PR-10, defensin and cold dehydrin genes are among those over expressed in *Oxytropis* (Fabaceae) species adapted to the arctic

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Abstract In many studied plants, typical responses to cold treatment include up-regulating the hydrophilic COR/LEA genes and down-regulating photosynthesis-related genes, carbohydrate metabolism, GDSL-motif lipase, hormone metabolism and oxidative regulation genes. However, next to nothing is known about gene expression in arctic plants, which are actually adapted to a harsh, cold environment. The molecular mechanisms behind the many specific adaptations of arctic plants, such as slow growth, well-developed root systems and short stature, are not well understood. In this study, we examine whole plantlet transcriptome differences between two arctic and two temperate *Oxytropis* (Fabaceae) species, grown under their respective controlled environmental conditions. Gene expression differences are analyzed using cDNA library subtraction followed by expressed sequence tags sequencing and annotation. Sequences from a total of nearly 2,000 clones cluster into 121 and 368 unique genes from the arctic and from the temperate plants, respectively. The predominant biological process for genes from the arctic-enriched library is “response to stimulus”. A concurrent overexpression of pathogenesis-related class 10 proteins (PR-10), plant defensin and cold dehydrin genes is a novel feature for species adapted to stressful growth environment. The temperate-enriched genes are involved in photosynthesis, translation and nucleosome assembly. Interestingly, both arctic and temperate-enriched libraries also contain genes involved in ribosome biogenesis and assembly, however of different types. Real-time reverse transcription PCR of cold dehydrin and two PR-10 genes, as well as the light harvesting complex b1 genes demonstrates that the gene expression is dependent on species and growth conditions.

Keywords Arctic · Plant · Gene expression · Oxytropis · Library subtraction · Defence response

Abbreviations
ADR6 Auxin down-regulated 6
BLAST Basic local alignment search tool
cDNA Complimentary DNA
ESTs Expressed Sequence Tags
ELIP Early Light Induced proteins
LEA Late Embryogenesis Abundant proteins
mRNA Messenger RNA
PCR Polymerase Chain Reaction
PDF1 Plant Defensin 1
PR-10 Pathogenesis-Related class 10 proteins
RT-PCR Reverse Transcription PCR
SSH Suppressive subtraction hybridization
STP Specific tissue protein

Introduction

The challenges to arctic plant life go beyond the severe winter cold temperatures—very short growing season, common summer frosts, strong winds and low light quality are limiting conditions to plant growth. These tough little
plants present a suite of morphological and physiological specializations compared to their temperate relatives. They have long life cycles (Gruulke and Bliss 1988), leaves or flower primordium development extend on for many growth seasons (Sørensen 1941) and they sport well-developed root systems (Bliss and Gold 1999). Furthermore, their photosynthetic and respiratory apparatus is more efficient at 10°C than at higher temperatures (Xiong et al. 1999; Semikhatova et al. 2007; Pyankov 1991), and they can tolerate freezing temperatures while still actively growing (Junttila and Robberecht 1993). However, the understanding of the molecular mechanisms behind these adaptations is at best fragmentary.

Because of the potential adaptive value of variation in gene expression (Swindell et al. 2007; Whitehead and Crawford 2006a, b), we explored differentially expressed genes between arctic and temperate plants. We chose the *Oxytropis* (Fabaceae) genus as our model system. *Oxytropis* is predominantly distributed in the temperate and boreal regions of North America and Northern Asia, and also includes 44 arctic species (Elven 2007). Although only eight species occur in the Canadian Arctic, this is the legume genus with highest species diversity in that area (Aiken et al. 2007). We used the suppression subtractive hybridization (SSH) technique to discover differences in the transcriptomes of two arctic *Oxytropis* species and two temperate *Oxytropis* species. Because it is an untargeted approach that enables novel gene discovery, the subtraction technique is especially appropriate for characterizing molecular features underlying the suite of arctic plant specializations. Expression of selected genes was validated by real-time reverse transcription polymerase chain reaction (PCR). The study is the first of its kind and we present several important differences in gene expression between the species.

Materials and methods

Plant material and RNA extractions

Seeds of the arctic species *Oxytropis maydelliana* and *Oxytropis arctobia* and the temperate species *Oxytropis splendens* and *Oxytropis campestris* subsp. *johannensis* were scarified, sterilized and stratified at 4°C on 1/2 MS Basal Medium (Sigma, Oakville, Ontario) agar plates. The seed sources are listed in Supplementary Table S1. Germinating seeds were placed in growth chambers mimicking the summer–fall conditions in temperate climates (16 h of light of 225 μmol/m²/s at 22°C and 8 h of darkness at 18°C) or in the low arctic (20 h of light of 150 μmol/m²/s at 10°C and 4 h of darkness at 10°C). RNA was extracted (Qiagen) from plantlets at the two leaves stage.

Suppressive subtraction cDNA library construction

Extracted RNA from two plantlets of a species were pooled prior to complimentary DNA (cDNA) synthesis, and 1 mg of this pool was used as template for cDNA synthesis using the SuperSmart PCR cDNA Synthesis kit (Clontech, Mountain View, CA). Genes specifically expressed in either the arctic or in the temperate species were isolated by applying the suppression subtractive hybridization strategy (Diatchenko et al. 1996) using the PCR-select cDNA subtraction kit (Clontech, Mountain View, CA). The subtraction compared a pool of plantlet cDNA from the two arctic species (*O. arctobia* and *O. maydelliana*) grown in simulated arctic environment, and a pool of plantlet cDNA from the two temperate species (*O. campestris* subsp. *johannensis* and *O. splendens*) grown in simulated temperate environment. The subtraction was performed in both directions resulting in two subtracted libraries: one “arctic-enriched” library and one “temperate-enriched” library. PCR products of arctic- and temperate-subtracted libraries were cloned non-directionally using a TOPO TA library. PCR products of arctic- and temperate-subtracted libraries were cloned non-directionally using a TOPO TA kit and transformed into ElectroMAX DH10B electro competent cells (Invitrogen, Carlsbad, California). White colonies were grown in a total of thirty 96-well plates containing SOC-ampicillin liquid medium, and subsequently screened by PCR using vector primers.

Sequence analysis and annotation of subtracted library clones

Clones with confirmed single inserts were sequenced single-pass (McGill University and Génome Québec Innovation Center). Sequences were base called using phred (Green 2002) and trimmed using SeqTrim (Falgueras et al. 2007). The sequences appear in public sequence databases under the GenBank accession numbers GW696871 to GW698115. The 1,108 arctic and 609 temperate processed expressed sequence tags (ESTs) sequences were assembled into contigs using Phrap (Green2002) and trimmed using SeqTrim (Falgueras et al. 2007). Eight ESTs that appeared to be chimaeras were excluded from further analysis. Blast2GO (Conesa et al. 2005; Gotz et al. 2008) was used to annotate and to assign Gene Ontology terms (Ashburner et al. 2000) to contigs and singlets representing unique genes. Presence of potential false positives (reported in Table 1) in each subtracted library was assessed by building two local basic local alignment search tool (BLAST) target databases, and evaluating sequence similarities between the two libraries using BLASTN and TBLASTX (Altschul et al. 1997). False positives are cDNAs retrieved from a subtracted library but that are not differentially expressed. *Oxytropis* unique gene sequences were manually classified into general categories considering the assigned Gene Ontology terms for biological process, the
MIPS functional categories (Ruepp et al. 2004), and the similarity (by BLASTX) to the complete Arabidopsis peptide sequence collection (TAIR8_pep_20080412). Annotations were manually verified and curated, based on information in the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto 2000), the Arabidopsis Information Resource (Swarbreck et al. 2008), the Plant Metabolic Network database (Zhang et al. 2005) or the NCBI Entrez Gene (Maglott et al. 2005).

Real-time reverse transcription PCR

Expression differences were confirmed for four genes with real-time reverse transcription PCR (RT-PCR) from cDNA. Specific primers (Supplementary Table S2) were designed in Geneious (Drummond et al. 2008) and manually adjusted to ensure that a primer anneals to a region that is conserved among the four species but that varies among the copies of a gene family. The different single PCR products from the four different Oxytropis genomes were sequenced and compared to other sequences of its gene family to confirm that primers pairs amplify a single gene. Plantlets from all four species (arctic and temperate) were grown from seeds in temperate and in arctic conditions (described above), RNA was extracted and 2 mg of RNA from single plantlets were reverse transcribed into cDNA using the QuantiTect kit (Qiagen, Mississauga).

Real-time RT-PCR reactions were performed using Brilliant SYBR Green dye (Supplementary Table S2) with two technical replicates per samples for each of three biological replicates, and data were analyzed with MxP3000 4.01 software (Stratagene). Normalization was carried out relative to actin gene expression (Simon 2003). An analysis of variance (ANOVA) using the general linear model (GLM) was performed on normalized relative expression ratio followed by Student–Newman–Keuls tests (SAS Institute 2004) between species in each growth conditions, separately.

Results

EST sequencing from subtracted libraries reveals different biological processes in arctic and temperate plants

In order to characterize potential adaptive differences in gene expression, we investigated transcriptome differences between arctic and temperate Oxytropis species. One arctic-enriched and one temperate-enriched subtracted library (by SSH) were constructed from Oxytropis plantlet mRNA. From the arctic-enriched library, 1,108 trimmed ESTs were assembled into 117 arctic unique genes; while from the temperate-enriched library 609 trimmed ESTs were assembled into 364 temperate unique genes. The compositions of the contigs are listed in Supplementary Table S3 (arctic) and S4 (temperate). Most Oxytropis unique genes received an annotation, except for 25.6% of the arctic-enriched and 7.6% of the temperate-enriched unique genes that have no similarity to any sequences in the public database. The temperate-enriched library comprises genes from a wide variety of processes and is especially rich in energy and photosynthesis related genes and in nucleosome assembly genes whereas the arctic-enriched library shows an important enrichment in “response to stimulus” and in novel genes (Table 1). Ribosomal genes of different types are present in both subtracted libraries. The results of similarity searches performed between the two libraries indicate that 21 of the 364 temperate unique genes and 19 of the 117 arctic unique genes are potential false positives.

The two subtracted libraries comprise numerous unique genes from several multigene families. Gene families retrieved from the arctic-enriched library include the pathogenesis-related class 10 proteins PR-10 (ten unique genes), defensins PDF1 (plant defensin 1; eight unique genes) and cold dehydrins (11 unique genes). Gene families retrieved from the temperate-enriched library belong to chlorophyll a/b binding proteins (12 unique genes), lipid transfer protein LTP (five unique genes), aluminium induced response ADR6 (three unique genes), ripening related protein (seven unique genes), specific tissue protein STP (four unique genes), vegetative storage protein-like (three unique genes) and metallothionein Type 1 (three unique genes).

Real-time RT-PCR of selected genes confirms differential gene expression and identifies a potential adaption to the arctic conditions

Expression of four genes was further investigated using real-time RT-PCR for all four species (O. arctobia, O. campestris subsp. johannensis, O. maydelliana and O. splendidens) each grown in arctic and in temperate simulated conditions (GenBank accession numbers HM107135 to HM107155; Fig. 1).

The cold dehydrin gene corresponding to arctic.contig47 (from the arctic-enriched library) is expressed in both arctic species in both the arctic and the temperate growth
In the temperate species, the gene is expressed twice as much in the arctic than in the temperate conditions. This cold dehydrin may therefore be constitutive in arctic species, but cold induced in temperate species. Arctic.contig61 and arctic.contig13/36 are two paralogs of the PR-10 family (pathogen-related proteins, class 10). Both are present in the genomes of all four Oxytropis species but they have different expression patterns. O. arctobia expresses the arctic.contig61 gene at a very high level under arctic conditions and at a reduced level under temperate conditions (Fig. 1b). In the other arctic species (O. maydelliana), the gene is almost not expressed in either growth condition. The two temperate species express the arctic.contig61 gene under arctic conditions but not under temperate conditions. The arctic.contig13/36 gene is expressed in the O. maydelliana arctic species and the two temperate species at a low level in arctic but not in temperate conditions, but is not expressed at all in O. arctobia (Fig. 1c).

The temperate.contig119 is a light harvesting complex from photosystem I type b and was retrieved from the temperate-enriched library. It is expressed in both conditions for all species, and slightly more in the temperate conditions (Fig. 1d). Expression is significantly higher in the temperate than in the arctic conditions only in O. splendens. The quantitative real-time RT-PCR data for these four genes provide validation of results found with the PCR-select method.

### Discussion

Arctic Oxytropis plantlets exhibit a lower expression for photosynthesis related genes, typical of cold acclimation.

In order to shed light on potential molecular adaptations that arctic plants have developed, we have sequenced and compared the subtracted plantlet transcriptomes of arctic and temperate Oxytropis legume species. These plants express distinct transcriptome signatures in their natural environment that shows both typical and novel features compared to latitudinal or altitudinal gradients in transcriptome variation in other plant lineages (Holliday et al. 2008; Swindell et al. 2007; Voelckel et al. 2008), or to plant species adapted to other abiotic stresses (van de Mortel et al. 2006; Lai et al. 2006; Knight et al. 2006; Hammond et al. 2006; Filatov et al. 2006; Brosche et al. 2005; Taji et al. 2004). Given that important constraints to plant growth in

### Table 1

| Gene category | EST ratio in arctic-enriched library (potential false positive) | EST ratio in temperate-enriched library (potential false positive) |
|--------------|---------------------------------------------------------------|---------------------------------------------------------------|
| No similarity | 5.85×10^-1 (5.91×10^-3)                                       | 5.84×10^-2 (0)                                                |
| Unclassified  | 3.55×10^-3 (0)                                               | 2.12×10^-1 (1.82×10^-3)                                       |
| ROS related   | 5.91×10^-3 (3.54×10^-3)                                       | 2.74×10^-2 (5.47×10^-3)                                       |
| Histones      | 1.18×10^-3 (1.18×10^-3)                                       | 2.92×10^-2 (1.82×10^-3)                                       |
| DNA-proteins  | 0                                                             | 2.00×10^-2 (0)                                                |
| RNA-proteins  | 2.01×10^-2 (9.46×10^-3)                                       | 7.66×10^-2 (3.65×10^-3)                                       |
| Response to   | 2.31×10^-3 (1.19×10^-3)                                       | 1.08×10^-1 (1.28×10^-2)                                       |
| Hormones      | 4.73×10^-3 (4.73×10^-3)                                       | 3.83×10^-2 (7.30×10^-3)                                       |
| Transport     | 2.36×10^-4 (1.18×10^-3)                                       | 4.01×10^-2 (7.3×10^-3)                                        |
| Nucleotides   | 1.18×10^-3 (1.18×10^-3)                                       | 1.82×10^-3 (0)                                                |
| Signaling     | 1.18×10^-3 (0)                                               | 1.82×10^-2 (1.82×10^-3)                                       |
| Protein       | 1.18×10^-3 (1.18×10^-3)                                       | 2.00×10^-2 (0)                                                |
| Nitrogen      | 1.18×10^-3 (1.18×10^-3)                                       | 2.00×10^-2 (5.47×10^-3)                                       |
| Lipid         | 7.57×10^-2 (7.47×10^-2)                                       | 1.46×10^-2 (3.65×10^-3)                                       |
| Carbohydrates | 1.18×10^-3 (0)                                               | 3.65×10^-2 (0)                                                |
| Energy        | 3.55×10^-2 (1.18×10^-4)                                       | 2.65×10^-1 (1.82×10^-3)                                       |

*ROS* reactive oxygen species

a The ratio of ESTs in a category is expressed relative to the total number of ESTs in the subtracted library of origin, as EST ratio=number of EST sequences in library in this category/total number of EST sequences in library

b Values in parenthesis report the potential false positive, identified using similarity of unique genes from one subtracted library to the unique genes of the other subtracted library.
the arctic are low summer temperature and frequent summer frosts (Savile 1972), arctic species are expected to show adequate expression of cold treatment genes. Gene expression reorganization following cold stress is now well characterized in model (Hannah et al. 2005) and agronomical (Cheng et al. 2007) plant species, and many of the differentially expressed genes between arctic and temperate Oxytropis species conform to this pattern. In Arabidopsis, genes up-regulated after long-term cold exposure are mainly of the stress response category, especially the hydrophilic COR/LEA proteins (Hannah et al. 2005), and some are found in the Oxytropis arctic-enriched library. Several genes down-regulated after long-term cold exposure in Arabidopsis, such as photosynthesis-related genes, carbohydrate metabolism, GDSL-motif lipase, hormone metabolism and oxidative regulation genes (Hannah et al. 2005) are found in the Oxytropis temperate-enriched library. Expression levels of the light harvesting gene lhcb1 calculated by real-time RT-PCR are higher in temperate conditions for all four Oxytropis species, suggesting that expression is dependent on growth conditions. This finding is consistent with the deterioration of photosynthetic capacity following cold exposure in most plants (Savitch et al. 2001; Stitt and Hurry 2002; Walters 2005) and with a plastic response of arctic plants that modify their optimum temperature for photosynthesis and carbon integration within a few days after being placed in warmer growth conditions (Pyankov and Vaskovskii 1994).

Defence response is a prominent characteristic of the arctic plantlet transcriptome

The constraints on plant growth imposed by the arctic environment extend beyond cold temperatures, and also include very short growing season, strong winds, low light intensity but long days. It is therefore expected to see a set of expressed genes in the arctic Oxytropis plantlets unique and different from a cold-treated temperate model plant. The most striking feature of the Oxytropis arctic-enriched library is its enrichment in genes of the “response to stimulus” category, indicating that under arctic simulated growth conditions, arctic Oxytropis express more of defensin (PDF1), pathogenesis-related proteins (PR-10), cold dehydrins, early light inducible (ELIP). These four genes are not as a group typical of those up-regulated during cold acclimation in temperate plants.

In other species, stress response genes show a substantial among taxa variation in gene expression (e.g. Chen et al. 2005),
Cold dehydrins is another “response to stimulus” gene family largely present in the arctic Oxytropis transcriptome. A certain level of constitutive expression for cold dehydrins was described (Boudet et al. 2006), in addition to its induction by heat, cold, drought, wounding and virus infection in soybean (Takahashi and Shimosaka 1997) and in Medicago (Chen et al. 2008; Pennycooke et al. 2008). Our finding that expression of a cold dehydrin gene is less responsive to the growth conditions in the arctic species than in the temperate species is in agreement with reports on a less-responsive expression of “response to stimulus” genes in stress-adapted plant species, (Brosche et al. 2005; Taji et al. 2004). The proposed role of cold dehydrins in drought tolerance (Close 1996) is desirable in an environment where water is limited (Aiiken et al. 2007) and where frequent summer frosts can induce ice formation in the apoplast leading to cellular dehydration.

A concurrent overexpression of the gene families PR-10, defensin and cold dehydrin, is a novel feature for plants adapted to adverse environmental conditions. Other “response to stimulus” genes are overexpressed in arctic Oxytropis, although with a less-striking expression difference, and several of these were also overexpressed in other stress tolerant species. The ELIP are overexpressed in arctic Oxytropis, a pattern described earlier (Lai et al. 2006), but that is not universal since they were also overexpressed in the lower altitude Pachycladon fastigata (Voelckel et al. 2008) or the Californian Picea (Holliday et al. 2008).

Ribosome biogenesis and assembly genes are differentially expressed between arctic and temperate species.

Results from the Oxytropis library subtraction suggest a differential expression for genes involved in ribosome biogenesis and assembly of a novel and not previously described pattern for arctic plants. Since arctic Oxytropis not only overexpress but also underexpress an important set of ribosome related genes; ribosome organization might play an important role in long-term adaptation of plants to the arctic, or in specific response to arctic conditions. In other species, it has been shown that several of these genes either increase (Kim et al. 2004; Saez-Vasquez et al. 2000) or decrease in (Berberich et al. 2000; Swindell et al. 2007) expression following stresses; or are differentially regulated during development (McIntosh and Bonham-Smith 2006; Whittle and Krochko 2009). Furthermore, differential expression of ribosomal genes among other population and species has been detected (Voelckel et al. 2008; Hammond et al. 2006; Filatov et al. 2006; Holliday et al. 2008), where, as in Oxytropis, more ribosomal genes are underexpressed than overexpressed in...
the species adapted to more stressful environment. This observation could reflect the observed slower growth of arctic plants (Bliss and Gold 1999; Aiken et al. 2007) in their natural habitat.

Nucleosome assembly genes are underrepresented in the arctic transcriptome

Histone genes are overrepresented in the temperate-enriched library, suggesting an underexpression in the arctic Oxytropis. Although a close look at differentially expressed genes in other plant species adapted to stressful conditions reveals anecdotic overexpression of some histone genes and underexpression of others (Filatov et al. 2006; Hammond et al. 2006; Holliday et al. 2008), we have not found examples where this category of genes is an important feature of a specialized transcriptome. In addition to the differential expression that we show here in Oxytropis for histone genes, previous findings describing that different histone genes can be differentially regulated during plant development (Huh et al. 1995) or stress response (Kapros et al. 1992) suggest that regulation of expression for genes related to chromatin assembly may also participate in plant adaptation to environmental conditions, rather than be a simple cause of slower cell division for arctic species (Meshi et al. 2000).

Conclusion

This is the first report to our knowledge on gene expression profiles of an arctic plant and our findings are supported by previous reports on plant adaptation to stressful environmental conditions. Arctic Oxytropis species, as opposed to temperate ones, over express stress response genes such as certain PR-10 genes, defensin and cold dehydrins and under express photosynthesis and histone genes. Real-time RT-PCR results also show that a cold dehydrin likely participated in adaptation to the Arctic because it is constitutive in the arctic species, and cold induced in the temperate species.

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