aligned using DalCBFa and AtCBF3 as reference (Figure 2). With the exception of DalCBFa none of the other CBF fragments contained the start codon. The degenerate primer designed to start amplification at the start codon did not work and new primers were developed further downstream (Figure 2).

All Draba CBFs showed the typical regions also found in Arabidopsis. These regions are the nuclear localization signal (NLS; 37-49aa), the AP2-binding domain (52-111aa), and the
acidic region (122-223aa). The potential nuclear localization signal as well as the entire DNA binding domain was conserved between the Draba CBF genes identified. All Draba CBF genes had the Val-14 and Glu-19 amino acids in the AP2-binding domain (Figure 2), crucial for the binding specificity of the DREB proteins, as shown by Sakuma et al. (2002). Pairwise DNA sequence comparison showed that the Draba AP2-binding domain shared an average sequence identity of 85.6%, 86.4%, and 86.6% with the same binding domain conserved in all three AtCBF genes (Appendix 3B). In contrast, pairwise DNA sequence comparison of the acidic region showed that Draba CBFs shared an average sequence identity of 68.7%, 66.7%, and 70% with that of AtCBF1, -2 and -3, respectively (Appendix 3C). The acidic region, thus, was the region with the highest level of variation. Similar to Arabidopsis none of the Draba CBF genes had introns interrupting their ORFs. All Draba species produced CBF transcripts that are polyadenylated at different positions (data not shown).

The differences between the various Draba CBF fragments were mainly due to amino acid substitutions in the acidic region that started at amino acid position 122. However, three CBF sequences; D. alyssoides CBFa, D. nivalis CBFc, and D. muralis CBFc showed additional differences. The full length DalCBFa sequence was characterized by a unique 12 amino acid indel at position 177-188. In the region between amino acid positions 150-153 DnCBFc, in turn, was characterized by a one amino acid indel and three amino acid substitutions compared to DalCBFa. In the same region DmCBFc had a two amino acid indel followed by two amino acid mutations. Both CBFc fragments further contained a unique five amino acid insert (DnCBFc) and a three amino acid insert (DmCBFc) between position 208 and 212.

Analysis of the Draba CBF gene sequences suggest clade-specific duplication events

A gene tree based on the acidic region was made to visualize the relatedness of the various CBF genes from Draba. As more sequence diversity is present at the nucleotide than amino acid level, the phylogenetic relationship within the acidic region is shown at nucleotide level only (Figure 3A). The results revealed three major CBF groups within the genus Draba. Bootstrap values higher than 70% supported the branch points of the different groups. Within the tropical-alpine Draba species studied CBFa formed a distinct group from CBFb. From the two D. nivalis CBF genes detected CBFa, showed a high homology to the tropical-alpine CBFa gene. The long branch length, however, indicated a high divergence between the tropical CBFa’s and the arctic DnCBFa. The third group CBFc comprised a rather heterogeneous group of D. nivalis, D. muralis, and D. verna CBFc. Here again, long branch lengths indicated the substantial evolutionary changes between the CBF genes that were isolated from these species.

To investigate if the above CBF gene tree maps onto the phylogeny and geographical origin of the different Draba species a phylogenetic analysis of the combined trnL-F and ITS sequences available from Genbank was conducted. This analysis placed the three tropical-alpine Draba species as sister to the arctic-alpine D. nivalis, the closest temperate species being D. verna and D. muralis. The latter is the most basal species included in the analysis (Figure 3B). The CBF gene tree and the Draba species tree were not congruent. Arctic-alpine D. nivalis shared both a CBF gene with the tropical-alpine Draba clade (CBFa), as a CBF gene with the temperate Draba clade (CBFc). D. nivalis, therefore, appeared to represent an intermediate between the temperate and the tropical-alpine CBF genes. In the trop-
 Calder-alpine Draba species an independent gene duplication appeared to have occurred resulting in the presence of CBFb. Whether independent gene duplications have also occurred within the temperate Draba species remains unresolved. The rooted phylogenetic tree of the Draba CBF genes also reflects this pattern (Figure 4). In addition, DmCBFc was now grouped separate (bootstrap value of 61%) from all other Draba CBF genes, reflecting its more distant relationship to the other Draba species.

The maximum likelihood tree of the acidic region of the CBF proteins reflects the basic evolutionary relationship in a wider Brassicaceae context (Figure 4; Schranz et al., 2007). In this broader phylogenetic comparison of the Brassicaceae family all CBF genes from Draba are placed in the Arabidae tribe, separate from the other two tribes. Thus, the various Draba CBF genes identified cannot be said to be homologous to a particular CBF gene known from one of the other tribes, since the homologies between CBF genes within the different species are higher than those between the clades. The main conclusion is that the CBF family has undergone independent duplication events in the different tribes.

**Figure 3. Three CBF clusters are present within Draba.** Maximum-likelihood phylogenetic tree analysis of the nucleotide sequence of the CBF acidic region (A) and the combined trnL-F and ITS sequences (B) of the studied Draba species. The nucleotide tree (A) was constructed based on the best-fit TN93 substitution model, and the combined trnL-F and ITS tree (B) on the best-fit GTR+$\gamma$ substitution model. Tree A is unrooted, tree B is rooted with Arabis alpina as an outgroup. Only bootstrap values $>60\%$ (based on 1000 replicates) are indicated above the branch points. Branch lengths indicate genetic distance. Presence of the genes CBFa, b, or c in the various Draba species is marked as a, b, or c in Figure B. Dar = D. aretioides, Ds = D. steyermarkii, Dh = D. hookeri, Dal = D. alyssoides, Dn = D. nivalis, Dm = D. muralis, and Dv = D. verna.
Divergence in the acidic region of the CBF gene

After the phylogenetic relationship between the Draba and Arabidopsis CBF genes had been clarified we set out to investigate the conserved and diverged regions within the CBF genes of Draba and Arabidopsis in more detail. Alignments of the Draba CBF amino acid sequences with Arabidopsis CBF genes demonstrated high similarities in the AP2-binding domain and the acidic region (Appendix 4). These similarities were investigated in more detail with use of a sliding window analysis (DNAsp v 5; Librado and Rozas, 2009). CBF genes of both A. thaliana and A. lyrata were included as a reference for the amount of variation present between these genes in the genus Arabidopsis.

The average nucleotide diversity in the acidic region of Draba and Arabidopsis CBF genes was higher (\(\pi = 0.21910\); Figure 5A) than in the AP2-binding domain (\(\pi = 0.10389\); Appendix 5A). The DNA binding domain is highly conserved in both Draba and Arabidopsis, precluding further analyses. In the acidic region more divergence between the different genes occurred. When comparing the two genera, the average diversity in the acidic region among the ten Draba CBF genes is somewhat lower than that between the six Arabidopsis CBFs (\(\pi_{\text{Draba}} = 0.11764\) versus \(\pi_{\text{Arabidopsis}} = 0.14438\); data not shown). The ratio of non-synonymous to synonymous substitutions is higher in this region when compared to the AP2 domain (\(\pi(a)/\pi(s)_{\text{acidic region}} = 0.230\) versus \(\pi(a)/\pi(s)_{\text{AP2 domain}} = 0.045\); Figure 5B and Appendix 5B). This increase in non-synonymous sites, as found in all 16 CBF genes, suggested that the acidic region is under less selective pressure than the AP2-binding domain.

Does selection, however, act on the same area within the acidic region in Draba and Arabidopsis? Draba CBF cDNA nucleotide sequences differ from Arabidopsis mainly in the middle N-terminal part of the acidic region of the CBF gene. The peak in non-synonymous differences (Figure 5B), identifying a ‘hotspot’ in the acidic region of Draba and Arabidopsis, is solely due to Draba CBF genes (Figure 5C). All ten CBF genes from Draba

![Figure 4](https://example.com/figure4.png)

**Figure 4. Draba CBF genes within a family-wide context.** Maximum-likelihood phylogenetic tree analysis of the nucleotide sequence of the CBF acidic region within the Brassicaceae. The unrooted tree was constructed based on the best-fit K80 substitution model. Only bootstrap values >60% are shown above the branch points. Branch lengths indicate genetic distance. Draba species as in Figure 3. Al = Arabidopsis lyrata, At = A. thaliana, Bj = Brassica juncea, Bn = B. napus, Br = B. rapa, Cbp = Capsella bursa pastoris, Ta = Thlaspi arvense, and Ts = Thellungiella salsuginea.
had a unique indel between nucleotide positions 111-114 (aa position 37 with the acidic region, Appendix 4), which was absent in all six Arabidopsis CBF genes. The peak in non-synonymous differences in Figure 5B is thus due to this difference. The analysis of the acidic region in Draba is based on 10 CBF sequences versus two times three sequences for A. thaliana and A. lyrata, making it less likely to detect a signal of selection in Arabidopsis.

In Arabidopsis the most divergent sites differed between the two species A. thaliana and A. lyrata. In A. thaliana the region of 60 to 90 base pairs (20-30aa; Figure 5D) contains the highest number of substitutions, while in A. lyrata this region is located between 145-160bp (48-54aa; Figure 5E). In Draba this region is located between 120-147bp (40-49aa; Figure 5C). Thus there appear to be distinct locations in the acidic regions where substitutions occur. However, these regions are not overlapping in the different species. It is therefore not clear whether these differences present in the N-terminal part of the acidic region within Arabidopsis and Draba concerned functional differences that are under selection.

Two classes of the CBF–target gene COR15 are identified in Draba
Homologs of the CBF-target gene COR15 were amplified in Draba using degenerate and non-degenerate primers and RT-PCR. In total 19 genes were cloned from cDNA and gDNA of eight different Draba species.

Similar to CBF, the cDNA was obtained from cold treated plants, the gDNA from control plants. The majority of the sequences were isolated with a non-degenerate forward

**Figure 5. Divergence between Arabidopsis and Draba CBF genes occurs within the acidic region.** Sliding window comparison of nucleotide diversity (A) and non-synonymous to synonymous substitution ratios (B) in the acidic region of Draba and Arabidopsis CBF genes. Pi(a)/Pi(s) ratios of Draba (C), A. thaliana (D) and A. lyrata (E) are represented independently. The sequences were aligned in correspondence to the coding region. All 10 Draba CBF genes (DalCBFa-b, DarCBFa-a, DhCBFa-a, DmCBFc, DnCBFa/c, DsCBFa, and DvCBFc) together with AtCBF1-3, and AlCBF1-3 were analyzed. The sequences were 172bp long, corresponding to the first 57aa of the acidic region. The analysis was done with use of DnaSP v5 software, with a window length of 10 sites and a step size of 5 sites.
primer including the start codon, responsible for the absence of variation at the start of the sequences. Most of the sequences are not full length but only miss the last six or seven amino acids before the stop codon as inferred from the *Arabidopsis COR15a* sequence (Figure 6).

Pairwise DNA sequence comparisons of *Draba COR15* with *AtCOR15a* and *AtCOR15b* showed that the *COR15* genes from *Draba* had the highest similarity to *AtCOR15b*

**Figure 6.** Alignment of the inferred amino acid sequences of *Draba COR15* with *Arabidopsis COR15a/b*. Alignment of *Draba* sequences *D. alyssoides* (Dal) *COR15a1/a2/c*, *D. hookeri* (Dh) *COR15a/c1/c2*, *D. aretioides* (Dar) *COR15a/c1/c2*, *D. steyermarkii* (Ds) *COR15a/c*, *D. nivalis* (Dn) *COR15a*, *D. muralis* (Dm) *COR15a1/a2*, *D. verna* (Dv) *COR15a1/a2/c*, *D. lactea* (Dl) *COR15a/c*, and *Arabidopsis thaliana* (At) *COR15a* (accession no. NM_129815) and *AtCOR15b* (accession no. NM_129814). Black shading indicates identical amino acids; grey shading similar amino acids; dashes represent gaps inserted to optimize alignment. The black arrow (↓) marks the position of the intron, the double-black line the consensus cleavage site sequence for chloroplast transit peptides and the dotted lines the repetitive 13-amino acid motif. Sequences were aligned using MUSCLE v3.7.
D. nivalis COR15a was most similar to the Arabidopsis genes: 84% identity with AtCOR15b and 80% identity with AtCOR15a.

All inferred protein sequences of the various COR15 genes were aligned using D. verna COR15c as reference (Figure 6). A. thaliana COR15a and b are included for reference. If a species had two very similar copies of one of both COR15 genes they were labeled COR15a1 and a2, or COR15c1 and c2. Similar to Arabidopsis, all cloned Draba sequences contained one intron, between amino acid positions 65 and 66, which varied in length between the different species (data not shown). Based on these alignments the sequences could clearly be divided into two groups: COR15a and COR15c. Representatives of both groups were isolated from all Draba species except D. muralis and D. nivalis (only COR15a).

The Draba COR15c genes distinguished themselves by the presence of a large insertion that did not contain an intron and in which no defining splice sites or branch points were detected. All but one had a 228 bp/76 amino acid insert in the region 120-196aa, resulting in an open reading frame of 217 amino acids, compared to 140 amino acids of the Draba COR15a. COR15c picked up from the arctic-alpine D. lactea had a shorter insert of 108 bp/36 amino acids. Apart from the insert numerous non-synonymous substitutions are present in COR15c when compared to COR15a. Nonetheless, all COR15a/c copies in Draba contained the consensus cleavage site sequence for chloroplast transit peptides (Val/Ile)-X-(Ala/Cys)↓Ala in the 49-52aa region. Only the final amino acid, neighboring the cleavage site, differed between Draba COR15a and COR15c. In Draba COR15c, identical to AtCOR15b, the last alanine was replaced by a valine. All Draba COR15a, in contrast, had a threonine in this position. Despite these differences, ChloroP analysis (ChloroP 1.1 server; www.cbs.dtu.dk/services/ChloroP) showed that Draba COR15a and COR15c both have putative chloroplast targeting signals.

Further analysis of all Draba COR15 genes revealed the presence of several imperfect repeats of about 13 amino acids in length (consensus: KA(K/S)D(Y/F)(V/I)(V/T)EK(T/G) KEA). These repetitive motifs were distributed irregularly throughout the coding region, occurring from positions 68-80, 86-98, 108-120, and 199-211 in Draba COR15a. In COR15c they occurred three additional times in positions 126-138, 148-160, and 166-178. With exception of D.verna COR15c where, due to its shorter insert, the motif was only repeated once more.

The gene tree of the Draba COR15a cDNA sequences with AtCOR15b as an outgroup reflected the underlying geographical origin of the species (Figure 7). The four tropical-alpine Draba species are placed sister to both arctic-alpine species, with the closest temperate species being D. verna while temperate D. muralis is basal to all other species. COR15a genes of tropical-, arctic-alpine or temperate origin all grouped together in a separate branch. This clustering was also found in a broader comparison with more Brassicaceae COR15 sequences, obtained from Genbank. In order to prevent differences in sequence length from affecting the analysis, all sequences were truncated to include only amino acids 13-210. The two COR15a and COR15c copies found in the eight Draba species studied remained distinct clusters within the larger phylogenetic COR15 context of the Brassicaceae family (Figure 8). Both clusters were supported by high bootstrap values of 94 and 100%. All Draba COR15a genes clustered together with the COR15a genes of D. alpina and D. draboides, as identified by Zhou et al. (2009). Members of the COR15b cluster identified by the same authors were not detected in our study. Pairwise DNA sequence analysis of D. alpina and D. draboides COR15a-b with AtCOR15a-b revealed that for both Draba species
their COR15a gene shared a higher similarity to AtCOR15b (84%; APPENDIX 3D) than to AtCOR15a (81%; APPENDIX 3D). All analyzed COR15c genes grouped together in their own cluster, without any known close relatives.

In summary, we have identified two COR15 homologs in Draba: COR15a, closely related to D. alpina and D. draboides COR15a, but distantly related to AtCOR15b and COR15c, unique to Draba.

**DISCUSSION**

The main goal of this study was to identify candidate genes involved in the cold tolerance pathway in Draba species originating from arctic-alpine, tropical-alpine and temperate regions. This step was essential for the later studies of gene expression, as discussed in Chapter 3. During the process a lot of sequence information was generated on the selected genes, and in this Chapter we summarize these findings. While we realize that our search was not exhaustive, important conclusions can already be drawn from the variation within the Draba genus, and differences to other species in the Brassicaceae family.

**Identifying CBF genes and their relationships in Draba**

Making use of degenerate primers followed by the design of more specific primers for Draba resulted in the amplification of two distinct CBF cDNAs from D. alyssoides, D. hookeri, and D. nivalis. From the other species studied, i.e., D. aretioides, D. muralis, D. nivalis, D. steyermarkii, and D. verna only one CBF copy has been isolated. Sequence and phylogenetic analysis of all copies revealed two distinct clusters within the tropical-alpine Draba species, labeled CBFa and CBFb. The cDNAs from temperate D. muralis and D. verna, CBFc, clustered together, albeit in a rather diverse group. The arctic-alpine D. nivalis had a CBFa ortholog in common with the tropical-alpine Draba species (76% bootstrap value, FIGURE 4) and a CBFc ortholog in common with the temperate Draba species. CBFb

**FIGURE 7. COR15a gene tree reflects the geographical origin of the Draba species.** Rooted maximum-likelihood phylogenetic tree of the nucleotide sequence of the Draba COR15a sequences with AtCOR15b as outgroup. The rooted tree was constructed based on the best-fit GTR+γ substitution model, as described in FIGURE 3.
was unique to the tropical-alpine Draba species, suggesting an independent CBF gene duplication within the tropical-alpine lineage. The divergence between the three CBFc genes is large, indicating that substantial evolutionary change has occurred in the CBFc genes of these different species. It is as yet not clear whether the divergence within the genus Draba is associated with the spread into new habitats, or is simply a signal due to a common phylogenetic background.

Based on the DNA gelblot analysis, it cannot be excluded that the temperate D. verna and D. muralis have more than the CBFc copy we cloned, whereas the tropical-alpine D. aretioides and D. steyermarkii may also have a CBFb gene. A DNA gelblot analysis cannot elucidate how many CBF copies exist exactly in the different Draba species. A single band may contain fragments from several gene copies and two bands fragments of a single gene, as shown for the three tandemly-repeated CBFs in Arabidopsis. Results did suggest, however, that it is unlikely that Draba species contain many more gene copies than Arabidopsis. Initially, CBF fragments that were found only once were excluded from further analysis, but later checks showed that the single fragments of D. verna did not resemble CBFa or CBFb, confirming the general pattern. One D. verna fragment resembled another single fragment of D. nivalis, both in the CBFc cluster, which may indicate the presence of additional CBFc like copies in these two species.

As all cDNAs were isolated from RNA of cold treated plants, we are dealing with a biased sample and some family members may not have been isolated.

**FIGURE 8.** Draba COR15 genes within a family-wide context. Maximum-likelihood phylogenetic tree of the nucleotide sequence of the COR15-like sequences within the Brassicaceae. The unrooted tree was constructed based on the best-fit LG+γ substitution model, as described in FIGURE 3. Dalp = Draba alpina, Dd = D. draboides, Dl = D. lactea, At = Arabidopsis thaliana, Bn = Brassica napus, Bo = B. oleracea, Br = B. rapa, Cbp = Capsella bursa pastoris, and Ts = Thellungiella salsuginea.
**CBF in a family-wide context**

The *Arabidopsis* CBF-family consists of six homologues, three of which; i.e. *CBF1*, *CBF2*, and *CBF3* are cold-related and organized in a tandem repeat on chromosome 4 (Gilmour et al., 1998; Shinwari et al., 1998; Medina et al., 1999; Sakuma et al., 2002). It is proposed that *CBF1-3* in *Arabidopsis* share a common origin and that they arose via two consecutive duplications of the ancestral gene followed by divergence through mutations (Medina et al., 1999; Gilmour et al., 2004). Zhou et al. (2007) investigated the colinear region of the *Thlaspi arvense* genome and found that there only one single *CBF* copy was present. They hypothesize that the duplication event in the ancestral *CBF* gene took place long before the different lineages within the Brassicaceae family arose. Subsequently, the common ancestor of *T. arvense* and *A. thaliana* would have had three *CBF* copies. In *T. arvense* two of these genes have since been lost, but were maintained in *A. thaliana*. The Brassicaceae-wide phylogenetic analysis of all known *CBF* genes showed that the *CBF* genes cloned from *Draba* did not group together with any of the other *CBF* genes. Within each cluster various copies of *CBF* genes were found per species, implying that the *CBF* family is characterized by relatively frequent and independent gene duplication events. With the increase in genome projects within the Brassicaceae family the evolutionary relationship of the *CBF* and other gene families may become more apparent.

The phylogenetic tree analysis of Brassicaceae *CBF* genes reflected a complex gene family structure. Similar patterns and dynamics of variation in genes arising from duplication and deletion events have also been found in disease-resistance (R) (Michelmore and Meyers, 1998) and methylthioalkylmalate synthase (MAM) genes (Benderoth et al., 2006). More detailed research would have to elucidate whether common evolutionary processes apply to these gene families. In all three cases the genes involved are tandemly arrayed genes which according to Rizzon et al. (2006) represent a powerful evolutionary force for plant adaptation. New genes arising from duplication events may diverge in function and therefore provide a new source for adaptation.

**Draba CBF genes contain all important functional domains**

Analysis of the structural organization of the *Draba CBF* encoded proteins showed that they all contained the characteristic nuclear localization signal (NLS), AP2-DNA-binding domain and acidic transcriptional activation domain. Comparative protein sequence alignment of *Draba*, *Arabidopsis thaliana* and *Arabidopsis lyrata* revealed the presence of conserved sequence blocks throughout the *CBF* coding region. The nuclear localization signal, which forms part of the *CBF* ‘signature signal’ PKK/RPAGRxKFxETRHP and DSAWR designated by Jaglo et al. (2001) is conserved among all species. Sliding window analyses showed that, despite some substitutions in *Arabidopsis*, the AP2-domain was highly conserved, while the acidic region showed the most variation. Within *Draba*, the entire AP2-binding domain was conserved. The fact that both the NLS and AP2 domains have been evolutionarily conserved in various members of the Brassicaceae family confirms the important functional role in DNA targeting (Stockinger et al., 1997; Jaglo et al., 2001; Lin et al., 2008). Furthermore, all *Draba CBF* genes have the Val-14 and Glu-19 amino acids in the AP2-binding domain crucial for the binding specificity of DREB proteins, as shown by Sakuma et al. (2002). Selective pressures on the acidic activation domain appear to be less stringent, as most divergent regions were found within this domain. When comparing the divergence within the acidic domain of *Draba*, *A. thaliana*, and *A. lyrata*, no common hotspots of vari-
ation were found. This suggests that within the two different genera selection acts upon different sites within the acidic transcriptional activation domain. A possible explanation could be that mutations in this domain do not necessarily result in less active activators (Wang et al., 2005). In addition, Lin et al. (2008) found nucleotide diversity in the promoter regions of the Arabidopsis CBF genes to be greater than those in the transcriptional activation domain. Nonetheless, all Draba CBF genes possess all the amino acids critical for the binding to their target genes (Figure 2).

**CBF-target COR15 is also present in Draba**

We have isolated two types of COR15-like cDNAs from the Draba species studied. Sequence and phylogenetic analyses of all copies showed the presence of two distinct COR15 types; COR15a and COR15c. From D. muralis and D. nivalis we cloned only the COR15a type. The gene tree of the COR15a cluster reflected the geographic origin of the different Draba species. The tropical-alpine Draba species are sister to the arctic-alpine Draba species, which in turn are sister to the temperate D. verna. D. muralis, also a temperate species, is the most basal species. This is in concordance with the phylogenetic relationship of these species found by Jordon-Thaden et al. (2010). DNA sequence comparison of the identified COR15a sequences from Draba with COR15a/b from Arabidopsis revealed that the full length Draba sequences were more than 80% identical to AtCOR15b and more than 75% identical to AtCOR15a. Draba COR15c shared a 48-50% identity with AtCOR15a and a than 50-53% identity with AtCOR15b. Both Draba COR15 genes are characterized by the presence of a single intron that occurs at the same position in the gene as that in AtCOR15a/b. COR15c has a characteristic 76 amino acid insert (36 in D. lactea) in its deduced amino acid sequence that is unique to Draba. Blastn results in the nucleotide collection (http://blast.ncbi.nlm.nih.gov/Blast.cgi) rendered no other known COR15-like, or any other genes with a similar insert.

Zhou et al. (2009) isolated two functional COR15-like sequences from five Draba species that were not used in this study. From two of these species COR15 sequences were available via Genbank and our analyses show that one of these genes, COR15a, shared a high similarity with our Draba COR15a genes (>87%). Surprisingly we did not detect any of the COR15b sequences identified by Zhou et al. (2009) in our Draba species. As our focus was on cDNA from cold treated plant material, while Zhou et al. looked at gDNA only, one explanation could be that this COR15b gene is not present or not expressed in our Draba species in response to cold. This could be checked by conducting a cold experiment with D. alpina and D. draboides.

**Both COR15 proteins contain a putative chloroplast targeting signal**

From Arabidopsis studies it has been shown that the mature COR15a polypeptide is located in chloroplasts as it contains features typical of a chloroplast transit peptide (Lin and Thomashow, 1992; Steponkus et al., 1998; Nakayama et al., 2007). Some of these features were also found within the Draba COR15 genes. For example, the loosely defined chloroplast cleavage site consensus sequence (Val/Ile)-X-(Ala/Cys)-Ala (Gavel and von Heijne, 1990) was partly conserved within Draba COR15a/c. All Draba COR15a peptides contained a threonine adjacent to the cleavage site rather than an alanine. Draba COR15c resembled AtCOR15b in that it had a valine bordering the cleavage site. In addition, the arginine residue present eight amino acids prior to the putative cleavage site was conserved...
between *Draba* and *Arabidopsis*. *Draba* COR15a/c proteins are presumably imported into the chloroplast as was determined via ChloroP analysis. Further sequence analysis showed that both COR15 proteins in *Draba* contain an irregularly repeated 13-amino acid motif (consensus: KA(K/S)D(Y/F)(V/I)(V/T)EK(T/G)KEA) throughout the coding region. In *Draba* COR15a, similar to *A. thaliana* COR15a/b (Wilhelm and Thomashow, 1993) and *Capsella bursa-pastoris* CORb (Liu et al., 2004) this motif is repeated four times. In *Draba* COR15c the motif is repeated an additional three times within its 76 amino acid insert. *D. lactea* has a shorter insert of 36 amino acids in which the motif is repeated once. Repeating amino acid sequences are not uncommon and are thought to occur in 14% of all proteins as a result of recombination and gene conversion (Marcotte et al., 1999). According to Dure et al. (1989), and cited by Wilhelm and Thomashow (1993), a variable repetition of an amino acid motif within a polypeptide is a characteristic of many late embryogenesis abundant (LEA) proteins. LEA proteins are involved in the drought response of plants and most COR genes have been shown to be responsive to drought (Hajela et al., 1990; Baker et al., 1994). Implications of these sequence similarities in terms of function and evolutionary significance remain to be determined.

With the isolation and identification of *CBF* and *COR15* genes we have shown that these components of the cold response pathway are conserved in *Draba*. In addition, our results indicate that the *CBF* and *COR15* genes isolated from *Draba* contain the necessary functional components known from other related species.
### APPENDIX 1. List of primers used for PCR amplification, cDNA synthesis, and sequencing.

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Abbreviations: Dal = *D. albus*, Dh = *D. hookeri*, Dm = *D. murielis*, Dn = *D. nivalis*, Dv = *D. verna*, and At = *A. thaliana*. Act = Actin.
APPENDIX 2. Overview of accession numbers of the different genes used.

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**APPENDIX 3. Identity matrices of Arabidopsis and Draba CBF and COR genes.**
APPENDIX 4. Alignment of the deduced amino acid sequences of *Draba* *CBFs* with the protein sequences of *Arabidopsis thaliana* *CBF1-3* and *Arabidopsis lyrata* *CBF1-3*. Alignment of *Draba* sequences; as described in FIGURE 1, with *Arabidopsis thaliana* (At) *CBF1-3*, and *A. lyrata* (Al) *CBF1-3*. Legend as in FIGURE 2.
APPENDIX 5. Sliding windows along the AP2 binding domain of *Draba* and *Arabidopsis CBF* genes. Sliding window of the nucleotide diversity within the genus *Draba* (A) and *Arabidopsis* (B). Nonsynonymous to synonymous substitution ratios of *A. thaliana* (C) and *A. lyrata* (D). The nucleotide position was placed in correspondence to the coding region. The sequences were 174bp long, corresponding to 58aa. For *Draba* eight different sequences (*DalCBFa*-b, *DarCBFa*, *DhCBFa*, *DmCBFc*, *DnCBFa*, *DsCBFa*, and *DvCBFc*) were analyzed. For *Arabidopsis* seven sequences: *A. thaliana* CBF1-3, and *A. lyrata* CBF2A-C and CBF3. The analysis was done with use of DnaSP v5 software, with a window length of 10 sites and a step size of 5 sites.