Acta Universitatis Latviensis, 2008, Vol. 745, Biology, pp. 87–101

Cloning and characterization of barley homologues of the *Arabidopsis LSD1* gene: putative regulators of hypersensitive response

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Abstract

Plant hypersensitive response (HR) is a highly controlled reaction to prevent spread of biotroph pathogens. Understanding the mechanisms of HR could potentially allow development of crop plant varieties with improved resistance to pathogens. HR and its molecular control mechanisms have been extensively studied in model organisms, such as *Arabidopsis thaliana*, allowing to identify numerous necrotic or lesion-mimic mutants that constitutively express the HR even in absence of pathogen. *Arabidopsis LSD1* gene encodes a zinc finger protein characterized as a central regulator of HR. Here we identified three genes homologous to the *LSD1* in barley and compared them to *LSD1* homologues in other grass species. Barley gene *CBC04043* appeared to be more similar to the *Arabidopsis LOL1* gene, however, the two other genes originating from gene duplication after the monocot - dicot divergence represented candidate genes for the *LSD1* orthologue. As the sequence analysis alone did not allow identification of the *LSD1* orthologue in barley, expression of the three genes was studied in barley necrotic mutants *nec1* and *nec3*.

Key words: *Arabidopsis LSD1* gene, barley, gene expression, lesion-mimic mutant, necrotic mutant, phylogeny.

Introduction

Incompatible plant-pathogen interactions cause series of stress response reactions involving ion fluxes, accumulation of reactive oxygen species and altered production of the endogenous signal molecules (Heath 2000). Biotic stress often triggers hypersensitive response (HR) – cell death in tissues directly surrounding infection site. HR is a highly controlled physiological process requiring specific initiation, propagation and restriction mechanisms directed by crosstalk of plant signalling pathways (Shirasu, Schulze-Lefert 2000; Lam et al. 2001). Alterations in HR have been shown to affect plant defense mechanisms by increasing disease resistance or rendering plants more susceptible to pathogen infection (Govrin, Levin 2000; Vleeshouwers et al. 2000; Kombrink, Schmelzer 2001). Despite the involvement of HR in plant defense mechanisms, knowledge concerning the genetic basis of HR is still scarce. Characterization of genetic disorders causing the lesion-mimic phenotype (constitutively expressed HR) or preventing pathogen-induced

cell death (inability to initiate HR) is a common approach to uncover the genetics behind the HR (Glazebrook 2001; Lorrain et al. 2003). Mutant plants exhibiting impaired HR are categorized into two groups depending on a stage of HR altered (Lorrain et al. 2003). Lesion-mimic mutants (LMM) belonging to the class of initiation mutants display spontaneous induction of HR. In contrast, LMM showing normal initiation of HR, but incapable of restricting propagation of programmed cell death belong to the propagation class of mutants. In order to obtain more thorough insight into the molecular mechanisms involved in HR, studies examining both types of LMM would be required.

One of the best characterized propagation LMM is *lsd1* (*lesion simulating disease 1*), which cannot restrict lesion propagation initiated in response to superoxide (Jabs et al. 1996). *LSD1* has been proposed to be a negative regulator of plant cell death involved in regulation of copper zinc superoxide dismutase expression (Kliebenstein et al. 1999; Epple et al. 2003). The role of LSD1 in reactive oxygen species (ROS) homeostasis regulation has also been confirmed by the fact that *LSD1* acts in acclimation to excess excitation energy operating through repression of *PAD4* and *EDS1*-dependent stomatal closure (Mateo et al. 2004). More recently, LSD1 has been implicated in control of lysigenous aerenchyma formation in *A. thaliana* roots in response to hypoxia (Muhlenbock et al. 2007). LSD1 function in regulation of ROS homeostasis has been shown to depend on antagonistic interaction with LOL1, a closely related protein also belonging to the class of LSD1-type Zn-finger domain-containing proteins (Epple et al. 2003). Although the precise mechanism by which LSD1 and LOL1 interaction affects PCD is not known, in *A. thaliana* LSD1 is considered to operate as a cellular hub ensuring retention of the pro-apoptotic transcription factor AtbZIP10 in the cytoplasm (Kaminaka et al. 2006). Functional involvement of *LSD1* in regulation of plant cell death and its central role in HR propagation control renders the gene interesting for HR studies. Apart from *A. thaliana*, *LSD1* has also been cloned from *Oryza sativa*, where it is proposed to be involved also in callus differentiation (Wang et al. 2005), and from *Brassica oleracea*, where AtLSD1 homologues have been shown to function during senescence-related PCD (Coupe et al. 2004). In this study we describe *A. thaliana LSD1* homologues in barley and characterize the phylogenetic relationships between *LSD1* gene homologues in different grass (*Poaceae*) species. Possible interactions between the identified genes and previously characterized HR related genes were studied by analyzing gene expression using real time quantitative PCR in barley initiation LMMs *nec1* and *nec3*.

Materials and methods

Identification and sequence analysis of barley homologues of Arabidopsis *gene* LSD1

The *Arabidopsis thaliana* LSD1 amino acid sequence AAC49660 was used as a query in TBLASTN search against the barley Expressed Sequence Tags (ESTs) at the NCBI GenBank database and against the barley EST unigene database HarvEST assemblies 21 and 35 (http://harvest.ucr.edu). Coding sequences of two barley homologues of the *A. thaliana LSD1, ABC10220* and *ABC06454*, were identified by sequencing cv. *Morex* cDNA clones HvCEa0008p08 and HvSMEb0007a07. The coding sequence of the third homologue, *CBC04043*, was predicted from the unigene sequence. Intron-exon structure of the barley genes was predicted by comparing the cDNA and unigene sequences with the respective genomic clones. cDNA and genomic sequences of the barley homologues of

the *Arabidopsis LSD1* gene have been deposited in the GenBank under accession numbers EU545232 and EU545233 for *ABC10220* gDNA and cDNA sequences respectively, EU 545231 and EU545234 for *ABC06454* gDNA and cDNA sequences respectively, EU545230 for gDNA sequence of *CBC04043*.

Phylogenetic analysis of LSD1 *homologues in* Poaceae

Coding sequences were predicted using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Predicted amino acid sequences of barley *LSD1* homologues were used in TBLASTN query of the NCBI EST-others database (search parameters: score > 100, E value < 0.00005) to identify homologous sequences in other *Poaceae* species. EST sequences showing the highest homology were translated using NCBI ORF Finder and aligned using ClustalX 1.81 (Thompson et al. 1997). The amino acid sequence alignment was manually edited and gene phylogeny was reconstructed with Maximum Likelihood method using the proml programme from the PHYLIP3.66 package (Felsenstein 1989). Bootstrap confidence levels were calculated from 100 iterations using the seqboot programme from the PHYLIP package. The phylogenetic tree was visualized using TreeView (Page 1996).

Plant material, cultivation and DNA extraction

Plants for DNA extraction and *LSD1* expression analysis were grown in a growth room at 22 °C under long-day (16 h day/ 8 h night) low light (ca. 130 μmol m^{-2} s^{-1}) conditions. DNA was extracted from fresh leaf tissue as described by Edwards et al. 1991.

The barley necrotic mutant *nec1* (GSHO 1284) containing mutation in the *Cyclic Nucleotide Gated Ion Channel 4* (*CNGC4*) gene (Rostoks et al. 2006) and necrotic mutant *nec3* (GSHO 2065) were used for the barley *LSD1* expression analysis. GSHO 1284 is described as a natural mutant in cv. *Parkland* (Fedak et al. 1972), while GSHO 2065 is a backcross of the *nec3.d* allele into a Bowman genetic background (Lundqvist et al. 1997).

RNA extraction

For RNA extractions 5 cm long segments of the first leaf from 2-week-old plants of necrotic mutants *nec1* and *nec3* and their parents, Parkland and Bowman, were snap-frozen in liquid nitrogen immediately after harvesting. Total RNA was extracted from frozen leaf tissues using a RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. Each RNA sample was extracted from a pool of five plants and three biological replicates of each barley line (15 plants in total) were used for expression analysis of the barley *LSD1* homologues in necrotic mutants and their respective parents. Integrity of the extracted RNA was monitored using non-denaturing agarose gel electrophoresis. One to two μg of the extracted RNA was treated with DNaseI (Fermentas, Vilnius, Lithuania) following manufacturer's instructions and afterwards purified using a QIAGEN RNeasy Plant Mini Kit. The quantity of purified total RNA was monitored using a spectrophotometer Ultrospec 3100 pro (Amersham Biosciences, Little Chalfont, UK).

PCR, RT-PCR and quantitative real-time PCR

Gene-specific primers (Table 1) were designed by Primer3 software (Rozen, Skaletsky 2000). PCR reactions were carried out in a 20 μL of total volume containing 50 - 100 ng genomic DNA, 0.5 μM primers, 1.8 mM $MgCl_2$, 0.2 mM dNTPs and 1 u Hot Start or TrueStart *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) used with manufacturer-

Table 1. Oligonucleotide primers for PCR, sequencing and quantitative real time PCR. *, primers used for quantitative real time PCR

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
ABC10220_L01	cgagggatttctcgtctgct	ABC06454_R03_b	caagattgactgtactgcaacatgagc
ABC10220_L02	cgtctgctcggttcctctcg	ABC06454_R04	gtgtacatcagcaaagttcggcaac
ABC10220_L03	atgcgtcggctgtcgaactt	ABC06454_R05	gtgcatgctttccttggtg
ABC10220_L04	ctctggtctcccccgtttgt	ABC06454_R06	ataagctgagccatttccactg
ABC10220_L05	caacgtcgtagtcggggtta	ABC06454_R07	ttgttaatccttcggaagtctgtgc
ABC10220_L07*	ccatacggagcatcttctgtcaagt	ABC06454_R08	atatcacgtgccactaaggtcttgc
ABC10220_R01	ccgcgctttgggtttttgtt	CBC04043_L01	cattccaactcatgttgttattctgag
ABC10220_R02	cacacttgacagaagatgctccgta	CBC04043_L02	gtccgcctcttcctctgaac
ABC10220_R03_b	gtgctggtctggcaaggttga	CBC04043_L03	gacgagcaggatttcatgtagag
ABC10220_R04	gggcccctctggggtagag	CBC04043_L04	agtctcgtccgcagcaac
ABC10220_R05	gacagctactccctccgttcc	CBC04043_L05	ctcaaagccaacttgtctgctc
ABC10220_R07*	gtggtaaccccgactacgacgtt	CBC04043_L06*	gagatggcgcagctagtttg
ABC06454_L01	gacccaggagccctctgtca	CBC04043_L07	aatcaggttgcgcatgtaaact
ABC06454_L02	tggttgccgaactttgctga	CBC04043_L08	atcatcgggtgcagagcag
ABC06454_L03_b	aaacgccatgtcacctgcac	CBC04043_R01	tgcccggttcagaggaagag
ABC06454_L04	catgtgccgagccatcacc	CBC04043_R02	gaagtcgaggggatgagaacagat
ABC06454_L05	ctgtacattcacagctgaatagtgg	CBC04043_R03	cgtgaagggcgcaggag
ABC06454_L06	tggtcagcaacgtcgtagtc	CBC04043_R04	ggaacagcggtcacggtact
ABC06454_L07	cagtggaaatggctcagctt	CBC04043_R05	cttccattgccaggttgacagt
ABC06454_L08	gtgtgattcatagttcgatgccatt	CBC04043_R06*	actgatgtcacgaaactgcagac
ABC06454_L09*	aatatagcccacgtgaattgtggtc	CBC04043_R07	ctatacatgtgcactcaaagcattc
ABC06454_R01	tcaggcagcaaccaatcacc	CBC04043_R08	caacgaagggagaaatggagag
ABC06454_R02*	cgtcgacagtcatagggttctcaa	CBC04043_R09	ggttcatgtatctgtctgacccagt

supplied buffers. PCR was carried out on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) as follows: initial denaturing step for 5 min at 95 °C, 7 cycles of touch down of 30 s at 95 °C, 30 s at 65 - 58 °C, 3 min at 72 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C, 3 min at 72 °C and final extension of 5 min at 72 °C.

cDNA was synthesized with oligo (dT)18 primer in a total volume of 15 µL containing 0.5 µg of total RNA using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

For real-time PCR aliquots of cDNA were amplified on an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany) in a total volume of 25 µL containing 2 µL of cDNA and 0.3 µM primers. Primers used for real-time PCR are listed in Table 1. Reaction was carried out as follows: initial denaturing step for 15 min at 95 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C (data acquisition step). Standard curves for the quantification of the transcript levels were calculated from serial dilutions of appropriate cDNA fragments amplified from cv *Morex*. Transcript levels of *LSD1* homologues were expressed as a percentage of *HvGAPDH* transcript amount in the same sample. Combined

Table 2. CAPS markers for linkage mapping of barley homologues of the *LSD1* gene

Gene	PCR Primers	Restriction enzyme	Restriction fragment size (bp)	
			OwbD	OwbR
ABC10220	ABC10220_L02 /ABC10220_R03b	NdeI	2350	2150
				200
ABC06454	ABC06454_L02 /ABC06454_R02	TaqI	720	620
			300	300
			230	230
				100
CBC04043	CBC04043_L04 /CBC04043_R05	SspI	1070	700
				370

values of two technical replicates of the three biological replicates (n = 6) were used to calculate the average values and standard deviations. Analysis of variance (ANOVA) of transcript abundance between the mutant and the corresponding parent was conducted using Microsoft Excel (Redmond, WA, USA).

Sequencing

LSD1 barley homologues were sequenced from cvs. *Morex* and *Steptoe*. PCR products were purified using DNA Extraction Kit (Fermentas, Vilnius, Lithuania) or treated with exonuclease I and shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) prior to sequencing. *LSD1* barley homologues were sequenced by subcloning PCR products into vector pTZ57R/T (Fermentas, Vilnius, Lithuania) or directly by primer walking. Sequencing reactions were carried out using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in 10-μL total volume containing 10 - 50 ng of purified PCR product, 1 μM primer, 1 μL BigDye and manufacturer-supplied buffer. Base calling and sequence assembly were done with Staden package (Staden 1996).

Linkage mapping

The Oregon Wolf Barley Dominant × Oregon Wolf Barley Recessive population (Costa et al. 2001) consisting of 94 doubled haploid lines was used for linkage mapping of the three barley *LSD1* homologues. Cleaved Amplified Polymorphic Sequences (CAPS) markers differentiating the parents of the mapping population were developed for each gene based on single nucleotide polymorphisms detected by sequencing (Table 2).

Results

Identification of barley homologues of Arabidopsis thaliana *gene* LSD1

TBLASTN query of the NCBI GenBank barley EST database and HarvEST EST unigene database with *Arabidopsis thaliana* LSD1 amino acid sequence AAC49660 identified three groups of barley ESTs represented by two HarvEST assembly 21 unigenes, *ABC10220*, *ABC06454*, and one HarvEST assembly 35 unigene, *CBC04043*. Sequences of the two cv. *Morex* cDNA clones, HvCEa0008p08 and HvSMEb0007a07, which matched unigenes

Fig. 1. Multiple sequence alignment of deduced amino acid sequences of *Arabidopsis thaliana LSD1* and the identified barley homologues. Zn-finger domains are denoted by black frame. aa acids identical in all four sequences are indicated by colored background.

ABC10220 and *ABC06454*, as well as the unigene sequence *CBC04043* were used to predict the encoded amino acid sequences. The three barley homologues showed approximately equal amino acid identity (ABC10220 – 54 %, ABC06454 – 55 %, CBC04043 – 57 %) with the *Arabidopsis* LSD1 (Fig. 1), however, the CBC04043 was more similar (86 % amino acid identity) to *Arabidopsis* LOL1.

Genetic mapping of barley LSD1 *homologues*

The Oregon Wolfe Barley Dominant by Recessive doubled haploid mapping population (Costa et al. 2001) was used for linkage mapping of barley homologues of the *LSD1* gene. Segregation data of the three CAPS markers (Table 2) was used for linkage mapping in Map Manager QTX software (Manly et al. 2001) relative to restriction fragment length polymorphism and simple sequence repeat markers (Costa et al. 2001). The *CBC04043* gene was mapped to chromosome 5(1H) between JS10C (bin09) and Bmac0113A (bin11) markers, *ABC10220* was mapped to chromosome 7(5H) between ABG395 (bin04) and NRG045A (bin05) and *ABC06454* was located on chromosome 7(5H) between BE456118C (bin11) and Tef3 (bin11 - 12).

Structure of barley LSD1 *homologues*

Pairwise alignment of cDNA and gDNA sequences identified the exon–intron structure of barley *LSD1* homologues (Fig. 2). Genes *ABC10220* and *ABC06454* showed highly conserved exon-intron organization, each comprising six exons of conserved size and sequence. Similarly to *Arabidopsis LSD1*, barley genes encode three putative Zn finger domains (pfam 06943) detected by conserved domain search of the Pfam 22.0 database (Finn et al. 2006). The position of Zn-finger domains is highly conserved in ABC10220

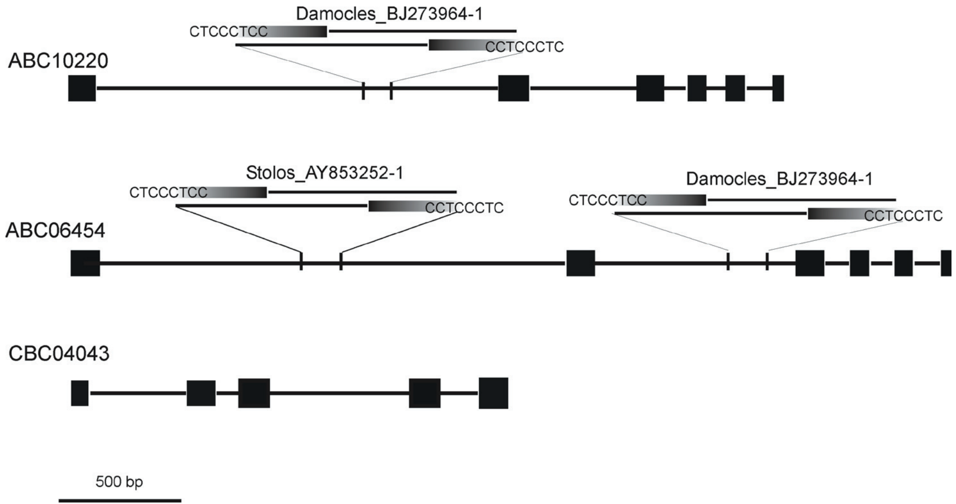

Fig. 2. Gene structure of LSD1 barley homologues with exons shown as black boxes. Stowaway MITE insertions are shown above the gene structure.

Table 3. List of LSD1 gene homologues from *Poaceae* species used for phylogenetic analyses

Barley *LSD1* homologue	Species (designation in phylogenetic tree, Fig. 3)	GenBank accessions
ABC10220	*Triticum aestivum* (2)	BJ268629
	Brachypodium distachyon (2)	DV489172
	Oryza sativa (2)	CF316414
	Zea mays (2)	DY39886
	Saccharum officinarum (2)	CA122488
	Sorghum bicolour (2)	CX609698
ABC06454	*Triticum aestivum* (1)	CJ689628
	Brachypodium distachyon (1)	DV487362
	Oryza sativa (1)	CT848680
	Zea mays (1)	EC872597
	Saccharum officinarum (1)	CA215734
	Sorghum bicolour (1)	CF486644
CBC04043	*Triticum aestivum* (3)	CJ603752
	Brachypodium distachyon (3)	DV477202
	Oryza sativa (3)	CI036551
	Zea mays (3)	DV505906
	Saccharum officinarum (3)	CA088897
	Sorghum bicolour (3)	BG241556
	Secale cereale (1)	BE705617
	Triticum monococcum (1)	BG607068

and ABC06454, in which the first three exons each contain a single Zn-finger domain. In addition, C-termini of ABC10220 and ABC06454 contained highly conserved Val rich regions.

Homology-based annotation of genomic sequences indicated presence of repetitive sequences within gene structure. BLASTN search against the TREP *Triticeae* Repeat database (http://wheat.pw.usda.gov) showed that the 1st intron of *ABC10220* and 1st and 2nd intron of *ABC06454* contained sequences sharing high homology with several Stowaway MITEs (miniature inverted repeat transposable elements) (Fig. 2).

Exon-intron organization and position of the conserved domains of *CBC04043* differed from that deduced for *ABC10220* and *ABC06454*. According to the pairwise alignment of cDNA and gDNA, the coding sequence of *CBC04043* consisted of five exons with Zn-finger domains positioned in the 2nd, 3rd and 4th exons.

Comparison of Arabidopsis LSD1 *with homologues in barley and other* Poaceae *species*

TBLASTN search with deduced amino acid sequences of barley LSD1 homologues was performed to identify homologues sequences in other *Poaceae* species (Table 3). Amino acid sequence alignment was used to reconstruct phylogenetic relationships among the barley *LSD1* homologues and related genes in other *Poaceae* species. Identified sequences clustered into three distinct groups, each including a single barley gene (Fig. 3). The cluster represented by the barley CBC04043 sequence showed a high degree of sequence conservation.

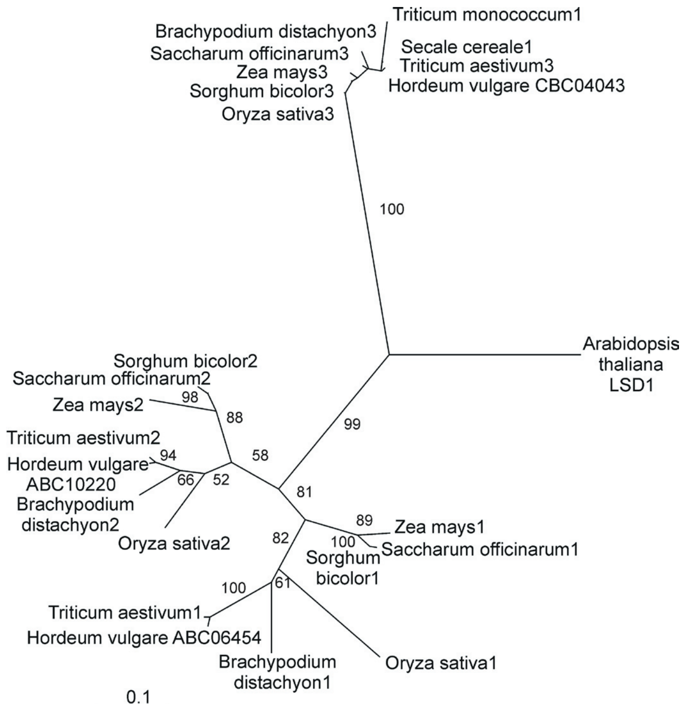

Fig. 3. Phylogenetic relationships among the barley *LSD1* homologues and related genes in other *Poaceae* species reconstructed with Maximum Likelihood method. Bootstrap confidence levels (calculated from 100 iterations) higher than 50% are shown.

Table 4. List of LSD1-like genes from *Arabidopsis thaliana*, *Oryza sativa* and *Hordeum vulgare* used for phylogenetic analysis in Fig. 4. a – designation consistent with TAIR (The Arabidopsis Information Resource) information; b – designation consistent with (Liu, Xue 2007); c – designation corresponds to HarvEST Assembly 21 unigene represented by a EST sequence

Designation in phylogenetic tree	*Arabidopsis* gene or GenBank accession
AtLOL1a	At1g32540
AtLOL2a	At4g216210
AtLOL3a	At1g02170
AtLSD1a	At4g20380
OsLOL1b	AK061509
OsLOL2b	AK111837
OsLOL3b	AK111569
OsLOL4b	AK120454
OsLOL5b	AK065375
OsLSD1b	AY525368
Hv14621c	CV063671
Hv15713c	BE421616
Hv2281c	AV933097

In order to better estimate the relationships of the three barley genes with rice and Arabidopsis LSD1 genes, more distant LSD1-like genes were identified in barley, rice and Arabidopsis using TBLASTN search. Three additional barley ESTs comprising LSD1-like Zn-finger domains were identified (Table 4) and used to reconstruct the phylogeny of barley, Arabidopsis and rice LSD1-like genes (Fig. 4).

Expression of barley LSD1 *homologues in lesion-mimic mutants* nec1 *and* nec3

Arabidopsis LSD1 is a negative regulator of cell death (Dietrich et al 1997). *lsd1* mutants are hypersensitive to cell death signals and unable to control the extent of cell death (propagation type of LMM). Expression of barley homologues of the *AtLSD1* was studied in initiation LMM *nec1* and *nec3* in comparison to parental varieties (Fig. 5).

A two-fold decrease (p = 0.0003) in transcript abundance of *CBC04043* was observed in *nec1* mutant, while in *nec3 CBC04043* expression showed a slight, but statistically significant (p = 0.015) increase. Although no remarkable difference in transcript abundance of *ABC10220* was detected between the LMMs and the respective parents, gene was slightly repressed in *nec1* (p = 0.003). Observed differences in expression of *ABC06454* were not statistically significant (Fig. 5).

Discussion

Three barley homologues of *A. thaliana LSD1* were identified based on sequence homology searches. All three genes encoded three Zn-finger-LSD1 domains and showed a very similar level of amino acid homology with the AtLSD1. Two of the barley homologues, *ABC10220* and *ABC06454*, showed highly conserved exon sizes and organization, as well as almost identical arrangement of the Zn-finger-LSD1 domains. The third homologue,

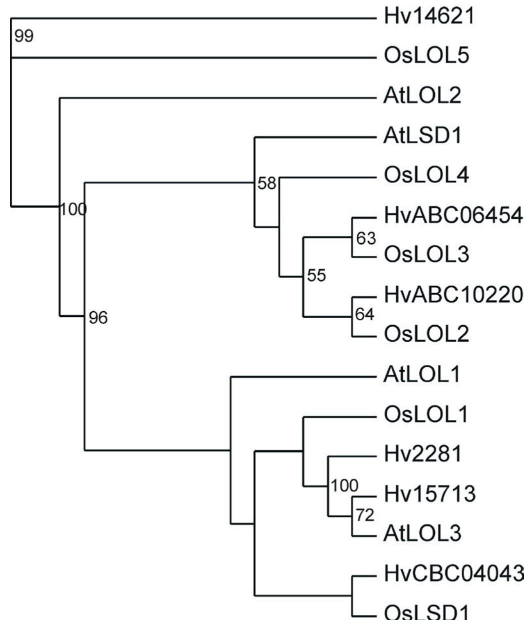

Fig. 4. Phylogenetic relationships among known *LSD1*-like genes and barley genes comprising LSD1-like Zn-finger domains. Phylogenetic tree was reconstructed with Maximum Likelihood method. Bootstrap confidence levels (calculated from 100 iterations) higher than 50 are shown. Barley genes are designated according to the corresponding HarvEST assembly 21 unigene number.

CBC04043, had substantially different exon-intron organization and distribution of the Zn-finger domains. The structural distinctiveness of the *CBC04043* and considerable similarity of *ABC10220* and *ABC06454* implied the putative divergence of the *CBC04043* from the common ancestor before the separation event of *ABC10220* and *ABC06454*.

Phylogenetic analysis of the barley *LSD1* gene homologues from other *Poaceae* species partitioned sequences into three clusters, each comprising homologues from all analyzed grass species including barley (Fig. 3). The two groups including barley genes *ABC06454* and *ABC10220* each included wheat, rice, sorghum and sugarcane genes, thus the apparent gene duplication must have happened after the monocot - dicot split, but before the divergence of Pooideae (barley, brachypodium and wheat), Panicoideae (maize, sorghum and sugarcane) and Ehrhartoideae (rice) (Kellogg 2001). The ancient origin and functional divergence of the *LSD1*-like gene family has also previously been described in rice (Liu, Xue 2007).

Monocots and dicots diverged about 140 - 150 million years ago (Chaw et al. 2004). Since then, *A. thaliana* has undergone whole genome duplication (Arabidopsis Genome Initiative 2000). Similar genome duplication has occurred in rice (Yu et al. 2005). There is emerging evidence confirming similar genome duplications in a lineage leading to barley (Stein et al. 2007). Following duplication, one of the copies can continue to perform the old function, while the selective pressure is now lifted from the second copy which can accumulate mutations and can become a pseudogene or acquire a novel function. Thus gene and genome duplications may complicate identification of functional orthologues in

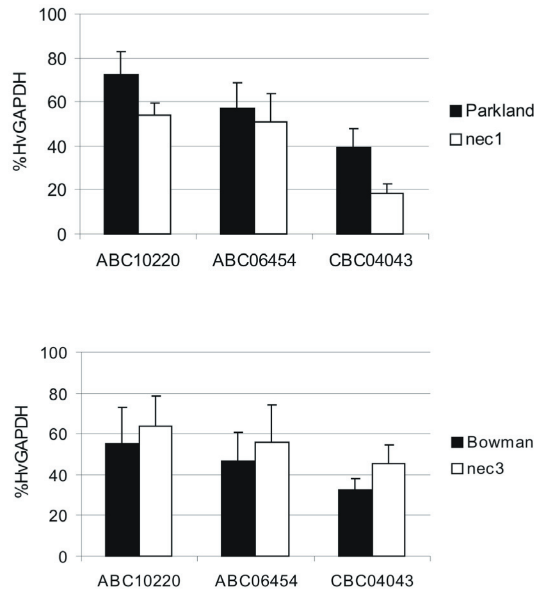

Fig. 5. Expression of barley homologues of *Arabidopsis LSD1* gene in barley necrotic mutants *nec1* and *nec3*. Gene expression levels are expressed as percent of HvGAPDH expression. Error bars represent standard deviations.

distant taxonomic groups based on sequence homology alone.

The phylogenetic analysis of more distant *LSD1*-like gene homologues from different monocots provide deeper insight into the putative origin and divergence time of the three barley homologues (Fig. 4). Analogy of rice and barley *LSD1*-like gene phylogeny suggests putative functional characteristics of studied barley homologues. According to the phylogenetic analysis, OsLOL2 and OsLOL3 are the closest rice homologues of ABC10220 and ABC06454 respectively, whereas the closest *A. thaliana* homologue of these genes was AtLSD1. Similar discrepancies between *A. thaliana* and rice homology with barley were also observed in the case of CBC04043, which clustered with OsLSD1, whereas the closest *A. thaliana* homologue is AtLOL1 (Fig. 4). Clustering of CBC04043 with AtLOL1 indicated that CBC04043 may be the functional homologue of LOL1 in barley. LOL1 is highly conserved throughout monocots, as well as dicots, and has been predicted to function as a positive regulator of plant cell death, probably taking part in developmental processes (Epple et al. 2003). Discrepancies in clustering of barley homologues with *A. thaliana* or rice *LSD1*-like genes prevented from an unambiguous designation of the ABC10220 and ABC06454 to a certain functional group of *LSD1*-like genes. ABC10220 and ABC06454 clustered with AtLSD1 but not with OsLSD1. As reported by Epple et al. (2003) and Wang et al. (2005), the rice gene AY525368 was designated as *OsLSD1* based on its functional characterization despite the fact that it showed higher sequence homology to the *AtLOL1* than to *AtLSD1*. Discrepancies in barley *LSD1*-like gene homology with rice and *Arabidopsis* could be caused by differences in stress regulation requirements of these species. It has been reported that AtLSD1 is involved in lysogenic aerenchyma

formation in roots of *A. thaliana* in response to hypoxia (Muhlenbock et al. 2007). Since growth habits of rice might be associated with higher stress of hypoxia compared to *A. thaliana* or barley, putative functional divergence of *LSD1*-like genes in these species could be anticipated. Considering different environmental pressure imposed on different species, it might be difficult to assign a correct functional annotation to the *LSD1*-like genes involved in plant stress response regulation based only on sequence homology and phylogenetic analysis. Therefore, in order to identify the functional homologue of *AtLSD1* in barley, further experiments were needed.

The expression analysis of the identified LSD1 homologues in barley initiation LMMs *nec1* and *nec3* showed that the *CBC04043* expression was significantly decreased in the *nec1* genetic background and slightly increased in the *nec3* background. In the case of *AtLOL1*, reduction of transcript abundance to 25 - 60 % of the wild type level was enough to increase susceptibility to virulent pathogen and alter HR elicitor treatment response (Epple et al. 2003). Therefore, a two-fold decrease in transcript abundance of *CBC04043* (putative *HvLOL1*) in nec1 could serve as indication for a functional link between *LOL1* and *NEC1* in barley. Because the expression of *CBC04043* was suppressed in *nec1* in spite of spontaneous HR, lesion formation in *nec1* may depend on other pro-apoptotic genes functioning in an alternative pathway. Of the other two barley genes, *ABC10220* and *ABC06454*, representing candidates for barley *LSD1* gene, only the *ABC10220* showed statistically significant reduction of expression in the *nec1* mutant, while its expression was not affected in the *nec3* genetic background. The finding that *LSD1*-like gene expression can be affected in the *nec1* mutant with a deficient *NEC1* gene served to suggest involvement of cyclic nucleotide regulated ion channels in a *LSD1*-related HR pathway. Intuitively it would have been expected that expression of pro-apoptotic genes like *LOL1* will be increased in necrotic mutants and that different patterns of expression of the *LSD1* homologues will be observed due to the antagonistic nature of *LOL1* and *LSD1*. Contrary to the initial hypothesis, the expression of all three barley *LSD1* homologues in *nec1* was slightly suppressed.

In conclusion we have identified three barley *LSD1* homologues. Based on the phylogenetic analysis one of the identified genes, *CBC04043*, can be designated as barley *LOL1*. Sequence analysis indicate that *ABC10220* and *ABC06454* genes represent the best candidates for homologues of the *LSD1* gene, however, further gene expression analyses may be required to identify the functional homologue of *LSD1*.

Acknowledgements

This study is supported by a grant ZP-59 from the University of Latvia to Dr. Rostoks and USDA NRI grant 2004-35301-14635 to Dr. Kleinhofs. Seed of the barley necrotic mutants and their parents was obtained from USDA National Plant Genetic Resource Collection, Aberdeen, ID, USA.

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Ar hipersensitīvo atbildi saistītā *Arabidopsis LSD1* gēna homologu identifikācija un raksturošana miežu genomā

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Kopsavilkums

Augu hipersensitīvā atbilde (HA) ir kontrolēta atbildes reakcija uz stresu, kura fenotipiski izpaužas kā lokalizēta šūnu bojāeja tiešā infekcijas vietas tuvumā. Lai gan HA ir cieši saistīta ar augu slimību izturību, izpratne par HA molekulārās norises mehānismiem ir nepilnīga. HA molekulāro mehānismu izpēte ir nozīmīga gan augu programmētās šūnu bojāejas izpētei, gan praktiskai jaunu slimībizturīgu lauksaimniecības augu šķirņu radīšanai. Līdzšinējie HA pētījumi lielākoties veikti modeļorganismos, pētot nekrotiskos mutantus ar traucētu HA norisi. Lai nodrošinātu praktisko pielietojamību, būtu nepieciešama iegūto zināšanu pārnese uz lauksaimnieciski nozīmīgiem augiem. Šajā pētījumā esam identificējuši un raksturojuši HA iesaistītā *Arabidopsis thaliana* gēna *LSD1* homologus miežos, kā arī raksturojuši identificēto gēnu ekspresiju miežu nekrotiskajos mutantos *nec1* un *nec3*.