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REGULATION OF HYPERSENSITIVE RESPONSE IN BARLEY

DOCTORAL THESIS

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Summary

Study aims at identification and characterization of genes involved in regulation of hypersensitive response in barley using barley lesion mimic mutants as a model system. Two known lesion mimic mutants – *nec1* and *nec3* – were chosen for the study. In addition, identification and characterization of previously unknown barley homologues of anti-apoptotic disease resistance regulator *AtLSD1* have been included in the study.

Results reveal, that *nec1* mutation does not affect either basal or *mlo-5* mediated penetration resistance to virulent powdery mildew. However, constitutive induction of *PR1*, over-accumulation of salicylic acid and hydrogen peroxide suggests, that *nec1* activates certain layer of barley immunity. Repressed powdery mildew micro-colony formation and inhibited multiplication of non-host bacteria *Pseudomonas syringae* pv. *tomato* in *nec1* confirm, that mutation imposes differential effect on barley disease resistance. Physiological characterization of *nec1* reveals, that mutant exhibits altered sensitivity to externally applied auxin. Effect of *nec1* on auxin signaling in barley is confirmed by repressed expression of auxin biosynthesis related genes and increased content of IAA of *nec1*. Therefore, differential disease resistance of *nec1* is likely attributed to distorted hormonal balance of salicylic acid and auxin in mutant.

Forward genetics approaches of transcript based cloning and map-based cloning are employed to identify *nec3* candidate gene. Although transcriptome analysis of *nec3* at two different developmental stages fails to identify *nec3* mutation, characterization of differentially expressed gene set unravels early onset of senescence in *nec3*, which is confirmed by physiological characterization of the mutant. Due to low polymorphism of target region, mapping of *nec3* mutation is restricted to 13 cM region of 6HS chromosome. Screen of barley homologues of rice genes, positioned syntenically to *nec3* target region, fails to identify *nec3* mutation.

Barley *LSD1* candidate genes – *ABC10220, ABC06454* and *CBC04043* are identified, based on sequence homology to *AtLSD1*. Sequencing, phylogenetic analysis and characterization of diurnal expression pattern of identified genes reveals that *CBC04043* is barley orthologue of *AtLOL1*, and does not represent a candidate of functional barley *LSD1*. *ABC06454* does not complement run-away cell death phenotype of *A. thaliana* lesion mimic mutant *lsd1-1*, and therefore is very unlikely to be functional barley *LSD1*. Reverse genetics approach of TILLING mutant population screening identified single non-synonymous G163E mutation in ABC10220, however more detailed characterization of identified mutation is delayed until backcrossing eliminating background mutations is completed.

In conclusion, identification and characterization of barley lesion mimic mutants has revealed previously unknown aspects of barley disease resistance. Successful identification and characterization of HR-related genes in barley requires integrated approach, involving application of forward (positional cloning, transcript-based cloning) and reverse (TILLING population screening, complementation of model-organism *A. thaliana*) genetics, as well as physiology and plant pathology – based experimental methods.

Key words: *A. thaliana*, hypersensitive response, *LSD1*, barley, *nec1*, *nec3*

Kopsavilkums

Pētījuma mērķis ir noskaidrot un raksturot gēnus, kas iesaistīti hipersensitīvās atbildes regulācijā miežos, izmantojot miežu nekrotiskos mutantus kā modeļsistēmu. Pētījuma veikšanai izmantoti divi iepriekš zināmi miežu nekrotiskie mutant – *nec1* un *nec3*. Pētījumā arī iekļauta iepriekš nezināmu anti-apoptotiskā slimību-izturības gēna *AtLSD1* miežu homologu identificēšana un raksturošana.

Pētījums atklāj, ka *nec1* mutācija neizmaina miežu bazālo un *mlo-5* nodrošināto penetrācijas rezistenci pret miežu miltrasu. Tomēr, konstitutīva *PR-1* indukcija, palielināta salicilskābes un ūdeņraža peroksīda koncentrācija *nec1* augos norāda uz noteiktu miežu imunitātes komponentu aktivāciju mutācijas ietekmē. Samazināta miltrasas mikrokoloniju veidošanās, kā arī ierobežota baktērijas *Pseudomonas syringae* pv. *tomato* vairošanās *nec1* augos apstiprina ideju, ka *nec1* mutācija atšķirīgi ietekmē dažādus miežu slimību-izturības komponentus. *nec1* mutantu fizioloģisks raksturojums atklāj izmainītu *nec1* augu jutību pret apstrādi ar auksīniem. *nec1* mutācijas ietekmi uz auksīna atbildes reakcijām miežos apstiprina izmainīta auksīna biosintēzes gēnu ekspresija *nec1* mutantos, kā arī būtiski paaugstināta indol-3-etiķskābes koncentrācija mutantos. Izmainīts fitohormonu līdzsvars *nec1* augos, iespējams, ir cēlonis mutācijas diferencētajai ietekmei uz miežu slimību-izturību.

nec3 kandidātgēnu identificēšana balstīta uz tiešās ģenētikas metodēm – pozicionālo klonēšanu un uz transkriptomu balstīto klonēšanu. Lai gan *nec3* mutanta transkriptomas analīze neidentificē *nec3* mutāciju, diferenciāli ekspresēto gēnu analīze atklāj mutanto augu ātrāku novecošanu, ko apstiprina arī *nec3* mutantu fizioloģisko atbildes reakciju raksturošana. *nec3* mutācijas kartēšanu ierobežo zemais polimorfisma līmenis attiecīgajā hromosomas rajonā. *nec3* pozīcija noteikta līdz 6HS hromosomas 13cM lielam rajonam. Balstoties uz sintēniju ar rīsu genomu, identificēti miežu homologi, kas potenciāli var atrasties 6HS hromosomas *nec3* kartēšanās mērķa rajonā. Šo gēnu pārbaude neatklāj *nec3* mutāciju.

Balstoties uz sekvenču homoloģiju ar *AtLSD1* gēnu, identificēti miežu *LSD1* kandidātgēni *ABC10220, ABC06454* un *CBC04043*. Pilnas sekvences noteikšana, filoģenētiskā analīze un gēna ekspresijas raksturošana atklāj, ka *CBC04043* ir *AtLOL1* ortologs. *ABC06454* nespēj komplementēt *A. thaliana* mutanta *lsd1-1* neierobežotas šūnu nāves fenotipu, tādēļ maz ticams, ka *ABC06454* ir miežu funkcionāls *LSD1*. *ABC10220* mutantu meklējumi, izmantojot reversās ģenētikas metodi, balstītu uz lokālu genoma bojājumu inducēšanu miežos, identificē nesinonīmu aminoskābju nomaiņu G163E gēnā *ABC10220*. Pirms šī mutanta tālākas raksturošanas jāveic atkrustošanu, kas nodrošina fona mutāciju ietekmes izslēgšanu.

Noslēgumā, miežu nekrotisko mutantu identificēšana un raksturošana atklāj iepriekš nezināmus miežu slimību-izturības aspektus. Sekmīgai miežu hipersensitīvās atbildes komponentu raksturošanai nepieciešams izmantot integrētu pieeju, kas ietver gan tiešās ģenētikas metodes (pozicionālo klonēšanu, uz transkriptomu balstīto klonēšanu), gan reversās ģenētikas metodes (TILLING populāciju pārbaudi, modeļorganisma *A. thaliana* mutantu komplementāciju), kā arī fizioloģijas un augu patoloģijas eksperimentālās metodes.

Atslēgvārdi: *A. thaliana*, hipersensitīvā atbilde, *LSD1*, mieži, *nec1*, *nec3*

Abbreviations

Gene abbreviations

APG5	autophagy related 5
BI-1	*Bax* inhibitor 1
CHS4	chilling sensitive 4
CNGC 4	cyclic nucleotide gated ion channel 4
COI1	coronatine insensitive 1
DND 2	defense no death 2
EDS1	enhanced disease susceptibility 1
EIN3	ethylene insensitive 3
EIL1	*EIN3*-like
FLS2	flagellin sensitive 2
HLM 1	HR-like lesion mimic
HSP90	heath shock protein 90
ICL	isocitrate lyase
LLS1	lethal leaf spot 1
LOL1	*LSD1*-like 1
LSD1	lesion simulating disease 1
MC1	metacaspase 1
ML	malate synthase
MLA1	mildew resistance locus A
MLO	mildew resistance locus O
NahG	salicylate hydroxylase
NEC1	necrotic leaf spot 1
NEC3	necrotic leaf spot 3
NECS	necrotic Steptoe
NIT2	nitrilase 2
NOS	nitric oxide synthase
NR	nitrate reductase
PAD4	phytoalexin deficient
PR1	pathogenesis related 1
RAR1	required for *Mla* resistance
RBOH	respiratory burst oxidase
RPG1	reaction to *Puccinia graminis* 1
SAG15	senescence associated gene 15
TAA	tryptophan aminotransferase of *Arabidopsis*
TPT	triose-phosphate/phosphate translocator
VPE	vacuolar processing enzyme
VT2	vanishing tassel 2
YUC1	yucca1

Other abbreviations

AA	amino acid
Affy	Affymetrix Barley GeneChip 1
At	*Arabidopsis thaliana*
Bgh	*Blumeria graminis* f.sp. *hordei*
BTH	benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester
CaM	calmodulin
CaMB	calmodulin binding domain
CaMV p35S	cauliflower mosaic virus 35S promoter
CAPS	cleaved amplified polymorphic sequences
cfu	colony forming unit
CNB	cyclic nucleotide binding domain
Col	Columbia
cv	cultivar
EEE	excess excitation energy
EMS	ethyl methanesulfonate
ET	ethylene

Other abbreviations (cont.)

ETI	effector triggered immunity
FA	fatty acids
GMO	genetically modified organism
GO	homology dependent gene silencing
HDGS	gene ontology
hpi	hours post inoculation
HPLC	high performance liquid chromatography
HR	hypersensitive response
Hv	*Hordeum vulgare*
IAA	indole acetic acid
IAN	indole-3-acetonitrile
INA	2,6-dichloro-isonicotinic acid
IAOx	indole-3-acetaldoxime
IPA	indole-3-pyruvic acid
JA	jasmonic acid
LHCII	light harvesting complex II
lmm	lesion mimic mutants
LPS	lipopolysaccharides
LRR-RLK	leucine-rich repeat receptor-like kinase
LTP	lipid transfer proteins
MBC	map-based cloning
MeJA	methyljasmonate
MS	Murashige and Skoog plant growth medium
NAA	naftil acetic acid
NBS-LRR	nucleotide-binding site, leucine-rich repeat domains
Os	*Oryza sativa*
PAMP	pathogen associated molecular patterns
PCD	programmed cell death
PQ	plastoquinone
PRR	pattern recognition receptors
PSI	photosystem I
Pst	*Pseudomonas syringae* pv. *tomato*
PTGS	post-transcriptional gene silencing
PTI	PAMP triggered immunity
PVX	potato virus X
QTL	quantitative trait locus
ROS	reactive oxygen species
SA	salicylic acid
SAG	salicylic acid β-glucoside
SAR	systemic acquired resistance
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
TAM	tryptamine
TBC	transcript based cloning
TGS	transcriptional gene silencing
TILLING	targeted induce local lesions in genomes
TNL	TIR-NB-LRR (Toll/Interleukin1 receptor–nucleotide binding site–leucine-rich repeat) gene
TTSS	type three secretion system
UTR	untranslated region
Ws0	Wassilewskija-0
wt	wild type
Zm	*Zea mays*

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Introduction

Rising demand for food and other agricultural production provokes intensified agriculture resulting in an increased anthropogenic pressure on environment. Crop improvement, based on employment of intrinsic yield and disease resistance potential of crop plants, can minimize adverse side effects of increased agricultural production. Development of improved cultivars, comprising enhanced intrinsic disease-resistance, requires detailed knowledge of plant immunity.

Accelerated and intensified defense responses of plant immunity can result in hypersensitive response – localized plant cell death at the site of attempted infection (Jones and Dangl 2006). Therefore, studies revealing molecular mechanisms of hypersensitive response can also reveal important aspects of plant disease resistance. Majority of discoveries concerning molecular mechanisms of hypersensitive response and plant disease resistance are based on dicotyledonous model plant *A. thaliana* (Somerville and Koornneef 2002). In order to practically apply these discoveries to plant breeding and agriculture, it would be required to find out, if the same disease resistance mechanisms underlie also immunity of crop plants. Four out of top five world's major agricultural crops are monocots; therefore monocot-based discoveries of plant physiology and molecular biology potentially provide significant contribution to science-based advancement of agriculture. Barley is one of the main monocot model-species (Schulte et al. 2009), and studies of barley have revealed many aspects of monocot disease resistance.

Studies of hypersensitive response in *A. thaliana* have significantly benefited from identification and characterization of mutations conferring lesion mimic phenotype (Lorrain et al. 2003). This allows for assumption, that studies of barley lesion mimic mutants can help in identification and characterization of molecular components of hypersensitive response of monocots. Although lesion mimic mutant class is usually well represented in barley mutant collections, only several mutations conferring lesion mimic phenotype have been identified or characterized in barley so far. Knowledge regarding molecular mechanisms of disease resistance comprises significant practical importance, therefore insufficiently studied barley lesion mimic mutants can become a source of scientifically as well as practically relevant discoveries regarding monocot disease resistance.

Aim of the study is **identification and characterization of genes involved in regulation of hypersensitive response in barley, using barley lesion mimic mutants as a model system.**

To achieve the aim three **tasks** are proposed:

1) Physiological and molecular characterization of barley lesion mimic mutant *nec1*.

2) Identification of new gene involved in hypersensitive response in barley, using transcript-based cloning and map-based cloning of *nec3* mutation triggering lesion mimic phenotype.

3) Application of *A. thaliana* as a model system to identification of barley *LSD1* gene.

1. Literature review

1.1. Hypersensitive response – form of programmed cell death in plants

Accelerated and intensified defense responses of plant immunity can result in hypersensitive response (HR) – localized plant cell death at the site of attempted infection (Jones and Dangl 2006). HR is the form of programmed plant cell death (PCD) (Reape et al. 2008, Mur et al. 2008), and, in contrast to necrotic cell death, usually caused by physical damage, induction of HR requires active transcription and translation (Heath et al. 2000). HR induction is preceded by activation of a certain set of genes including genes specific to this phenomenon – so called *hsr* (hypersensitive response) or *hir* (hypersensitive-induced reaction) genes (Lacomme and Roby 1999, Rostoks et al. 2003). Similarly to animal cell during apoptosis, plant cells undergo DNA laddering, cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling and cytochrome c release (Mittler et al. 1997, Geenberg and Yao 2004, Mur et al. 2008). Although plant PCD shares common characteristics with animal apoptosis (Lam et al. 2001), morphological specificity of plant cell defines several substantial differences between plant PCD and animal apoptosis. For example, presence of chloroplasts offers an additional ROS source for plant HR and presence of plant cell wall precludes phagocytosis, which is characteristic to apoptosis (Williams and Dickman 2008). Another substantial difference is caused by lack of plant caspases – indispensable element of apoptotic PCD. Despite the lack of direct caspase homologues, plants have been reported to comprise caspase-like activity, and HR can be successfully inhibited by expression of caspase inhibitors (del Pozo and Lam 1998, Chichkova et al. 2004). In addition, animal caspases can alter plant disease resistance and cause DNA laddering, if expressed in transgenic plants (Dickman et al. 2001). Although no plant genes share high sequence homology with caspases, Uren and colleagues (2000) have identified plant protein family of high structural similarity to caspases. However, the functional homology of plant metacaspases to animal caspases is questioned (Bonneau et al. 2008). Taking into account these differences plant cell death is viewed as apoptotic-like PCD, rather than true apoptosis (Danon et al. 2000). HR has also been shown to share common characteristics with autophagic animal cell death (Liu et al. 2005), therefore it has been argued, that plant HR is not an equivalent of any of the forms of animal PCD, but rather is a unique form of cell death (Mur et al. 2008).

1.2. HR and plant immunity

1.2.1. Position of HR in plant immunity network

Strategies, employed by plants to prevent infection, can be categorized under two large categories – PAMP triggered immunity (PTI) (formerly defined as basal disease resistance) and effector triggered immunity (ETI) (earlier termed as R-gene mediated resistance) (Jones and Dangl 2006) (Figure 1). PTI is the initial immune response, activated upon the first plant-pathogen contact (Boller and He 2009). PTI is based on the ability of plant pattern recognition receptors (PRRs) to recognize pathogen derived molecules called pathogen associated molecular patterns (PAMPs) – usually vital structural elements, that are common for wide

range of pathogens (Nicaise et al. 2009, Schwessinger and Zipfel 2008). Successful recognition of PAMP activates MAP kinase signaling (Asai et al. 2002), leading to induction of defense mechanisms such as cell wall fortification, cytosolic ion fluxes, ROS production and induction of systemic acquired resistance (SAR) (McDowell and Dangl 2000). SAR ensures, that signal induced by local primary infection is spread through-out the plant, triggering resistance of distal tissues to secondary infection (Durrant and Dong 2004). Induction of SAR by PAMPs at least partially is achieved through salicylic acid (SA) signaling (Tsuda et al. 2008).

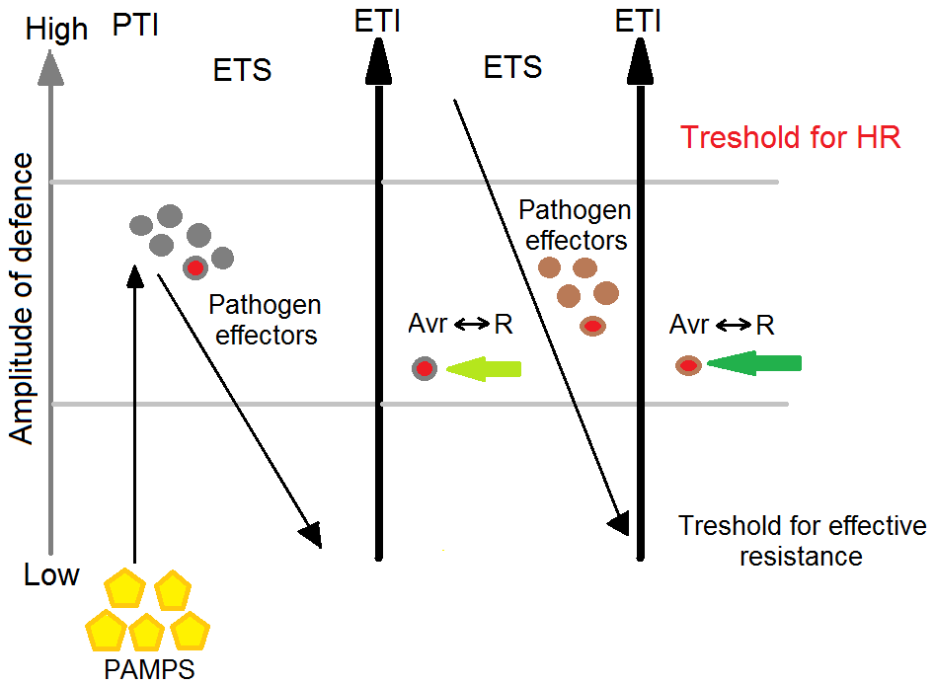


Figure 1. Plant immunity representing zigzag model developed by Jones and Dangl 2006. Model describes interaction between two levels of plant immunity – PAMP triggered immunity (PTI) and effector triggered immunity (ETI) and pathogen derived molecules – either pathogen associated molecular patterns (PAMPS) or pathogen effectors causing effector triggered susceptibility (ETS). Model reflects evolutionary "arms-race" between plant and pathogen defined by development of new effectors and corresponding R genes. The model also sets the position of hypersensitive response (HR) in overall plant immunity network.

Formerly PTI was considered to be a weak and ineffective form of defense. In order to establish a successful infection, pathogens develop effector molecules suppressing PTI (Jones and Takemoto 2004). Effectors suppress PTI through interaction with either PAMP recognition receptors or downstream components of PTI signaling (Boller and He 2009). Suppression of PTI employs various strategies – for example, PTI can be suppressed by inhibiting kinase activity of PRRs (Xiang et al. 2008) or through altering PRR interaction with downstream signaling molecules (Shan et al. 2008). Molecular mechanisms, used by effectors to suppress PTI, involve enhanced host protein degradation, by affecting protein ubiquitination or directly cleaving host proteins, transcriptional regulation of host genes and inhibition of kinases, leading to disrupted signal transduction (Block et al. 2008).

Development of effectors, allowing for suppression of PTI and successfully surpassing R-genes, is thought to be the main factor underpinning pathogen virulence (Cui et al. 2009).

Plants in their turn have evolved resistance proteins (R-proteins), sensing interaction of effectors with their targets (Martin et al. 2003, Nimchuk et al. 2003). R-protein dependent recognition of effector and a subsequent defense response is base of effector triggered immunity (ETI) (Jones and Dangl 2006).

ETI is hypothesized to act through derepression of PTI (Schwessinger and Zipfel 2009), and success of plant immunity to withstand pathogen invasion, likely, depends on speed and intensity of resistance response induction (Tao et al. 2003). Thus, R-proteins are thought to amplify PTI signaling, involving ROS production and ion fluxes, leading to enhanced defense gene expression, and often leading to HR (Nimchuk et al. 2003).

Based on the occurrence of HR, ETI is clearly separated from PTI, however there are also remarkable similarity between regulatory mechanisms triggering ETI and PTI. Transcriptional profiling has revealed a significant overlap between gene sets involved in flg22 response of *A. thaliana* and R-gene mediated response to *Pseudomonas* in tobacco, suggesting, that PTI and ETI might employ largely similar molecular mechanisms (Navarro et al. 2004). The difference, probably, is defined by the quantitative, rather qualitative expression of plant defense mechanisms (Nimchuk et al. 2003).

Inability of the pathogen to overcome PTI is thought to underlay non-host resistance (Nurnberger and Lipka 2005, Niks and Marcel 2009). A distinct but very important type of resistance – non-host resistance involves both – PTI as well as ETI components (Jones and Dangl 2006, Niks and Marcel 2009). Based on transcriptional profiling of *A. thaliana* interaction with host and non host *Pseudomonas syringae*, a significant overlap between molecular mechanisms, underlying different types of resistance, has been suggested (Tao et al. 2003). Analogy with ETI (R-gene mediated resistance) and non-host resistance can be based on the fact, that some plant-non-host pathogen interactions involve induction of localized cell death at the infection site, resembling HR of ETI. Based on presence or absence of cell death during plant interaction with non-host pathogen, non-host resistance is classified into two categories – Type I non-host resistance has no apparent phenotypic effect, whereas Type II non-host resistance is accompanied by HR (Mysore and Ryu 2004). However, parallels between ETI need to be taken with certain sense of precaution, since cytological observations suggest different molecular basis for host and non-host HR (Christopher-Kozjan and Heath 2003). Alternatively, non-host resistance might be a superior form of PTI, based on the pathogens inability to produce effectors, successfully overcoming basal defense mechanisms (Niks and Marcel 2009). In support of close link between PTI and non host resistance, Jafary and colleagues (2008) have identified significant overlap of QTLs, responsible for both types of resistance in barley.

1.2.2. Role of HR in plant disease resistance

Although HR usually accompanies ETI, it is doubted if HR is indispensable for ETI (Jones and Dangl 2006). Several examples of R-gene mediated resistance, proceeding independently of cell death, allow to hypothesize, that HR can be uncoupled from plant disease resistance. For example, *Rx* gene conditioned resistance to potato virus X and *Rrs1*

mediated *A. thaliana* resistance to *Ralstonia solanacearum* proceed without development of HR (Bendahmane et al. 1999, Deslandes et al. 2002). Similarly, barley gene *Rdg2a* confers barley resistance to leaf stripe, caused by *Pyrenophora graminea,* without initiating HR (Bulgarelli et al. 2010). There are also reverse examples, where HR is induced regardless of the outcome of plant-pathogen interactions - oat gene *Rih* ensures HR induction in susceptible as well as resistant oat plants (Yu et al. 2001). These examples suggest, that induction of HR can be uncoupled from disease resistance, and both processes are likely regulated by independent genetic mechanisms. This is supported by the fact, that certain mutations disrupt HR, while not affecting or even enhancing disease resistance. Silencing metacaspase *AtMC1* in *lmm lsd1* suppresses cell death, but does not affect mutant's enhanced disease resistance (Coll et al. 2010). Similarly, mutations *dnd1* and *dnd2* confer resistance to wide range of pathogens, at the same time enabling *A. thaliana* to induce hypersensitive response (Clough et al. 2000, Jurkowski et al. 2004). Using two tobacco species with different response to Cauliflower mosaic virus (CaMV), Cole and colleagues (2001) were able to show, that in tobacco resistance to CaMV is inherited independently from HR. Altogether these evidence suggest, that HR can be genetically and physiologically uncoupled from disease resistance.

Despite the above mentioned facts, the majority of R-gene mediated plant-pathogen interactions are accompanied by HR, suggesting that HR plays a certain role in plant disease resistance. HR and its suppression is a subject of plant-pathogen arms-race. Several pathogen effectors are known to specifically target genes, regulating plant cell death or suppressing HR, suggesting that at least for some plant-pathogen interactions PCD might be important (Jamir et al. 2004, Nomura et al. 2005). Besides, successful infection is often associated with suppressed HR (Abramovitch et al. 2003). The outcome of the plant-pathogen interaction is often defined by the plants ability to rapidly induce HR, and delayed onset of HR allows establishment of infection, suggesting that at least in some plant-pathogen interactions HR is vital for resistance. For example, success of *Mla-1* mediated barley resistance to *Bgh* has been shown to depend on plant's ability to induce HR rapidly and without delays (Vanacker et al. 2000). Similarly, resistant potato cultivars elicited fast HR in response to *Phytophthora infestans* infection, on contrary to susceptible lines, where HR onset was delayed (Vleeshouwers et al. 2000).

The above described contradictory opinions on the role of HR in plant disease resistance can be merged under the third, combined hypothesis. This hypothesis suggests, that the same molecular mechanisms are employed during disease resistance, provoking HR, and disease resistance, proceeding without HR, the only difference being timing and severity of defense mechanisms employed. It has been suggested, that infection is counteracted without cell death, if plant defense response is fast enough to effectively stop pathogen spread before HR, but for slower defense responses HR is ubiquitous (Greenberg and Yao 2004). Interestingly, several R-gene mediated disease resistance responses, occurring without HR (for example, *Rx* mediated resistance to potato virus X (Bendahmane et al. 1999), *HRT* mediated resistance to turnip crinkle virus (Cooley et al. 2000)) indeed are described as very fast and extreme resistance. Thus, probably, in case of HR-less R-gene mediated resistance cell-to-cell signal does not amplify strongly enough, to exceed minimal threshold, required for induction of cell death, as proposed by the so called plant immunity zigzag model, according to Jones and Dangl 2006 (Figure 1).

1.3. Control of HR

1.3.1. Induction of HR- recognition of pathogen derived signal

Traditionally HR is associated with ETI (Jones and Dangl 2006), and initiation of HR requires plant to recognize pathogen effectors, and R-genes to counteract effectors, to avoid suppression of immunity (Abramovitch et al. 2006). Effectors are proteins, specifically targeted for interaction with plant immunity, and, depending on different pathogens, they can either be delivered directly into plant cell, using secretion systems, or released into extracellular space-apoplast (Göhre and Robatzek 2008). One of the most important bacterial systems, employed for delivery of effectors into host cell, is type three secretion system (TTSS) (Alfano and Collmer 2004). Successful delivery of effectors to host cell is essential for establishment of HR - this is supported by the fact, that pathogens, comprising non-functional secretion systems, are unable to elicit HR (Degrave et al. 2008).

Although in the majority of plant-pathogen interactions HR induction is triggered by effectors, there are also some exceptions. A specific case of molecules, eliciting HR, are harpins, which are not considered to be effectors due to the lack of host specificity (Alfano and Collmer 2004). Besides, unlike true TTSS effectors, harpins induce HR without entering host cell (Tampakaki and Panopoulos 2000). Another exception is flagellin, which, being a typical elicitor or PAMP, and thus eliciting PTI rather than ETI, still can elicit HR in certain pathosystems (Naito et al. 2008). Interestingly, it has been shown that HR inducing ability of flagellin depends on its N-terminal domain (Naito et al. 2007) and post-translational modification of the protein (Taguchi et al. 2003). HR inducing capacity of certain elicitors might be determined by their ability to trigger comparatively fast and strong response of plant immunity. For example, in *A. thaliana* HR inducing elicitor harpin triggers significantly faster plant immunity reactions compared to HR non-inducer LPS (Livaja et al. 2008).

Thus, although the accepted model of plant immunity claims, that the threshold of HR is surpassed only during ETI, whereas PTI proceeds without programmed cell death (Jones and Dangl 2006), several evidence suggest, that HR can be triggered by either specific effectors or general pathogen derived elicitors, or PAMPs, implying, that HR might not be exclusively ETI related phenomenon. This is also supported by lessons learned from non-host resistance, which is suggested to be an extreme version of PTI (Niks and Marcel 2009). Indeed it is difficult to explain, why different plant defense levels largely overlapping in transcriptome (Navarro et al. 2004), would dramatically differ in ability to elicit HR.

Regardless of type of pathogen derived molecule inducing plants immunity, successful induction of HR requires a plant to detect and recognize intrusion. In case of PTI such recognition is established by pattern recognition receptors (PRR), whereas in ETI effector presence is recognized by resistance proteins (R-proteins). Both recognition systems differ in their strategy of sensing presence of pathogen– PRR sense “non-self” molecules, by detecting molecular patterns, which are not present in plants and are exclusively characteristic for pathogens, whereas R-protein sensing system relies on detecting “missing-self” or sensing alterations in host proteins caused by effectors (Gómez-Gómez 2004).

So far there are two types of PRRs, known to participate in PAMP recognition - receptor-like kinases (belonging to LRR-RLK or LysM-RLK subfamilies), and receptor-like proteins (Segonzac and Zipfel 2011). Ability to recognize PAMP by PRRs is case specific – recognition of a specific PAMP requires presence of a corresponding PRR (Boller and He 2009). In addition, recognition of PAMP by a specific PRR is absolutely crucial for induction of downstream signaling (de Jonge et al. 2010). Despite such PAMP-PRR recognition specificity, downstream signaling events seem to share a common transcript profile among different PAMP-PRR interactions (Zipfel et al. 2006), and most likely, even among different plant species, as proved by heterologous expression of PRRs (Lacombe et al. 2010).

In case of ETI, HR induction depends on R-protein mediated ability to sense PTI repression by effectors (Abramovitch et al. 2006). Effectors and R-proteins are thought to be the main subject of co-evolution or arms-race between host and pathogen (Bergelson et al. 2001, Chisholm et al. 2006). Majority of R-proteins belong to NBS-LRR domain protein class (Nimchuk et al. 2003). Since R-proteins show quite large specificity usually counteracting only a specific pathogen effector, plant genomes encode comparatively large number of R-genes (Ellis et al. 2001). *A. thaliana* genome is estimated to comprise ~150 and rice genome ~600 loci with sequence homology to NBS-LRR domain (Tan et al. 2007, Goff et al. 2002). Successful recognition of effector by R-protein, most likely alters intramolecular interaction of different R-protein domains, eliciting downstream signaling, triggering HR (Moffett et al. 2002).

1.3.2. Signal transduction downstream of pathogen recognition, leading to HR induction

1.3.2.1. Signal transduction during PAMP triggered immunity (PTI)

Recognition of PAMP by PRR elicits downstream signaling cascade (Schwessinger and Zipfel 2008), mounting the first-line plant immune response - PTI (Jones and Dangl 2006). PRRs interact with intracellular partners, to trigger downstream signaling pathway. One of such proteins, converting signal detected at cell wall by PRR into intracellular message, is BAK1 (Heese et al. 2007). Although BAK1 is not a universal PRR signal transducer, it participates in several PTI pathways (Shan et al. 2008). Interestingly, BAK1, which interacts with FLS2, to amplify signal induced upon interaction with PAMP flg22 (Chinchilla et al. 2007), has also been shown to regulate ROS accumulation and HR related cell death (Heese et al. 2007). Several PRRs have recently been shown to require pass through endoplasmic reticulum quality control for proper folding and accumulation, and mutations disrupting proper PRR processing, inhibit oxidative burst and MAPK signaling in response to appropriate elicitors (Nekrasov et al. 2009).

Early molecular events, following the initial signal perception, involve activation of ion channels, which ensure anion efflux (El-Maarouf et al. 2001) and Ca^{2+} influx (Lecourieux et al. 2002, Ma et al. 2009 a). Ca^{2+} increase in response to PAMPs is most likely mediated by plant CNGCs (Ali et al. 2007). Recently PRR AtPepR1 has been shown to comprise guanylyl cyclase activity, enabling AtPepR1 to activate Ca^{2+} conducting CNGC2, suggesting that at least some PRRs might employ cNMPs for downstream signal activation (Qi et al. 2010).

Mutations, affecting CNGC2, prevent cGMP mediated Ca^{2+} rise in response to PAMPs (Qi et al. 2010). However, requirement for CNGC2 is not ubiquitous, since it has been shown, that flg22 induced cell membrane depolarization can proceed also in the absence of functional CNGC2 (Jeworutzki et al. 2010). Increase in intracellular Ca^{2+} levels is indispensable for cell death induction in response to PAMPs, for example, inhibition of Ca^{2+} influx by Ca^{2+} channel blockers significantly represses cell death in response to cryptogein (Binet et al. 2001). Importance of Ca^{2+} import for PTI is supported by the fact, that plant pathogens counteract PTI by inhibiting Ca^{2+} influx with extracellular polysaccharides, chelating apoplastic Ca^{2+} ions (Aslam et al. 2008). Rise of cytosolic Ca^{2+} activates MAPK signaling cascade (Lecourieux et al. 2002), and over-expression of certain MAPK kinases enhances cell death in response to elicitor (Gao et al. 2008), suggesting, that MAPK cascade is indispensable for cell death induction in response to elicitors (Pitzschke et al. 2009).

Activation of MAP kinase signaling cascades is followed by oxidative burst in response to elicitor recognition (Ren et al. 2002, Liu et al. 2007, Zhang et al. 2007) Extracellular alkalinization and oxidative burst follows within the first minutes after elicitor treatment (Zipfel et al. 2006, Nekrasov et al. 2009, de Jong et al. 2010). On contrary to oxidative burst during ETI (described in the next paragraph), there is only one strong peek of ROS production in response to PAMPs, usually reaching a maximum at 10-15 min after elicitor treatment (Gerber et al. 2004, Krause and Durner 2004, Gimenez-Ibanez et al. 2009, de Jong et al. 2010). Oxidative burst in response to flg22 in *A. thaliana* has been shown to depend on respiratory burst oxidase AtRbohD (Zhang et al. 2007), however, it is not yet known, if RbohD participates in ROS production also downstream of other PAMP-PRR recognition. Transcriptional profiling of immunity signaling in response to different PAMPs shows different requirement for Rbohs (Livaja et al. 2008). PAMP induced immune signaling also involves sharp increase in Ca^{2+}, calmodulin and cAMP dependent NO production (Ali et al. 2007, Ma et al. 2008). Although NO production in response to elicitors appears to be ubiquitous for PTI (Gerber et al. 2004, Krause and Durner 2004), the source of NO in plants is still a matter of debates. NO production might be mediated by nitrate reductase (NR), since elicitors have been shown to induce NR expression and nitrite dependent NO production (Yamamoto et al. 2003). However, it has also been shown, that lipopolysacharrides – typical PAMP in animal pathosystems – trigger nitric oxide synthase (NOS) dependent NO production, which is required for subsequent defense gene induction (Zeidler et al. 2004).

Primary oxidative burst, involving NO over-production, triggers stomatal closure in response to PAMP recognition by PRRs (Melotto et al. 2006, Zeng et al. 2010). Although the molecular mechanisms, inducing stomatal closure, has not been described in details, it is known that NO/ROS induced stomatal closure in response to PAMPs requires inhibition of plasma membrane H+-ATPases, which interacts with RIN4, to ensure stomatal reopening in response to PAMPs (Liu et al. 2009). Given that stomata are the main regulators of gas exchange in plants, stomatal closure significantly affects ROS production, and consequently it has been suggested to serve as an important regulatory factor for HR related cell death (Mühlenbock and Karpinski 2005). In parallel to stomatal closure, which restricts CO_2 uptake, HR inducing signals also inhibit CO_2 fixation in chloroplasts, leading to production of excess excitation energy (Liu et al. 2007). Structural changes of chloroplasts, occurring as a response

to elicitor treatment, have even been suggested to serve as a marker for early stages of HR (Boccara et al. 2007).

Chloroplasts are not the only source of ROS inducing HR – certain elicitors are known to impair mitochondrial functions by altering membrane potential, decreasing ATP production and causing ROS over-accumulation (Krause and Durner 2004). Importance of sustained ATP production for HR induction is confirmed by the fact, that plants attempt to ensure steady ATP production by balancing alternative oxidase, cytochrome oxidase and antioxidative system activity, in order to maintain the HR inducing capacity in response to elicitors (Vidal et al. 2007). Certain TTSS effectors, suppressing PTI, are targeted to chloroplasts or mitochondria after injection into plant cell suggesting, that functional chloroplasts and mitochondria might be essential for PTI (Jelenska et al. 2007, Block et al. 2010).

Apart from intracellular sources of ROS, plant cells are also able to accumulate ROS in apoplastic space. Apoplastic ROS production in response to PAMPs is accomplished by plasma membrane respiratory burst oxidases (Rbohs) (Mersmann 2009), which produce superoxide by electron transfer from NADPH to O_2 (Torres and Dangl 2005). Thus all three pathways (chloroplastic, mitochondrial, plasma membrane), ensuring ROS production in response to PAMPs, are sensitive to NADPH or ATP. This allows cell to adjust ROS production, according to defense requirements and available resources.

These early signaling events lead to transcriptional reprogramming, which results in cell death in response to certain PAMPs (Livaja et al. 2008). Pro-apoptotic effect of ROS and NO has been shown to result from induction of a specific pro-death program, rather than from a direct oxidative damage (Delledonne et al. 2003, Wagner et al. 2004). ROS/NO interaction affects ascorbate/glutathione antioxidative cycle, and, as a consequence, alters redox balance of the cell (de Pinto et al. 2002). Altered redox balance affects protein S-nitrosylation, and thereby modifies protein activity (Wang et al. 2006 b). Therefore S-nitrosylation of pro-apoptotic proteins is a likely mechanism linking NO to HR induction. For example, nitrosylation of metacaspase AtMC9 inhibits its autocleavage, and thereby also its pro-apoptotic activity (Belenghi et al. 2007). Metacaspase mediate downstream signaling, leading to execution of pro-apoptotic machinery occurs in response to several elicitors. Victorin, elicitor of fungal origin, induces signaling cascade, involving serine proteases, comprising caspase-like activity, resulting in Rubisco proteolysis and plant cell death in oat (Coffeen and Wolpert 2004). Similarly, harpin induced HR has been shown to depend on vacuolar processing enzyme (VPE) – cysteine proteinase (Zhang et al. 2010), which has been proposed to trigger HR trough activation or release of vacuolar hydrolytic enzymes into cytosol (Hara-Nishimura et al. 2005).

1.3.2.2. Signal transduction in effector triggered immunity (ETI)

Only several PAMPs trigger cell death in host plants (Naito et al. 2008, Livaja et al. 2008), whereas successful recognition of effectors by R-proteins during ETI is very often accompanied by HR. Despite of this difference in HR initiation, transcriptome of ETI and PTI largely overlaps (Navarro et al. 2004) implying, that signaling, leading to HR induction, might be largely similar. Indeed, many signaling components, participating in PTI, are also required for HR induction in ETI. Similarly to PAMP perception by PRRs, effector

perception by R-proteins elicits cAMP and calmodulin dependent rise in intracellular Ca^{2+} (Ma et al. 2009 a). Requirement of cAMP and calmodulin for Ca^{2+} rise in response to effector recognition by R-proteins suggests a possible involvement of CNGCs in HR induction during ETI. In support of this idea, mutants, comprising impaired CNGC2 or CNGC4, display 'defense no death' phenotype and are unable to elicit HR related cell death (Clough et al. 2000, Jurkowski et al. 2004).

Although empirical evidences have for long suggested a causal relationship between rise of cytosolic Ca^{2+} and over-production of plant stress and defense related phytohormone SA, molecular mechanisms ensuring this link have only recently started to emerge. Ca^{2+} / calmodulin binding has been shown to activate transcription factor SR1, which represses EDS1 (Du et al. 2009). EDS1 is required for R-gene mediated HR induction, and its interaction with PAD4 ensures potentiation of SA signalling (Feys et al. 2001). Previously EDS1 was considered to act upstream of SA in HR induction, but recently it has been suggested that EDS1 and SA function redundantly, and induction of both components can initiate HR (Venugopal et al. 2009). Although molecular pathway connecting EDS1 and HR cell death has not been elaborated in details, EDS1 most likely triggers SA mediated ROS over-accumulation and consecutively alters redox balance of the cell (Wiermer et al. 2005).

Thus, rise in intracellular Ca^{2+} levels leads to accumulation of ROS (Grant et al. 2000), which most likely is achieved through induction of SA mediated signalling. During ETI ROS production and the effect of oxidative burst on cell death has been shown to depend on Rbohs (Torres et al. 2002). Over-accumulation of ROS at infection site can be indispensable for HR induction in certain pathosystems (Hückelhoven et al. 2000). In addition, inhibition and alteration of certain components of plant antioxidative system can trigger development of *lmm* phenotype and enhance plant disease resistance (Pavet et al. 2005). Pro-apoptotic effect of ROS most likely depends on ROS capacity of altering intracellular redox balance, rather than on direct cytotoxic effect of ROS *per se* since well developed antioxidative system of plants permits survival of comparatively high ROS concentrations (Pastori and Foyer 2002, Hong et al. 2008). Change of cell redox balance often acts as a trigger for activation of certain downstream signaling pathways. For example, reducing agents trigger monomerization and thereby also activation of NPR1 protein (Mou et al. 2003) which is a key component regulating HR induction downstream of SA (Rate and Greenberg 2001).

Similarly to PTI, apoptotic effect of ROS during ETI is regulated by NO (Hong et al. 2008). Pro-apoptotic action of NO during ETI has recently been shown to depend on S-nitrosylation, and consequently activation of ROS producing capacity of AtRbohD (Yun et al. 2011). Simultaneously elevated concentration of ROS and NO induces HR through either transcriptional reprogramming (Delledone et al. 2003, Zago et al. 2006) or regulation of protein turnover (Belenghi et al. 2003). Similarly to PTI, NO has also been shown to induce caspase-like activity in cells, undergoing gene-for-gene resistance related HR (Clarke et al. 2000), and caspase-like activity is required for HR induction during incompatible plant-pathogen interactions (del Pozo and Lam 1998).

Release of hydrolytic enzymes from vacuoles is considered to be one of the main hallmarks of HR related cell death in plants (Hatsugai et al. 2006). Depending on a type of plant-pathogen interaction, there are two modes of hydrolytic enzyme release from vacuoles during HR. Interaction with viral pathogens invading cytosol, involves dezintegration of

vacuolar membranes, allowing release of hydrolytic enzymes into cytosol, whereas release of hydrolytic enzymes in apoplast, promoted by fusion of vacuolar and plasma membrane, is applied in case of extracellular bacterial infection (Hara-Nishimura and Hatsugai 2011). Along with degradation of main proteins, triggered by release of proteases, plant cells are reported to undergo DNA fragmentation during HR related cell death. For example, tobacco cells infected either with tobacco mosaic virus or *Pseudomonas syringae* pv. *phaseolicola* trigger fragmentation of nuclear DNA in 50 kb fragments (Mittler et al. 1997).

Induction of pro-apoptotic molecular mechanisms results in morphological changes of plant cell, undergoing HR-related cell death. One of the early morphological characteristics of HR-related cell death in plants is cessation of cytoplasmic streaming (Heath 2000), vacuolization of the cytosol (Mur et al. 2008) and reorganization of cytoskeleton (Smertenko and Franklin-Tong 2011). HR-related plant cell death shares some morphological characteristics with apoptotic cell death of animals. Similarly to apoptosis, HR-related cell death triggers cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling (Mur et al. 2008) and loss of mitochondrial membrane potential (Yao et al. 2004). However, in contrast to animal cells undergoing apoptosis, plant cells subjected to HR relate cell death retain nuclear integrity until late stages of cell death (Mittler et al. 1997).

1.3.3. Genetic and molecular mechanisms restricting spread of HR

One of the characteristics, defining HR, is constraint of cell death to direct proximity of infection site. This implies existence of regulatory mechanisms restricting spread of cell death into distal tissues and preventing so called runaway cell death. Such restriction of HR PCD has been suggested to involve mechanisms homologous to animal autophagy (Liu et al. 2005) - type of cell death operating by formation of autophagosomes delivering cell particles to vacuole for further degradation. Although an autophagy, as a phenomenon important for certain processes in plants, has previously been well known (Bassham 2007), evidence suggesting importance of autophagy for plant disease resistance and HR related PCD emerged only lately (Liu et al. 2005, Patel and Dinesh-Kumar 2008, Hofius et al. 2009). Tobacco plants comprising non-functional *BECLIN1* gene or *ATG* genes involved in autophagy are not able to restrict spread of HR PCD (Liu et al. 2005) and *A. thaliana* antisense lines of *AtATG6* are unable to prevent spreading of HR PCD beyond the site of infection (Patel and Dinesh-Kumar 2008). However, the precise role of autophagy in HR related cell death is currently equivocal, since recently several *ATG* genes and vacuolar processing enzyme (VPE- cysteine proteinase) have been reported to participate in HR as pro-death agents (Hofius et al. 2009, Zhang et al. 2010). Currently only very few components of autophagy/HR PCD regulatory genes have been identified, but previously characterized propagation *lmm* (plants displaying normal induction of HR, but unable to restrict further spread of HR) suggest, that there are more regulatory genes, participating in restriction of HR PCD spread.

1.3.4. Homology of molecular mechanisms of disease resistance and HR between A. thaliana and barley

The enormous financial and intellectual investments into *A. thaliana* research have yielded a substantial amount of information, concerning genetic and molecular characteristics of the model species (Somerville and Koornneef 2002). From the resource management point of view research focusing on model organism characterization is justified by the concept, that knowledge obtained in model plant studies can be transferred to agriculturally important species. On contrary to the main model plant *A. thaliana*, the main commercial plant species, such as rice and wheat, are monocots. This raises a question of value of conclusions, derived from *A. thaliana*, for practical application in monocots, therefore it is important to understand the level of physiological and genetical homology between monocots and dicots. Based on different estimates, the last common ancestor of monocots and dicots is dated back to 140-200 Myr years (Laroche et al. 1995, Chaw et al. 2004). Characterization of rice and *A. thaliana* genome colinearity has shown, that synteny between both genomes is rather low and homology can be observed over short DNA segments (Wang et al. 2006 a). This implicates significant difficulties on map based cross-species gene identification in case of grasses and *A. thaliana* (Devos et al. 1999). The situation is more promising within grass family, where knowledge of model species, such as barley and rice, can successfully be applied in characterization of more complicated genomes such as wheat (Devos and Gale 2000)

Although the whole genome studies might indeed be difficult to build upon *A. thaliana* and monocot comparison, it does not exclude the possibility that homology of individual genes between *A. thaliana* and grasses is still high enough, to apply knowledge of *A. thaliana* genetics for identification of a particular monocot genes. Based on the sequence homology of the well-characterized disease resistance related genes between rice and *A. thaliana*, it has been predicted, that disease resistance mechanisms might be relatively conserved between dicots and monocots (Goff et al. 2002). Over-expression of the monocot gene in wt *A. thaliana* or complementation of the *A. thaliana* mutants with the corresponding monocot genes appears to be a successful approach to functional characterization of new rice and barley genes (Whipple et al. 2004, Moreno-Risueno et al. 2008, Vannini et al. 2004, Kojima et al. 2002).

There are contradictory evidence of the resemblance of molecular mechanisms of disease resistance between monocots and dicots. In a broad sense, the immunity of the main monocot species is similar to that described for *A. thaliana* – monocots also employ PAMP triggered immunity, consisting of non-specific basal resistance mechanisms, as well as have developed specific resistance genes, ensuring effector triggered immunity (Ayliffe and Lagudah 2004). However, when the particular signaling pathways or molecular components of a certain disease resistance mechanisms are considered, the homology between monocots and dicots becomes more indistinct. Similarly to dicots, monocots recognize PAMP through PRRs which mainly belong to receptor-like kinases. For example, rice homologue of *A. thaliana* flagellin receptor FLS2 is able to complement *A. thaliana* mutant *fls2* (Takai et al. 2008). Similarly, receptor-like kinase CERK1 is required for chitin perception in *A. thaliana* (Miya et al. 2007) and rice (Shimizu et al. 2010) suggesting, that PAMP recognition mechanisms are at least partially conserved between monocots and dicots. Although there are

also *A. thaliana* PRRs, which do not comprise a homologue in monocots and *vice versa*, the fact that many PAMP recognizing proteins in monocots and dicots belong to the same LRRXII-RLK protein family supports evolutionary link between monocot and dicot PAMP perception systems (Morillo and Tax 2006). Despite the general similarity, response to certain elicitors may significantly differ in monocots and dicots. For example, PaNie elicitor and lipoplysaccharides induce cell death in dicots but not in monocots suggesting that PTI response to a particular elicitor can significantly differ between monocots and dicots (Veit et al. 2001, Desaki et al. 2006).

Signal transduction downstream of PAMP recognition also shares a certain level of homology between monocots and dicots. For example, *A. thaliana* LRR-RLK BRI1 and rice LRR-RLK Xa21 have been shown to employ the same downstream signaling. Chimeric protein, containing extracellular (signal perceiving) domain and transmembrane domain from *A. thaliana* BRI1 and kinase domain from rice Xa21, was able to elicit downstream signaling in rice cells (He et al. 2000). Downstream of PRRs monocot as well as dicot immune response employs WRKYs type transcription factors to up- or down-regulate defense related genes. Thus, rice protein Xa21 interacts with OsWRKY62 and over-expression of OsWRKY62 in Xa21 comprising rice compromised resistance to *Xanthomonas oryzae* (Peng et al. 2008). Similarly, HvWRKY1 and HvWRKY2 have been shown to repress PTI in barley (Shen et al. 2007), and in *A. thaliana* numerous WRKYs have been implicated in PTI related signaling (Xu et al. 2006). Despite the general similarity at signal pathway level, conservation of particular genes involved in PTI is less evident and can be misleading. For example, *A. thaliana POL* genes are involved in regulation of plant organ development (Gagne and Clark 2007), whereas homologous protein XB15 in rice interacts with Xa21 and regulates disease resistance related responses (Park et al. 2008).

In order to ensure more specific and thereby also more efficient defense, plants have developed ETI, which is based on R-gene mediated pathogen recognition (Jones and Dangl 2006). Majority of R-genes belong to the family of NBS-LRR proteins, and, based on the presence of coiled-coil or Toll/interleukin-1 receptor like motifs, NBS-LRR proteins are categorized into TNS (TIR-NBS-LRR) or CNS (CC-NBS-LRR) subfamilies (McHale et al. 2006). TNS class of R-genes is thought to have evolved after monocot-dicot split since it is not represented in the major monocot species (Pan et al. 2000, Tarr and Alexander 2009). Large diversity and lack of colinearity or synteny of R-genes within grass family suggests, that monocot R-genes are subjected to more rapid evolution than the rest of the genome (Leister et al. 1998). However, despite the above mentioned differences, R-gene mediated signaling pathways are suggested to be largely conserved among monocots and dicots (Ayliffe and Lagudah 2004). Thus, for example, RAR1 mediated signaling is required for a particular race-specific *Bgh* resistance in barley (Shen et al. 2003, Bieri et al. 2004), as well as for RPS2 mediated *A. thaliana* resistance to *Pseudomonas syringae* (Takahashi et al. 2003). Besides, functionality of both AtRAR1 and HvRAR1 depends on the ability to interact with HSP90, and the mechanism of the interaction is highly conserved among *A. thaliana* and barley (Takahashi et al. 2003). Cooper et al. (2003) were able to identify *A. thaliana* proteins involved in AtSGT1 mediated pathogen response network, based on the sequence homology with rice proteins interacting with OsSGT1. However, only very few R-genes have been

identified in monocots, which makes it difficult to understand the true level of conservation of R-gene mediated signaling across plant classes (Martin et al. 2003).

Examples of resistance pathways, sharing high monocot-dicot homology at level of particular genes and mechanisms are rare. Resistance to biotrophic fungus - causal agent of powdery mildew – is an excellent evidence for highly conserved resistance pathway. *A. thaliana* gene *PEN1,* implicated in establishment of basal penetration resistance against non-host pathogen *Blumeria graminis* f.sp. *hordei* in *A. thaliana,* is able to complement barley mutant *ror2-1,* impaired in *mlo* mediated and also basal *Bgh* resistance (Collins et al. 2003). Evolutionary conservation of signaling pathways ensuring basal and race non-specific powdery mildew resistance between barley and *A. thaliana* is also confirmed by the fact, that functional AtMLO in *A. thaliana,* similarly to HvMLO in barley, is required for susceptibility to virulent powdery mildew (Consonni et al. 2006). Recent studies have confirmed, that the homology of MLO mediated signaling between *A. thaliana* and barley extends beyond the main components, such as ROR or MLO, and involves numerous other proteins (Humphry et al. 2010).

The primary infection can lead to establishment of resistance to subsequent infections in distal tissues – phenomenon called systemic acquired resistance (SAR). Increased resistance to secondary infections has been reported in several monocots – rice, wheat and barley (Cho and Smedegaard-Petersen 1986, Stenzel et al. 1985, Smith and Metraux 1991, Krishnamurthy and Gnanamanickam 1998) suggesting, that similarly to dicots, SAR operates also in monocots. SAR can also be induced artificially using chemical inducers mimicking SA action (Conrath 2006). Chemical inducers of SAR such as BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester) or INA (2,6-dichloro-isonicotinic acid) have successfully been applied to induce disease resistance in both – dicots as well as monocots (Görlach et al. 1996, Morris et al. 1998, Vallad and Goodman 2004).

Despite the fact, that SAR operates in dicots, as well as monocots, there are several substantial differences in mechanisms, underlying SAR between both plant classes (Kogel and Langen 2005). One of the main differences is defined by the role of SA for SAR establishment. Accumulation of salicylic acid (SA) is a molecular marker, characterizing onset of SAR (Durrant and Dong 2004). SA mediated signaling is crucial for dicot defense, whereas the role of SA in monocot disease resistance is ambiguous (Vlot et al. 2009). In *A. thaliana* bacterial and fungal infections are usually accompanied by increased endogenous SA level (Alvarez 2000), whereas, for example, in barley SA concentration does not increase after race-specific interaction with fungal pathogen *Blumeria graminis,* but inoculation with non-host pathogen *Pseudomonas syringae* triggers SA accumulation (Hückelhoven et al. 1999). In rice endogenous level of SA does not rise after bacterial or fungal infection, and PR gene induction is SA independent, suggesting that SA in rice does not act as a messenger molecule for systemic resistance (Yang et al. 2004). Such invariability of endogenous SA level in rice has often been explained by the fact, that rice constitutively comprises very high basal level of SA (Chen et al. 1997). Ineffectiveness of exogenously applied SA on induction of PR genes has also been observed in barley (Vallelian-Bindscheldler et al. 1998) and wheat (Molina et al. 1999). However, overexpression of PR genes in transgenic wheat triggers lesion formation and over-accumulation of SA (Anand et al. 2003), suggesting that the knowledge of the role of SA in wheat PTI is ambiguous. Despite the fact, that SA is unlikely to operate as a

signaling molecule in rice resistance to bacterial pathogens (Silverman et al. 1995), overexpression of SAR related *A. thaliana* gene *NPR1* enhanced disease resistance of rice (Chern et al. 2001), suggesting that signaling pathways downstream of SA accumulation might be conserved between both groups of plants. On the contrary to rice, overexpression of *A. thaliana NPR1* in maize did not affect disease resistance, and SA deficiency did not compromise pathogen response (Balint-Kurti and Johal 2009). NahG transgenic rice has been shown to over-accumulate ROS and develop HR like lesions, suggesting that SA might have an antioxidative function in rice (Yang et al. 2004). Antioxidative effect of SA has also been observed in wheat (Agarwal et al. 2005). Thus, although SAR operates in monocots, it employs signaling mechanisms at least partially distinct from those described in dicots.

Recently it has been observed that acquired resistance in barley, induced by avirulent *Pseudomonas syringae* pv. *tomato*, is restricted to adjacent leaf area, in contrast to *A. thaliana*, where acquired resistance expands systemically (Colebrook et al. 2012). Despite such spatial differences, transcriptional profiling identified substantial overlap in gene sets, differentially-regulated in response to avirulent pathogen in adjacent tissues of barley and systemic tissues of *A. thaliana* (Colebrook et al. 2012). Taking into account these similarities, it might be expected, that mechanisms, regulating immunization of uninfected tissues in monocots, might be more similar to dicotyledonous plants, than previously thought, although this issue still requires further research.

1.4. Methodological approaches to identification of HR related genes

Identification of molecular mechanisms and genes, regulating HR, is a popular topic of plant pathology research. Studies, performed to identify and characterize HR related genes, apply wide range of methodological approaches including both – forward as well as reverse genetics.

By far the most common approach to identification of disease resistance or HR related genes is based on cloning of genes, underlying mutant phenotype displaying altered disease resistance or HR. Map based cloning (MBC) is a traditional approach, allowing identification of genes and mutations responsible for mutant phenotype. MBC utilizes information about inheritance of studied phenotypic trait and its linkage to known molecular markers in mapping population, to identify genome region, comprising mutation, underlying mutant phenotype. MBC has successfully been applied to identify genes, underlying *lmm* phenotype in *A. thaliana* (for example, *CNGC4* (Jurkowski et al. 2004), *LSD1* (Dietrich et al. 1997), *CPR11/12* (Yoshioka et al. 2006)), rice (*SPL28* (Qiao et al. 2010), *SPL7* (Yamanouchi et al. 2002), *SPL11* (Zeng et al. 2004)), barley (*MLO* – Brüschges et al. 1997). Although MBC is widely applied in model plants, it has several requirements restricting its application. The outcome and success of MBC largely depends on density of polymorphisms, and thereby also molecular markers in target region. Whole genome sequencing has significantly fostered polymorphism identification, and specific databases containing information on whole genome polymorphisms have been developed in *A. thaliana* (Jander et al. 2002) and rice (Shen et al. 2004). Although considerable efforts of numerous scientists have been applied to develop new markers and increase marker density in barley (Wenzel et al. 2006, Marcel et al. 2007, Stein et al. 2007, Close et al. 2009), average marker density in barley (2.7 SNP per 1cM (Close et

al. 2009) or one marker per 0.99cM (Muñoz-Amatriaín et al. 2011)) still is significantly lower, than that of *A. thaliana* (one SNP per 3.3kb (Jander et al. 2002)) and rice (one SNP per 268 bp (Shen et al. 2004)).

Comparatively lower marker density and large genome size makes positional cloning in barley a challenging task. Anticipated barley genome sequence will undoubtedly facilitate map-based cloning of barley genes in future. Currently map-based cloning in barley can be accelerated using information about gene synteny in related species, comprising whole genome sequence, such as rice, *Brachypodium* or *Sorghum*. However, only 13%, 14% and 20% of barley genes are estimated to be syntenically positioned in sorghum, rice and *Brachypodium* respectively (Mayer et al. 2011), rendering synteny based gene identification in barley applicable to only certain barley genes.

Alternatively, genes, underlying *lmm* phenotype, can be identified using transcript based cloning (TBC). In case, when mutations conferring mutant phenotype affect transcript abundance of mutated gene, comparison between mutant and wild type transcriptome can identify gene underlying mutant phenotype (Mitra et al. 2004). This approach can be particularly useful, if mutants, comprising large deletions or premature stop codons, altering mRNA abundance or stability, are available (Mitra et al. 2004). TBC has so far been successfully applied to identify mutation underlying necS *lmm* phenotype (Zhang et al. 2009 b), as well as to identify candidate genes for barley stem rust resistance suppressor gene *Rpr1* (Zhang et al. 2006 a) and *bcd1* mutant (Xi et al. 2009). In all of these studies TBC has been applied to mutations, caused by ionizing radiation. Fast neutron mutants can be especially advantageous for TBC, since this type of mutants comprise large deletions, usually spanning for several kb (Li et al. 2001).

Previously described methods of HR related gene identification are based on forward genetics approach, when genes underlying mutant phenotype are searched. On contrary to forward genetics, reverse genetics approaches help to assign molecular functions to known genes. Therefore, reverse genetics approaches can be applied, to find out, if known barley genes are involved in HR. Barley genes potentially involved in HR regulation can be identified based on sequence homology to major model plants – *A. thaliana* or rice. Studies of molecular mechanisms, underlying plant disease resistance, suggest that at least major principles of plant immunity might be conserved among plants (discussed in chapter 1.3.4). Therefore, molecular mechanisms, underlying HR in widely studied model organisms, can potentially help in identification of HR related genes also in barley. Rostoks et al. (2003) was able to identify several barley HR related genes based on sequence homology with maize *hypersensitive-induced reaction* (*HIR*) genes. Similarly, educated guess, based on knowledge, obtained from *A. thaliana* CNGC4 mutation *hlm1* studies, allowed identification of CNGC4 mutation in barley fast neutron mutants FN085 and FN338 (Rostoks et al. 2006).

However, identification of mutant, bearing mutation in studied gene, is not always straight forward, and may require screening of large mutant populations. Mutant identification, using TILLING (targeting induced local lesions in genomes) procedure is one of solutions. Generation and maintenance of TILLING population is time and resource consuming, however, TILLING screening can be performed in collaboration with laboratories specialized in TILLING. There are several barley TILLING populations currently publicly available (Kurowska et al. 2011). TILLING screening has several advantages. First, large

mutant populations increase chances of identification of series of allelic mutants, which can be used to reveal functional role of protein domain or particular amino acid residues (Weil 2009). Second, since mutants are maintained in population, instead of isolating a single line, lethal recessive mutations can be maintained in heterozygotic state. Despite these advantages examples of TILLING application to barley mutant identification of known genes (reverse genetics approach) are rare and mainly restricted to initial characterization of newly developed barley TILLING populations and to genes, which already comprise characterized mutants (Caldwell et al. 2004, Talamé et al. 2008, Lababidi et al. 2009, Gottwald et al. 2009). To my knowledge, there are only two examples of successful TILLING-based identification of mutations in barley genes, for which previously no mutations have been reported - identification of *ahas* mutant, comprising imidazolinone herbicide resistance (Lee et al. 2011) and starch metabolism related mutant discovery (Bovina et al. 2011). However, taking into account that majority of barley TILLING populations have been developed in comparatively recent past, it can be expected that the number of studies applying TILLING technique to barley mutant identification will only grow.

1.5. Lesion mimic mutants – plants with impaired HR

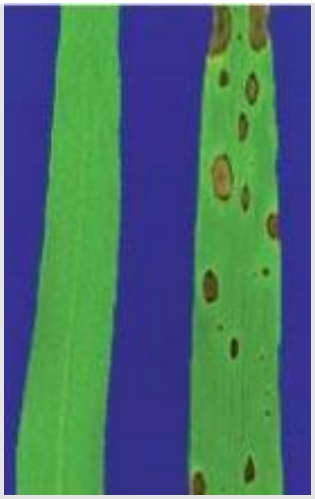
Studies, aiming at identification of molecular mechanisms regulating HR, have significantly benefited from characterization of mutations, misregulating HR related cell death. Mutant plants, displaying impaired HR phenotype, are generally referred to as lesion mimic mutants (*lmm*) in *A. thaliana* and maize, spotted leaf mutants (*spl*) in rice and necrotic mutants in barley (Moeder and Yoshioka 2008, Huang et al. 2010 a, Søgaard and Wettstein-Knowles 1987). *lmm* is a very diverse group of mutants – it encompasses mutants displaying various phenotypical appearances and derived by application of different mutagens. Necrotic leaf mutants of barley are represented among barley, mutagenised by different types of radioactive radiation - fast neutron radiation (Wright et al. 2007), X-ray and gamma rays (Lundqvist et al. 1997), chemical mutagens - ethyl methanesulfonate (Caldwell et al. 2004) and sodium azide (Talamé et al. 2008), as well as naturally occurring mutants (Rostoks et al. 2006). Although currently only several mutations responsible for necrotic phenotype in barley have been characterized or mapped to a particular chromosome (Table 1), many more *lmm* of barley are reported from barley mutant populations (Talamé et al. 2008, Caldwell et al. 2004).

Depending on a specific mutation, lesions of *lmm* can be expressed at various stages of plant development – for example, *nec1* phenotype becomes apparent on 7-14 days old seedlings whereas lesions on barley mutant *nec2* appear on 3-6 weeks old plants or in case of *nec7*, even after heading (Franckowiak and Lundqvist 1997). Besides, genetic background also has a significant effect on timing of lesion formation, for example *nec3* mutant in Steptoe (mutant FN362) develops lesions two weeks after germination whereas *nec3* mutation in cv. 'Villa' becomes apparent only shortly before heading (Franckowiak and Lundqvist 1997). Although in general the timing of lesion development seems to be genetically determined, at least for certain mutants phenotypical appearance can be triggered and enhanced by a specific environmental conditions (Johal 2007). For example, exposure to high intensity light or long day growth conditions promote *lmm* phenotype of *A. thaliana lsd1* and *acd11* (Mateo et al. 2004, Brodersen et al. 2002), maize *lls1* (Gray et al. 2002), rice *blm* (Jung et al. 2005). Lesion

formation can also be triggered by high temperature treatment, as demonstrated for rice *spl* mutants (Matin et al. 2010).

Depending on the actual phenotypic appearance and timing of HR-like spots, *lmm* are referred to as either initiation or propagation *lmm* (Lorrain et al. 2003). Initiation *lmm* display restricted localized necrotic spots, whereas on propagation *lmm* necrotic spots spread throughout the leaf, resulting in complete leaf collapse. Occurrence of two types of *lmm* suggests, that plants have evolved molecular mechanisms, regulating initiation of HR or signal perception, leading to HR, and also mechanisms, regulating further spread of cell death and probably cell-to-cell communication, directing this spread (Dangl et al.1996, Moeder and Yoshioka 2008).

Table 1. Characteristics of barley lesion mimic mutants of known map position (Lundqvist et al. 1997) .

Locus	*Necrotic leaf spot 1*	*Necrotic leaf spot 2*	*Necrotic leaf spot 3*	*Necrotic leaf spot 4*	*Necrotic leaf spot 5*	*Necrotic Steptoe*
Symbol	*nec1*	*nec2*	*nec3*	*nec4*	*nec5*	*necS*
Mutagen*	gamma-rays and diethyl sulphate	EMS	X-ray	X-ray	EMS	EMS
Chromosome	1H	6H	6H	3H	3H	3H
Phenotype		N.A.		N.A.	 (Saisho and Takeda 2011)	 (Zhang et al. 2009b)

* – Mutagen used to induce original mutation (allele)

N.A. – photo not available

1.5.1. LSD1

1.5.1.1. LSD1-Zn finger domain containing protein

LSD1 belongs to a class of *LSD1*-like genes, defined by the presence of conserved *LSD1* type Zn-finger (zf-*LSD1*) domain CxxCxRxxLMYxxGASxVxCxxC (PF06943) (Dietrich et al. 1997). *LSD1*-like genes are plant specific and are represented in flowering plants, as well as mosses and algae - suggesting an ancient origin of this class of proteins in plants (Liu and Xue 2007). *A. thaliana* genome contains six *LSD1*-like genes – multiple zf-*LSD1* domain containing genes - *LSD1* (At4g20380), *LOL1* (At1g32540), *LOL2* (At4g21610) and single zf-*LSD1* domain containing genes - *LOL3* (or *AtMCP1* - At1g02170), *AtMCP2* (At4g25110) and *AtMCP3* (At5g64240) (Epple et al. 2003, Coll et al. 2010). In rice genome eight *LSD1*-like genes have been identified, including single as well as multiple zf-*LSD1* domain containing genes (Liu and Xue 2007). Diversification of *LSD1*-like proteins is thought to have occurred before monocot and dicot divergence as a result of two distinct duplications, yielding two sub-classes of *LSD1*-like genes – single zf-*LSD1* domain containing and multiple zf-*LSD1* containing genes (Liu and Xue 2007).

Proteins, belonging to both subfamilies (single or multiple zf-*LSD1* domain containing), have been implicated in plant cell death regulation. Single zf-*LSD1* domain containing proteins comprise also peptidase-C14 domain (PF00656) at their C-terminus. These proteins participate in plant cell death regulation during embryogenesis (Suarez et al. 2004), and are homologous to yeast caspase-1 (Yca1), regulating apoptosis in yeast (Watanabe and Lam 2005). Multiple zf-*LSD1* containing proteins have also been assigned cell death related functions in plants, regulating PCD in either pro- or anti-apoptotic manner, depending on a specific gene (Epple et al. 2003, Coupe et al. 2004).

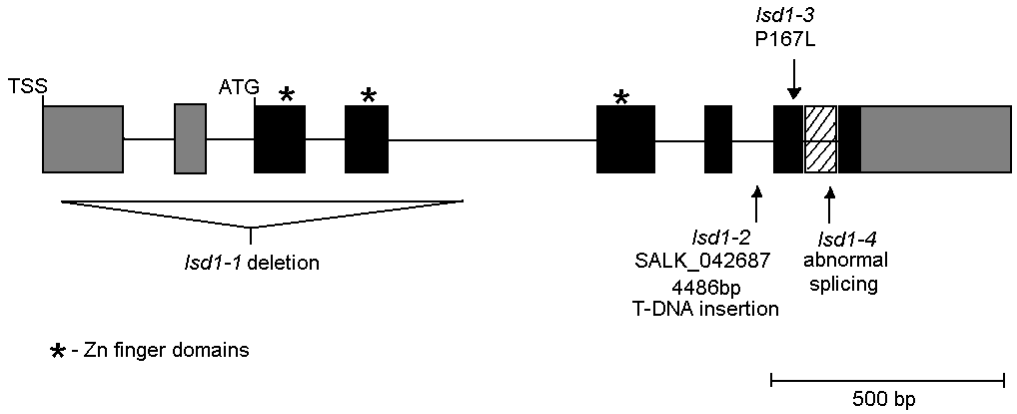


Figure 2. Structure of *AtLSD1* and position of *lsd1* mutations. Black boxes represent exons of coding part of the gene, grey boxes represent non-translated part of mRNA. TSS-transcription start site, ATG-translation start site, asterix marks location of *LSD1* type Zn finger domains. Deletion causing *lsd1-1* mutation, T-DNA insertion site of *lsd1-2* mutation and *lsd1-3* amino acid substitution P167L are indicated. *lsd1-4* mutation caused by 29 amino acid insertion due to abnormal splicing is depicted as striped box.

AtLSD1 (At4g20380) is 189aa long Zn finger protein, which was cloned and first described in 1997 by Dietrich and colleagues. The gene contains three zf-*LSD1* domains, which are located in the first three exons of the protein (Figure 2). *LSD1* forms homodimers, and it is localized in cytoplasm and nucleus (Walter et al. 2004). According to Genevestigator data (Zimmermann et al. 2004), *LSD1* is constitutively expressed in all tissues with particularly high expression in mature pollen, and it is up-regulated upon drought and *Pseudomonas* infection, whereas down-regulated in etiolated seedling upon shift to light.

1.5.1.2. lsd1 – propagation lesion mimic mutant

Four allelic *A. thaliana lsd1* mutants have been characterized so far. *lsd1-1* mutation in the Ws-0 background comprises deletion, encompassing first two exons of the *AtLSD1* (At4g20380) (Dietrich et al. 1997) (Figure 2). *lsd1-2* is a T-DNA insertion line, generated in Col-0 background using *Agrobacterium tumefaciens* mediated transformation with plant binary vector pROK2 (Alonso et al. 2003). *lsd1-3* and *lsd1-4* comprise EMS induced SNPs in *LSD1*, causing either amino acid substitution P167L or 29AA insertion close to C-terminal end of the *LSD1* (Huang et al. 2010 b). *lsd1-1* is a null mutation, whereas *lsd1-3* and *lsd1-4* are still able to produce mutant *LSD1* protein, although at a diminished concentration, compared to a wt protein in Columbia (Huang et al. 2010 b).

lsd1 belongs to a broad class of lesions simulating disease resistance response mutants. Lesion formation in *lsd* mutants can either be spontaneous or conditionally induced, and it is usually accompanied by expression of disease resistance related responses, such as induction of PR genes and elevated level of SA production (Dietrich et al. 1994). *lsd1* is propagation *lmm*, meaning that lesion initiation in the mutant does not occur spontaneously, but it requires triggering factors, however, once induced, lesions spread unrestricted, causing phenomenon known as runaway cell death. Lesion formation in *lsd1* can be induced by either pathogen infection (Dietrich et al. 1994) or by certain abiotic factors, promoting excess excitation energy formation in plant (Mateo et al. 2004). Long day conditions (or continuous photoperiod) or increased light intensity (>450 μmol m^{-2} s^{-1}) trigger cell death in *lsd1-1*, starting from a single lesion, which spreads and kills the entire leaf within 2-4 days (Dietrich et al. 1997) (Figure 3). Experiments with various wavelength light application and introduction of PSII associated mutation *cao* into *lsd1-1* have proved , that *lsd1-1* phenotype is related to activity of PSII rather than PSI (Mateo et al. 2004).

The actual biochemical activator or the initial signal, causing cell death in *lsd1* is uncertain. It has been suggested, that H_2O_2 generated during photorespiration might be the elicitor of cell death in *lsd1* (Mateo et al. 2004), however, external application of H_2O_2 does not cause unrestricted lesion spread in *lsd1* (Jabs et al. 1996). Indirect evidences suggest, that superoxide could be the causal agent of *lsd1-1* phenotype, since treatment with superoxide generating system xanthine and xanthine-oxidase induced lesion formation in *lsd1* (Jabs et al. 1996). Devastating effect of superoxide on *lsd1-1* might be attributable to impaired regulation of Cu Zn superoxide dismutase in the mutant (Kliebenstein et al. 1999).

The source of superoxide causing runaway cell death phenotype of *lsd1-1* is yet uncertain. Although diphenyleneiodonium (DPI) – potent inhibitor of NADPH oxidases – significantly reduced lesion formation in *lsd1* (Jabs et al. 1996), this alone does not prove the

NADPH oxidases as a source of lesions triggering superoxide due to unspecific inhibitory effect of DPI affecting all flavoenzymes. Besides, *lsd1*/*atrbohD/atrbohF* triple mutants had more severe phenotype than *lsd1-1*, whereas overexpression of *AtRbohD* in *lsd1-1* attenuated mutant phenotype, suggesting that superoxide generating plasma membrane NADHP oxidases restrict, rather than promote runaway cell death in *lsd1* (Torres et al. 2005). Although the mechanism of LSD1 and AtRboh interaction is yet unknown, it has been suggested, that both proteins act as negative cell death regulators in the same signaling pathway, adjusting ROS levels through induction of antioxidative enzymes (Torres et al. 2005). DPI inhibitory effect on *lsd1-1* runaway cell death phenotype might be attributable to interference with other, yet unidentified flavon-containing enzyme. DPI is known to inhibit nitric oxide synthase (EC 1.14.13.39) – NO generating enzyme in mammals. Nitric oxide synthase (AtNOS1) and nitrate reductase (NR) (EC 1.6.6.1-3) are considered to be the most likely candidates for NO source in plants (Wendehenne et al. 2001, del Río et al. 2004). Both enzymes have also been implicated in plant pathogen response signaling (Yamamoto et al. 2003, Zeidler et al. 2004). ROS pro-apoptotic effect has been shown to depend on ROS/ NO balance (Delledonne et al. 2001, Zago et al. 2006, Hong et al. 2008), suggesting that DPI might affect *lsd1* phenotype through inhibition of NO generating flavoenzyme. However, unlike animal NOS, AtNOS1 does not require FAD as cofactor (Guo et al. 2003) and is unlikely to be inhibited by DPI. Therefore the question regarding DPI effect on *lsd1-1* phenotype remains yet unresolved.

On contrary to *lsd1-1*, *lsd1-3* and *lsd1-4* does not differ phenotypicaly from wt Col plants at 20-22°C, even if grown at long day conditions. Lesion formation on *lsd1-3* and *lsd1-4* requires low temperature treatment (Huang et al. 2010 b).

lsd1 has gained an interest of plant researchers not only for its misregulated cell death phenotype, but also for its altered disease resistance. Induction of runaway cell death is accompanied by constitutive activation of SAR like responses, such as over-accumulation of SA and over-expression of *PR-1* in *lsd1-1* (Dietrich et al. 1994). SA over-production and *PR-1* over-expression has also been observed for *lsd1-3* and *lsd1-4* (Huang et al. 2010 b). *lsd1-1* mutation renders *A. thaliana* plants more resistant to biotrophic oomycete *Peronospora parasitica* and to biotrophic bacteria *Pseudomonas syringae* pv. *tomato* (Rustérucci et al. 2001, Aviv et al. 2002).

Figure 3. *lsd1-1* mutant (left) and wt Ws-0 (right). Both plants are grown at long day (16h day/ 8h night) conditions for four weeks.

1.5.1.3. Physiological and molecular functions of LSD1- position of LSD1 in plant stress signaling pathways

Based on *lsd1* runaway cell death phenotype, it has been suggested, that *LSD1* might act as a negative regulator of cell death in plants by adjusting the threshold level of ROS, required to initiate cell death (Dietrich et al. 1997). Induction of *lsd1* lesions by superoxide (Jabs et al. 1996), and inability of *lsd1* to induce CuZn superoxide dismutase (CuZnSOD) (Kliebenstein et al. 1999) suggests, that *LSD1* might be required for proper action of plant antioxidative system. This is also confirmed by the fact, that *lsd1* is sensitive to photorespiratory conditions, and it comprises reduced catalase activity and consequently also elevated accumulation of H_2O_2 upon stress treatment (Mateo et al. 2004, Mühlenbock et al. 2007). Apart from above mentioned, *LSD1* has also been implicated in H_2O_2 mediated PCD regulation in response to hypoxic conditions (Mühlenbock et al. 2007) and ROS mediated cold acclimation (Huang et al. 2010 b). Thus, *LSD1* is involved in a wide range of stress responses, requiring ROS signaling, suggesting that *LSD1* might be required at an early stage of universal stress signal transduction upstream of divergence of specific stress response pathways.

It has been shown, that anti-apoptotic effect of *LSD1* in response to ROS is achieved through prevention of CuZnSOD down-regulation (Kliebenstein et al. 1999), which is probably mediated by pro-apoptotic Zn finger protein LOL1 (*LSD1* like 1) (Epple et al. 2003). Although the precise molecular mechanisms of LSD1/LOL1 antagonistic control of cell death are yet unknown, it has been hypothesized, that both genes compete for the same transcription factors, regulating cell death (Kaminaka et al. 2006). Transcription factor AtbZIP10 acts as a positive regulator of cell death, and its pro-apoptotic effect depends on nuclear localization of the protein. Studies of LSD1/AtbZIP10 interaction have shown, that LSD1 retains AtbZIP10 in cytosol, thus preventing AtbZIP10 from transcription initiation of pro-apoptotic signaling pathway in nucleus (Kaminaka et al. 2006). Using yeast two-hybrid system, *LSD1* has been shown to directly interact with a related Zn-finger domain protein LOL1 (Epple et al. 2003), with pro-apoptotic metacaspase AtMC1 (Coll et al. 2010) and also with anti-apoptotic protein GILP (He et al. 2011). Thus, LSD1 likely executes its role of an anti-apoptotic regulator through direct interaction with numerous cell death regulatory proteins.

Anti-apoptotic activity of LSD1 is involved in plant disease resistance related cell death regulation, since *lsd1* null mutant shows altered disease resistance phenotype, over-accumulates SA and over-expresses *PR-1* (Dietrich et al. 1994). LSD1 has been shown to operate downstream of *EDS1* and *PAD4* in SA mediated signaling (Rustérucci et al. 2001). EDS1 and PAD4 interact to ensure TIR-NB-LRR type R-gene mediated plant disease resistance, and this interaction amplifies SA mediated defense signaling (Feys et al. 2001). Role of LSD1 in disease resistance seems to be evolutionary conserved also in monocots. Recently *HvLSD1b* – a barley homologue of *AtLSD1*- has been shown to co-locate with race-non-specific *Bgh* resistance QTL in barley (Spies et al. 2012). Expression of rice *OsLOL2* (*LSD1*-like) in tobacco induces *PR* gene expression and renders plants more resistant to bacterial wilt (Bhatti et al. 2008, Bhatti et al. 2011), whereas antisense lines of *OsLOL1* over-express *PR1* and develop lesion mimic phenotype (Wang et al. 2005). Besides, recently bamboo (*Bambusa oldhamii*) homologue of *AtLSD1* has been shown to respond to pathogen

infection and exogenous treatment with hydrogen peroxide and functional analogue of SA – INA (Yeh et al. 2011).

LSD1 has also been implicated in plant excess excitation energy (EEE) acclimation (Mateo et al. 2004). Knowledge of dual effect of LSD1 (involvement in SA mediated disease resistance pathways and EEE acclimation) has facilitated discovery of details of molecular mechanisms, linking SA signaling and light acclimation in plants. EEE triggers redox changes in plastoquinone (PQ) pool– redox sensor of photosynthetic electron transport chain - which than leads to induction of ROS stress signaling in plants (Walters 2005). LSD1 is thought to act as a negative regulator of ROS accumulation in response to PQ redox change (Mühlenbock et al. 2008), which most likely is achieved by up-regulating CuZnSOD and catalase (EC 1.11.1.6) (Kliebenstein 1999, Mateo et al. 2004). Thus, LSD1 acts as a safety-valve for cellular ROS responses, by inhibiting stress signal propagation in case, when the signal does not exceed certain threshold. LSD1 has been suggested to participate in light acclimation also in monocots, since rice *OsLSD1* expression is induced in response to light and suppressed by dark (Wang et al. 2005).

It is yet unknown, how do the cell differentiates between situations, which require suppression of ROS spread, to prevent unnecessary stress response, causing unrestricted spread of cell death, and situations, where LSD1 inhibition of ROS accumulation might cause a dangerous delay in stress response, by precluding induction of systemic resistance. One of the possible mechanisms, determining outcome of LSD1/LOL1 antagonism, might depend on glutathione level, since *lsd1* lesion formation and hypersensitivity to low temperature treatment have been shown to require glutathione biosynthesis (Senda and Ogawa 2004, Huang et al. 2010 b). ROS balance and redox state of cellular antioxidant pools have also been shown to influence activity of NPR1 – protein required for induction of *PR* genes during SAR (Mou et al. 2003). *lsd1* runaway cell death requires NPR1, and *lsd1* constitutively over-expresses *PR1*, suggesting that LSD1 acts as a negative regulator of NPR1 signaling (Aviv et al. 2002).

1.5.2. Cyclic nucleotide gated ion channel 4 (CNGC4)

1.5.2.1. Structure of plant cyclic nucleotide gated ion channels (CNGCs)

The fact, that cNMPs regulate wide range of cellular processes, has created scientific interest in finding molecular targets of cNMP signaling in plants (Bridges et al. 2005). CNGCs are good candidates for cNMP mediated signaling in plants, since CNGCs contain cyclic nucleotide binding (CNB) domain, and their permeability can be regulated by cNMPs (Leng et al. 2002). *CNGC* gene family comprises 16 members in rice (Bridges et al. 2005) and 20 members in *A. thaliana* as a result of gene duplication (Mäser et al. 2001). Like their animal counterparts, plant CNGCs contain six transmembrane domains, with a pore region located between the 5^{th} and 6^{th} transmembrane domain (Hua et al. 2003 a). Although the general structure of animal and plant CNGCs is similar, there is a major difference in the position of regulatory cNMP binding (CNB) and calmodulin binding (CaMB) domains. In plant CNGCs CNB domain and CaMB domain overlap and are located in the C-terminal part of the protein, whereas animal CNGCs comprise their CaMBD at the N-terminal part of the

protein (Köhler and Neuhaus 2000). CNBD is also predicted to interact with C-linker (sequence between CNBD and pore region), thus stabilizing conformation of the channel. Mutation, causing change of highly conserved AA in C-linker of CNGC11/12, leads to suppression of the characteristic phenotype, suggesting that C-linker is important for the normal functioning of CNGCs (Baxter et al.2008).

Intrinsic functional characteristics of a particular plant CNGC are largely determined and affected by the sequence of pore domain, consisting of three specific regions– pore helix, selectivity filter and flanking helix from 6^{th} transmembrane domain (Talke et al. 2003). Pore helix is thought to stabilize cation, while it is being conducted through the pore (Hua et al. 2003 b). The functional significance of the pore helix can be illustrated by the fact, that SNP, causing G to D change within the conserved part of pore helix of CNGC4, resulted in lesion mimic phenotype in barley (Rostoks et al. 2006). Selectivity filter is considered to be the part of the pore domain, determining the type of ions conducted by a specific CNGC. Selectivity filter is usually composed of three AA, located within the P-loop part of the pore domain (Leng et al. 2002). Mutations, disrupting AA triplet of AtCNGC2 selectivity filter, impair channel's ability to discriminate between K+ and Na+ (Hua et al. 2003 b). According to phylogenetic analysis, based on the pore region sequence, CNGCs can be grouped into four major groups – CNGCs from group I and II comprise conserved GQN triplet in P-loop, III group CNGCs P-loop triplet is GQG, but IV, the most distinctive group of plant CNGCs has either AND or AGN as a selectivity filter (Mäser et al. 2001).

1.5.2.2. Mechanisms of action/ regulation of CNGCs

Due to technical difficulties, conductivity and ion selectivity of plant CNGC has been preferentially studied, applying yeast mutant complementation approach, rather than heterologous expression (Kaplan et al. 2007). Studies employing complementation of cation transport deficient yeast strains for functional characterization of CNGCs have had various success. The first characterized plant CNGC HvCBT1 failed to complement yeast mutant CY162 deficient in K+ uptake (Schuurink et al. 1998), whereas successful complementation of CY162 by AtCNGC1 required application of mutated AtCNGC1 form, comprising deletion of CaMBD, due to inhibitory effect of yeast calmodulin (Ali et al. 2006). Until now electrophysiology of only four plant CNGCs – CNGC1, CNGC2, CNGC4 and CNGC10 has been studied, using heterologous expression in *Xenopus laevis* oocytes or HEK cells (Leng et al. 1999, Leng et al. 2002, Balagué et al. 2003, Christopher et al. 2007) (Table 2). According to these studies, cation selectivity varies among different plant CNGCs. CNGC10 is known to conduct K^+ (Christopher et al. 2007), CNGC1 and CNGC4 has been shown to be permeable to both – Na^+ and K^+ (Balagué et al. 2003, Hua et al. 2003 b), whereas CNGC2 conducts K+ and Ca^{2+}, but is non-permeable to Na+ (Leng et al. 1999, Leng et al. 2002). Although cation selectivity seems to be a specific characteristic of a particular plant CNGC, all CNGCs are subjected to the same molecular mechanisms, regulating channel permeability. cNMP binding to CNGC activates the channel, while CaM binding and high extracellular Ca^{2+} concentrations reverse cNMP mediated activation (Leng et al. 2002, Hua et al. 2003 a).

Table 2. Application of yeast mutant complementation approach to plant CNGC studies.

Plant CNGC	Yeast mutant	Success of complementation +/-	Reference
HvCBT1	K^+ transport deficient CY162 (*trk1/trk2*)	-	Schuurink et al. 1998
AtCNGC2	K^+ uptake deficient CY162 (*trk1/trk2*)	+ (Only in the presence of cAMP)	Leng et al. 1999
AtCNGC1	K^+ uptake deficient *trk1* and *trk2*,	+ (only with deletion of CaMBD),2,4	Ali et al.2006
AtCNGC2	K^+ uptake deficient *trk1* and *trk2*,	+ (only with deletion of CaMBD),2,4	Ali et al.2006
ATCNGC4	Ca^{2+} uptake deficient *mid1 cch1*	+ (only with deletion of CaMBD)	Ali et al.2006
AtCNGC3	G-19 hypersensitive to Na+ due to impaired Na+ extrusion	-	Gobert et al. 2006
	K^+ transport deficient CY162 (*trk1/trk2*)	Partial +	
	Ca^{2+} uptake deficient *mid1 cch1*	-	
AtCNGC10	K^+ uptake deficient CY162 (*trk1/trk2*)	+	Li et al. 2005
AtCNGC11	K^+ uptake deficient CY162 (*trk1/trk2*)	+	Yoshioka et al. 2006
AtCNGC12	K^+ uptake deficient CY162 (*trk1/trk2*)	+	Yoshioka et al. 2006
ATCNGC11/ 12	K^+ uptake deficient CY162 (*trk1/trk2*)	+	Yoshioka et al. 2006

Functional animal CNGCs have been shown to form heterotetramers (Kaupp and Seifert 2002). Although the *in vivo* structure of functional plant CNGCs is yet unknown, several evidence suggest that the hypothesis of heterotetramer formation cannot be excluded. First, the structure of the pore region of analyzed plant CNGCs does not preclude formation of ion conduction pathway, if the channel would consist of four subunits (Hua et al. 2003 b). Second, mutations in related ion channels CNGC2 and CNGC4 cause highly similar phenotype (dwarfed stature, impaired HR, increased disease resistance and spontaneous lesion formation), suggesting a possible involvement of these proteins in the formation of a common ion channel (Jurkowski et al. 2004). Besides, *dnd1*/*dnd2* double mutants are infertile, of low viability and comprise 'extreme' dwarf phenotype (Jurkowski et al. 2004). In addition, mutations affecting plant CNGCs often only partially impair cytosolic Ca^{2+} rise, suggesting

that absence of one channel component does not completely abolish channel formation (Qi et al. 2010).

According to publicly available gene expression data from Genevestigator (Zimmermann et al. 2004), plant *CNGCs* are expressed in different types of tissues, with no specific expression pattern characteristic for phylogenetic groups, defined by Mäser and colleagues (2001) (Figure 4). Subcellular localization of only very few plant CNGCs has been studied so far. HvCBT1, AtCNGC3, AtCNGC10, AtCNGC18 are known to reside in plasma membrane (Schuurink et al. 1998, Gobert et al. 2006, Christopher et al. 2007, Frietsch et al. 2007).

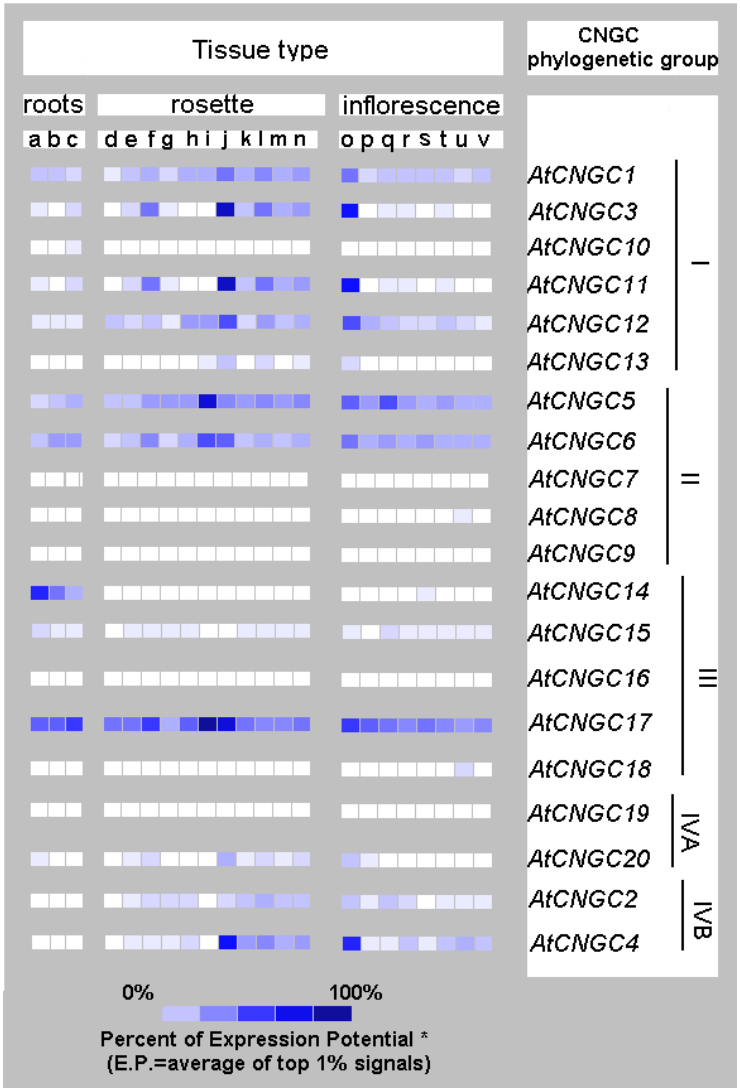


Figure 4. *A. thaliana CNGC* expression in various parts of plant according to Genevestigator (Zimmermann et al. 2004). CNGCs are grouped into phylogenetic groups according to Mäser et al. 2001. a-lateral roots, b-primary roots, c-roots; d-shoot apex, e-axillary shoot, f-axillary bud, g-stem, h-leaf primordia, i-hypocotil, j-senescent leaf, k-petiole, l-adult leaf, m-juvenile leaf, n-rosette; o-cauline leaf, p- shoot apex, q-node, r-stem, s-seed, t- silique, u-flower, v-inflorescence.

1.5.2.3. Functions of CNGCs in plants

Although it has been hypothesized, that plant CNGCs might be involved in ion homeostasis regulation, requiring cNMP and Ca^{2+} /CaM signaling (Talke et al. 2003), the precise molecular role has been assigned to very few plant CNGCs. Ascribed functions involve plant growth and development, disease resistance and abiotic stress response (Chin et al. 2009).

The most apparent role of plant CNGCs is related to their cation conductivity. CNGC3 has been suggested to participate in ion transportation, since *cngc3* mutants are more resistant to elevated Na^+ and K^+ concentrations (Gobert et al. 2006). Mutation disrupting CBP4 in *Nicotiana tobaccum* and CNGC1 in *A. thaliana* increased tolerance to Pb^{2+} (Sunkar et al. 2000), whereas over-expression of the NtCBP4 rendered transgenic plants more resistant to Ni^{2+} (Arazi et al. 2000). CNGC19 and CNGC20 have been hypothesized to participate in re-allocation of monovalent cations within plant, contributing to salt tolerance (Kugler et al. 2009). Together these findings support the role of CNGCs in plant cell ion homeostasis regulation.

Hypotheses considering CNGCs' role in plant growth and development are mainly based on the fact, that mutations, disrupting certain CNGCs, cause aberrant or retarded growth. Mutations disrupting CNGC2 and CNGC4 cause dwarfed stature in *A. thaliana* (Clough et al. 2000, Jurkowski et al. 2004). CNGC2 mutation also affects plant fertility, since mutant plants display abnormal flower development and impaired pollen tube growth at elevated Ca^{2+} conditions (Chaiwongsar et al. 2009). Impaired fertility has also been observed for *cncg18* mutants, which display altered pollen tube growth, most likely caused by disruption of general mechanisms, determining polarized cell growth (Frietsch et al. 2007). AtCNGC10 has also been implicated in plant growth regulation, since antisense lines of AtCNGC10 showed reduced root elongation, smaller leaf size and delayed gravitropism (Borsics et al. 2007).

Disrupted HR and spontaneous lesion mimic phenotype of several plant CNGC mutants has served as an indication of a possible role of CNGCs in plant immunity. Mutations disrupting CNGC2 and CNGC4 elicit spontaneous HR like phenotype and affect *A. thaliana* resistance to *Pseudomonas syringae*, through activation of SA mediated pathway, and resistance to *Botrytis cinerea*, through activation of ethylene mediated pathway (Genger et al. 2008). Mutation resulting in fusion of two distinct genes, encoding *CNGC11* and *CNGC12*, induces pathogenesis related genes in *A. thaliana*, and renders mutant plants more resistant to oomycete *Hyaloperonospora parasitica* (Yoshioka et al. 2006). Together these observations suggest that plant CNGCs might be involved in multiple signaling pathways regulating plant-pathogen interaction.

1.5.2.4. CNGC4 in barley

HvCNGC4 (AY972619) gene is localized on 5 (1H) chromosome, and it consists of four exons (Rostoks et al. 2006). HvCNGC4 protein is predicted to be 686 AA long, and, based on sequence analysis, it is expected to comprise six transmembrane domains, regulatory cyclic nucleotide binding domain and calmodulin binding domain (Rostoks et al. 2006). Although

HvCNGC4 shares only 67% overall AA identity with AtCNGC4 (At5g54250), some parts of the protein are highly conserved between barley and *A. thaliana*. Thus, the region including pore helix and selectivity filter is fully identical between *A. thaliana* and barley at AA level, whereas CNBD of HvCNGC4 shares 83% identical and 92% similar AA with AtCNGC4 (Figure 5).

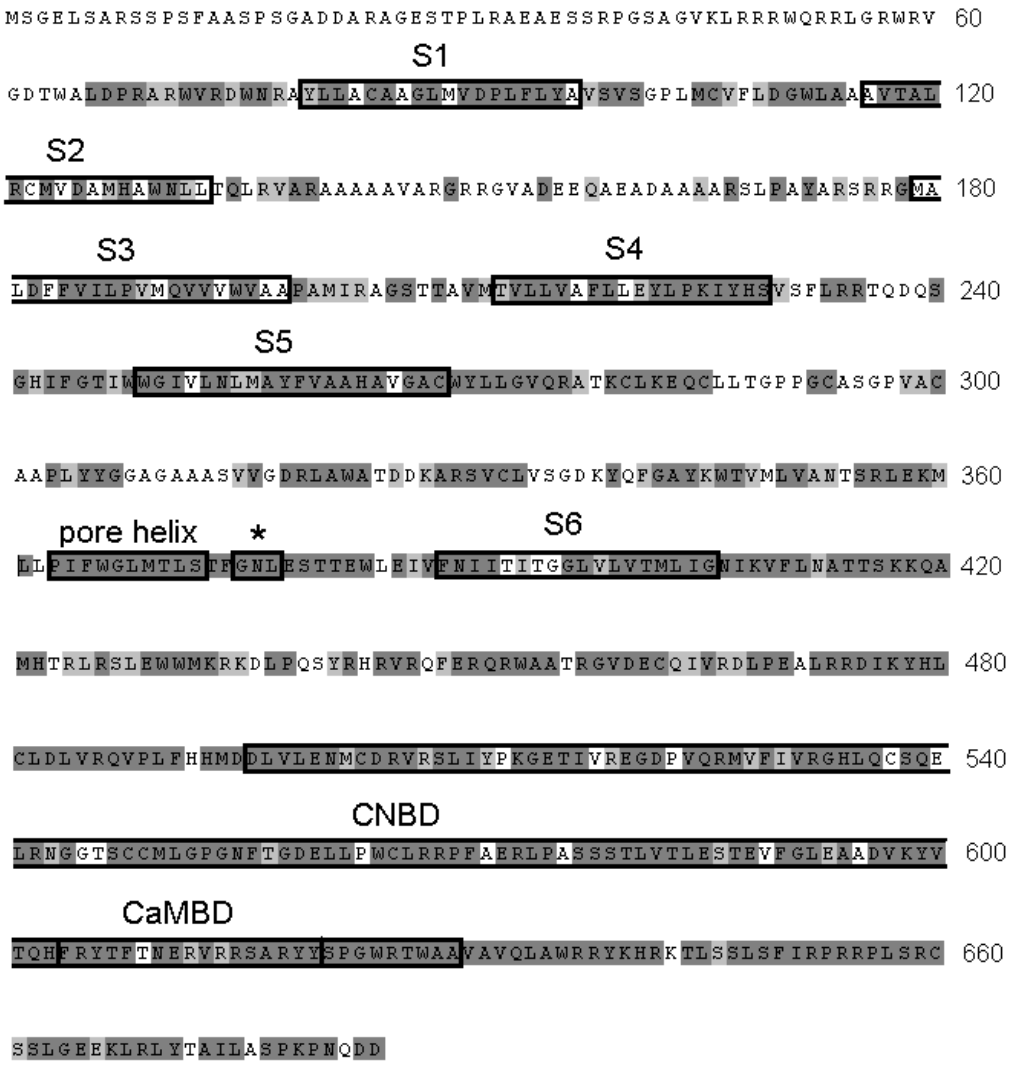


Figure 5. Amino acid sequence of HvCNGC4 – structure and homology with AtCNGC4. AA identical or similar to AtCNGC4 are highlighted with dark and light shading respectively. S1-S6 – membrane spanning regions, *-selectivity filter, CNBD – cyclic nucleotide binding domain, CaMBD – calmodulin binding domain.

1.5.2.5. dnd2/hlm1/nec1 – mutations of CNGC4, causing initiation lesion mimic phenotype

dnd2 and *hlm1* are the best characterized CNGC4 mutants in *A. thaliana*. *dnd2* was initially isolated in the screen of Col-0 EMS mutants, showing altered HR in response to activation of avrRpt1-RPS2 pathway (Yu et al. 2000). *dnd2* comprises G269A point mutation,

causing Trp change to premature Stop codon, located in the first transmembrane domain of *CNGC4* (Jurkowski et al. 2004). *hlm1-1* is a T-DNA insertion line generated in Ws-4 background, and it contains T-DNA insertion in the first intron of *AtCNGC4* (Balagué et al. 2003).

Phenotypically *dnd2* and *hlm1* are essentially identical. Both mutants comprise dwarfed stature, spontaneous microscopic lesions on leafs, absence of HR in response to avirulent pathogen, over-expression of *PR-1*, elevated levels of SA and altered disease resistance (Balagué et al. 2003, Jurkowski et al. 2004). The effect of *dnd2/hlm1* mutation on *A. thaliana* disease resistance is pathogen specific. Thus, despite the absence of HR, *dnd2* retains the same level or in certain cases (RPS4 mediated resistance) even elevated resistance to avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) (Jurkowski et al. 2004). Growth of virulent *Pst* on *dnd2* is also restricted, although to a lesser extent than that, observed for avirulent pathogens (Jurkowski et al. 2004). *hlm1* also exhibits elevated resistance to virulent, as well as to a majority of avirulent strains of *Pst* and *Xanthomonas campestris*, however, *RPS4* mediated gene-for-gene resistance is impaired in *hlm1* (Balagué et al. 2003). Apart from pathogenic bacteria, *dnd2* has also been shown to comprise elevated resistance to oomycete *Hyaloperonospora parasitica* and necrotrophic fungus *Botrytis cinerea* (Genger et al. 2008).

Although barley mutant *nec1*, displaying lesion mimic phenotype, was identified in 1970's (Fedak et al. 1972), identification of the actual gene, comprising the mutation was reported only in 2006 (Rostoks et al. 2006). There are several allelic *nec1* mutants in barley (Table 3), however, in this study *nec1* mutation has been characterized using spontaneous mutant GSHO1284 (further referred to as *nec1*), comprising MITE insertion in the second intron of the gene, causing frameshift and alternatively spliced gene product (Rostoks et al. 2006). *nec1* displays lesion mimic phenotype, which becomes visible at two to three leaf stage (7-14 days post germination) (Figure 6B), however, unlike *dnd2* in *A. thaliana*, *nec1* mutation in barley does not cause severe dwarfing (Figure 6A and 6C). Although *nec1* over-expresses *PR-1* (Rostoks et al. 2006), disease resistance of *nec1* has not previously been studied.

Table 3. HvCNGC4 mutants (Lundquist et al. 1997, Rostoks et al. 2006).

mutant / *nec1* allele	Mutation	Mutagen	Parental cv.
FN085	deletion of the gene	fast neutron irradiation	Steptoe
FN338	deletion of the gene	fast neutron irradiation	Morex
FN370	Gly367Asp	fast neutron irradiation	Steptoe
GSHO1284/ *nec1*.b	frameshift - MITE insertion	spontaneous mutation	Parkland
GSHO 989/ *nec1*.a	Trp130Stop	gamma-rays and diethyl sulfate	CarlsbergII

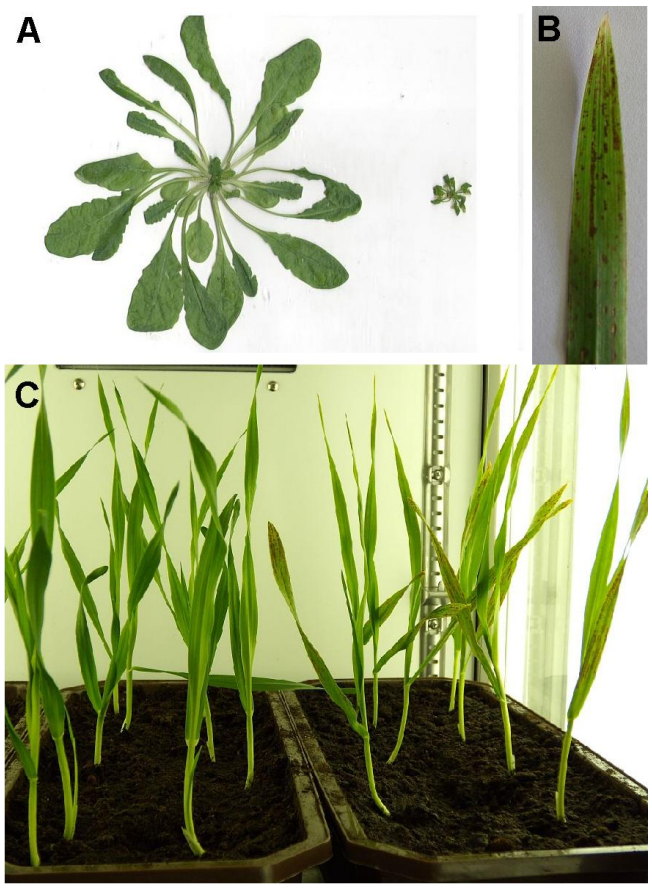


Figure 6. Mutations affecting *CNGC4* in *A. thaliana* and barley. A – *CNGC4* mutation in *A. thaliana*, wild type *Col* plant (left) and mutant *dnd2* (right); **B** – lesions on *nec1* leaf from 14 day old barley plant, C – *CNGC4* mutation in barley, wt Parkland plants (left) and *nec1* (right).

1.5.2.6. Position of CNGC4 in plant disease resistance signaling pathways

CNGC4 mutants display enhanced disease resistance and constitutive activation of SA mediated signaling (Balagué et al. 2003, Jurkowski et al. 2004), therefore it might be expected, that functional CNGC4 acts as a negative regulator or repressor of SA mediated plant defense responses. Introduction of bacterial *salicylate hydroxylase* gene (*NahG*) into *dnd2* background abolishes resistance to *Pst*, conferred by the mutation, suggesting that SA indeed might be required for *dnd2* phenotype (Jurkowski et al. 2004). However, *NahG* effect can be misleading, since introduction of *NahG* gene has also other physiological effects, apart from those caused by just a removal of SA (Glazebrook et al. 2003). Introduction of mutations, affecting SA signaling, into *dnd2* background has given a more reliable conclusions, concerning interaction between *dnd2* and SA. Mutations *sid2* and *pad4* impair SA mediated disease resistance - *SID2* encodes SA biosynthesis enzyme isochorismate synthase (Wildermuth et al. 2001), whereas *PAD4* acts upstream of SA (Wiermer et al. 2005). Disease resistance studies of *dnd2/sid2* double mutant show, that *dnd2* resistance to *Pst* requires functional *SID2* (Genger et al. 2008). Similarly, elimination of *PAD4* in *dnd2* background abolishes *dnd2* resistance to *Pst*, confirming that *dnd2* resistance to biotrophic bacteria is based on activation or de-repression of SA mediated pathway (Jirage et al. 2001).

SID2 and *PAD4* along with *EDS1* are required for TIR-NB-LRR (TNL) type R-gene mediated disease resistance (Wiermer et al. 2005), suggesting that *DND2* also might be linked to TNL resistance. *DND2* along with *DND1* have recently been shown to be repressed by *SNC1* - TIR/NB/LRR type R-gene acting as a transcription factor (Zhu et al. 2010). SNC1 requires TPR1 (Topless related 1) for its functional activity. Constitutive over-expression of *TPR1* induces similar phenotype, to that observed for *dnd2* and *dnd1* – dwarfed stature, over-accumulation of SA and constitutive expression of *PR* genes (Zhu et al. 2010).

Taken together, above mentioned evidences suggest, that *DND2* acts as a negative regulator of SA mediated plant defense, including R-gene resistance. Mechanisms employed by *DND2* to suppresses SA mediated defense are yet unknown, however, based on analogy to mammalian *CNGC*s, it might be expected that *DND2* employs Ca^{2+} /CaM signaling, to perform its physiological function. Recently a direct link between SA and Ca^{2+} /CaM signaling has been established by characterization of transcription factor SR1, which binds Ca^{2+} /CaM, to inhibit *EDS1* expression and hence SA mediated defense responses (Du et al. 2009).

AtCNGC4 expression is up-regulated in response to methyl jasmonate (Balagué et al. 2003). Positive regulation of *DND2* by JA and negative regulation by SA related transcription factors agrees with generally accepted concept of tight interaction between SA and JA signaling pathways in plant defense response regulation (Balbi and Devoto 2008). The character of SA/JA interaction depends on concentration of both phytohormones – at low concentrations SA has been shown to act synergistically to JA, whereas at high concentrations SA inhibits JA signaling; besides JA/SA both applied at high concentrations can induce ROS dependent cell death (Mur et al. 2006). However, the role of *DND2* in SA and JA signaling is unlikely to be explained simply by pro-JA and anti-SA model, since elimination of functional *DND2* seems also to induce JA and this effect is likely suppressed by overproduction of SA (Jurkowski et al. 2004, Genger et al. 2008).

Neither removal of SA, using *nahG*, nor disruption of SA signaling with *sid2* or *pad4* mutation did not affect dwarfed stature or absence of HR in mutant plants, suggesting that other signaling pathways, apart from those mediated by SA, are activated in *dnd2* (Jirage et al. 2001, Jurkowski et al. 2004, Genger et al. 2008). Interestingly, JA has been suggested to share common signaling components with auxin signaling (Tiryaki and Staswick 2002, Balbi and Devoto 2008), whereas SA has been demonstrated to suppress auxin signaling for induction of plant defense (Wang et al. 2007). *DND2* has been shown to be negatively regulated by TPR1 – TF closely related to TPL-auxin dependent transcriptional repressor (Zhu et al. 2010). In addition, *dnd2* exhibits dwarfed phenotype and altered pattern of branching, which indicates possible link to auxin signaling (Sherman and Fromm 2009). Therefore, it might be expected, that functional *DND2* participates in auxin signaling, and that changes in JA and SA pathways in *dnd2* are caused by alterations in auxin signaling.

CNGC4 comprises cNMP and Ca^{2+} /CaM binding domains, suggesting that signaling pathways incorporating *DND2* are likely to employ cNMP and Ca^{2+} /CaM as secondary messengers. Closely related CNGC2 has recently been reported to trigger NO production during leaf senescence (Ma et al. 2010) and to act upstream of NO production in plant defense signaling (Ali et al. 2007). NO is known to interact in positive feedback loop manner with SA (Zottini et al. 2007, Vlot et al. 2009). Interestingly, NO triggered activation of *PR-1* and *PAL*

incorporates also cNMP and Ca^{2+} signaling (Klessig et al. 2000). Taken together, these observations suggest, that plant *CNGC*s might serve as an intermediates linking SA signaling to NO production in response to Ca^{2+} /CaM.

1.5.3. Barley lesion mimic mutant nec3

nec3 is a barley lesion mimic mutant, developing large dark brown or orange lesions on leaf blades (Franckowiak and Lundqvist 1997). Appearance of lesions and timing of lesion formation in *nec3* varies, depending on a specific allele and genetic background. *nec3* lesions can become apparent on the first weeks after germination, in case of *nec3*.e allele, or just before heading for *nec3*.d allele. There are several allelic *nec3* mutants available in different genetic backgrounds (Table 4 and Figure 7). *nec3* is known to reside on short arm of chromosome 6H, however the high resolution mapping of *nec3* has not yet been done.

Table 4. *nec3* mutants (Franckowiak and Lundqvist 1997).

mutant / *nec3* allele	Mutagenic factor of the original mutation	Parental cv
GSHO2065/ *nec3*.d	X-ray	Bowman
GSHO2423/ *nec3*.e		Villa
FN362	fast neutron	Steptoe
FN363	fast neutron	Steptoe

Figure 7. Lesions on leaf blades of allelic barley necrotic mutants carrying *nec3* mutation (photo: Dr. Nils Rostoks).

1.6. Manipulating HR – a perspective approach to crop improvement

Lessons gained from plant disease resistance studies can be successfully applied to plant breeding (Varshney et al. 2006). The most promising biotechnology based strategies include improvement of plant-pathogen recognition through modification or introduction of plant PRRs or R-genes, artificial activation of plant resistance, using synthetic or pathogen derived compounds, and suppression of pathogen virulence by inactivation or removal of plant susceptibility genes (Gust et al. 2010).

Manipulation of HR or plant cell death related signaling also offers a potentially powerful tool for crop improvement (Melcher and Stuiver 2000). Currently at least 37 patents have been issued in USA, and at least 20 patents registered in European Patent Register, describing hypersensitive response employment for plant disease and pest resistance improvement (The Unated States patent and trademark office 2012). Although the majority of the patents describe novel HR elicitors from various pathogens, or consider application of certain elicitors for plant immunity priming, there are also a limited number of patents concerning direct application of lesion mimic mutants in crop breeding (Ryals et al. 1998, Johal et al. 2002) or induction of *lmm* phenotype, to trigger plant disease resistance (Mori et al. 2009).

It has been suggested, that mutations, disabling host genes, required for establishment of pathogen infection, might be a perspective strategy in plant breeding (Pavan et al. 2010). Among the so called susceptibility genes are also several mutations conferring *lmm* phenotype (such as *dnd1, dnd2, lsd1, mlo*), suggesting that plant breeding might exploit mechanisms regulating HR for crop improvement (Pavan et al. 2010). Over-expression of anti-apoptotic gene *BI-1* in barley rendered plants more resistant to necrotrophic pathogen *Fusarium graminearum*, serving as an example of successful disease resistance improvement, based on exploitation of cell death regulation (Babaeizad et al. 2008). Similarly, heterologous expression of anti- apoptotic *p35* gene, derived from baculovirus, increased tomato resistance to fungal and bacterial pathogens (Lincoln et al. 2002). Unfortunately, suppression of pro-apoptotic signaling in plants can lead to enhanced susceptibility to biotrophic pathogens (Glazebrook 2005), however this could potentially be overcome by using pathogen-inducible or tissue specific promoters. For example, heterologous expression of pathogen elicitor cryptogein under control of pathogen-inducible promoter ensured rapid activation of HR upon pathogen infection and consecutively also restricted pathogen spread (Keller et al. 1999).

However, direct application of *lmm* in agriculture is doubtful, since finding a single mutation, conferring resistance to broad spectrum of pathogens, is unlikely (Hammond-Kosack and Parker 2003). Besides, most mutations, inducing spontaneous lesion formation, also negatively affect agricultural traits (Matin et al. 2010). This is not surprising, since induction of defense responses is cost demanding and may have an adverse effect on plant growth and fitness (Brown 2003, Berger et al. 2007, Walters and Heil 2007). Thus, practical application of *lmm* would be effective only in case of a mutation, which has non-significant effect on yield and confers resistance to widespread and very harmful pathogen. *mlo* – lesion mimic mutation, ensuring powdery mildew resistance of barley, is currently the only example of successful application of *lmm* to crop improvement. Although *mlo* mutation has slightly

negative effect on barley yield (Kjær et al. 1990), it is widely applied in barley breeding in regions, where powdery mildew is economically significant disease. Theoretically *mlo* can be uncoupled from low yield by breeding, since it has been shown, that yield loss of *mlo* plants might be caused by low yield coding QTL, located close to *mlo* (Hackett et al. 2001). In addition, the same lesion mimic mutation can have more or less severe *lmm* phenotype, depending on a genetic background. For example, rice *lmm spl11*, conferring enhanced resistance to rice blast fungus and bacterial blight, developed less lesions, if introgressed into susceptible cultivar CO39 (Yin et al. 2000). Alternatively, negative effect of mutations, causing lesion mimic phenotype, can be overcome by application of pathogen inducible promoters, ensuring targeted induction of a required phenotype (Gurr and Rushton 2005). Thus, uncoupling of agriculturally unfavorable traits from enhanced disease resistance in *lmm* can be achieved at least for some *lmm*, suggesting that practical application of *lmm* as a resistance source for crop breeding programs cannot be excluded.

2. Material and methods

Description of experimental part of the thesis in chapters Material and methods, Results and Discussion is structured according to the three main tasks of the thesis – characterization of barley necrotic mutant *nec1*, identification of *NEC3* gene and identification of barley *LSD1* gene. Scheme describing structure of the experimental part of the thesis is represented in Figure 8.

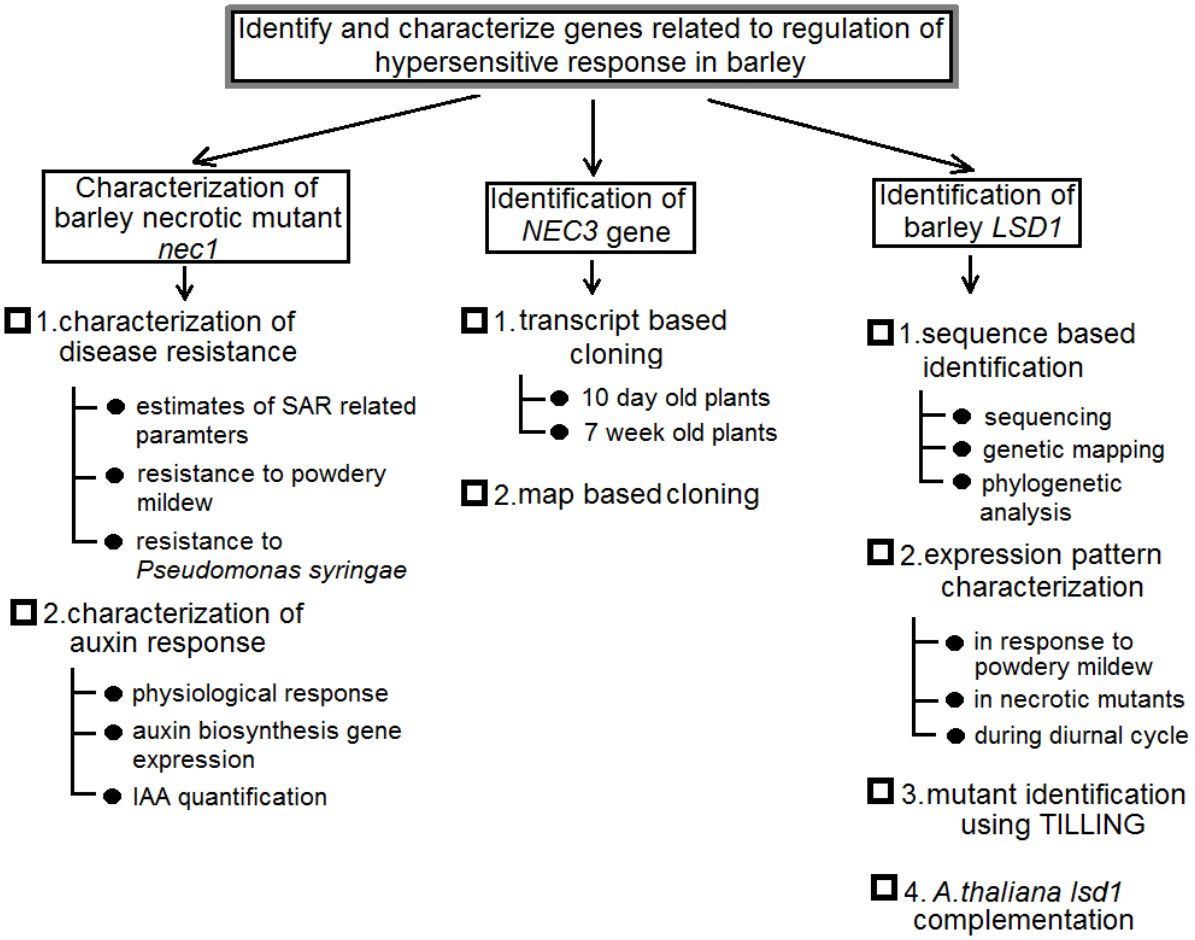


Figure 8. Structure of experimental part of the thesis. Thesis is structured according to the three main tasks - characterization of barley necrotic mutant *nec1*, identification of *NEC3* gene and identification of barley *LSD1* gene. Scheme represents also methodological approaches taken to achieve each of the proposed tasks.

2.1. Characterization of barley necrotic mutant *nec1*

2.1.1. Plant material

The barley *nec1* allele in cultivar Parkland was initially described as a natural mutation (Fedak et al. 1972), which was confirmed by identification of a MITE insertion in intron of the *NEC1* gene, that caused alternative splicing and a predicted non-functional protein (Rostoks et al. 2006). The *nec1* mutant line GSHO 1284 and a parental variety Parkland were genotyped with DArT markers - only 2.2% of 1131 DArT loci were polymorphic, suggesting that the mutant is essentially isogenic to Parkland (Dr. Nils Rostoks unpublished data).

If not stated otherwise, all experiments performed for characterization of barley necrotic mutant *nec1* were carried out with accession GSHO 1284. Barley accessions GSHO 1284 and Parkland were obtained from USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA).

mlo-5 and *nec1* double mutant was obtained by crossing accession GSHO1284 with NGB9276, carrying the *mlo-5* allele in cv. Carlsberg II background (Jorgensen 1992). Plants homozygous for *nec1* and *mlo-5* alleles were confirmed by genotyping the respective mutations, and F_4 plants were used for all experiments. NGB9276 was obtained from Nordic Genetic Resources Center (Alnarp, Sweden).

For HPLC based quantification of IAA two more allelic *nec1* mutants were used – GSHO 989 and FN085. Mutation in GSHO 989 has been introduced using combined treatment with gamma-rays and diethyl sulfate (Lundquist et al. 1997). GSHO 989 contains premature Stop codon at Trp 130 in CarlsbergII background. FN085 is Steptoe fast neutron mutant containing deletion of CNGC4 (Lundquist et al. 1997).

If not specified, plants for all experiments were grown in environmental growth chamber at 22°C under long-day (16 h day/ 8 h night), medium light (ca. 150 μmol $m^{-2}s^{-1}$) conditions.

2.1.2. Characterization of nec1 disease resistance

2.1.2.1. Characterization of systemic acquired resistance related indicators in nec1

Spectrofluorimetric analysis of whole-plant H_2O_2 content

Hydrogen peroxide was quantified spectrofluorometrically, as described by Jimenez et al. (2002). Briefly, 1 g of freshly harvested leaves from two-week-old barley plants were frozen in liquid nitrogen and ground in 50 mM Hepes-KOH buffer, containing 1 mM EDTA and 5 mM $MgCl_2$ (pH 7.5). After centrifugation for 10 min at 14’000 rpm, supernatant was transferred to a new centrifuge tube, and equal volume of chloroform and methanol (2:1, v/v) solution was added. After centrifugation for 3 min at 14’000 rpm, upper aqueous phase was transferred to a new centrifuge tube, and the 50 mM Hepes-KOH buffer solution (pH 7.5), containing 0.5 mM homovanillic acid and 15 U horseradish peroxidase VI, was added to a final volume of 3 ml. Samples were incubated at room temperature for 30 min before fluorescence measurements (excitation at 315 nm, emission at 425 nm). Fluorescence was

measured with spectrofluorometer FloroMax3 (Horiba Scientific, Japan). For quantification H_2O_2 standard curve (1 nM-100 μM) was applied. Sample correction for quenching was done by adding known sample amount to a 10 nM H_2O_2 solution.

Quantification of salicylic acid using HPLC

The SA content in leaf tissue extracts was analyzed using reversed-phase high performance liquid chromatography system. For SA quantification leaf samples of two week old barley plants were used. Each sample contained leaf tissue from three plants. Samples were prepared essentially as described by Aboul-Soud et al. (2004). As an internal standard for SA recovery correction, samples were selectively spiked with 50 μg^{-1} FW 3-hydroxy benzoic acid (3-HBA).

Briefly, 0.45 g barley leaf tissue was homogenized in liquid nitrogen and sequentially extracted, using 90% and 100% methanol. Extraction was repeated twice, and two supernatant fractions were then pooled and dried. The residue was resuspended in 1 mL of 5% acetic acid.

For the quantification of free SA 1 mL of ethylacetate:cyclopentane:isopropanol (50:50:1) was added. Sample was thoroughly vortexed, and the upper phase (approximately 1 mL) was transferred to a new microcentrifugation tube. The aqueous phase was then re-extracted, as described previously, and the both organic phases were pooled. The resulting solution was dried and thoroughly resuspended in 0.9 mL of mobile phase. This suspension was filtered through a 0.20 μm filter.

The aqueous phase, containing the SAG fraction, was acidified with HCl to pH 1 and boiled for 30 min, to separate free SA from conjugated SA. The released SA was then extracted with the organic mixture and treated as above.

Chromatographic analysis was performed on a modular HPLC system, Agilent 1100 series, consisting of quaternary pump, autosampler, column thermostat and both UV and fluorescence detectors (Agilent Technologies, Germany). Separation was achieved on Zorbax Eclipse XDB-C18 (Agilent Technologies, Germany) column 4.6x250 mm, 5 μm. Column temperature was controlled at 40 °C. Acetonitrile : 20 mM NaH_2PO_4 (pH 3.0 with acetic acid), in a volume ratio 25/75 was used as a mobile phase. The mobile phase flow rate was 1.0 mL min^{-1}. Injection volume was 100 μL. UV/VIS detector was set to 237 nm and 303 nm and fluorescence detector Ex=297 nm Em=407 nm respectively. Results were evaluated by a ChemStation Plus (Agilent, Germany).

2.1.2.2. Characterization of nec1 resistance against Blumeria graminis f.sp. hordei

Expression analysis of powdery mildew response related genes in *nec1*

For RNA extractions first leaf from 2 week old plants of necrotic mutant *nec1* and parental line Parkland, were snap frozen in liquid nitrogen immediately after harvesting. Total RNA was extracted from frozen leaf tissues, using Trizol-like reagent, as described by Caldo et al. (2004). 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 *Bax*

Inhibitor, *MLO*, *HvRacB* and *HvRbohA* in *nec1* and Parkland. Integrity of the extracted RNA was monitored, using non-denaturing agarose gel electrophoresis. Two µg of the extracted RNA was treated with DNaseI (Fermentas, Vilnius, Lithuania), following manufacturer's instructions, and afterwards purified, using chloroform-ethanol extraction. Quantity of purified total RNA was monitored, using spectrophotometer NanoDrop ND-1000 (NanoDrop products, USA).

cDNA was synthesized with oligo $(dT)_{18}$ primer in a total volume of 10 µl, containing 1 µg of total RNA, using RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

For real-time PCR aliquots of cDNA were amplified on ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA), using Applied Biosystems SYBR Green PCR kit in a total volume of 20 µl containing 2 µl of cDNA and 0.3 µM primers. Primers, used for real-time PCR, are listed in Table 5. 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 45 s at 72 °C (data acquisition step). Standard curves for estimates of amplification efficiency and for the quantification of the transcript levels were calculated from serial dilutions of appropriate cDNA fragments, amplified from cv Parkland. Transcript levels of the studied genes were expressed as a percentage of *HvGAPDH* transcript value in the same sample. Combined 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 done in Microsoft Excel.

Table 5. Primers used for resistance related gene expression analysis in *nec1*.

Primer	Sequence	Reference
HvBI_cw1	CGATGATCTCCTGCGTGTCG	This study *
HvBI_ccw1	TACCTCGGTGGCCTGCTCTC	
HvGAPDH_cw1	CGTTCATCACCACCGACTAC	Horvath et al. 2003
HvGAPDH_ccw1	CAGCCTTGTCCTTGTCAGTG	
*MLO*_F1	GTCGAGCCCAGCAACAAGTTCTTC	This study *
*MLO*_R1	ACCACCACCTTCATGATGCTCAG	
HvrbohA_F1	CCGATCAGATGTATGCTCCA	Trujillo et al. 2006
HvrbohA_R1	CAGAAGGCATTGAAGCCAGT	
HvRACB_L01	GGTAGACAAAGAACAAGGGCGAAGT	This study *
HvRACB_R01	CACAAGGCAGGAAGAAGAGAAATCA	

* Primers were designed using Primer 3 software using the following gene sequences as a template: HvBI (HarvEST21 Unigene 3323; AJ290421); *MLO* (HarvEST21 Unigene 6351; Z83834) ; HvRacB (HarvEST21 Unigene 5202; AJ344223)

Plant inoculation and characterization of *Bgh* penetration efficiency

Two week old plants of *nec1* and cv Parkland were inoculated with 10-20 conidia per mm^2 from mixed population of powdery mildew cultivated on cv Parkland. For penetration efficiency characterization infected barley leaves were harvested 48 h post inoculation and cleared for 24 h in 98% ethanol. Penetration efficiency was calculated as a ratio of interaction sites with haustoria formation and the total number of spores with developed appresoria. The overall penetration efficiency for the particular barley line is an average from three replicates, containing at least 100 interaction sites each.

2.1.2.3. Characterization of nec1 resistance against Pseudomonas syringae

To study *nec1* non-host resistance against *Pseudomonas syringae* pv. *tomato,* leaves of 14 day old *nec1* plants were vacuum infiltrated with a bacterial suspension in 10 mM $MgCl_2$. Bacterial suspension was applied at normal concentration $8*10^4$ and high concentration $6*10^7$ cfu ml^{-1}, since low concentration inoculum, typically applied for infection of host plants, can have minor or no effect on non-host species. For mock inoculation 10 mM $MgCl_2$ was used for infiltration. Immediately after infiltration plants were covered with plastic bags, to maintain high humidity, and kept in dark for 1 h. After an hour plants were transferred to growth conditions, described above. Bacterial growth was monitored at day 3 post inoculation, by dilution plating of homogenized plant tissue. Leaves were briefly sterilized with 70% ethanol before homogenization. *Pseudomonas syringae* pv. *tomato* was obtained from the German microbial type collection (accession 50315).

Cell death was quantified by electrolyte leakage assay, performed as described by Dellagi et al. (1998), with minor modifications. In brief, plants were vacuum infiltrated with *Pst,* as described above, and incubated in dark at high humidity for an hour. Five mm leaf disks were collected and washed with distilled water for 1 h and then transferred to a tube with 6.5 ml distilled water. Conductivity was measured with conductivity meter handylab LF11 (Schott Instruments). Each sample contained 4 leaf disks from 4 plants, and at each data point 4 independent replicates were measured.

2.1.3. Characterization of nec1 auxin response

2.1.3.1. Physiological tests for nec1 auxin response

Coleoptile elongation was assayed, as described by Kotake et al. 2000 with minor modifications. Briefly, 5 mm long coleoptile segments (3 mm below the tip of the seedling) were excised from 3 day old barley seedlings, grown in dark at 22 °C. Excised segments were floated in dark for 4 h in 5 μM NAA solution, containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES, pH 6.5.

We used leaf impression method (Khazaie et al. 2011), to determine epidermal cell size.

Root gravitropic response of *nec1* and wt was assessed as angle of root curvature after gravistimulation at 90° to the vertical. Seedlings were grown for 3 days vertically and then rotated by 90°. Measurements were done one day after rotation.

Root growth in response to exogenous auxin was assessed by germinating *nec1* and wt seeds on filter paper, wetted with IAA solutions of following concentrations 10 μM, 50 μM, 100 μM. Root measurements were done after seeds were incubated for 3 days at 4 °C and 1 day at 23 °C. In total, 18 plants per genotype were assessed.

2.1.3.2. Gene expression analysis using real time qRT-PCR

For RNA extraction, segments of first leaf from two week old plants of necrotic mutant *nec1* and wild type plants were frozen in liquid nitrogen. RNA extraction, cDNA synthesis and quantitative real-time PCR were performed as described in paragraph 2.1.2.2. Primers, used for qRT-PCR analysis, are listed in Table 6. Relative quantification was performed, using $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmitgen 2001. Transcript levels of the studied genes were normalized to *HvGAPDH* transcript value in the same sample.

Table 6. Primers used for auxin biosynthesis gene expression analysis in *nec1*.

Gene/ HarvEST 35 Unigene	*A. thaliana* homologue	Primer sequence 5'-3'
YUCCA1	*AtYUC1*	F:ATGGAGGTCTCCCTGGACCTGT
6837	AT4G32540	R:TCACCTTGTCCACGAACCAGAG
VT2	*AtTAA1*	F:GTATCCTGCCGTGACGGACTTC
11280	AT1G70560	R:ACTGCGGCCAGTAGTAGGCAAG
NIT2	*AtNIT1*	F:GTACCTGGGTAAGCACCGCAAG
17606	AT3G44310	R:GTGCTGTCCTTAACAGTGGCATC

2.1.3.3. IAA detection and quantification using HPLC

For IAA extraction seedlings were grown in soil at 22 °C under long-day (16 h day, 8 h night), medium light (ca. 150 μmol m^{-2} s^{-1}) conditions. Leaf samples were taken from 14 days old plants.

Extraction and purification for auxin was performed, as described by (Dobrev and Kaminek 2002), with minor modifications (Nakurte et al. 2012). Briefly, the plant material was ground in liquid nitrogen and extracted with 100 % methanol. Samples were pre-concentrated by solid phase extraction (SPE) using AccuBOND II ODS-C18 200 mg 3 ml SPE (Agilent). Chromatographic analysis was performed on a modular HPLC system, Agilent 1100 series (Agilent Technologies, Germany). HPLC separations were achieved by using a reverse-phase Zorbax Eclipse XDB-C8 (Agilent Technologies, Germany) column 4.6x150 mm, 5 μm. Mobile phase was composed of methanol and 1% acetic acid (60:40 v v^{-1}) in isocratic mode at a flow rate of 1 ml min^{-1}. The detection was monitored at 282 nm (Ex) 360 nm (Em) (IAA, IPA). The developed method was validated in terms of accuracy, precision,

linearity, limit of detection, limit of quantification and robustness. Standards of indole-3-acetic acid (> 99%) and indole-3-pyruvic acid (99%) were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Identification of barley *NEC3* gene

2.2.1. Plant material

Experiments, related to transcript based cloning of *NEC3,* were performed with two allelic *nec3* mutants – FN362 and FN363. Barley fast neutron mutants FN362 and FN363 were kindly provided by Dr. Andris Kleinhofs (Washington State University, Pullman, Washington, USA). FN362 and FN363 are fast neutron mutants, isolated from a cv. Steptoe seeds, irradiated with fast neutrons at the IAEA Seibersdorf facility in Austria. FN362 and FN363 are allelic to known *nec3* mutants GSHO 2423 and GSHO 2065 (allelism tests was done by prof. A. Kleinhofs research group at Washington State University).

nec3 mapping and physiological characterization of *nec3* mutant was performed, using accessions GSHO 2423 and GSHO 2065 respectively. Barley accession GSHO 2423 (parental cv. Villa) and accession GSHO 2065 (parental cv. Bowman) were obtained from USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA).

2.2.2. Transcript based cloning of NEC3 - Affymetrix Barley1 GeneChip analysis of nec3

Two independent Affymetrix Barley1 GeneChip experiments were performed. Both experiments differed in age of *nec3* plants, used for analysis – transcriptome of either 10 days old or 7 week old plants were analyzed. According to the plant age, used for analysis, the experiments will be further referred to as 10d and 7w experiment. For 10d experiment Affymetrix microarray analysis was done at Washington State University. For 7w experiment Affymetrix microarray analysis was done at ATLAS Biolabs, Germany.

2.2.2.1. RNA extractions for nec3 transcriptome analysis

For 10d experiment transcriptome analyses RNA was isolated from 10 days old cv. Steptoe, FN362 and FN363 plants, as described (Zhang et al. 2006 a). Each sample consisted of five plants. For 7w experiment RNA was isolated, using Trizol-like reagent from 7 week old cv. Steptoe, FN362 and FN363 plants, as described by Caldo et al. 2004. Each sample consisted of three plants. Extracted RNA was treated with DNaseI (Fermentas, Vilnius, Lithuania), following manufacturer's instructions, and afterwards purified using Qiagen RNeasy Plant Mini kit, according to manufacturer's instructions. Integrity of the extracted RNA was monitored using non-denaturing agarose gel electrophoresis.

For Affymetrix data validation with qRT-PCR, cDNA was synthesized from the same RNA, prepared for Affymetrix experiment. cDNA synthesis and qRT-PCR was performed as described in paragraph 2.1.2.2.

2.2.2.3. Affymetrix microarray analysis

Two independent biological replicates of Steptoe, and FN362 and FN363 mutants were subjected to Affymetrix Barley1 GeneChip analysis, as described (Zhang et al. 2006 a). Probeset summary data was obtained, using Affymetrix Expression Console 1.1 and the MAS 5.0 processing algorithm (Affymetrix, Santa Clara, CA, USA). The GeneChip data have been submitted to NCBI GEO database under accessions GSE23775. The resulting data were exported into Microsoft Excel, where all the subsequent analyses were performed. Briefly, two-tail t-test was used to identify significant ($p<0.05$) differences in expression for each probeset between the control (Steptoe) and both mutant. Two–fold reduction of expression in mutant was used as a cut-off for identification of *nec3* candidate genes.

2.2.2.4. PCR screen of nec3 candidate-genes, RT-PCR and quantitative real-time PCR for microarray data validation

Gene specific primers (Table 7) were designed by Primer3 software (Rozen and Skaletsky 2000). PCR reactions were carried out in a 20 µl of total volume, containing 100 ng genomic DNA, 0.5 µM primers, 1.8 mM $MgCl_2$, 0.2 mM dNTPs and 1 u Hot Start *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), used with manufacturer-supplied buffers. PCR was carried out as follows: initial denaturing step for 5 min at 95 °C, 35 cycles of 30 s at 95°C, 30 s at 60 °C, 2 min at 72 °C and final extension of 5 min at 72 °C.

2.2.2.5. Analysis of differentially expressed genes from nec3 Affymetrix Barley1 GeneChip experiment

Functional categorization of the genes, differentially expressed in *nec3* mutant, was performed using Gene Ontology service (Gene Ontology Consortium 2012). Analysis was based on *Arabidopsis* homologues of the *nec3* differentially expressed genes. Correspondence between barley Affymetrix probes and *A. thaliana* Affymetrix probes was established using PlexDB microarray platform translation tool (PlexDB Group 2011). GoSlim and GO Term enrichment tool was applied for analysis of representation of different gene groups in *nec3* transcriptome in comparison to whole genome data. Analysis was based on *Arabidopsis* and rice homologues of *nec3* differentially expressed genes, using correspondingly TAIR and GRAMENE database as background data.

In order to determine particular metabolic pathways, overrepresented among differentially regulated gene set of 10d and 7w, *nec3* was analyzed using MapMan 3.5.1R2, adapted for Affymetrix Barley1 GeneChip data (Sreenivasulu et al. 2008). MapMan derived results were also verified, using GENECODIS web based analysis tool (Tabas-Madrid et al. 2012), allowing annotation and classification of any analyzed gene set, according to different background data (Nogales- Cadenas et al. 2009, Tabas-Madrid et al. 2012). KEGG pathway data (Kyotto Encyclopedia of Genes and Genomes pathway database) were selected as background data.

Table 7. Oligonucleotide primers for *nec3* candidate-gene PCR screening and quantitative real time PCR.

Primer	Sequence 5'-3'
ABC3257_L01*	TCAGGAGCTAGCTATCGATGGAGAA
ABC3257_R01*	GAAAGGTCGTTGGCTGGAGGAC
ABC4521_L01*	GCTCGTGGACCACTCCATTGT
ABC4521_R01*	GGTTGTACGACGAGTCCATATCGTG
ABC14229_L01*	GGTCCGACGTACAGTCACTCGTT
ABC14229_R01*	CCAGCGATCAACACATTAAGAAGGA
ABC1954_L01	GCACGTCGCCCTAGAGAAACT
ABC1954_R01	ATAATACTACGCCTGCTCTGCTGTG
ABC2279_L01*	GTCTTCTGCTTGCAAGTTTGACATC
ABC2279_ R01*	CAACGCCTTATTACAGTGAGGTACG
ABC3448_L01	CTACAACAAGAAGATGAAGCCATGC
ABC3448_ R01	GATGCAGAAGCCTCTTTACATTTGA
ABC4024_L01*	ACGGAAATATTGGAGACAAGAGGAG
ABC4024_ R01*	TCAAATGTACACAGAGTTGCAATGG
ABC6708_L01	ATCCTTCAAGGCCTATCTGAATGAC
ABC6708_ R01	GGCAGGAAGAGTTGCAAACTAGAAT
ABC7098_L01	CTATACGTTGTTCGGTTCAATCAGC
ABC7098_ R01	TGGGATACTACGATCATGGACAGTT
ABC7285_L01	CGTGTACCATTCTCCTGTAGGTTCT
ABC7285_ R01	CAAGGTTACACGATACAAGGAAACG
ABC7377_L01*	AGATCATCCTCACCTTCTCCCTTCT
ABC7377_ R01*	ATTTGCTTTCCTAAAAGTCCCAACC
ABC16209_L01*	GAAAACCATGGGAGTAAATGGAAC
ABC16209_ R01*	TACGTATACACCGTACACAGGATGC
ABC18830_L01	CAGGAGCAGGCTCTCAACAAAC
ABC18830_ R01	CGGATCTTATTGTCTCATACGTGTC
ABC19204_L01	CAAGGCCTACCTCAACCGCTAC
ABC19204_ R01	GAAGGCTCCCTCGAAATCAATC
ABC20556_L01	ACGCAAGTGAAAGTGACCAAGAA
ABC20556_ R01	CTCTTCTTCTTCTCGAGCGTCTTTT
ABC21141_L01	GCACCGTGAATATTTGGTTTAATGA
ABC21141_ R01	GCATCTAGTCCTCCTCTAGCCACTC
HVSMEl0012A13f_L01	GCACGATCTTCACAGGTATCACTTT
HVSMEl0012A13f _ R01	CAAAAGATGGGTCTCCTTCCATAAC
EBpi01_SQ004_C23_L01	GCCCAAGGGACTGTCTAGTG
EBpi01_SQ004_C23_ R01	TCTAGACTAGGGCTTGCATAAGG
HD05F08r_L01	ATGGTGTGTGTGCCTCAGATGT
HD05F08r _ R01	CGCTACAAGCTGGTATCATAAGGAG
ABC17652_L01	TCCAGAATTTGCAAGTCATCTTCAT
ABC17652_ R01	TTTGCTGGGATGACAAAAGATGTAT
ABC431_L01	TGTTCACTGGGGAGTGTAAGGAATG
ABC431_ R01	ACAGACTTTATCAAGGGGAGCCTCA
ABC12590_L01	CTGCTAAGCGAGTCCGAGTTCCT
ABC12590_ R01	GTTGAGGTCGAACCGGCAGAT
ABC14129_L01	CTTTACTGGAGAGGCTTTCGCTCAT
ABC14129_ R01	AGGGTCTGACGAAAGCTGGAGTT
ABC12169_L01	GTGTATCAAATGAGCTCGGTGCTG
ABC12169_ R01	CAGGTCATCAAACAAGAATGTGACG
ABC5163_L01	TCCTAAGGGAAAACAAGGACCAGC

Table 7 (continued). Oligonucleotide primers for *nec3* candidate-gene PCR screening and quantitative real time PCR.

Primer	Sequence 5'-3'
ABC5163_R01	TCCAGAACCATAGCATCAGTCAACC
ABC19029_F	CAAGGATTTGGCCGACATGAAC
ABC19029_R	GGCCGTTGAAGTTTAGGCACTG
ABC26350_F	AAGCGCCTACAAGTACAAACACAGG
ABC26350_R	CCCAGCTCGAAGCCAGTTAGTTAAG
ABC5710_F	CAGGCTGCTGTTACTCTCCTTCG
ABC5710_R	AACAGTGTAAGCTCCACCGGC
baak32n13_F	CATGTTCTCCATGTTTGGCTTCTTC
baak32n13_R	AGCATCCATCAGCAATGAAGTTGTC
ABC4948_sL01	CCGGAAGAACAGCAGAAGAAGTAGAG
ABC4948_sR01	TATATCGGCCTATGGCCTCATGG
ABC2489L01	GAGGACCTTGCCAACAAGAGTGA
ABC2489R01	ATTCCCTTAGGGCCACCTCTAGC
HB25K17L01	GAGAGGCTACCGAACGATGTTG
HB25K17R01	AATTACAACAACGCACCAGATAACTT
ABC7285L01	TGCTACCACAGATGAGCTTGTCG
ABC7285R01	CACGCCGTCGAAGCTTATATTCA
ABC12472_F	CCGCTGTATAGGTGGTGTGCTG
ABC12472_R	ACCATGCACGCGTTACATTCTG
ABC8703_F*	AGTGGTCGGGTGCCAACTTC
ABC8703_R*	CCTTCTCAACCTTCATCACATCCTG
ABC11998_F*	AAGATAAGGTGGAGAAGACGGCAG
ABC11998_R*	TTATGGATTAGTGCCTCGGCTTTC
ABC5710_F*	CAGGCTGCTGTTACTCTCCTTCG
ABC5710_R*	AACAGTGTAAGCTCCACCGGC
ABC19029_F*	CAAGGATTTGGCCGACATGAAC
ABC19029_R*	GGCCGTTGAAGTTTAGGCACTG

*-primers used for quantitative real time PCR

2.2.2.6. Comparison of nec3 transcriptome with transcriptome changes in barley under biotic and abiotic stress

We compared differentially expressed (at least two-fold up- or down-regulated) probe sets from our experiment with the expression of the same probe sets in a following set of publicly available barley GeneChip experiments from PlexDB database (Wise et al. 2008), representing barley transcriptome change in response to abiotic and biotic factors: drought1 and drought2 – BB77, chilling and freezing – BB81, *NecS* – BB54, senesc– BB50, *mlo-5* – BB7, *Mla1/6/13* – BB4, *Rpg1* – BB49. For comparison of *nec3* transcriptome data with publicly available *A. thaliana* Affymetrix experiment data, correspondence between barley Affymetrix probes and *A. thaliana* Affymetrix probes was established using PlexDB platform translation tool. Following *A. thaliana* Affymetrix experiments were included in analysis: ColMeJA/coiMeJA/einMeJA – AT98, thrips/ aphid/caterpillar - AT49, whitefly - AT63, aphid saliva – AT90, *Pst* DC3000 – AT13, *Bc* (*Botritis cinerea*)– AT51, dark/high light – AT123, sucrose starvation – AT8, *tpt* high light – AT16, uvA/uvB - AT54, *icl/ml* - AT11. Only genes, differentially expressed in at least one dataset, were included in analysis. Complete linkage hierarchical clustering, based on absolute correlation of relative expression

values of differentially expressed genes, was performed using Cluster3.0 and Java TreeView 1.1.5r2 (Eisen et al. 1998).

2.2.2.7. Characterization of nec3 response to carbohydrate or nitrogen starvation

Detached leaves of four week old *nec3* and Bowman plants were subjected to prolonged dark. Chlorophyll content of detached leaves was measured every 24 hours for eight days, using Minolta SPAD-502 chlorophyllmeter. Each datapoint contains data from at least 10 plants.

Seeds of *nec3* and Bowman plants were germinated on filter paper, wetted with solution, containing 4 mM $CaCl_2$, 3 mM KCl, 1.8 mM H_2PO_4, 1 mM $MgSO_4$ $7H_2O$, 45 µM $FeSO_4$ 7 H_2O, pH 5.8 (modified from Hohe and Reski 2002). $Ca(NO_3)_2$ was substituted with $CaCl_2$, to ensure nitrogen deprived conditions. Seeds were incubated at 4 °C for 3 days and than transferred to 16 h/ 8 h light/ dark conditions at 21 °C. After 3 days root length was measured.

2.2.3. Mapping of nec3

nec3 was mapped, using two barley F2 mapping populations: FN388 x GSHO2423 (*nec3*.e), consisting of 58 individuals, and GSHO2423 (*nec3*.e) x GSHO1284 (*nec1*.c), consisting of 53 individuals. Fragments from HarvEST unigenes and molecular markers, which are known to map at 6HS, were sequenced from parental lines Villa, Parkland and Morex, for identification of polymorphisms, potentially useful for *nec3* mapping in two mappig populations. Screened HarvEST unigenes and markers with corresponding primers are listed in Table 8. Sequencing was done essentially as described in 2.3.2.1. paragraph.

Since majority of screened unigenes did not comprise any polymorphism, distinguishing in parental lines, only subset of unigenes were used for mapping. CAPS markers, that have been used for final mapping, are listed in Table 9. Segregation data of the three CAPS markers was used for linkage mapping in Map Manager QTX software (Manly et al. 2001). Positions of loci, used for CAPS markers in barley consensus map, were derived from GrainGenes website Genetic marker database (Agricultural Research Service of USDA 2008 a).

Table 8. HarvEST unigenes, loci and primers, used for polymorphism search useful for *nec3* mapping.

Locus	Primers	Ref. for primers
U35_1037	F:GCTTGCTTGCTCTCTTCCTCCTC R:GCTCCACGTTCTTCTTGATCTCG	*
U35_3329	F:TCTGGTCCAAAGTCAAAGGCTTG R:CAAGCAAGAGGGTCAAGATCCAG	*
U35_11467	F:AAGCACACTAGCCAGCAGCTCAG R:CCATGATCGAAGCTCCTCACG	*
U35_14470	F:CCACTGCTGCTGCTATTGCCTAC R:GTCCTTGGTCATGCTACGCTCAC	*
U35_15050	F:TGGTGAAGCACAACAACGTCATC R:TCTTGAGCCTCTGGATGTTGGAC	*
U35_14773	F:CGGCTTCAAGAACAACCACTACG R:TCGACCACATGATCCACAGTAGC	*
U35_17450	F:TGCATCGGTTCCTGGTGGTATAG R:CCACATTCACTTGTGCCTTCCTC	*
U35_18248	F:CAACCTTCAATAGCGACCTGCAC R:TCTCATCCACCTGACGAAGAACC	*
U35_47882	F:GGAGGAAGAAGAAGCTCCAGACG R:TGGTCAAAGTCCTCACCTCCAAG	*
U32_106	F:TCGAGATTATCGCCAATGACCAG R:GTAGGCAATAGCAGCAGCAGTGG	*
U32_1769	F:GACGGATACACCGACGAGGAC R:GTGCGAGATCCAGTCCAGATACG	*
U32_7624	F:AGCACGGTGGTCTTGATGAATGT R:GGCTGGAACTTGATAAGGTGGTG	*
ABC07351	F:TACTCCCCCGGCGGACTTAT R:CCCAGCTAGCAGCAAGGGAA	Rostoks et al. 2005 b
ABC01964	F:ACCTCGGAGTCCACGTGAGG R:CAAGTGGGAAAGGGGTGTCG	Rostoks et al. 2005 b
GBS0520	F:TCGTGCAGTTACATTTCGGG R:CCCAAGCTGGAGTTCTGAAGAG	Stein et al. 2007
U32_384	F:CTACCGGGACTGGGTTCAGCTAC R:GACCATATATGCTGCCATGCAAG	*
U32_1914	F:AAGAAGGGCCTACTGGGCAAGAT R:GCATAATGCCCAGCAATAAACCA	*
U32_2047	F:GGACTCGGCCAAGTACACCAAG R:TGCATATACAACCTGCAAGAACC	*
U32_5993	F:AACGTCGACATGTCTTATGAAGTCC R:GAAGATGATAACAAGTTTCAGGTCACA	*
U32_2152	F:ATCGAACTGGCCAACGATACTC R:ACTACAAAGGCCGGCACACAG	*
U32_3436	F:ATCGCTGGAGCGAAGGTTAGAG R:ACGCCATTGTTGTTGTTGAGGA	*
U32_4396	F:ACCGTCGTCACAAGGATCCAG R:GATCCAGCACAAGGTGCAATGT	*

Table 8 (cont.). HarvEST unigenes, loci and primers used for polymorphism search useful for *nec3* mapping.

Locus	Primers	Ref. for primers
U32_1490	F:AAGAAGCCAGAGAACTGGGCACT R:TCTCCTTGGGCAATAGGATCACA	*
U32_3831	F:TGCAGGCAGCTGTTTCAATTACA R:AGTCGGCTTGCTCCATGTCACT	*
U32_1041	F:TGGAAGCCCAAGTTTGATGTTG R:AACAGCACTGGGCTTCTTGGTC	*
U32_6854	F:CTGCATGACTGAGAACTTCACC R:ATCCAATCAATCCATTATTCTTTGT	*
U32_979	F:GGTGTCCTACAGGGAAGAAATG R:CCATAAACATGATTCAACATACTCA	*
U32_3658	F:CTGAGGTCGACATGGCTGAAG R:ATACTGGAATTGCGCTGGTTGA	*
Bmac0316	F:ATGGTAGAGGTCCCAACTG R:ATCACTGCTGTGCCTAGC	Ramsay et al. 2000
scssr09398	F:AGAGCGCAAGTTACCAAGC R:GTGCACCTCAGCGAAAGG	Ramsay et al. 2004
Bmag0500	F:GGGAACTTGCTAATGAAGAG R:AATGTAAGGGAGTGTCCATAG	Ramsay et al. 2000
GBM1270	F:TGCGTCTTACAACTTCGTGG R:AGGCTGCTGTTAGTGGTGGT	Varshney et al. 2007
GBM1215	F:ATGACCAGAAAACGCCTGTC R:GGATTCTGCACACACGAGAA	Varshney et al. 2007
GBM1355	F:ATCCGTCGTATTCGCATCTC R:GCTGGTACTGGGAGAAATGG	Varshney et al. 2007
GBM1212	F:TGTTGCAAGAAGCAAGGATG R:GCGCTTACTCTCTCGTCGTC	Varshney et al. 2007
Lth	F:TGGAACGAGGGAAAAAACAG R:CATATCTCATTCTACTTGAG	GG1
Nar1	F:ATGTCCTCCGTCACAAACCC R:AGTACTTCCGTCCATGCTCG	GG1
ABG378	F:TTA GTC ATA GAA TCC CTG TT R:AAA ATT CGC CTG TGC TGT GT	GG1
MWG966	F:GATCAAGCAAGCTAGCTCCA R:CACAGATGCCACACCGGAAT	GG2
MWG573	F:CTGCACTCATGTCAGCAAGA R:GACCAACGGGAGATGCTCGT	GG2

* - primers designed in this work. Based on HarvEST Assembly32 Unigenes, using Primer 3 software;
GG1 – information submitted by A.Kleinhofs and T.Blake to GrainGenes genetic marker database (Agricultural Research Service 2008 a);
GG2 - information submitted by A.Graner to GrainGenes genetic marker database.

Table 9. CAPS markers used for mapping of *nec3* mutation in barley.

Locus/ HarvEST unigene	Primers	Restriction enzyme	Cuts allele in*	Reference for primers (p) restr. enzyme (r)
ABG466	CGTTGCAAATGGACGTGCCA GGAGGGAAGCGGCTGATAAT	*HaeIII*	Villa	p: GG; r:***
HARV32_1872	TAAGTCGGGACGTCTCTGCTTCTC AGGTCAAGGGTATGGCTGATCGTA	sequenced	-	Muñoz-Amatriaín et al. 2011 p: ***
HARV32_5771	GCAGATGTTGCCGTTGCTGT TCCTTCCATCTCACCCAAGAACA	*HpaII*	Morex	**
HARV32_3164	ACCACAAATGGGGGCAAAAA GGGTCTCATGGGGAAGCAGA	*Bsp1407I*	Morex	**
HARV32_12210	TTCCAGTTCCCCTGCTGCTC GGGCACGTCCTTCTGCTCAT	*HhaI*	Morex	**
HARV32_6964	CCCCACGATCCAAATCGAAA GAGGCTGGTGGCTGATTGCT	*AluI*	Morex	**
HARV32_1490	TCTCCTTGGGCAATAGGATCACA AAGAAGCCAGAGAACTGGGCACT	*PsuI*	Villa	***
HARV32_239	GCGGTACTCGATCCATGACG GGAGATTTAGCCCGGCTGCT	*Mph1103I*	Morex	**
HARV32_1852	AGCACGCTGCTGAACACAGG GGCCATCAGGAGGAAGACCA	*MunI*	Villa	**
HARV32_4547	TACGCTGCAAGGAGCGCATA GGCCATGAGACCCATGAACA	*PvuII*	Parkland	**
CMWG652a	CACACCTTCTTCTTCCTCTT GAGCTGCTCGTTCTCGTTGA	*NlaIII*	Villa	p: Abu Qamar et al. 2008 r: Tupiņa 2012

* - parental lines of mutants used for mapping – Villa (GSHO2423), Parkland (GSHO1284), Morex (FN338); ** - Nils Rostoks unpublished data; *** - markers designed in this work; GG – information submitted by A.Kleinhofs and T.Blake to GrainGenes genetic marker database (Agricultural Research Service 2008 a)

2.2.4. Screening of barley HarvEST21 unigenes homologous to rice genes positioned syntenically to nec3 region

In order to find out, if any of candidate genes comprise large deletions in *nec3* mutant plants, PCR was performed on cDNA synthesised from RNA of two week old FN362, FN363 and wild type plants, using gene specific primers (Table 10).

Table 10. Primers used for expression analysis of barley homologues of rice genes positioned syntenically to chromosome region comprising *nec3*.

HarvEST21 Unigene	Primer sequence
1360	F:GACGTCGTCAAGAACATCGAGAAG R:TTCATAGCGGAGTTCATCTCATTCA
1610	F:CAAGTCTGAGAAGATCACCATCACG R: CTTCTCCTCGTAGTCCTCCTTCTCG
10453	F:TCACTGACCACAACGGTGACTTC R:ATGGTCCAGTGAATGCCTCACAT
11021	F:GCCTGGAGAAGCACTCTCTGTTG R:ACTACCTATCGAACGGCGTGGTC
18494	F:TCCATCCTTACCGTGCAACAACT R:GTCAACGATGGCCATAACACCTC
23335	F:CACATGCCACACCACAGATTAGG R:AGTAGACCTGTCCACCGCAATCC
49137	F:TTTCCGCATCGAGATGGATTTGT R:TTGGTGTGCTTGTGGACGAACTT
49394	F:AACAAGGGTGTGAACCCTGATGA R:CAGAAAGGTCAAACTTGCCGAGA
22597	F:CCCTTCCTGACTTTAGGGCCAAG R:GCCTTTCTGGATCTGCACAGAGA
49543	F:TGCAACTGGATAAGGCATGTGAG R:CGAGGCTTCGAGAGACAATCAAG
21172	F:CCTGATCGTTTAGGCGTGTCG R:CACCGAATACTCTTCCTTCCCAAC
37524	TF:CGAGATCGCAAGGCAACTC R:CCTATTGTGTGCATAACTGAATTGACG
24301	F:TTTCTGATGTTACCTGTCCCTTCTAC R:TTGCTATTTGAGTATCCCAACCAAC
33461	F:CATGGTACCTGTTCGGTTATACGTG R:TGTACATGTGCATGCTTCTCAGTT
33462	F:CGTTGCTACCAGAGTTCTTCAGCTC R:GGAAGGCAACTCTCCTCCTATGGTA
2229	F:GAGTCCACAGCGAGGAGCTG R:GGCCTTCTGCCATCACTTCA
9642	F:TCAACTCCAACGGCAGCATC R:CCTCGCGGTGTCCTCATAGC
1430	F:AGGAGGAGATCGGCCAGTCC R:GGAGCAGATGGACAGCAGCA
7800	F:GGTACAAGTTCCTTCCATCTCACC R:TTCGTCCGCACATCATAAGCA

2.3. Identification of barley *LSD1* gene

2.3.1. Plant material and growth conditions

Plant lines, used for identification and characterization of barley *LSD1* homologues, are described in Table 11.

If not stated otherwise, *H. vulgare* lines were grown in environmental growth chamber at 22°C under long-day (16 h day/ 8 h night), medium light (ca. 150 μmol $m^{-2}s^{-1}$) conditions and *A.thaliana* lines were grown in environmental growth chamber at 23°C/ 20°C under long-day (16 h day/ 8 h night), medium light (ca. 150 μmol $m^{-2}s^{-1}$) conditions.

Table 11. Plant lines used for identification and characterization of barley LSD1 homologues.

Plant line	Accession	Species	Obtained from
lsd1-1		*A. thaliana*	Dr. Petra Epple, Department of Biology, North Caroline University
Wassilewskija-0	N1603	*A. thaliana*	The European Arabidopsis Stock Center
nec1	GSHO 1284	*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)
nec3	GSHO 2065	*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)
Parkland		*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)
Bowman		*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)
Morex		*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)
Steptoe		*H. vulgare*	USDA ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA)

2.3.2. Sequencing and phylogenetic analysis of A. thaliana LSD1 barley homologues

2.3.2.1. Identification and sequence analysis of barley homologues of Arabidopsis gene LSD1

Arabidopsis thaliana LSD1 amino acid sequence AAC49660 was used as a query in TBLASTN (Gertz 2005) 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 (Wanamaker et al. 2011). Coding sequence of two barley homologues of the *Arabidopsis LSD1*, *ABC10220* and *ABC06454*, were identified by sequencing cv. Morex cDNA clones HvCEa0008p08 and HvSMEb0007a07. Coding sequence of the third homologue, *CBC04043*, was predicted from the unigene sequence.

Table 12. Primers used for *ABC10220, ABC06454* and *CBC04043* sequencing.

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
ABC10220_L01	cgagggatttctcgtctgct	ABC06454_R03_b	caagattgactgtactgcaacatgag
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*	ccatacggagcatcttctgtcaag	ABC06454_R08	atatcacgtgccactaaggtcttgc
ABC10220_R01	ccgcgctttgggtttttgtt	CBC04043_L01	cattccaactcatgttgttattctgag
ABC10220_R02	cacacttgacagaagatgctccg	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	ctgtacattcacagctgaatagtg	CBC04043_R03	cgtgaagggcgcaggag
ABC06454_L06	tggtcagcaacgtcgtagtc	CBC04043_R04	ggaacagcggtcacggtact
ABC06454_L07	cagtggaaatggctcagctt	CBC04043_R05	cttccattgccaggttgacagt
ABC06454_L08	gtgtgattcatagttcgatgccatt	CBC04043_R06*	actgatgtcacgaaactgcagac
ABC06454_L09*	aatatagcccacgtgaattgtggt	CBC04043_R07	ctatacatgtgcactcaaagcattc
ABC06454_R01	tcaggcagcaaccaatcacc	CBC04043_R08	caacgaagggagaaatggagag
ABC06454_R02*	cgtcgacagtcatagggttctca	CBC04043_R09	ggttcatgtatctgtctgacccagt

*-primers used for quantitative real time PCR

Full sequences of *LSD1* barley homologues were determined by sequencing from cvs. Morex and Steptoe. DNA was extracted from fresh leaf tissue, as described by Edwards et al. 1991. Gene specific primers (Table 12), for sequencing of barley *LSD1* homologues, were designed by Primer3 software (Rozen and 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-supplied buffers. PCR was carried out in 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.

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).

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*.

2.3.2.2. Phylogenetic analysis of LSD1 homologues in Poaceae

Coding sequences of barley *LSD1* homologues were predicted, using NCBI ORF Finder (United States National Library of Medicine 2012). 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. Sequences, included in phylogenetic analysis, are represented in Table 13. 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 proml program from PHYLIP3.66 package (Felsenstein 1989). Bootstrap confidence levels were calculated from 100 iterations, using seqboot program from PHYLIP package. Phylogenetic tree was visualized using TreeView (Page 1996).

Table 13. List of LSD1 homologues from *Poaceae* species used for phylogenetic analysis.

Barley *LSD1* homologue	Species (designation in phylogenetic	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

Phylogenetic analysis was also performed, to reconstruct phylogenetic relationships between barley *LSD1* homologues and previously characterized *LSD1* homologues from *A.thaliana* and rice. 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 14) and used, to reconstruct phylogeny of barley, *Arabidopsis* and rice *LSD1*-like genes. Phylogenetic reconstruction was performed essentially as described above.

Table 14. List of *A. thaliana, O. sativa* and *H. vulgare LSD1*-like genes used for phylogenetic analysis.

Designation	Gene or GenBank accession
AtLOL1	At1g32540
AtLOL2	At4g216210
AtLOL3	At1g02170
AtLSD1	At4g20380
OsLOL1	AK061509
OsLOL2	AK111837
OsLOL3	AK111569
OsLOL4	AK120454
OsLOL5	AK065375
OsLSD1	AY525368
Hv14621	CV063671
Hv15713	BE421616
Hv2281	AV933097

Table 15. CAPS markers for linkage mapping of barley homologues of the *LSD1* gene in Oregon Wolfe barley (Owb) mapping population.

Gene	PCR Primers	Restriction enzyme	Restriction fragment size (bp)	
			OwbD	OwbR
ABC10220	F:cgtctgctcggttcctctcg	*Nde*I	2350	2150
	R:gtgctggtctggcaaggttga			200
ABC06454	F:tggttgccgaactttgctga	*Taq*I	720	620
	R:cgtcgacagtcatagggttctcaa		300	300
			230	230
				100
CBC04043	F:agtctcgtccgcagcaac	*Ssp*I	1070	700
	R:cttccattgccaggttgacagt			370

2.3.2.3. Linkage mapping

Oregon Wolf Barley Dominant x Oregon Wolfe 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 (SNP), detected by sequencing (Table 15). Segregation data of the three CAPS markers was used for linkage mapping in Map Manager QTX software (Manly et al. 2001) relative to restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers (Costa et al. 2001).

2.3.3. Characterization of expression pattern of barley LSD1 homologues

2.3.3.1. RNA extraction

To determine the effect of lesion mimic mutations on expression of barley *LSD1* homologues, expression of *ABC10220, ABC06454* and *CBC04043* was analyzed in two barley lesion mimic mutants – *nec1* and *nec3*. Expression of barley *LSD1* homologues in response to powdery mildew infection was analyzed in cv Morex and during diurnal cycle – in cv Parkland.

For RNA extractions 5-cm-long segments of first leaf from 2-week-old barley necrotic mutants *nec1* and *nec3* and corresponding parental lines were snap frozen in liquid nitrogen, immediately after harvesting. For diurnal expression analysis, leaves were harvested at 2^{nd}, 6^{th}, 10^{th} and 16^{th} hour of light cycle and at 18^{th} 22^{nd} and 24^{th} hour of dark cycle. For analysis of expression in response to powdery mildew infection, two week old plants of cv Morex were inoculated with 10-20 conidia per mm^2 from mixed virulent population of powdery mildew, and leaves for RNA extraction were harvested 12 h post inoculation. Total RNA was extracted from frozen leaf tissues, using 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. 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 QIAGEN RNeasy Plant Mini Kit. Quantity of purified total RNA was monitored, using spectrophotometer Ultrospec 3100 pro (Amersham Biosciences, Little Chalfont, UK) or NanoDrop ND-1000 (NanoDrop products, USA)..

2.3.3.2. PCR, RT-PCR and quantitative real-time PCR

cDNA was synthesized with oligo $(dT)_{18}$ primer in a total volume of 15 μl, containing 0.5 μg of total RNA, using RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). For real-time PCR aliquots of cDNA were amplified on ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA), using 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 12. 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 amplification efficiency estimates were calculated from serial dilutions of appropriate cDNA fragments,

amplified from cv Steptoe. Relative quantification was performed using $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmitgen 2001. Transcript levels of the studied genes were normalized to *HvGAPDH* transcript value in the same sample.

2.3.4. Identification of induced mutations in barley genes ABC10220 and ABC06454 using TILLING

2.3.4.1. Selection of analyzed gene fragments

Region most suitable for TILLING analysis was delineated using program CODDLE (Choosing codons to Optimize Discovery of Deleterious LEsions), allowing identification of DNA region with the highest probability of occurrence of missense or truncation mutations. For *ABC10220* 998 bp fragment, starting at C2020, and for *ABC06454* 996 bp fragment, starting at G2628, were analyzed (Figure 9). Primers for amplification of the analyzed region were designed using Primer3 software (Rozen and Skaletsky 2000) (Table 16).

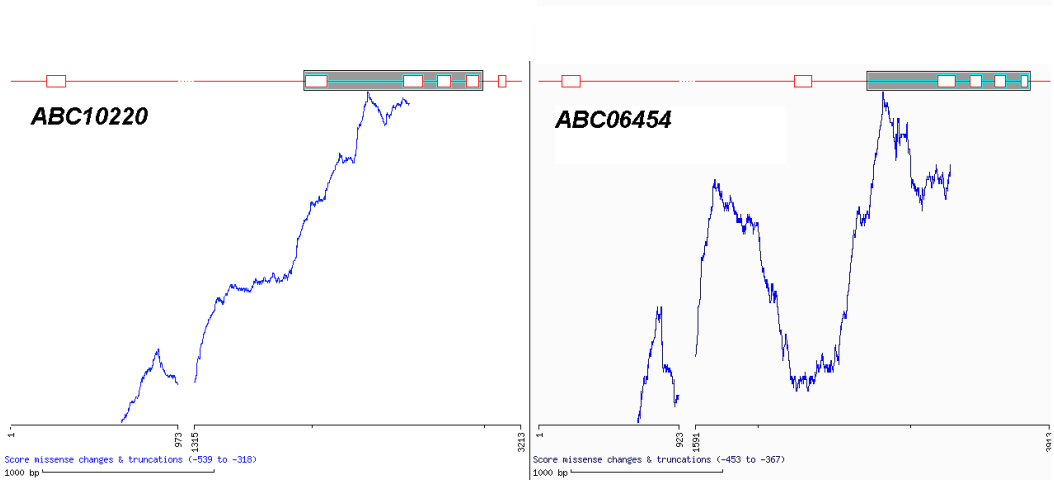


Figure 9. Output from CODDLE program indicating exon–intron structure representation of *ABC10220* and *ABC06454* and probability of occurrence of phenotypically deleterious mutations. Probability is indicated by blue line. Screening window is delineated with grey box.

Non-redundancy of the selected fragments was also verified by Southern blotting, using DNA from 6 barley cultivars (Steptoe, Morex, Barke, Optic, Parkland, Bowman), digested with 3 different restriction enzymes (*Hind*III, *BamH*I, *Xba*I). Briefly, 20 µg DNA was treated with restriction enzyme, separated by gel electrophoresis and transferred to hybridization membrane by alkaline transfer using 0.4 M NaOH and 0.6 M NaCl solution. Prehybridization was done overnight at 60 ºC in prehybridization buffer, containing 1xHSB, 10xDenhardt's III solution and carrier DNA 0.5mg ml^{-1}. ABC10220tilF1_R1 fragment and ABC06454tilF2_R2 fragments, amplified from Parkland cDNA (100 ng), were used as a probe. Probe labeling with 32P was done using RadPrime DNA Labeling System (Invitrogen), following manufacturers recommendations. Hybridization was done overnight at 60 ºC in hybridization buffer, containing 1xHSB, 10xDenhardt's III solution and carrier DNA 0.5 mg ml^{-1}.

Hybridized filter was washed twice with 2xSSC and 1%SDS solution and once with 1xSSC/0.5%SDS solution.

Table 16. Primers used for ABC10220 and ABC06454 TILLING screening.

Primer	Sequence	Labeling
ABC10220tilF1	CGCAATGCAACTACTGTCAGATGCTCA	IrDye700
ABC10220tilR1	CGAATGCAGACTGATTGGAAGGTGGTA	IrDye800
ABC06454tilF2	GCAAGCGGGAGCTACATGCAC	IrDye700
ABC06454tilR2	AAACAATCATTCAATTTTTCCCAGGTGT	IrDye800

2.3.4.2. TILLING population screening

For identification of mutants, comprising mutations in *ABC10220* and *ABC06454*, two barley TILLING populations were screened – TILLING population of University of Silesia and population, developed at University of Bologna (Table 17). Screening of Silesia mutant population was performed by author of the thesis, whereas screening of Bologna TILLING population was performed by Dr.Valentina Talamé at University of Bologna.

Selected fragments of analyzed genes were amplified using IrDye700/800 labeled primers. Amplification was done in total volume of 20 µl reaction, containing 300 ng genomic DNA, 0.5 µM primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 2 u Yellow Taq DNA polymerase (EURx, Poland), used with manufacturer-supplied buffers. PCR was carried out in Biometra T1 thermal cycler as follows: initial denaturing step for 5 min at 95 °C, 4 cycles of touch down of 30 s at 95 °C, 30 s at 67-63 °C, 1.5 min at 72 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 63 °C, 1.5 min at 72 °C and final extension of 5 min at 72 °C.

Pooled samples, containing DNA from 8 individuals, were screened from both – forward and reverse pools, to relieve identification of mutations. DNA fragments, amplified with PCR, were further subjected to the following temperature treatment: 95 ºC 5 min, 80 ºC 1 s 55 ºC 20 s 45 ºC 1s, 25 ºC 1s, 15 ºC, in order to ensure heteroduplex formation. Obtained heteroduplexes were treated with Surveyor nuclease (*Cel* nuclease family) from SURVEYOR mutation detection kit (Transgenomic, USA) for 15 min at 42 ºC. Digested samples were purified, using ethanol/sodium acetate precipitation. Samples were analyzed by polyacrylamide gel electrophoresis on Li-Cor 4300 DNA analyzer.

Table 17. Characteristics of screened TILLING populations.

TILLING population	Method of mutagenesis	Nr of mutant plants*	Parental cultivar	Average frequency of mutations	Reference
University of Silesia	Sodium azide and N-nitroso-N-methylurea	6000	Sebastian	1 per 235kb	Kurowska et al. 2011
Tillmore (University of Bologne)	Sodium azide	4906	Morex	1 per 374kb	Talamé et al. 2008

*Nr of plants at the time of analysis

2.3.4.3. Sequencing and sequence analysis of the identified mutants

Mutations, detected by Cel nuclease, were confirmed by sequencing. PCR products, amplified from identified mutants and corresponding parental line, were treated with exonuclease I and shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) prior to sequencing. 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).

Obtained sequences were analyzed with software PARSESNP, to identify the location and type of mutation. DNA sequences, comprising mutations in the non-coding part of the genes, were analyzed, using NetGene2 server (Hebsgaard et al. 1996), to identify mutations, potentially affecting RNA splicing.

To verify the effect of mutations on RNA splicing, full length coding sequence was amplified and sequenced from analyzed plants.

2.5. Complementation of *A. thaliana lmm lsd1* using homologous barley genes

All experiments described in this part of the study have been carried out with the GMO handling permission 1-24/91, issued on 28.12.2009 by Food and Veterinary Service, Ministry of Agriculture of Latvia.

2.5.1. Plant lines used for transformation

Arabidopsis thaliana mutant line *lsd1-1* (Dietrich et al. 1997) was used for complementation tests. Seed of *lsd1-1* were kindly provided by Dr. Petra Epple from Dr. Jeffery L. Dangl laboratory at Department of Biology, University of North Carolina. *lsd1-1* mutant comprises deletion, encompassing two out of six exons of the *AtLSD1* (At4g20380) (Dietrich et al. 1997). *lsd1-1* mutation was verified by PCR amplification, using either three primers, yielding products of different length in mutant and parental line, or two primers amplifying PCR product only from parental line (Table 18).

Table 18. Primers used for confirmation of *lsd1-1* mutation.

Primers	Expected result		Reference
	mutant	wt	
PCR product obtained by primer triplet: F1:ACCTAACAAAAAGAAAAGTGTGTGAGG F2:ATAATAAACCCTACTAGCTCTAACAAG R:CTGCTACTTTCATCCAAAC	600bp	940bp	Rusterucci et al. 2001
or			
F: GTG TGT GTT TGG ATG AAA GTA GCAG R: GCT AAA TGA CAA CAG CTT AGA CGC	fragment absent	fragment present	Mateo et al. 2004

2.5.2. Plant growth conditions

Seeds from *A. thaliana* mutant line *lsd1-1* were surface sterilized, incubated at 4 ºC for 3 days and germinated on half strength MS medium. *lsd1-1* seedlings were transferred to soil and grown under 8 h day (23 ºC)/ 16 h night (20 ºC), to avoid induction of run-away cell death.

2.5.3. Plasmid DNA constructs used for lsd1-1 complementation

To establish the functional *HvLSD1*, two *AtLSD1* barley homologues – *ABC10220*, and *ABC06454*, were tested for their ability to complement *A. thaliana mutant lsd1-1*. For constructs full length cDNA of *ABC10220* or *ABC06454* from cv Steptoe was amplified with gene specific primers, containing appropriate restriction sites (Table 19) for further subcloning into plant binary vector pMOA36, described by Barrell and Conner (2006). Each barley gene was subcloned under the promoter of *AtLSD1* (Figure 10). P_{AtLSD1} was amplified from *A. thaliana* Col and included 1052 bp sequence upstream of *AtLSD1* start codon.

Table 19. Components of vector constructs used for *lsd1-1* complementation.

Fragment	Sequence Gene Bank ID	Primers	Restriction sites for subcloning
ABC10220	EU545233	F:TTTTTCATATGCAGAGCCAGATCGTGTGC R:TTTTTACTAGTCTACTTTTTCCCGCCGGTGG	*NdeI/SpeI*
ABC06454	EU545234	F:TTTTTCATATGCAGAGCCAGATCGTGTGC R:TTTTTACTAGTTCAATTTTTCCCAGGTGTAATTCCG	*NdeI/SpeI*
P_{AtLSD1}	Chr4:11003971..11005023	F:TTTTTGAGCTAGAAATAAACCGATAAAGACTTC R:TTTTTCATATG CCAAACACACACAAATTGCTTC	*SacI/NdeI*
pMOA36	DQ869007	T-DNA flanking F:GATATCGTTTACCCGCCAT R:TTCTACGTGTTCCGCTTCCT	*SacI/SpeI*

For amplification of all PCR derived fragments, *Pfu* polymerase was applied. PCR fragments were run on gel electrophoresis and purified using silica precipitation. Purified fragments were than digested with appropriate restriction enzymes (Fermentas, Lithuania), following manufacturer's recommendations. Digested fragments were purified, using chlorophorm, and precipitated with 3 M sodium acetate 95 % ethanol in v/v 1:20.

Vector DNA was digested with appropriate enzymes (Fermentas, Lithuania), following manufacturers recommendations. Digested vector was dephosphorilated with 3 u of shrimp alkaline phosphatase for 1h and separated using gel electrophoresis. Required vector fragment was excised and purified using silica precipitation.

Prepared fragments of the promoter and gene were ligated into digested vector, using T4 ligase. Ligation was carried out in 20 µl reaction containing 1 u T4 ligase with manufacturer's supplied buffer (Fermentas, Lithuania), 100 ng of vector DNA and required amount of gene

and promoter fragments. Vector : insert DNA was ligated in 1:3 molar ratio. Ligation mix was incubated at 22 ºC for 2 h and afterwards inactivated in 65 ºC for 15 min. Obtained constructs were verified by PCR with gene or promoter specific primers in combination with primers from vector T-DNA flanking region (Table 19).

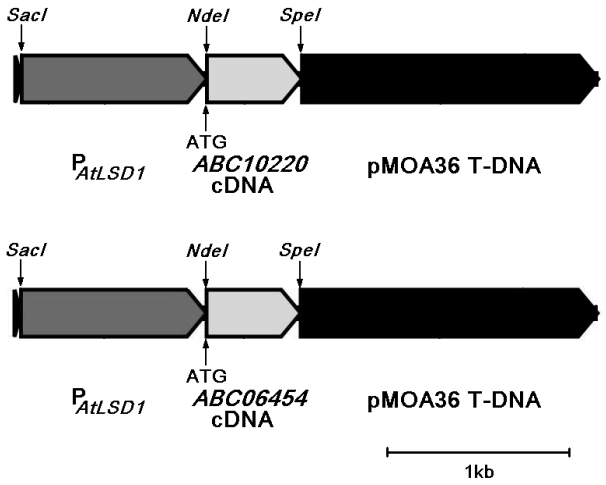


Figure 10. Schematic representation of T-DNA region of vector constructs used for complementation of *lsd1-1*.

2.5.4. Bacterial transformations

2.5.4.1. Transformation of E.coli

Obtained constructs were cloned in *E.coli*. Transformation was done according to Inoue et al. 1990. In brief, for preparation of competent cells, *E.coli* DH5α was grown at 20 ºC and 200 rpm until OD_{600}=0.6. Afterwards cells were chilled on ice and harvested by centrifugation. Pelletted cells were than resuspended in transformation buffer, containing 55 mM $MnCl_2$ $4H_2O$, 25 mM $CaCl_2$ $2H_2O$, 250 mM KCl, 10 mM PIPES. After two cycles of centrifugation cells were resuspended in ice-cold transformation buffer, containing 10% DMSO and snap-frozen in liquid nitrogen.

For transformation 100 µl of competent cells were melted on ice and 2 µl of ligation mix was added. After 30 min incubation on ice cells/plasmid mix was subjected to 42 ºC for 45 sec, after which 1 ml of pre-warmed SOC medium (tryptone 20 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 9 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, $MgSO_4$ 10 mM, 20 mM glucose, pH7) was added. Cells were incubated at 37 ºC, 200 rpm for 45 min and afterwards harvested by centrifugation and sown on solid LB medium containing 100 µg ml^{-1} spectinomycin. Colony PCR was performed with combination of insert and vector specific primers, in order to identify colonies, carrying desired construct. Selected colonies were used as inoculum for over-night cultures, from which plasmid DNA was later extracted. Aliquots of the culture were stored in 15% glicerol at -80 ºC. For plasmid DNA extraction a standard miniprep protocol was applied. In brief, after alkaline lysis of over-night culture, plasmid DNA was precipitated using sodium acetate/isopropanol precipitation.

Constructs were verified by sequencing insert with primers from plasmid T-DNA flanking region. PCR products, amplified by combination of insert and plasmid specific primers, were treated with exonuclease I and shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) prior to sequencing. 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).

2.5.4.2. Transformation of A. tumefaciens

Verified constructs were transferred to *A. tumefaciens* by electroporation. For preparation of electrocompetent *A. tumefaciens*, AGL-1 was grown at 28 ºC temperature, 250 rpm until log phase (OD_{600}=0.8-1.0). Cells were chilled on ice for 30 min, harvested by centrifugation and resuspended in ice-cold sterile water. Centrifugation/ resuspension was repeated twice, after which cells were resuspended in sterile 10 % glycerol and snap-frozen in liquid nitrogen.

Electroporation was done with MicroPulser electroporator (Bio-Rad) 200 ng of plasmid DNA was added to 40 µl of electrocompetent cells. Cells were subjected to electroporation by 2.2 kV for 5.8 msek in 0.2 cm gap cuvettes. After electroporation cells were immediately transferred to prewarmed YM medium (0.04 gL^{-1} yeast extract, 1 gL^{-1} glucose, 1.7 mM NaCl, 0.8 mM $MgSO_4$ $7H_2O$, 2.2 mM K_2HPO_4 3 H_2O), incubated for 3h at 28 ºC, 250 rpm and afterwards harvested by centrifugation and sown on solid YM medium containing 100 µg ml^{-1} spectinomycin. Colony PCR was performed with combination of insert and vector specific primers, in order to identify colonies carrying desired construct. Selected colonies were used as inoculum for over-night cultures. Aliquots of the culture were stored in 15% glicerol at -80 ºC.

2.5.5. Plant transformation

Plant transformation was done according to Zhang et al. 2006 b. Briefly, *Agrobacterium* cultures, harboring the appropriate construct, were grown at 28 ºC to the stationary phase (OD_{540}=1.5-2.0). Cells were collected by centrifugation and resuspended in 5% sucrose solution with 0.02% Silwet L-77 surfactant added. *Arabidopsis* mutants (10 plants per transformation), containing flowering stems, were fully dipped into *Agrobacterium* solution for 10 s and afterwards allowed to dry for 5 s. Transformed plants were covered with plastic bags and kept in dark for 24 h. Plants were repeatedly transformed after 7 days, to increase transformation efficiency.

2.5.6. Screening of primary transformants

Seed from transformed plants were harvested, surface sterilized and germinated in half strength MS medium, containing phosphinothricin 10 mg L^{-1}. After 2 weeks healthy, green plants were transferred to autoclaved soil where they were grown at short day conditions for 2

weeks. Afterwards long day conditions were applied, to induce flowering and seed formation in T_1 plants.

2.5.7. Confirmation of transformation

Presence of transgene was verified by PCR on genomic DNA (primers are indicated in Table 19). For DNA extraction, leaf samples were taken from well developed T_2 plants. DNA was extracted from fresh leaf tissue as described by Edwards et al. 1991.

Expression of transgene was evaluated with *ABC10220*, *ABC06454* and *BAR* (PPT resistance) specific primers (Table 20) in RT-PCR. RNA extraction and cDNA synthesis was performed according to methodology described in paragraph 2.3.3.1. and 2.3.3.2.

Table 20. Primers used for transgene expression assessment.

Analyzed gene	Primers
BAR	F:cgcaacgcctacgactggac
	R: agctgccagaaacccacgtc
ABC10220	F:atgggtgcaggagcgttctactcta
	R:ggtctggcaaggttgactgtatcac
ABC06454	F:catgtgccgagccatcacc
	R:gcaacatgagcatctcacagtgtct

2.5.8. Characterization of transgenic ABC06454/lsd1-1 plants

Confirmed transgenic *ABC06454/lsd1-1* plants were characterized for tolerance to long-day light conditions. Transgenic plants and wt Ws0 plants were grown at short day light conditions (8 h light/16 h dark) at 23 °C/20 °C day/night temperature. After four weeks plants were transferred to long day (16 h light/ 8 h dark) conditions. Three days after transfer run-away cell death phenotype of leaves was evaluated, using trypan-blue staining. Detached leaves were submerged into ethanol : glacial acetic acid solution (3:1) for 24 hours. After distaining leaves were washed in sterile distilled water and stained for 30 minutes with solution, containing 60% lactic acid, glycerol, trypan blue (10 mg ml^{-1}) and phenol in equal volumes. Stained leaves were washed with distilled water to remove excess stain.

ABC06454/lsd1-1 plants were also characterized for their response to exogenously applied salicylic acid. Transgenic plants, *lsd1-1* and wt Ws0 plants were grown at short day light conditions (8 h light/16 h dark) at 23 °C/20 °C day/night temperature. After two weeks plants were sprayed with 0.5 mM salicylic acid solution. Plants were uprooted and transferred to 10 ml of distilled sterile water 24 hours after spraying. Cell death was evaluated with trypan-blue staining, as described previously and quantified by electrolyte leakage assay, as described by Dellagi et al. (1998), with minor modifications. Conductivity was measured with conductivity meter handylab LF11 (Schott Instruments) every 12 hours. Each datapoint contained data from at least 6 plants. Data were expressed as percentage of total conductivity. Total conductivity was estimated as conductivity of sample after freeze/thaw treatment.

3. Results

3.1. Characterization of barley lesion mimic mutant *nec1*

3.1.1. nec1 mutant exhibits constitutive activation of systemic acquired resistance related parameters of barley mutant nec1

Since it is known, that *nec1* significantly over-expressed pathogenesis related genes (Rostoks et al. 2006), it was investigated, whether *nec1* plants spontaneously display also other SAR-related signals, such as altered accumulation of reactive oxygen species and over-accumulation of salicylic acid (SA). Spectrofluorimetric analysis of whole-leaf extracts of two week old *nec1* plants with a fully developed lesion mimic phenotype and the parental line Parkland showed a three-fold higher overall level of H_2O_2 in the mutant (data not shown).

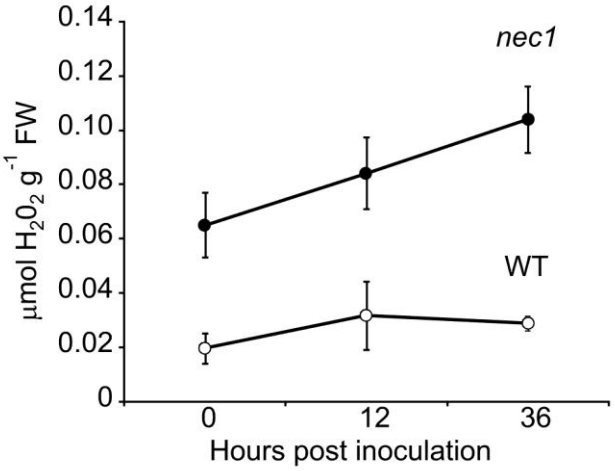


Figure 11. Time course of whole leaf H_2O_2 accumulation in *nec1* and wt plants after *Blumeria graminis* f.sp *hordei* (*Bgh*) infection. *nec1* mutation triggers H_2O_2 over-accumulation in barley in the absence of pathogen infection, but it does not alter time course of H_2O_2 production in response to *Bgh* infection. Error bars represent the standard deviation of means (n = 5 per data point).

To ascertain, whether the elevated overall amount of H_2O_2 in *nec1* plants affected H_2O_2 accumulation during *Bgh* infection, overall H_2O_2 amount in *nec1* and wt plants was assessed at 12 h and 36 h after inoculation with a virulent mixed population of *Bgh*. The analysis did not reveal considerable changes in the H_2O_2 content of wt plants during the first 36 h after inoculation, whereas *nec1* mutants showed a slight, statistically non-significant increase in H_2O_2 levels at 36 h after inoculation (Figure 11).

H_2O_2 accumulation and *PR-1* expression is known to be associated with SA dependent signaling. Therefore, the SA content of *nec1* and wt plants was also measured. HPLC assay confirmed that levels of free SA and conjugated SA were four- and fifteen-fold higher, respectively, in *nec1* than in wt plants (Figure 12).

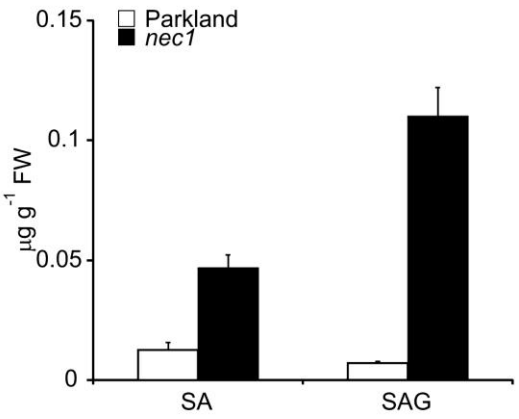


Figure 12. Level of free salicylic acid (SA) and salicylic acid β-glucoside (SAG) in *nec1* and wt plants. *nec1* mutant contains significantly higher level of conjugated, as well as free SA compared to parental cv. Parkland. SA content was analyzed using reverse-phase high performance liquid chromatography in leaf tissue extracts of 14 day old plants. Average values from three biological replicates are presented, each consisting of three technical replicates. Error bars represent standard deviation.

3.1.2. Resistance of the nec1 mutant to Pseudomonas syringae

Barley resistance to the non-host bacterial pathogen *Pseudomonas syringae* likely employs SA-mediated defense pathway (Vallelian – Bindschedler et al. 1998). Therefore, the constitutive activation of SA signaling in *nec1* might contribute to its non-host resistance. *nec1* plants were inoculated with *P. syringae* pv. *tomato* (*Pst*) at two inoculum densities – 10^5 and 10^8 cfu ml^{-1} using vacuum infiltration technique. At day 3 after infiltration with 10^8 cfu ml^{-1} of *Pst* the amount of bacteria in *nec1* was reduced, whereas Parkland had accumulated ca. 6-fold higher amount of *Pst* making the difference in bacterial growth between wt and *nec1* statistically highly significant ($p = 0.01$, Student's *t*-test) at this stage of infection (Figure 13). Inoculation with *Pst* at lower inoculum density (10^5 cfu ml^{-1}) did not reveal any differences in *Pst* resistance between *nec1* and wt plants (Figure 13A).

Ion leakage measurements were also performed to characterize the effect of *Pst* infection on *nec1* and Parkland. Vacuum infiltration with *Pst* at lower inoculum density (10^5 cfu ml^{-1}) did not elicit cell death in either *nec1* or Parkland (Figure 13B). On contrary to lower *Pst* density which did not reveal any difference in resistance between *nec1* and wt plants, inoculation with *Pst* at 10^8 cfu ml^{-1} elicited differential response in *nec1* and wt. Tissue samples from *nec1* plants inoculated with *Pst* at 10^8 cfu ml^{-1} displayed more pronounced ion leakage suggesting an enhanced cell death in *nec1* after infection (Figure 13B).

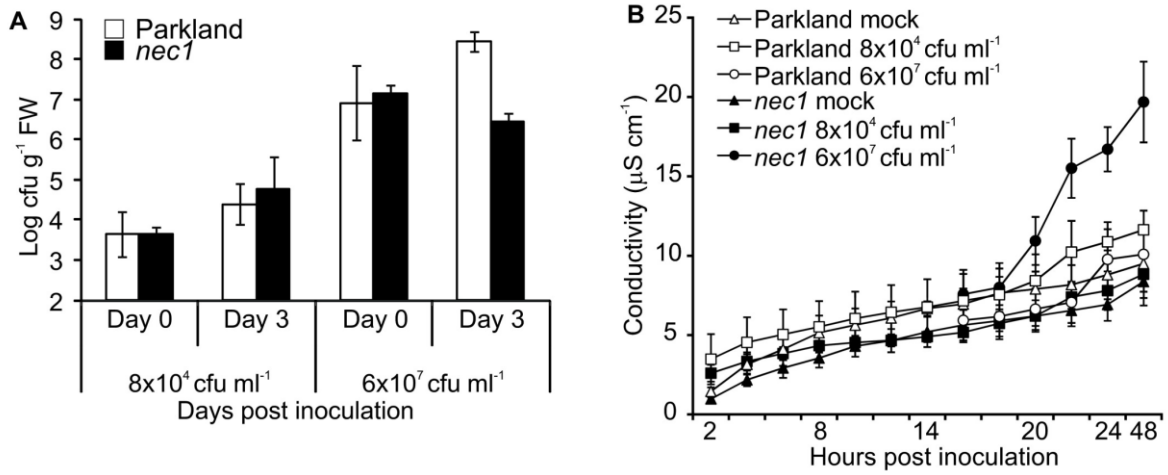


Figure 13. Response of barley lesion mimic mutant *nec1* to non-host pathogen *Pseudomonas syringae* pv. *tomato* applied at low and high inoculum densities. A - Growth of *Pseudomonas syringae* pv. *tomato* (Pst) in *nec1* and parental cv. Parkland was monitored immediately and 3 days after vacuum infiltration with Pst applied at inoculum densities of 8×10^4 or 6×10^7 cfu ml^{-1}. For mock inoculation plants were infiltrated with 10 mM MgCl2. Infection was expressed as number of colony forming units (cfu) per gram of fresh leaves (FW). Due to the high between-experiment variation, results of one representative experiment out of four independent experiments are shown. Error bars represent standard deviation. At high inoculum density (6×10^7cfu ml^{-1}) bacterial cfu number in *nec1* at the day 3 was significantly ($p < 0.01$, Student's t-test) lower than in wt. **B -** Progression of cell death in *nec1* and Parkland after infection with *Pseudomonas syringae* pv. *tomato* in the experiment shown in panel A. *nec1* mutation showed increased electrolyte leakage in barley inoculated with non-host bacteria Pst at 6×10^7 cfu ml-1. Measurements of electrolyte leakage were taken every two hours during 24 hour period and at 48 hours after inoculation. Error bars represent standard deviation.

3.1.3. Resistance of nec1 mutant to powdery mildew Blumeria graminis f.sp. hordei

Since *nec1* plants exhibited constitutively active defense responses, the role of *nec1* in basal resistance against *Bgh* was assessed. Due to their basal resistance, even susceptible barley cultivars are able to restrict infection to some extent. In order to assess the effect of *nec1* mutation on basal *Bgh* resistance, microcolony formation was examined. *nec1* supported formation of significantly ($p < 0.001$, *t*-test) smaller number of *Bgh* colonies compared to wt plants (Figure 14). To further test, if restricted formation of *Bgh* microcolonies on *nec1* derived from the rapid and effective localized response precluding fungal penetration or from post-invasive defense impeding further fungal development, we examined *nec1 Bgh* penetration resistance. The effect of *nec1* mutation on *Bgh* penetration resistance was characterized as the proportion of interaction sites that had formed *Bgh* haustoria to the total number of *Bgh* spores that had germinated at 48 hpi. *nec1* plants permitted almost identical entry and haustoria establishment rate of *Bgh* as the parental line (71% and 74% *Bgh* penetration efficiency respectively, $p = 0.64$, Student's *t*-test).

Basal *Bgh* resistance has been shown to be tightly linked to the molecular mechanisms of race-specific *Bgh* resistance triggered by different *Mla* alleles (Caldo et al. 2006, Shen et al. 2007). *HvRbohA* and *HvRacB* are known to participate in basal as well as race-specific

Bgh resistance (Schultheiss et al. 2002, Trujillo et al. 2006, Wong et al. 2007). The expression of these genes was characterized using real-time quantitative PCR. Relative mRNA abundance of the analyzed genes was not affected by *nec1* mutation (Figure 15), serving as additional evidence for the independence of *nec1* from effector-triggered immunity ensuring rapid localized *Bgh* resistance.

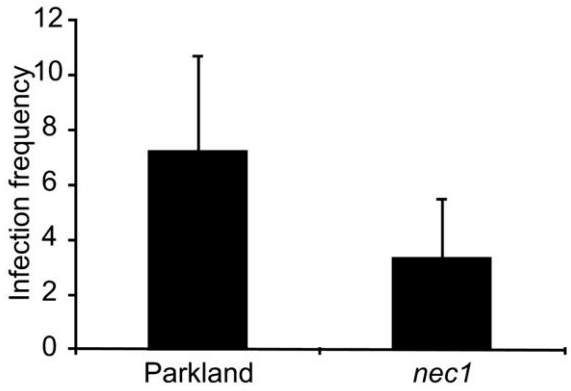


Figure 14. *Blumeria graminis* f.sp *hordei* (*Bgh)* microcolony formation on *nec1* and wt plants. Excised segments of barley leafs were inoculated with a virulent *Bgh* isolate. Microcolony formation was inspected microscopically 4 days post infection and infection rate was expressed as a number of microcolonies per cm^{-2} leaf area. Figure reflects data from two independent experiments. Error bars represent standard deviation. Infection frequency significantly differs between *nec1* and Parkland ($p < 0.001$, t-test).

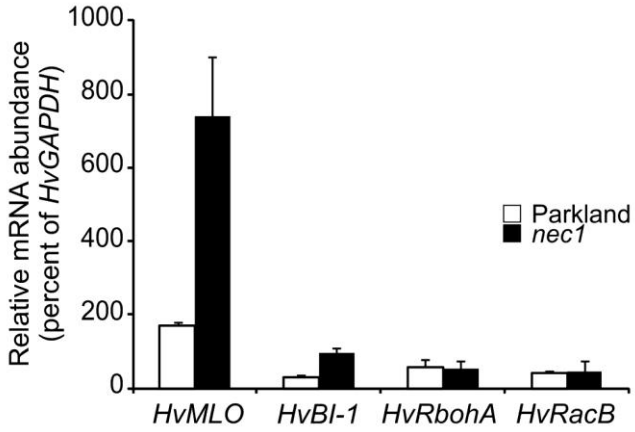


Figure 15. Effect of *nec1* mutation on expression of powdery mildew resistance related genes. Transcript abundance of powdery mildew resistance related genes in *nec1* mutants was determined by quantitative real time PCR. mRNA abundance of *HvMLO* and *HvBI-1* is significantly increased in *nec1*. Error bars represent standard deviation.

3.1.4. nec1 mutation alters expression of BI-1 and MLO, but does not affect mlo-5-triggered race non-specific powdery mildew resistance

To find out, if *nec1* mutation affected *mlo*-triggered race non-specific *Bgh* resistance, the expression of *MLO* and *BI-1* genes was analyzed using real-time quantitative PCR. Significant over-expression of both *MLO* and *BI-1* in *nec1* plants was observed (Figure 15). To further test whether *nec1* mutation had any effect on race non-specific powdery mildew resistance conferred by *mlo-5* mutation, *Bgh* penetration resistance of *nec1*/*mlo-5* double mutants was characterized. Similar to *mlo-5* mutants, *nec1*/*mlo-5* plants were almost fully resistant to *Bgh*, allowing establishment of fungal haustoria only at less than 2% of interaction sites (Figure 16). In addition, the H_2O_2 content of whole-leaf extracts from *nec1*/*mlo-5* double mutants was analyzed. While the *nec1* mutant showed markedly increased accumulation of H_2O_2 compared to wt *NEC1* plants, the experiment did not reveal a significant effect of *mlo-5* mutation on H_2O_2 over-accumulation in *nec1* (Figure 17).

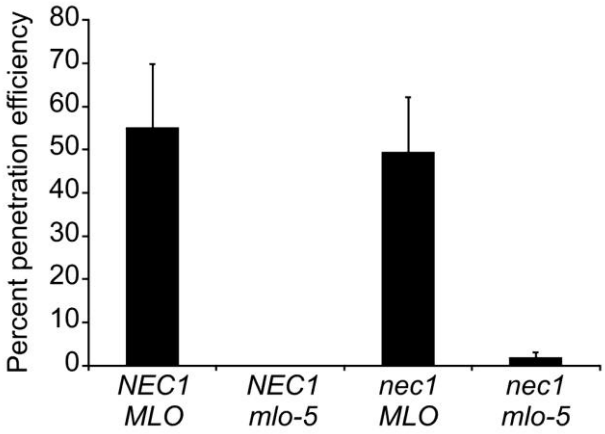


Figure 16. Effects of *nec1* mutation on *mlo-5* triggered *Blumeria graminis* f.sp *hordei* (*Bgh)* penetration resistance. Fourteen days old plants were inoculated with 10-20 conidia per mm^2 and at 48 h post inoculation infected leaves were harvested and *Bgh* penetration efficiency was assessed. At least 100 interaction sites per variant were observed. Error bars represent standard deviation.

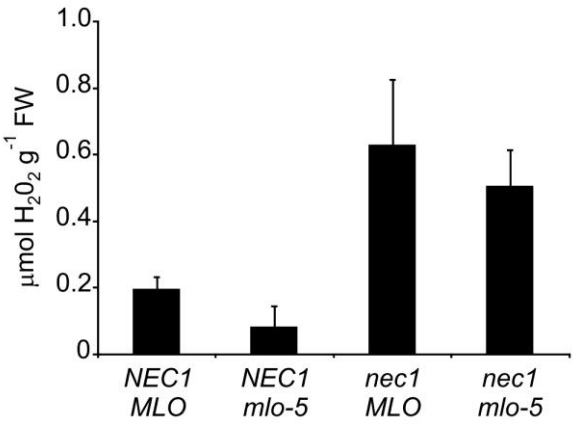


Figure 17. Effect of *mlo*-5 mutation on H_2O_2 accumulation in barley mutant *nec1*. *mlo-5* mutation does not affect overaccumulation of H_2O_2 in *nec1* mutant. H_2O_2 content was determined spectrofluorimetrically in leaf extracts of wt, *nec1*, *mlo-5* and *nec1/mlo-5* double mutants. Each datapoint is based on five samples each containing tissues from three plants. Error bars represent standard deviation.

3.1.5. Induction of systemic acquired resistance related markers in nec1 might be associated with altered auxin signaling

3.1.5.1. nec1 exhibits altered sensitivity to exogenously applied auxin

To find out if *nec1* mutation affects auxin signaling in barley three physiological responses traditionally serving as indicators for altered auxin signaling – root gravitropic response, cell size and coleoptile elongation were evaluated in *nec1*. Endogenous auxin regulates cell expansion (Perrot – Rechenmann 2010). We used leaf impression method (Khazaie et al. 2011) to measure size of epidermal cells from middle part of adaxial surface. Unlike *dnd2* in *A. thaliana nec1* does not exhibit dwarfed stature. However, average perimeter of epidermal cells in *nec1* was statistically significantly smaller compared to Parkland epidermal cells (Student's t-test p=3.5 E-07) (Figure 18 A). Root tip curving in response to gravistimulation at 90° to the vertical was not significantly altered in *nec1* (Figure 18 B). Coleoptile elongation is one of the tests routinely used for characterizing auxin sensitivity in plants. Exogenously applied auxin triggers excised coleoptile elongation in barley in dose dependent manner (Kotake et al. 2000). Detached coleoptile test showed differential response to NAA in *nec1* plants as the *nec1* coleoptiles showed higher increase in length following application of exogenous auxin (Figure 18 C). Thus, *nec1* showed significantly different response to exogenously applied auxin whereas physiological responses controlled by internal auxin did not show significant effect of the mutation.

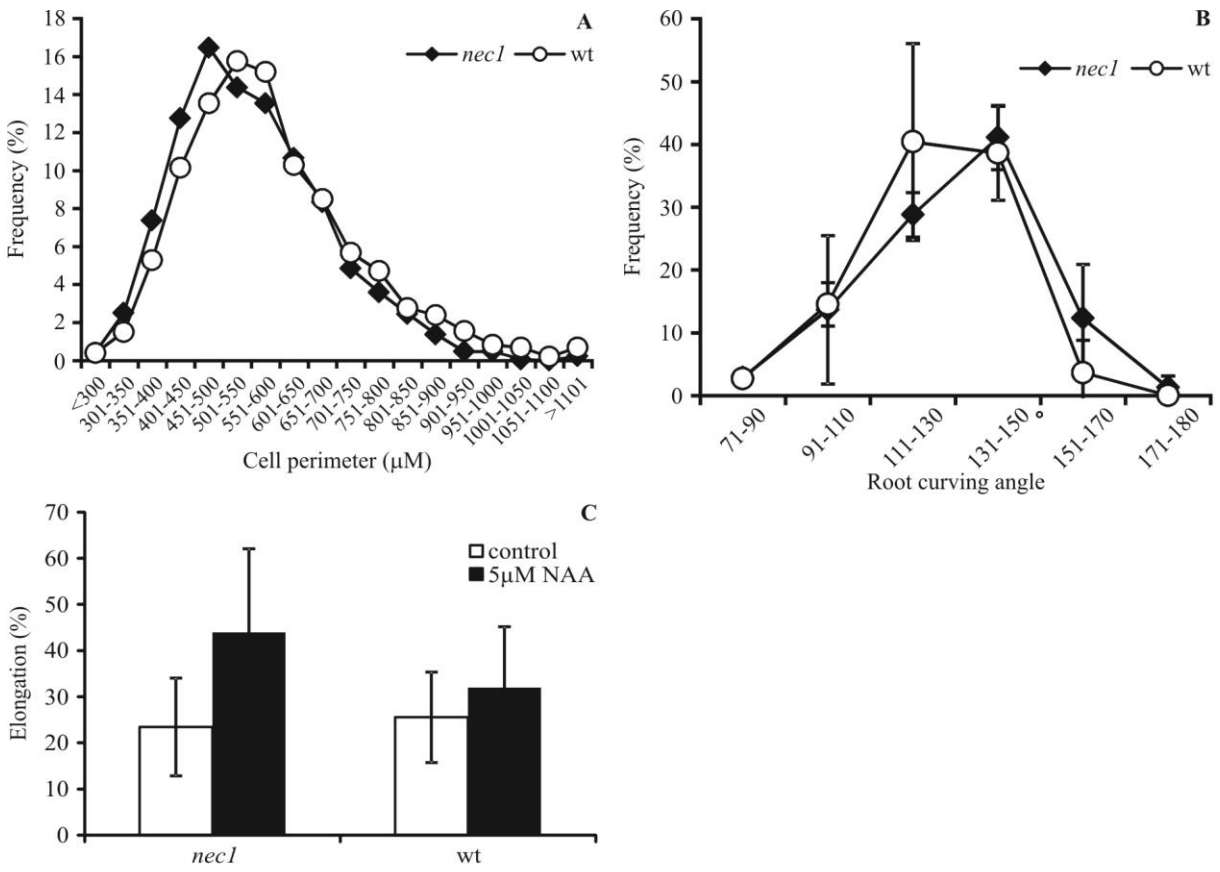


Figure 18. Physiological indicators of endogenous auxin response in barley mutant *nec1*. **A**-Cell size of *nec1* and wt plants. Perimeter of epidermal cells (n>1500) from 10 plants from *nec1* and wt was measured. Based on Student's t-test two datasets differ very highly significantly (p=3.5 E-07). **B**-Angle of root curvature after gravistimulation at 90° to the vertical. Root gravitropic response of *nec1* and wt does not differ significantly. Seedlings were grown for 3 days vertically and then rotated by 90°. Error bars represent the standard deviation of means (n=20 in each – *nec1* and wt). **C**-Exogenously applied naftil acetic acid (NAA) differentially affects coleoptile elongation in *nec1* and wt plants. Results are expressed as percent increase compared to initial segment length. Error bars represent the standard deviation of means.

Auxin is also known to affect root formation and elongation in plants. Root elongation in response to externally applied IAA was tested in *nec1* and wt plants. Lower IAA concentrations showed significantly stronger inhibitory effect on root elongation in *nec1* (Figure 19) whereas roots of untreated control plants did not differ between *nec1* and wt suggesting that *nec1* exhibits increased sensitivity to auxin applied at lower concentrations.

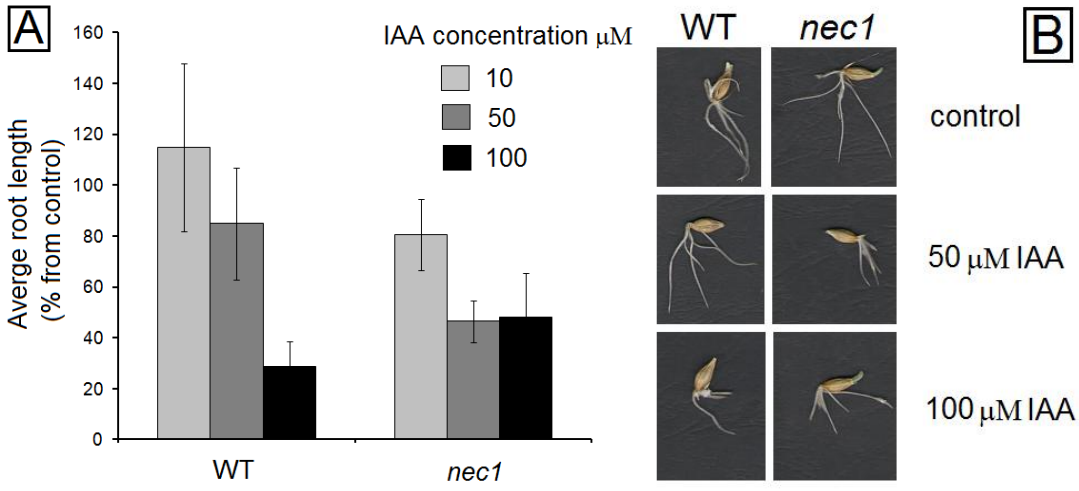


Figure 19. Root elongation in barley mutant *nec1* and wt plants in response to exogenously applied IAA. A – average root length of *nec1* and wt plants in response to exogenously applied IAA. Root length was estimated 3 days after germination. Each datapoint represents average value from at least 15 seedlings. Error bars represent standard deviation. Experiment was repeated twice with similar results. **B** – representative seedlings from experiment in A.

3.1.5.2. nec1 mutation affects expression of auxin signaling related genes

To gain a better understanding of molecular basis of the observed differences in *nec1* physiological auxin response, the expression of several key genes involved in auxin biosynthesis were studied using quantitative real-time PCR.

YUC1, *VT2* and *NIT2* were chosen as genes representing different auxin biosynthesis pathways. The expression of *VT2* and *YUC1* genes was significantly repressed (Figure 20) suggesting that some of the auxin responses in *nec1* mutant might be altered due to changed expression of auxin biosynthesis related genes.

3.1.5.3. IAA concentration in nec1 is significantly increased

Since significant changes in auxin biosynthesis gene expression were identified in *nec1* mutant, it was tested, if there are any changes in phytohormone concentration in the *nec1* mutant. Amount of internal IAA in three allelic *nec1* mutants and wt plants was evaluated with HPLC as described by Nakurte et al. 2012. All allelic *nec1* mutants showed significant increase in auxin concentration (Figure 21) suggesting that functional CNGC4 in barley is also required for salicylic acid and auxin hormonal balance in barley.

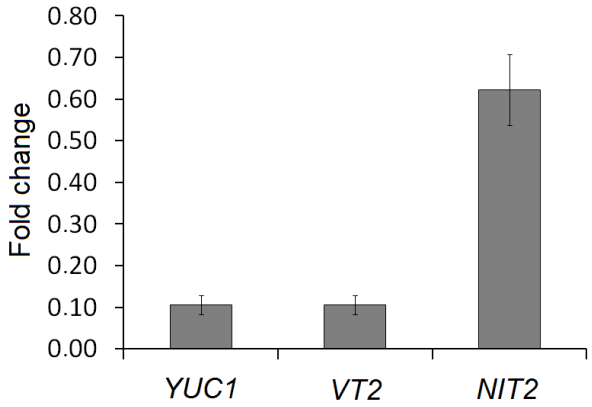


Figure 20. Expression of auxin biosynthesis genes in barley mutant *nec1* compared to wt plants. Expression was assessed using real time qRT-PCR. Value represents difference between *nec1* and wt plants. Data are average values of 5 biological replicates each containing tissues of 5 plants. Error bars represent standard deviation.

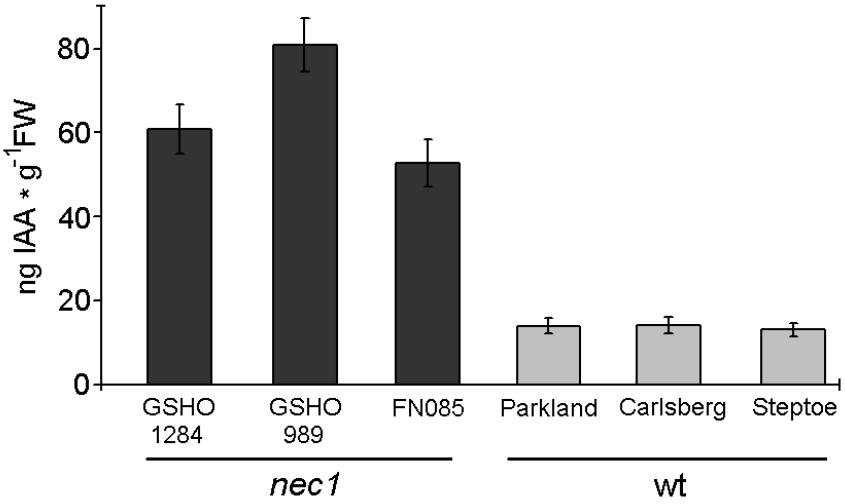


Figure 21. IAA concentration in three allelic barley *nec1* mutants and corresponding wt plants. IAA concentration was assessed with HPLC. Each datapoint contains data from three replicates. Error bars represent standard deviations.

3.2. Identification of barley *NEC3* gene

3.2.1. Transcript based cloning of nec3 candidate genes using Affymetrix barley DNA GeneChip 1

Fast neutron irradiation is known to cause large deletions in plant genomes (Li et al. 2001), which may cause complete or partial deletion of one or several genes and, consequently, lack of the corresponding mRNA in the plant. Thus, comparison of transcriptome between mutant and wt plant may identify candidate genes for the mutant phenotype, assuming that the microarray contains the probes for the deleted gene and that the appropriate tissue type and developmental stage, where the gene is expressed, are sampled.

The *nec3* mutation causes development of tan and brown necrotic spots on barley leaves (Lundqvist et al. 1997). We used two allelic fast neutron irradiated recessive mutants with necrotic spots FN362 (*nec3.l*) and FN363 (*nec3.m*) and parental cv. Steptoe to identify the defective gene responsible for the necrotic phenotype in *nec3*. FN362 and FN363 were isolated at Washington State University (Pullman, WA, USA) by prof. Andris Kleinhofs. FN362 and FN363 are allelic to described *nec3* mutants – GSHO2065 (*nec3*.d) and GSHO2066 (*nec3*.e) (prof. Andris Kleinhofs, unpublished data). Either 10 day or 7 week old plants were used for two independent Affymetrix chip experiments to identify *nec3* candidate gene. For sake of simplicity these two experiments will further be referred to as 10d (10 day old plants) and 7w (7 week old plants) experiments.

Out of 22 791 probe sets represented on Affymetrix Barley1 GeneChip, expression of 10 507 probe sets in 10d and 10 973 in 7w experiment were detected as present and exceeding a threshold signal level of 50 in at least one mutant or parental line. Gene expression values significantly correlated between FN362 and FN363 in any of experiments (Figure 22).

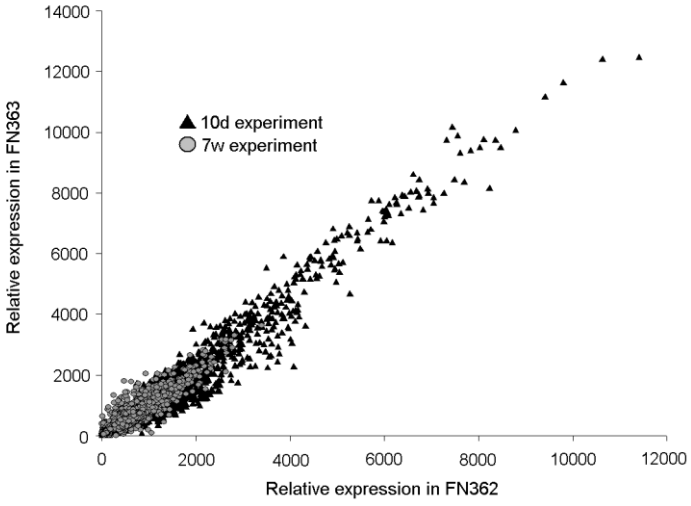


Figure 22. Correlation between transcriptomes of barley lesion mimic mutant *nec3* allelic mutants FN362 and FN363. Data represent relative transcript abundance assessed by Affymetrix Barley GeneChip1 analysis. Only genes with transcript abundance above threshold of detection are included.

Only a small proportion of genes were down-regulated in any of the mutants (Figure 23). *nec3* candidate genes were selected based on criteria that probe sets were at least two-fold down-regulated in 10d experiment or at least 5-fold down regulated in any mutant of 7w experiment. All candidate genes from both experiments were screened using genomic PCR to identify potential deletions. PCR fragments from all candidate genes of predicted length were present in both mutants and the wild type (Table 21 and Table 22), thus, none of the candidate genes appeared to be deleted to a detectable extent in the FN362 and FN363 mutants.

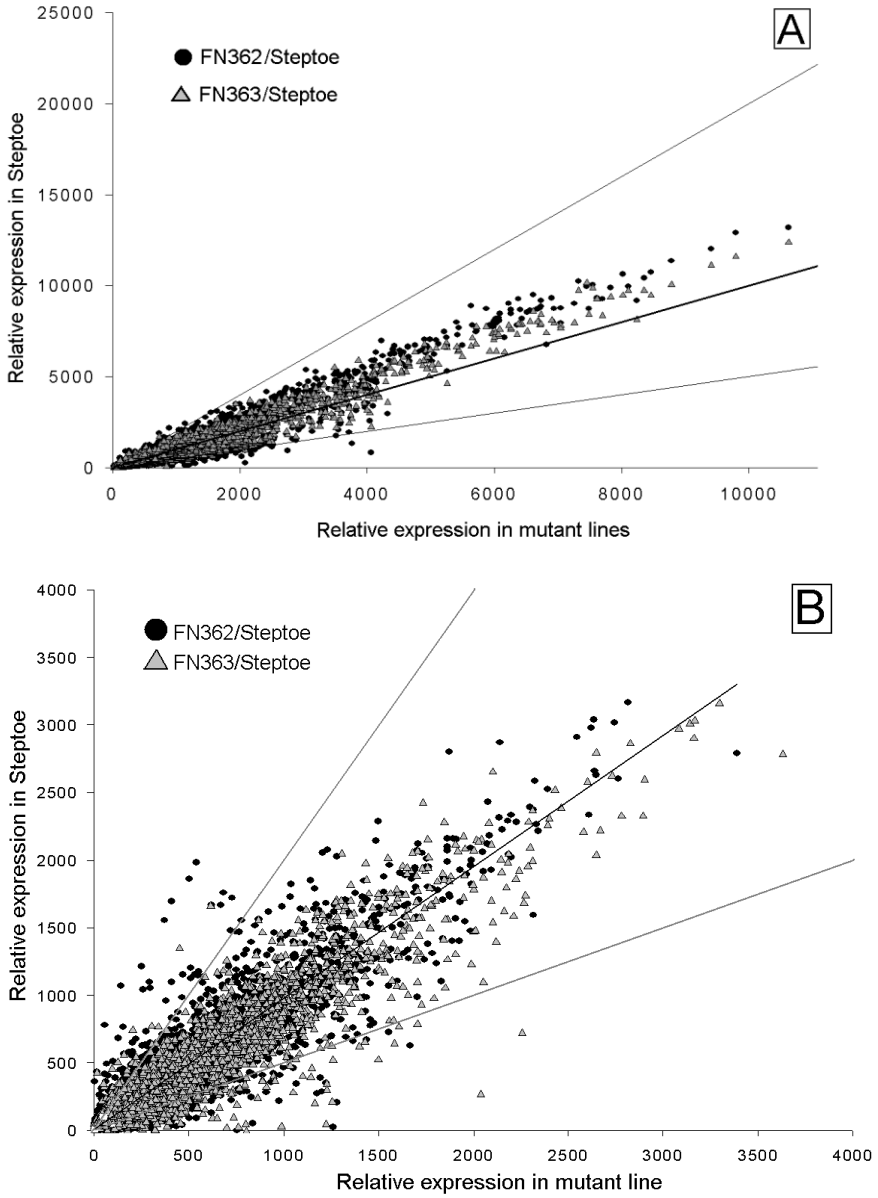


Figure 23. Transcriptome comparison between allelic barley lesion mimic mutants FN362 and FN363 containing *nec3* mutantion and parental line Steptoe. Data represent relative transcript abundance assessed by Affymetrix Barley GeneChip1 analysis. **A**-trancriptome comparison of 10 day old plants; **B**-transcriptome comparison of 7 week old plants. Only genes with transcript abundance above treshold of detection are included. Genes outside the trendlines are two-fold up- or down-regulated.

Table 21. Candidate genes of barley *NEC3* selected in Affymetrix Barley GeneChip1 analysis of 10 day old barley allelic mutants of *nec3* – FN362 and FN363.

Affy Contig	Log2 (FN362/Steptoe)	Log2 (FN363/ Steptoe)	Expression in 7w experiment	gDNA ST FN362 FN362
Contig17652_at	-2	-1	↓362; =363	
Contig18830_at	-1.5	-1	↓362; ↓363	
Contig431_at	-2	-2	A	
Contig1954_at	-1	-1	↑362; =363	
Contig2279_at	-2	-1	↓362; =363	
Contig3448_at	-1	-1	↓362; =363	
Contig4024_at	-1	-1	↓362; =363	
Contig6708_at	-1	-1	↓362; ↓363	
Contig7098_at	-1	-1	↓362; =363	
Contig7377_at	-1	-1	↓362; ↓363	
Contig19204_at	-1	-1	↓362; =363	
Contig20556_at	-1	-1	=362; ↓363	
Contig21141_at	-1	-1	↓362; =363	
EBpi01_SQ004_C23_at	-1	-1	↓362; =363	
HD05F08r_at	-1	-1	↓362; =363	
HVSMEl0012A13f_at	-0.6	-1	=362; ↓363	
Contig12590_at	-1	-2	A	
Contig14129_at	-1	-2	A	
Contig16209_at	-1	-1	↓362; =363	
Contig12169_at	-1	-1	A	
Contig7285_at	-1.5	-1	A	
HB25K17r_at	-1	-1.5	A	

↑-overexpressed in mutant line;
↓-underexpressed in mutant line;
A-absent from all analyzed samples;
=-expression does not differ from parental line

Table 22. Candidate genes of barley *NEC3* selected in Affymetrix Barley GeneChip1 analysis of 7 week old barley allelic mutants of *nec3* – FN362 and FN363.*

Affy Contig	Log2 (FN362/ Steptoe)	Log2 (FN363/ Steptoe)	Expression in 10d experiment	gDNA ST	gDNA FN362	gDNA FN362
Contig5710_at	-7	0	↑			
baak32n13_at	-5	-5	A			
Contig19029_at	-4	-1	↓362; =363			
Contig12472_at	-4	1	↓362; ↓363			
Contig26350_at	-3	0	A			
Contig5163_at	-3	-1	↓362; =363			
Contig4948_s_at	-3	-2	↓362; =363			
Contig2489_at	0	-3	↓362; =363	n.a.		

↑-overexpressed in mutant line; ↓-underexpressed in mutant line

A-absent from all analyzed samples; =-expression does not differ from parental line

* - underexpressed probes in 7w experiment that have been detected as present and non-repressed in 10d experiment were not further analyzed

n.a. – amplification could not be achieved in either of samples

3.2.2. Characterization of differentially expressed genes in barley lesion mimic mutant nec3

nec3 phenotype was expressed at late developmental stage – five to seven weeks after germination, therefore, it might be expected that the effect of *nec3* mutation on barley transcriptome largely depends on plant developmental stage. Number and identity of differentially regulated genes, as well as fold change of induction/repression significantly differed between both experiments. In total 203 genes (detected as present and exceeding a threshold of 50 in at least one sample) were differentially regulated in *nec3* ten days after germination and 274 genes – seven weeks after germination. Unfortunately no overlapping differentially regulated genes satisfying threshold limits (present and exceeding threshold of 50 in at least one sample) were identified between 10d and 7w experiments. No correlation in extent of up- or down-regulation of analyzed genes was observed in either mutant between both experiments (Figure 24) confirming that the effect of *nec3* mutation on barley transcriptome is strongly affected by plant developmental stage.

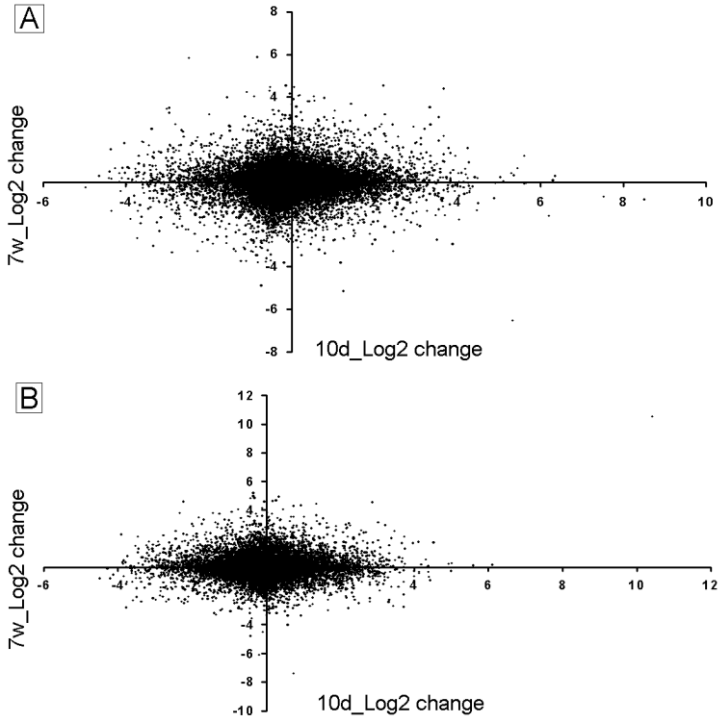


Figure 24. The effect of plant developmental stage on barley transcriptome affected by *nec3* mutation. Comparison of level of induction/repression of analyzed genes in *nec3* sampled either 10 days or 7 weeks after germination. **A** – FN362; **B** – FN363.

3.2.2.1. Characterization of differentially expressed genes overlapping between 10d and 7w

Since none of the differentially regulated genes overlapping between 10d and 7w satisfied previously set threshold limits a new analysis was performed. Second screen included all genes detected as present in at least one sample with no minimal expression threshold set. If no threshold was applied for the analyzed data 149 differentially regulated genes were identified as overlapping between both mutants in both experiments. Expression pattern of the majority of these genes differed between both experiments – *i.e.* genes up-regulated in both mutants in 10d experiment were down-regulated in 7w experiment and *vice versa*. Only 55 genes had the same regulation pattern (either up- or down-regulated) in both experiments (Figure 25 A). Cluster analysis of differentially regulated genes showing the same regulation pattern in both experiments distinguished two distinct clusters comprising roughly equal gene number (Figure 25). Although genes in either of clusters did not comprise clear regulation pattern, it can be observed that cluster I predominantly comprises genes of stronger up/down-regulation in 10d experiment and in FN363 in both experiments whereas genes included in II cluster were more up/down regulated in 7w experiment and in FN362. Cluster analysis of differentially regulated genes comprising variable regulation in both mutants at both developmental stages sorted genes into five distinct clusters. Cluster II (Figure 25 B) representing the largest number of genes can be characterized as comprising genes of opposite regulation in 10d and 7w. Remaining clusters comprised genes showing distinct regulation in a single mutant at one developmental stage – e.i. - I comprised genes of

distinct regulation in FN363_7w, III – FN362_7w, IV – FN363_10d and V – FN362_10d. Comparatively small proportion of genes comprising distinct regulation in a single mutant serves as an additional evidence for *nec3* mutation being the primary cause of transcript change in FN362 and FN363.

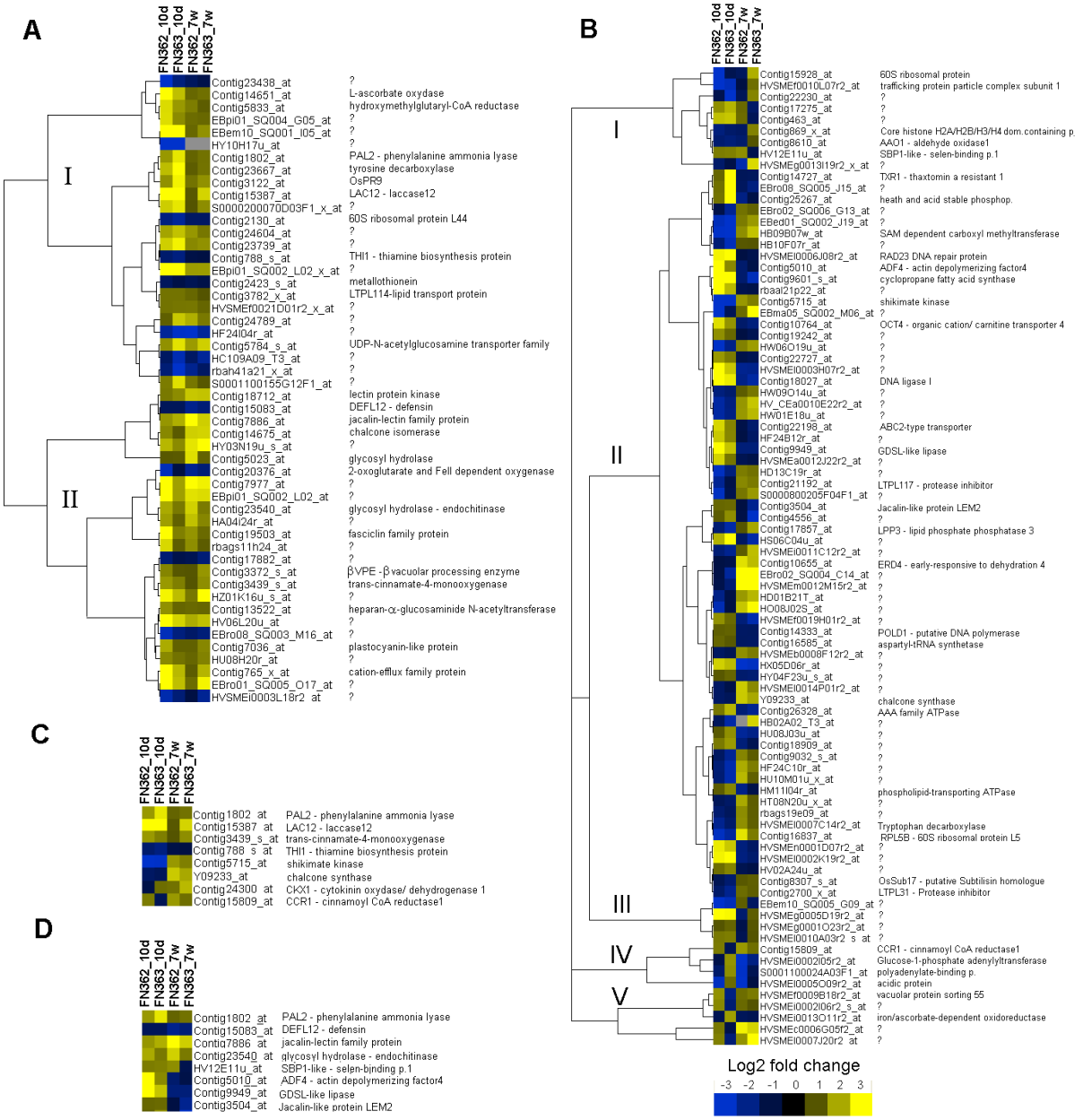


Figure 25. Heath map of differentially regulated genes overlapping between 10 days old and 7 weeks old plants of barley *nec3* lesion mimic mutants FN362 and FN363. **A** - genes showing the same pattern of regulation at 10d and 7w, **B** - genes showing inconsistent pattern of regulation at 10d and 7w, **C** – genes related to aromatic compound metabolism differentially regulated in *nec3*. **D** – genes related to plant defense reactions. ? – gene cannot be annotated based on homology to barley/rice/*A. thaliana* ESTs. Clustering parameters: absolute correlation (uncentered) and complete linkage.

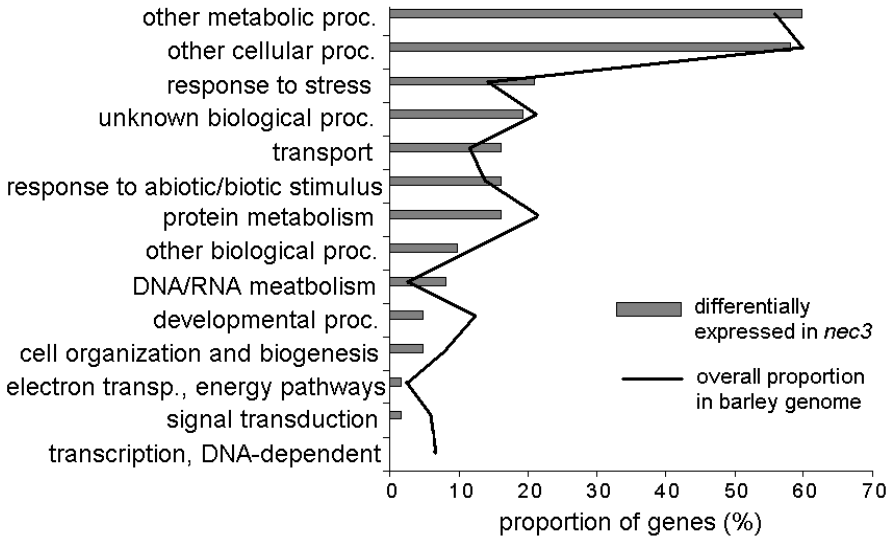


Figure 26. Functional categorization of genes differentially regulated in both barley *nec3* lesion mimic mutants FN362 and FN363. Analyzed data set combines genes differentially regulated in 10 day old and 7 week old *nec3* plants. Analysis based on TAIR gene ontology terms of biological processes.

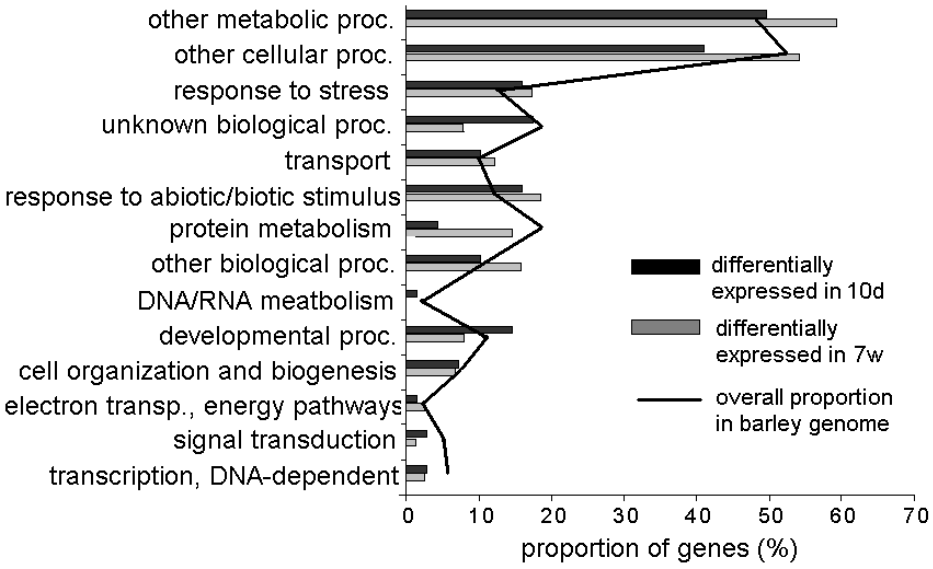


Figure 27. Functional categorization of genes differentially regulated in barley lesion mimic mutants of *nec3* at 10 days or 7 weeks after germination developmental stages. Analysis based on TAIR gene ontology terms of biological processes.

In order to asses if genes grouped by hierarchical clustering can be assigned to certain functional groups analyzed genes were annotated. HarvEST database (Close et al. 2007) and BLASTX (Gertz 2005) homology search against barley/rice/*A. thaliana* were used to identify annotations of the genes differentially regulated in *nec3* at both developmental stages. According to annotations no clear predominance of any gene functional groups could be observed for any cluster rejecting the assumption that genes differentially regulated in *nec3*

are representatives of certain functional group. Assigning gene ontology (GO) terms to analyzed genes by GOSlim did not reveal statistically significant overrepresentation of any parental GO term in analyzed gene set (Figure 26). However, more detailed analysis of differentially regulated genes using GO term Enrichment revealed significant overrepresentation (p=0.003) of genes associated with aromatic compound metabolism (GO:0006725) (Figure 25 C). More thorough screen of differentially expressed genes revealed a considerable number of genes previously associated with plant defense reactions (Figure 25 D). This observation does not contradict with GoEnrichment results since many plant disease resistance mechanisms involve or depend on aromatic compound metabolism – such as fortification of cell wall, biosynthesis of secondary metabolites of antifungal activity or biosynthesis of resistance related phytohormone salicylic acid. Although set of differentially expressed genes in *nec3* comprises many genes related to aromatic compound metabolism and plant defense no specific regulation pattern could be observed for either of these functional groups (Figure 25 C and D).

3.2.2.2. Characterization of genes differentially regulated in 10d or 7w

In order to get more insight into *nec3* effect on barley transcriptome datasets from both experiments were analyzed separately. Analyzed data set included genes differentially regulated in either of experiments and satisfying threshold limitations (present in at least one sample and exceeding minimal value of 50). For functional categorization of the differentially expressed genes, TAIR gene ontology tool (Berardini et al. 2004) was applied using *Arabidopsis* gene set homologous to differentially expressed genes from *nec3*. This categorization did not reveal any GO term biological process parental category to be overrepresented in analyzed data set (Figure 27).

GoSlim analysis offers categorization into very broad sense categories therefore more thorough analysis was performed using GO Term Enrichment tool. GO term enrichment tool allows for identification of common characteristics of a gene set and also identifies gene groups which are overrepresented in the analyzed dataset compared to the whole genome data (Carbon et al. 2009). Since whole genome data are not yet available for barley, we annotated barley Affymetrix probes to *A. thaliana* homologues using PlexDB Microarray cross-reference and searched the differentially expressed gene list against TAIR databases using GO term enrichment tool. 68 out of 203 and 71 out of 274 genes differentially regulated in 10d and 7w experiments respectively could be annotated to *A. thaliana* probes. GoEnrichment analysis of 7w differentially regulated probes revealed overrepresentation of genes in category metabolic process (Figure 27). According to GO classification this category involves anabolic and catabolic processes, including biosynthesis and degradation of small molecules as well as macromolecules. Detailed inspection of differentially regulated genes from 7w revealed significant number of genes related to senescence (Figure 28). Senescence in plants and in barley particularly involves accumulation of sugars, remobilization of N, suppression of growth associated hormones (such as cytokinins) and induction of senescence associated hormones (such as ethylene and jasmonic acid) (van der Graaff et al. 2006, Parrott et al. 2007). Induction of numerous proteolytic, nucleolytic and lipolytic enzymes and transport genes as well as up-regulation of pheophorbide a oxygenase involved in chlorophyll

breakdown indicates up-regulation of recycling of important metabolites characteristic for plant senescence in *nec3* at 7w. This is supported by observed alterations in hormonal balance of *nec3* - induction of JA signaling/biosynthesis related genes and genes involved in degradation of cytokinin and gibberellin. Moreover, *nec3* also up-regulates enzymes of glyoxylate pathway (isocitrate lyase, malate synthase) implicated in either gluconeogenesis or replenishment of malate pool for Krebs cycle during carbohydrate starvation induced senescence (Chen et al. 2000).

Surprisingly also 10d *nec3* differentially regulated gene list comprises numerous genes that might be associated with senescence. Although 10 days old *nec3* plants do not display *lmm* phenotype or any other characteristics of early senescence transcriptome analysis revealed that *nec3* mutation up-regulates proteolytic and lipolytic enzymes as early as 10 days after germination. However, predominance of genes encoding catabolic enzymes is much less evident compared to 7w (Figure 28 A). 10d differentially expressed gene list comprises considerably less enzymes degrading peptides and nucleases than 7w. Besides, induction of hormonal regulators of senescence and cell death associated genes observed in 7w dataset was not identified in 10d. Taking into account these differences it might be expected that accelerated senescence in *nec3* at 7w is rather side effect than a direct consequence of *nec3* mutation.

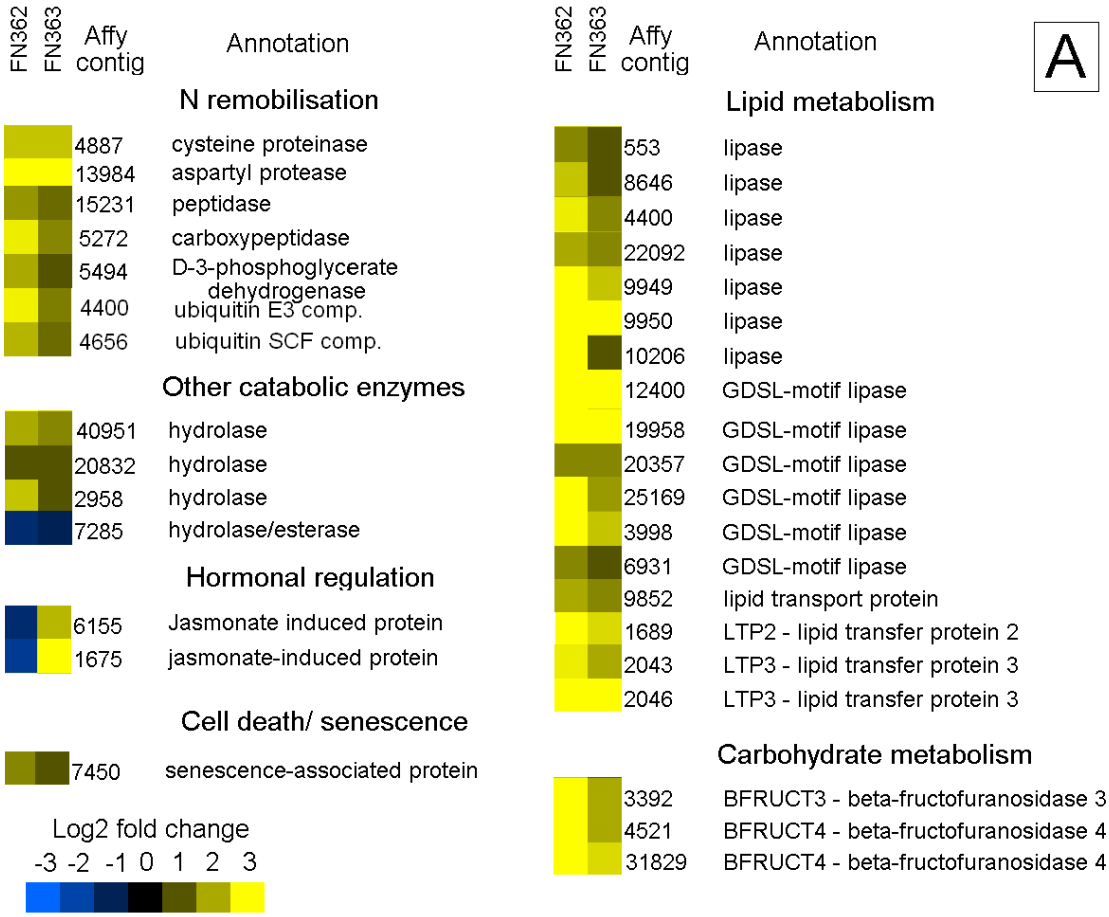


Figure 28 A. Heath map of senescence related genes differentially regulated in barley *nec3* lesion mimic mutants FN362 and FN363 at 10 days after germination.

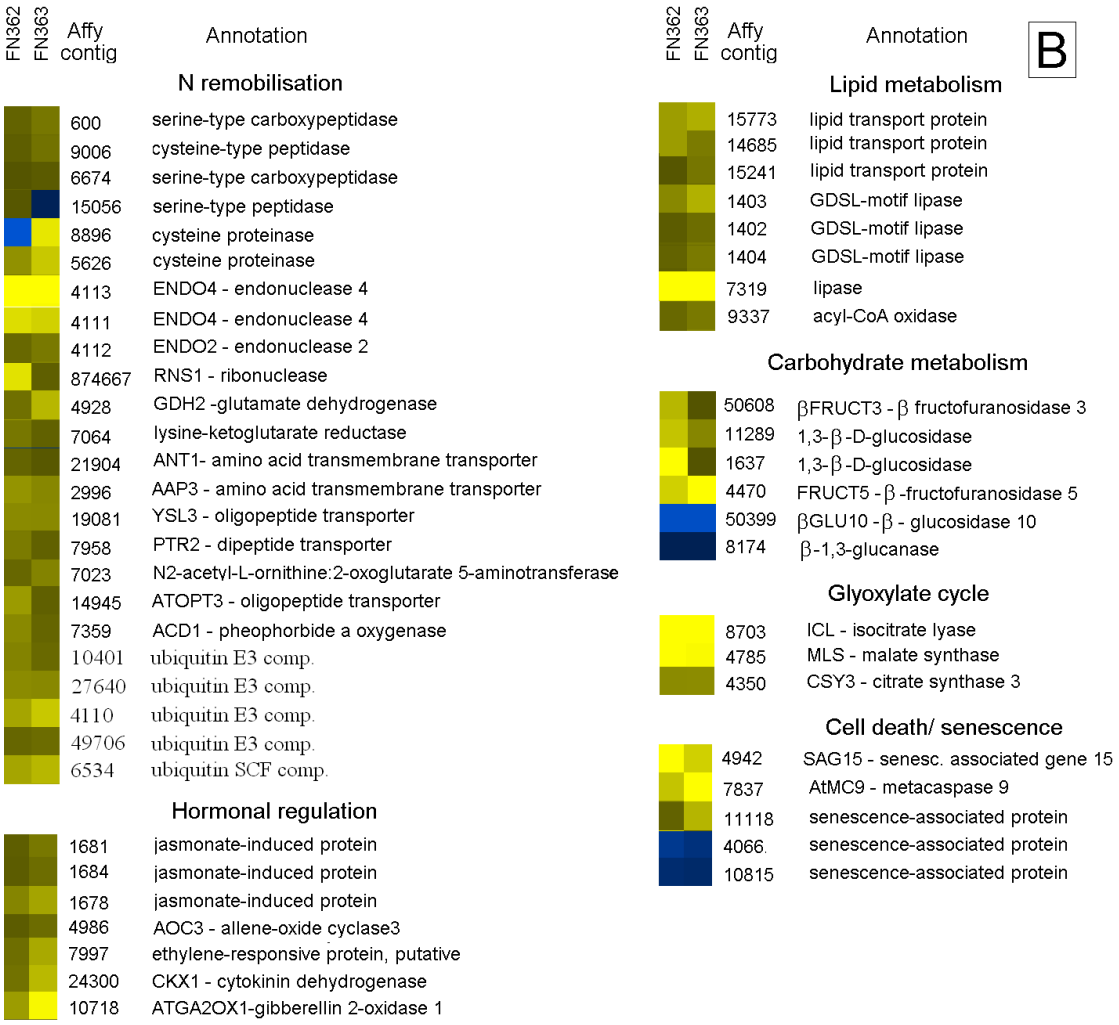


Figure 28 B. Heath map of senescence related genes differentially regulated in barley *nec3* lesion mimic mutants FN362 and FN363 at 7 weeks after germination.

Despite described differences between *nec3* at 10d and 7w with regard to overrepresented functional gene groups there are also some commonalities shared between two experiments. Looking for the common characteristics of *nec3* transcriptome at 10d and 7w significant overrepresentation of lipid metabolism related genes has been noticed. Although the initial analysis using GoEnrichment tool did not reveal significant overrepresentation of lipid metabolism related genes in 7w (Table 23), MapMan screen of 7w dataset revealed induction of several key genes involved in lipid biosynthesis and metabolism (Table 24). MapMan analysis also revealed differential regulation of a significant proportion of genes involved in secondary metabolism and particularly in phenylpropanoid biosynthesis pathway in *nec3* (Figure 29). Both datasets of differentially regulated probes comprise genes involved in lignin biosynthesis implying that *nec3* might affect cell wall lignification. Based on GoEnrichment analysis differentially expressed gene set of 10 day old *nec3* comprises significant proportion of genes involved in cell wall related biological processes (Table 23). More detailed inspection of genes categorized in this group reveals that *nec3* mutation significantly affects genes related to cell wall adhesion/ response to water stress or osmotic stress as well as genes required for cell wall fortification (Table 25).

Table 23. Functional categories overrepresented in differentially expressed gene set of barley lesion mimic mutant *nec3* at 10 days and 7 weeks after germination as assessed with GoTerm Enrichment Tool.

	Plant age	GO term	p-value	Sample freq. %	Back ground freq. %
Biological process	10d	GO:0052546 cell wall pectin metabolic process	1.46E-02	4.4	0
		GO:0006629 lipid metabolic process	2.31E-02	14.7	3
		GO:0009664 plant-type cell wall organization	4.49E-02	5.9	0.3
	7w	GO:0055114 oxidation-reduction process	7.84E-03	21.1	5.8
		GO:0008152 metabolic process	1.80E-02	67.6	43.1
Cellular component	10d	GO:0005618 cell wall	1.07E-04	16.2	2.1
		GO:0031225 anchored to membrane	1.23E-03	10.3	0.8
		GO:0009505 plant-type cell wall	2.06E-03	10.3	0.9
		GO:0044464 cell part	1.52E-02	60.3	35.2
	7w	GO:0016020 membrane	7.31E-03	38	16.5
		GO:0044464 cell part	2.15E-02	59.2	35.2
Molecular function	10d	GO:0003824 catalytic activity	1.16E-03	57.4	29.5
		GO:0003979 UDP-glucose 6-dehydrogenase activity	2.28E-02	2.9	0
	7w	GO:0003824 catalytic activity	2.35E-06	63.4	29.5
		GO:0016491 oxidoreductase activity	2.69E-04	23.9	5.7

Table 24. List of lipid biosynthesis and metabolism related genes differentially regulated in barley lesion mimic mutants of *nec3*-FN362 and FN363 identified using MapMan analysis (genes involved in lipid hydrolysis have been eliminated to avoid overlapping with Figure 28).

Plant age	MapMan Bin code – Metabolic process	Affymetrix probe	Blast based annotation	Log2 FN362	Log2 FN363
10 days	11.1.3 – lipid metabolism. FA synthesis and FA elongation. ketoacyl ACP synthase	contig6642_at	AAF61730.1 e-124 beta-ketoacyl-ACP synthetase I [*G. max*]	2.934	1.196
	11.1.9 – lipid metabolism. FA synthesis and FA elongation. Long chain fatty acid CoA ligase	contig11308_at	NP_175368.2 4e-85 (NM_103833) acyl CoA synthetase	4.503	3.079
	11.1.10 – lipid metabolism. FA synthesis and FA elongation. Beta ketoacyl CoA synthase	contig5663_at	CAC01441.1 e-105 putative fatty acid elongase [*Z. mays*]	2.256	1.154
	11.8 – lipid metabolism. 'exotics' (steroids, squalene etc)	contig19855_s_at	CAC84558.1 1e-81 (AJ311789) beta-amyrin synthase [*A. strigosa*]	1.96	1.028
	11.8 – lipid metabolism. 'exotics' (steroids, squalene etc)	contig9601_s_at	AAL57657.1 3e-74 (AY065013) AT3g23510/MEE5_5 [*A. thaliana*]	6.205	3.703
	11.8 – lipid metabolism. 'exotics' (steroids, squalene etc)	hvsmeg0018f23r2_at	AAC34989.1 1e-27 (AF042333) 24-methylene lophenol C24(1)methyltransferase [*O. sativa (japonica cultivar-group)*]	4.238	2.748
	11.6 – lipid metabolism. Lipid transfer proteins	contig1689_at	AAF14232.1 3e-55 lipid transfer protein [Hordeum vulgare]	3.651	2.53
	11.6 – lipid metabolism. Lipid transfer proteins	contig2043_s_at	Q42976 6e-36 Non-specific lipid-transfer protein 4 precurson (LTP 4) pir||T03297 lipid transfer protein precursor - rice (fragment)	2.804	1.898
	11.6 – lipid metabolism. Lipid transfer proteins	contig2046_at	Q42976 3e-36 Non-specific lipid-transfer protein 4 precurson (LTP 4) pir||T03297 lipid transfer protein precursor - rice (fragment)	5.109	3.182

Table 24 (continued). List of lipid biosynthesis and metabolism related genes differentially regulated in barley lesion mimic mutants of *nec3*-FN362 and FN363 identified using MapMan analysis.

Plant age	MapMan Bin code – Metabolic process	Affymetrix probe	Blast based annotation	Log2 FN362	Log2 FN363
7 weeks	11.1 – lipid metabolism. FA synthesis and FA elongation	contig8914_at	NP_564467.1 2e-21 (NM_103301) acetyl-CoA carboxylase	-1.59	-1.715
	11.1.8 – lipid metabolism. FA synthesis and FA elongation. Acyl CoA ligase	contig15150_at	AAM65672.1 4e-48 (AY088127) 4-coumarate-CoA ligase-like protein [*A. thaliana*]	1.385	1.433
	11.1.8 – lipid metabolism. FA synthesis and FA elongation. Acyl CoA ligase	hs05n06r_s_at	AAM65672.1 3e-37 (AY088127) 4-coumarate-CoA ligase-like protein [*A. thaliana*]	1.073	1.116
	11.8.1 – lipid metabolism. 'exotics' (steroids, squalene etc).sphingolipids	contig15701_at	NP_191408.1 2e-15 (NM_115711) putative protein; protein id: At3g58490.1 [*A. thaliana*]	-1.214	-1.031

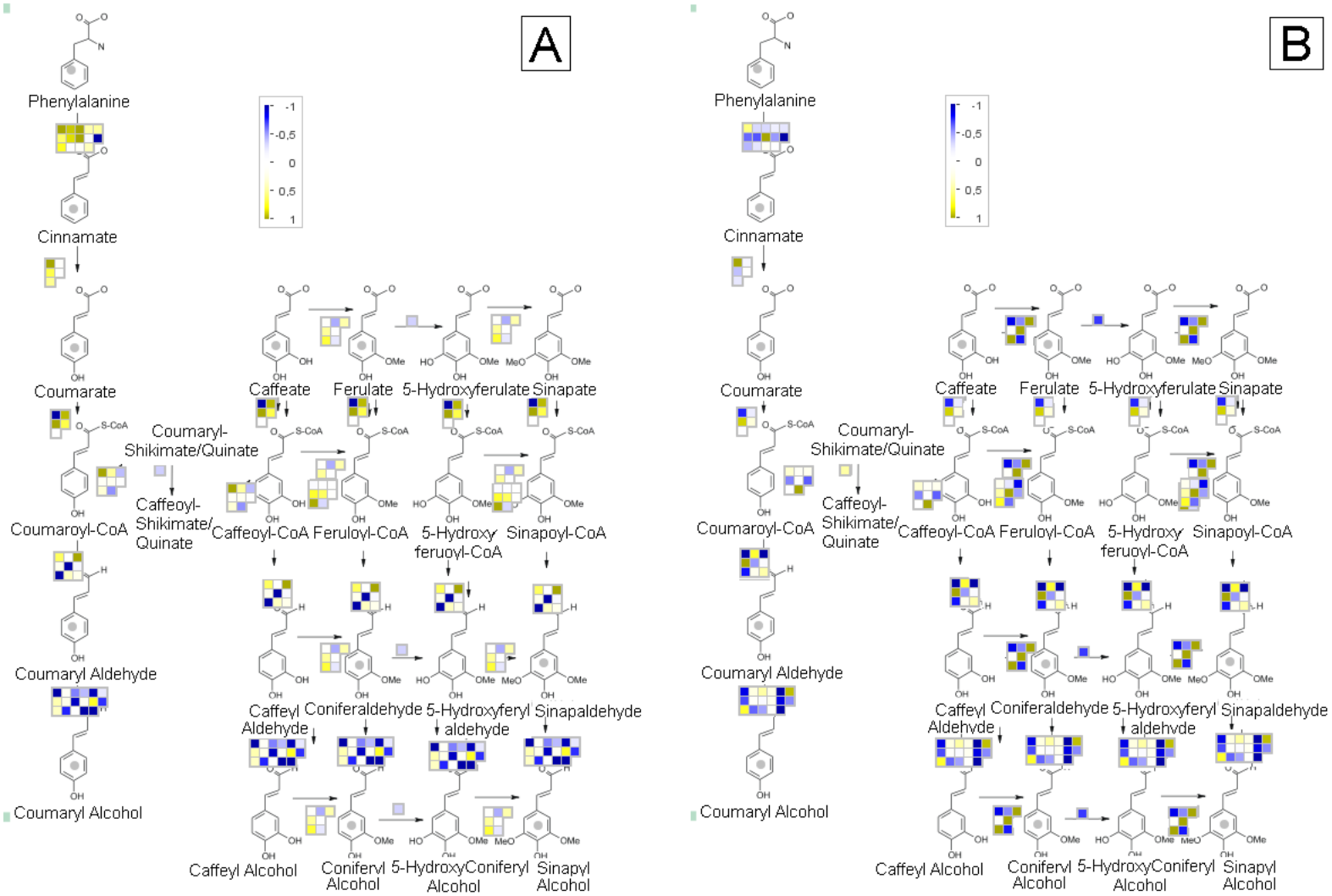


Figure 29. Functional representation of differentially regulated secondary metabolism related (phenylpropanoid synthesis) genes in barley *nec3* mutant as derived from MapMan analysis. A – differentially regulated gene set in 7 week old *nec3* plants; **B** – differentially regulated gene set in 10 day old *nec3* plants. Colored squares each represent a single gene differentially regulated in analyzed dataset.

Table 25. Cell wall related genes differentially expressed in the *nec3* mutant.

Plant age	Affymetrix Barley GeneChip probe set	Best BlastX hit (Uniprot Accession)	Function in cell wall modification	Fold-change FN362; FN363
10 days	HZ01K16u_s_at	UDP-glucose dehydrogenase A2YAR2	Cell wall formation, enzyme is regulated by the osmotic state of the cell (Johansson et al. 2002)	7;5
	Contig10778_s_at	polygalacturonase isoenzyme 1 beta subunit homolog Q6ZA27	Cell wall hydrolytic enzyme involved in fruit softening, plant development and organ senescence (Kim et al. 2006)	3;2
	Contig2873_s_at	Expansin EXPB2 Q6QFA2	Cell wall extensibility (Cosgrove et al. 2002)	5;3
	Contig7055_at	Expansin A5 Q6ZGU9		12;3
	Contig4124_s_at	Extensin HvEx1 O49870	Cell wall modifying enzyme (Sturaro et al. 1998)	18;3
	Contig2957_at	Xyloglucan endo-transglycosylase/hydrolase Q5JZX2	Cell wall polysaccharide modification (Minic and Jouanin 2006)	5;3
	Contig19503_at	Fasciclin FLA4 like protein Q06IA2	Fasciclin FLA4 mutation alters salt stress sensitivity of *Arabidopsis* (Shi et al. 2003). Involved in cell wall adhesion.	8;3
	Contig7789_at	Fasciclin FLA12 like protein Q06I94		4;2
	Contig12191_at	PRX52- peroxidase 52 homologue	involved in plant defense reaction in apoplast and cell wall	5;3
	Contig19815_at	feruloyl-CoA transferase	required for suberin synthesis	2;2
	Contig17985_at	CCR1 – cinnamoyl CoA reductase	involved in lignification	32; 7
7 weeks	contig1800_x_at	PAL - phenylalanine ammonia-lyase CAA89005	involved in lignin biosynthesis	2;3
	contig6770_s_at	N-hydroxycinnamoyl/benzoyl transferase CAC09504	involved in lignin biosynthesis	2.5; 2
	HVSMEb0003G21r2	extensin family protein	Cell wall modifying enzyme	2;2

In order to determine particular metabolic pathways overrepresented among differentially regulated gene set of 10d and 7w *nec3* GENECODIS analysis tool was applied (Nogales- Cadenas et al. 2009, Tabas-Madrid et al. 2012). This tool allows detection of statistically significantly overrepresented functional categories (metabolic pathways) among analyzed gene set compared to background data available for particular species. Since this application is available only for *A. thaliana* genome, genes differentially regulated in *nec3* were translated into *A. thaliana* gene IDs using homology based search through PlexDB microarray platform translation tool. KEGG pathway data (Kyotto Encyclopedia of Genes and Genomes pathway database) were selected as background data. GENECODIS analysis identified significant overrepresentation of pathways related to biosynthesis of secondary metabolites in both datasets thus confirming observations previously derived by MapMan analysis.

3.2.3. Comparison of nec3 transcriptome with barley transcriptome under biotic and abiotic stress

Analysis of gene functional groups overrepresented in *nec3* transcriptome using MapMan and GoEnrichment tool indicated that *nec3* might be related to stress responses inducing sink type metabolism or JA related biotic stress responses. In order to gain more evidence supporting or opposing this prediction we performed a comparative analysis of *nec3* transcriptome with barley transcriptome under various stress treatments using Barley GeneChip1 data publicly available at PlexDB database (Wise et al. 2008).

We chose a set of barley GeneChip experiments representing barley transcriptome change in response to four abiotic factors – chilling or freezing temperature, drought and wounding causing senescence; and five biotic factors – powdery mildew resistance of specific *Mla* alleles, effect of *mlo-5* mutation, stem rust resistance of transgenic Golden Promise containing *Rpg1* gene and response to lesion mimic mutation *necS*. The analysis was based on probes differentially regulated (at least 2 fold up- or down-regulated) in at least one dataset. Hierarchical cluster analysis (HCA) was based on complete linkage clustering of whole transcriptome data compared with absolute correlation similarity matrix. HCA grouped analyzed datasets into three major clusters each comprising roughly equal number of datasets. Clustering of abiotically stress barley transcriptomes from different, independent experiments (drought treatment and chilling/freezing treatment) into one cluster indicates that the applied approach can successfully identify groups of functionally related barley transcriptomes. Initially unexpected clustering of *mlo-5, necS* and senescence transcriptomes might be explained by initiation of cell death in all three cases – *mlo-5* and *necS* are lesion mimic mutants both expressing necrotic lesions on leaves whereas senescence is also known to activate molecular mechanisms characteristic to cell death. HCA revealed that among the datasets included in the analysis *nec3* 10d transcriptome shares the highest overlap with transcriptome of abiotically stressed barley whereas 7w transcriptome is more similar to barley undergoing senescence (Figure 30). However these data are more indicative rather than decisive since correlation between *nec3* transcriptome and analyzed datasets was not significant. In order to get a deeper insight into characteristics of gene set determining *nec3* similarity with abiotically stressed or senescing barley transcriptome the overlapping gene sets were subjected to cluster analysis. Analyzing gene set overlapping between 10d_*nec3* and abiotically stressed barley it was revealed that majority of these genes comprise opposite

regulation in *nec3* – genes which are induced during abiotic stress conditions are repressed by *nec3* and *vice versa* (Figure 31 A). Although some genes had opposite pattern of regulation in 7w_*nec3* and either of senescence associated transcriptomes, majority of overlapping genes were similarly regulated in all three datasets (Figure 31 B). Homology based annotation of overlapping gene set did not reveal predominance of any gene functional groups.

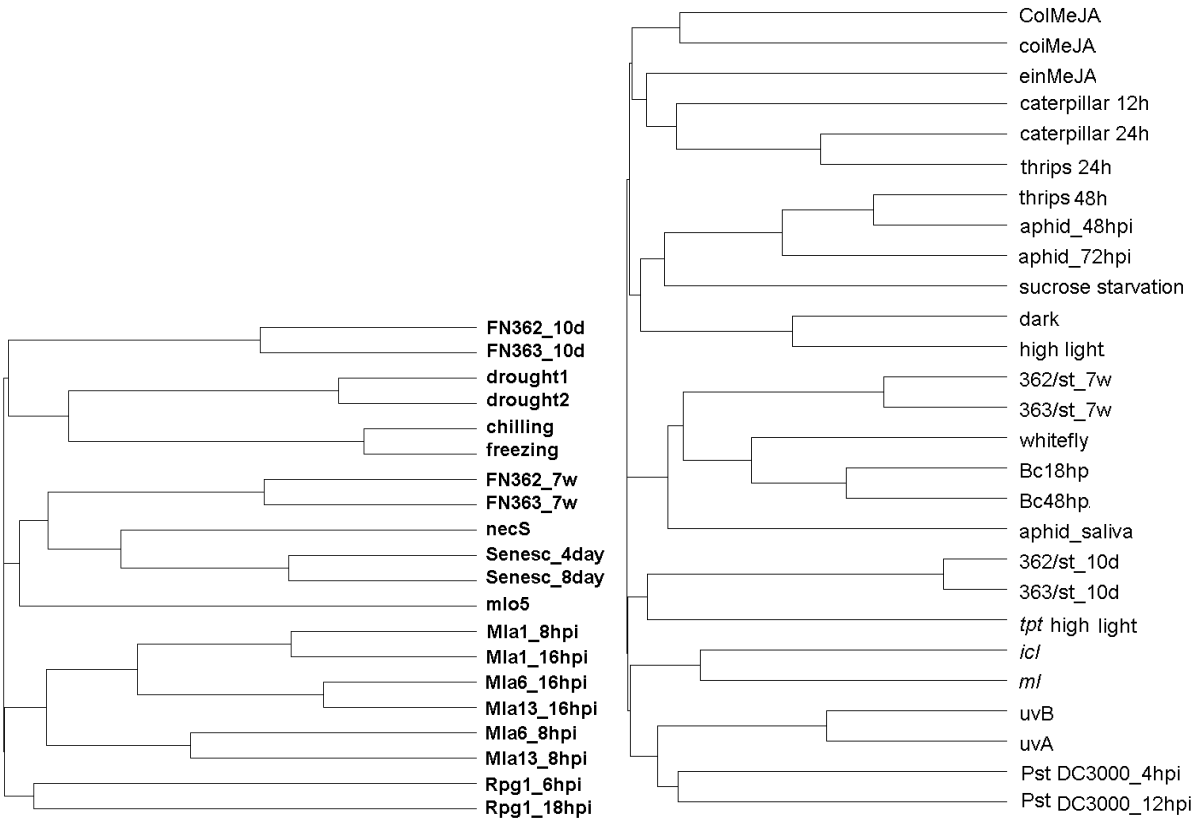


Figure 30. Hierarchical clustering of transcriptomes of 10 days and 7 weeks old plants of barley lesion mimic mutants FN362 and FN363 with barley transcriptomes under various stress treatments from data available at PlexDB database. Left – *nec3* transcriptome comparison to *H. vulgare* transcriptomes in response to different treatments. Data from following PlexDB experiments are included in analysis: drought1 and drought2 – BB77, chilling and freezing – BB81, NecS – BB54, senesc– BB50, mlo5 – BB7, Mla1/6/13 – BB4, Rpg1 – BB49; **Right** - *nec3* transcriptome comparison to *A. thaliana* transcriptomes in response to different treatments. Data from following PlexDB experiments are included in analysis: ColMeJA/*coi*MeJA/*ein*MeJA – AT98, thrips/ aphid/caterpillar - AT49, whitefly - AT63, aphid saliva – AT90, Pst DC3000 – AT13, Bc – AT51, dark/high light – AT123, sucrose starvation – AT8, *tpt* high light – AT16, uvA/uvB - AT54, *icl/ml* - AT11.

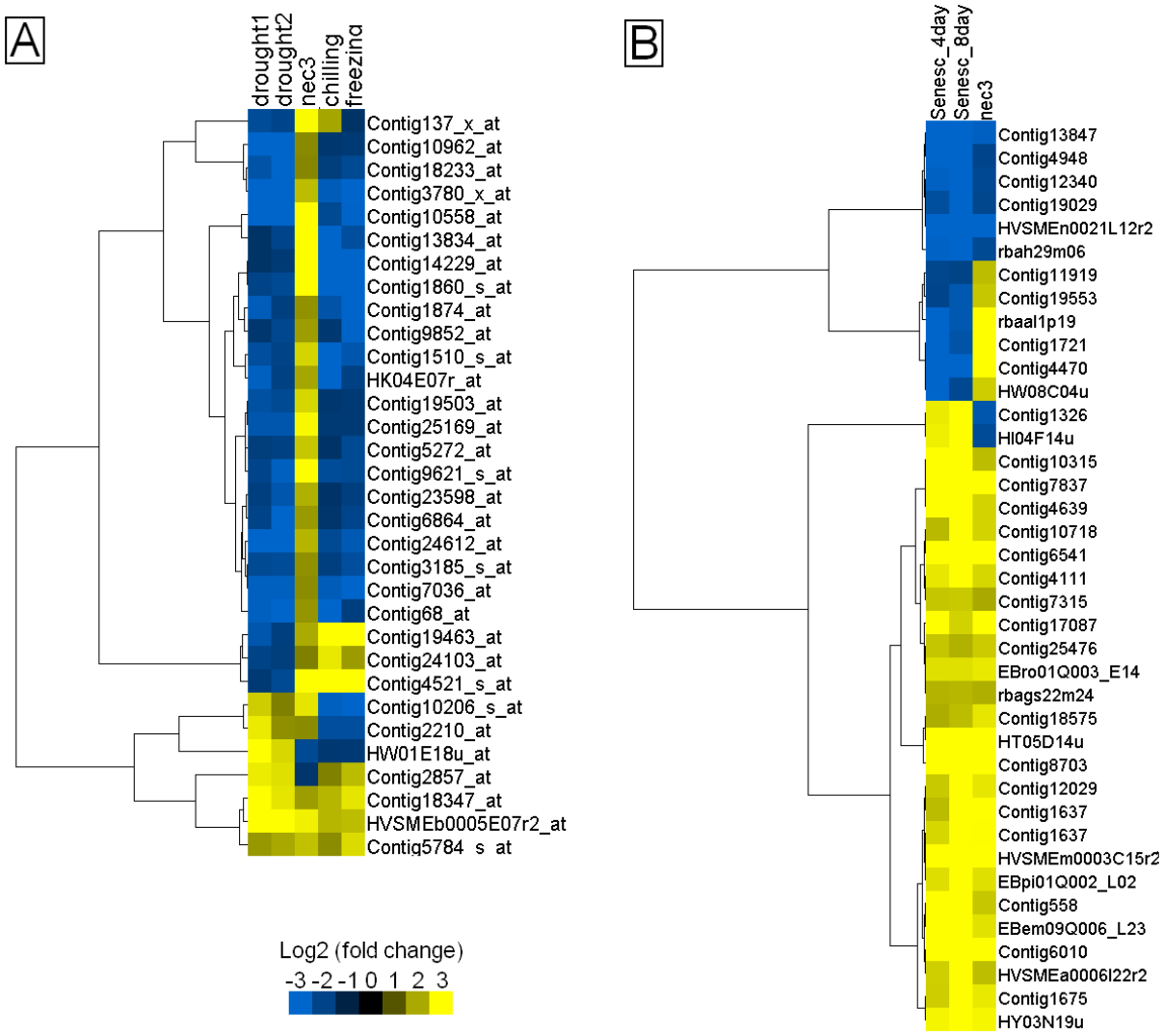


Figure 31. Heath map of differentially regulated gene set overlapping between transcriptome of barley lesion mimic mutant *nec3* and the most similar barley transcriptomes from figure 30. Only genes overlapping between all data sets and of Log2 value larger than 1.5 in A and 2 in B are included. **A** – comparison of differentially regulated gene set from 10d *nec3* and abiotically stressed barley transcriptomes, **B** - comparison of differentially regulated gene set from 7w *nec3* and barley transcriptome affected by artificially triggered senescence.

Unfortunately number of publicly available Barley GeneChip1 experiments is rather low and restricted mainly to biotic interactions with powdery mildew. Therefore, in order to understand if *nec3* transcriptome might share common characteristics with plant transcriptome under JA mediated response to biotic stress conditions or abiotic stress triggering carbohydrate starvation *nec3* transcriptome was compared to *A. thaliana* transcriptome data available at PlexDB database (Figure 30). Correspondence of Barley GeneChip1 probes to *A. thaliana* Affymetrix probes was established using PlexDB platform translation tool. 5324 probes out of 10 507 probe sets (detected as present and exceeding threshold of 50) in 10d and 5328 probes out of 10 973 in 7w could be annotated to *A. thaliana* Affymetrix probes. Expression values of the set of annotated *nec3* probes were compared to

A. thaliana transcriptome in response to biotic stressors - herbivore invasion (leaf chewing caterpillar, thrips or aphid invasion, treatment with aphid saliva, whitefly invasion), pathogenic bacteria *Pseudomonas syringae* pv. *tomato* DC3000 infection, necrotrophic fungus *Botrytis cinerea*; and also to abiotic stressors – treatment with UV light, prolonged treatment with dark or high intensity light conditions, sucrose starvation of cultured cells; treatments affecting JA mediated responses (JA response mutant coi1 and ET response mutant *ein3/eil1* and wt Col plants treated with MeJA); metabolic mutants related to lipid or carbohydrate metabolism (*icl* – isocitrate lyase and *ml* – malate synthase knock-out mutations impairing glyoxylate cycle, *tpt* - triose-phosphate/phosphate translocator mutant unable to supply enough triose-phosphates for sucrose production matching high rate CO_2 fixation at high light conditions). HCA grouped analyzed datasets into four major clusters each comprising roughly equal number of datasets. According to HCA *nec3* 10d transcriptome is slightly similar to *A. thaliana tpt* mutant impaired in carbohydrate metabolism although the level of correlation between *tpt* and *nec3* transcriptomes is non-significant. Hierarchical clustering also grouped *nec3* 7w transcriptome with *A. thaliana* transcriptome in response to whitefly invasion or necrotrophic fungus *Botrytis cinerea* infection.

3.2.4. nec3 mutants are hypersensitive to conditions artificially triggering early senescence

Since *nec3* transcriptome showed certain common characteristics of barley transcriptome undergoing senescence it was interesting to see if *nec3* response to conditions artificially triggering senescence would differ from wild type plants. High carbohydrate to nitrogen ratio caused by nitrogen stravation triggers early senescence in barley (Parrott et al. 2010). Germination of *nec3* and wt plants under nitrogen deprived conditions revealed significant difference between both genotypes. *nec3* plants developed considerably shorter roots compared to wt plants (Figure 32) suggesting that *nec3* causes increased sensitivity of barley to nitrogen starvation.

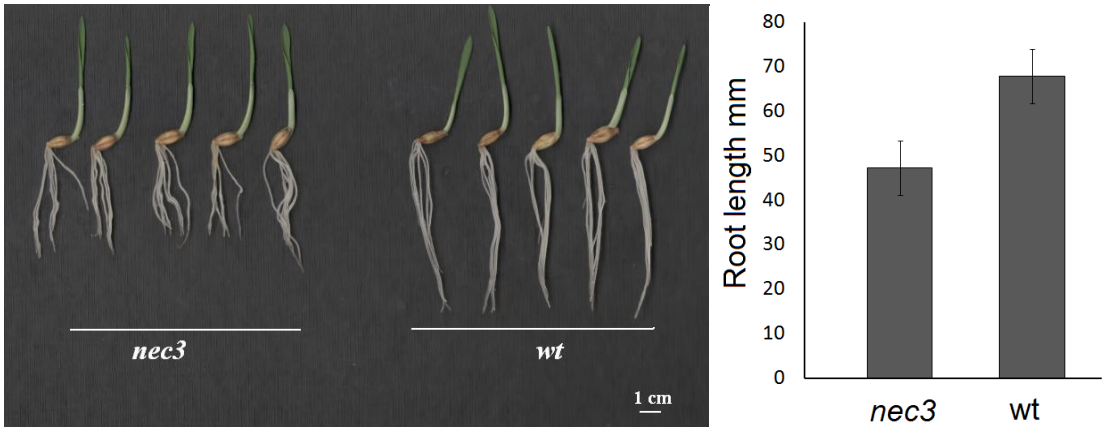


Figure 32. Root length of wt and barley lesion mimic mutant *nec3* plants under N depleted conditions. Difference between both genotypes is statistically very highly significant (*t* test p=1.03E-08). Experiment has been repeated twice with similar results. Photo depicts representative plants from one of experiments.

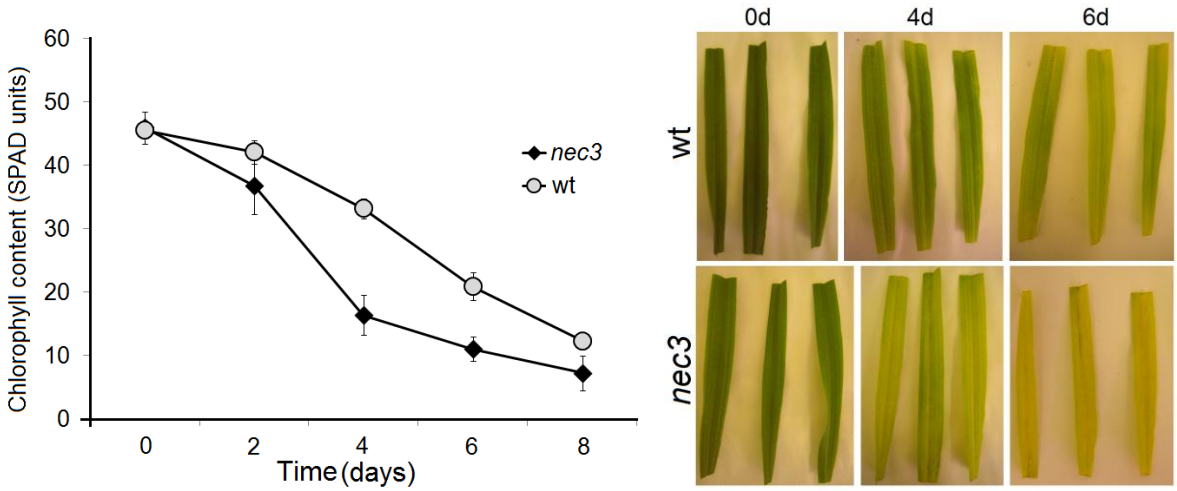


Figure 33. Dark induced senescence of barley lesion mimic mutant *nec3* and wt leaves under carboxydrate starvation. Excised leaves of *nec3* and wt plants were incubated in dark for indicated time points. **Left –** effect of carbohydrate starvation on chlorophyll content of wt and *nec3* leaves. Each datapoint contains measurements from at least 10 plants. **Right –** phenotype of dark-induced senescence in leaves of *nec3* and wt plants. Detached leaves have been incubated in dark for indicated time points.

Carbohydrate starvation caused by prolonged absence of light can also trigger early senescence in plants. Extended darkness triggers remobilization of carbohydrates and nitrogen rich substances to either developing parts of plant or seeds and flowers. Remobilization of proteins releases chlorophyll compounds which can be potentially harmful. Therefore, degradation of chlorophyll is one of the indicators of senescence. Chlorophyll content of *nec3* plants started to decreased already two days of dark treatment whereas in wild type plants visible indications of senescence could be detected only 4 days of dark treatment (Figure 33). Besides, on contrary to wild type plants which degraded chlorophyll gradually during the whole course of dark treatment, majority of chlorophyll in *nec3* was degraded within first four days after onset of darkness.

3.2.5. Towards map-based cloning of *nec3*

3.2.5.1. nec3 mapping

Since transcript analysis of *nec3* mutants FN362 and FN363 did not allow identification of *NEC3* it was decided that further pursuit of the candidate gene will be based on map based cloning. It has previously been reported that *nec3* is located on 6HS 29cM from *rob* and 16.7cM from *msg36* genes (Francowiak and Lundqvist 1997). Above mentioned markers are positioned distally from *nec3* therefore it was required to find markers mapping proximally to *nec3* to delineate location of *nec3* more precisely. *nec3* mapping was performed in F_2 mapping population: FN388 (*nec1*) x GSHO2423 (*nec3*.e). *nec3* was mapped between markers 1872 and 5771. In order to determine *nec3* position more precisely *nec3* was repeatedly mapped in another mapping population: GSHO2423 (*nec3*.e) x GSHO1284

(*nec1*.c). Based on data from both mapping populations *nec3* is located between CMWG652a and 5771 (Figure 34). More precise *nec3* mapping was precluded by the low number of polymorphic markers in target region – out of 55 previously published and newly developed markers (see Materials and methods paragraph 2.2.3.) only 11 were polymorphic in screened mapping populations. Depending on mapping population CMWG652a position is estimated around 16.3-26.5 cM from 6HS telomere whereas 5771 is located 39.8 cM from telomere (Agricultural Research Service of USDA 2008a). According to Mayer et al. 2011 in rice there are 55 genes (Os02g0114200 to Os02g0124700) positioned syntenically to 16-39 cM region of barley 6HS.

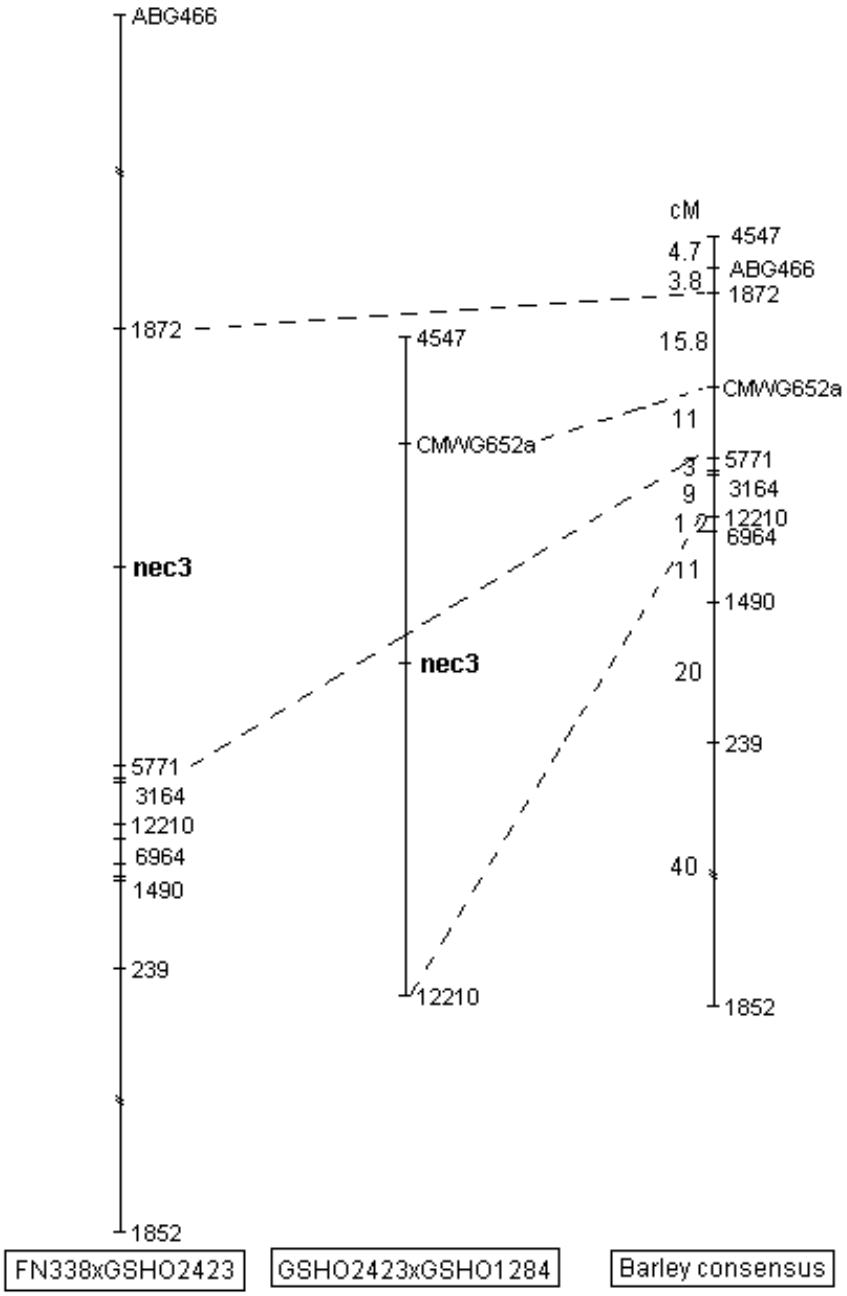


Figure 34. Estimated position of barley *nec3* mutation in 6HS chromosome based on mapping in two F_2 mapping populations. Position of analyzed markers in analyzed mapping populations is compared to position on barley consensus map.

3.2.5.2. Screening of barley unigenes corresponding to syntenic rice chromosome region for presence of deletion in FN362 or FN363

Functional annotation revealed that syntenic region in rice includes several genes that have previously been implicated in either regulation of senescence or associated with lesion mimic phenotype (Table 26). Using HarvEST Blast server HarvEST unigenes with the highest homology to these rice genes were identified. Majority of identified barley unigenes were not represented on Affymetrix Barley GeneChip1 suggesting that mutations of these genes could not be detected using transcript based cloning. Affymetrix Barley GeneChip1 probes representing several of the genes were detected as present in FN362 and FN363 in microarray experiments; nevertheless these genes were still included in further analysis to confirm Affymetrix data.

In order to find out if mRNA abundance of these genes is altered in *nec3* semi-quantitative PCR was performed on cDNA from FN362, FN363 and wild type plants (Figure 35).

Table 26. Screened subset of genes positioned syntenically to *nec3* target region in rice.**

Rice gene	Functionl annotation	HarvEST21 Barley unigene
Os02g0115900	heath shock protein 70 (HSP70)	1610/49394
Os02g0116500	protochlorophyllide reductase	37524/ 24301
Os02g0117800	autophagy related protein 5 (ATG5)	22597/21172/33462/33461
Os02g0120600	ubiquitin-protein ligase (UPL)	7800*/1430*/2229*/ 9642*
Os02g0116600	Proteasome-activating nucleotidase	10688*/ 49543
Os02g0117500	Glutamate receptro 3.3 precursor	18494/ 23335
Os02g0122000	Sin3 associated polypeptide p18 (SAP18)	6471*
Os02g0121700	Isoprenoid biosynthesis enzyme	11021/ 10453
Os02g0119400	Syntaxin 52	3933*
Os02g0123200	Peroxisomal membrane protein 16 (Pex16)	11483*
Os02g0121300	Cyclophilin 2	1360

* - probes detected as present and with unaltered expression in either of Affymetrix experiments

**** -** genes were selected based on potential involvement in plant senescence, cell death or stress response

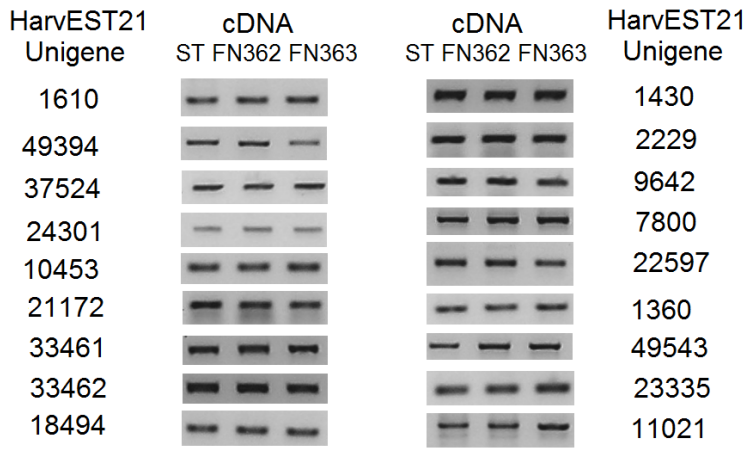


Figure 35. Expression of barley homologues of rice genes positioned syntenically to chromosome region comprising *nec3*. Semiquantitative RT-PCR was performed on cDNA of two week old wt Steptoe and *nec3* mutants FN362 and FN363.

3.3. Barley lesion mimic mutations *nec3* and *nec1* likely employ the same signaling pathway to trigger cell death in barley

Barley mutants *nec3* and *nec1* characterized in this study represent two classes of *lmm* – *nec1* comprising comparatively small lesions with clear istinct edge belongs to initiation *lmm* whereas *nec3* comprising large coalescing lesions likely represents propagation *lmm* class. If both mutations employ the same signalling pathway to trigger cell death, it can be expected that double mutant would comprise more severe, enhanced lesion mimic phenotype. Therefore, it was interesting to see if *nec3* mutation would affect spread of cell death in HR related lesion mimic mutant *nec1*.

In *nec1* lesions develop at two leaf stage and size of lesions is usually less than a square milimeter, whereas in *nec3* lesions are expressed much later – at heading and lesion size reaches several mm2 (Figure 36). A putative double mutant *nec1/nec3* developed lesions at the same time as *nec1* suggesting that spontaneous lesion initiation is likely triggered by *nec1* and not affected by *nec3*. Besides, average lesion size and total lesion area in double mutant exceeded that observed for *nec1* as well as for *nec3* suggesting that *nec3* mutation impairs control of cell death in *nec1*.

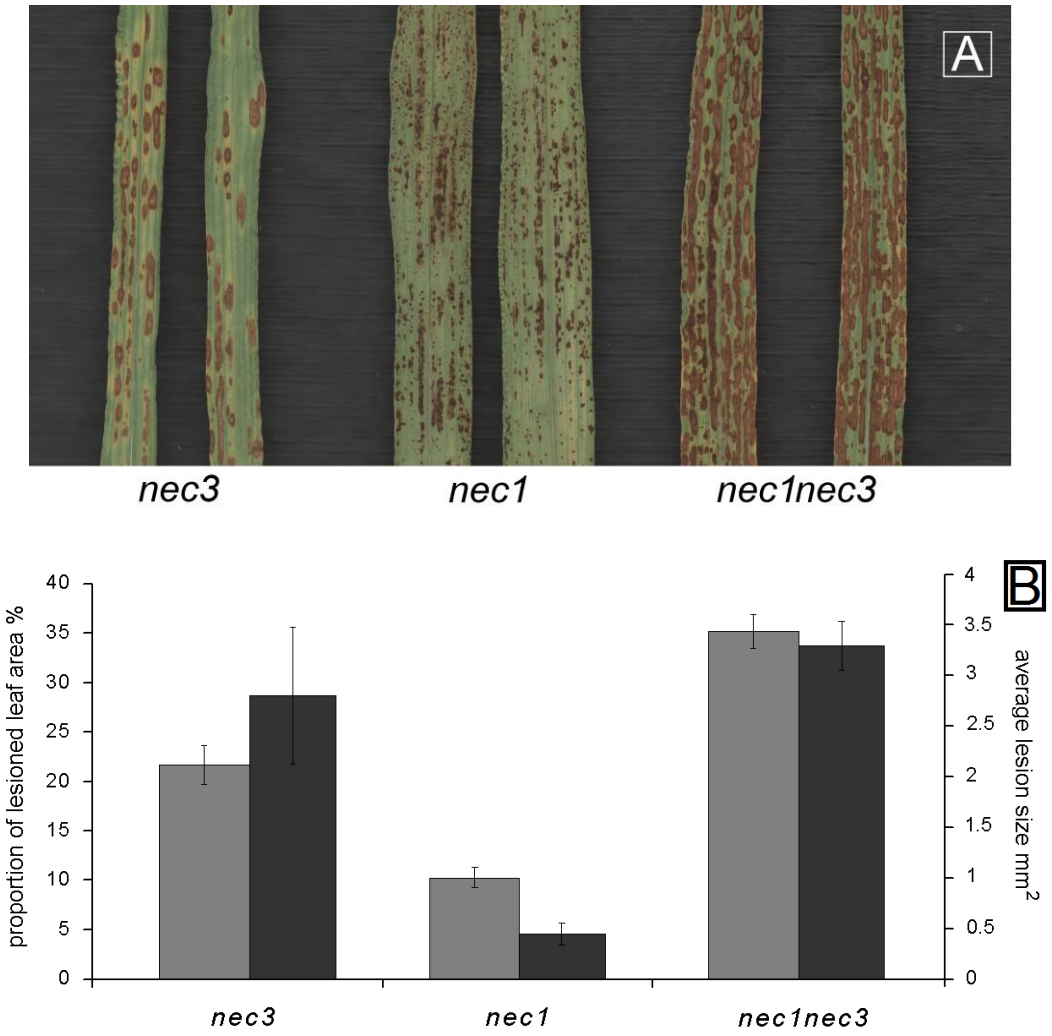


Figure 36. Effect of *nec3* mutation on cell death spread in barley lesion mimic mutant *nec1*. A – Lesion mimic phenotype of *nec3*, *nec1* and double mutant *nec1/nec3* (light gray area); **B –** total area of lesions and average lesion size in three studied mutants (dark gray area).

3.4. Identification and characterization of barley homologues of *AtLSD1*

3.4.1. Barley comprises two highly homologous AtLSD1 orthologues

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

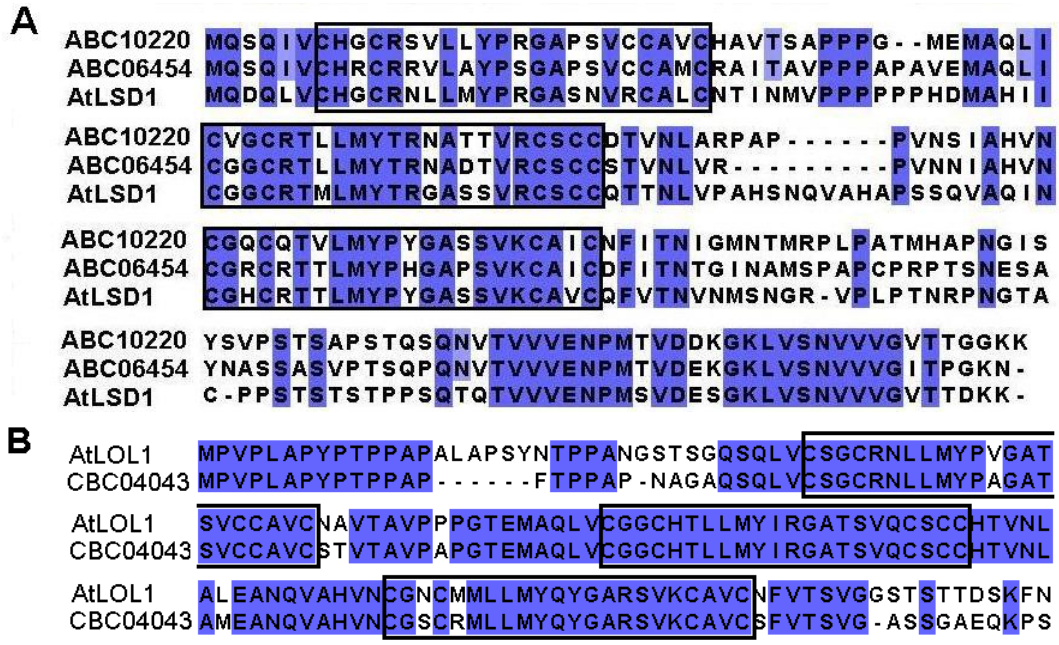


Figure 37. Sequence alignment of deduced amino acid sequences of *Arabidopsis thaliana LSD1*, LOL1 and identified barley homologues. **A**–alignment of AtLSD1 and corresponding barley homologues; **B**–alignment of AtLOL1 and corresponding barley homologue. Zn-finger domains are denoted by black frame. Identical amino acids are indicated by colored background.

Similarly to *Arabidopsis LSD1*, barley genes encode three putative Zn finger domains (pfam 06943) detected by conserved domain search of Pfam 22.0 database (Finn et al. 2006). Position of Zn-finger domains is highly conserved in *ABC10220* and *ABC06454*, in which the first three exons each contain a single Zn-finger domain. ABC10220 and ABC06454 are highly homologous (71% AA identity). Regions of the highest homology include predicted Zn-finger domains and C-terminal end of the predicted proteins (Figure 37).

3.4.2. Genetic mapping of barley LSD1 homologues

Oregon Wolfe Barley (OWB) Dominant by Recessive doubled haploid (DH) mapping population (Costa et al. 2001) was used for linkage mapping of *ABC10220, ABC06454* and *CBC04043*. CAPS markers allowing differentiation of alleles from both parental lines were developed based on genomic sequences of *ABC10220, ABC06454* and *CBC04043*.

Screening of OWB mapping population with CAPS markers was done at Washington State University by Dr. Upinder Gill. *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).

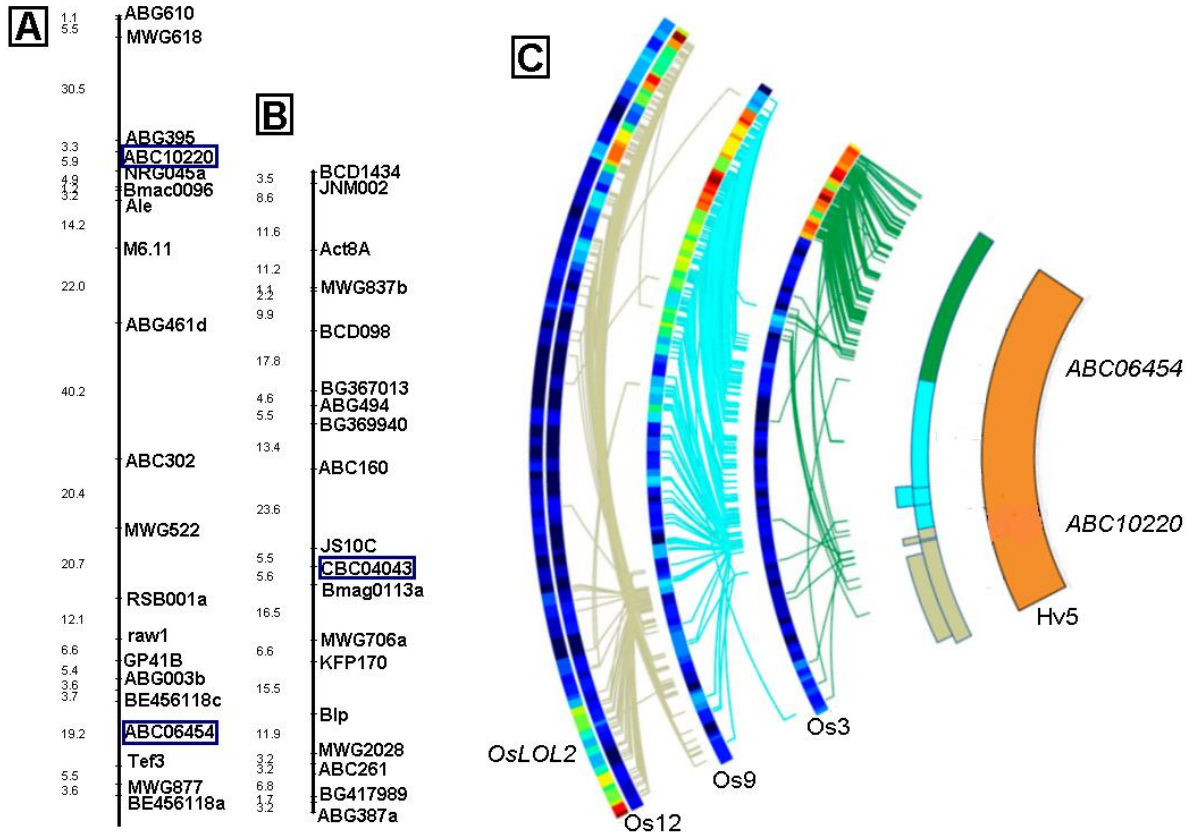


Figure 38. Position of AtLSD1 barley homologues on barley genetic map. A–fragment of 5H genetic map containing *ABC10220* and *ABC06454*; **B**–fragment of 1H genetic map containing *CBC04043*; **C**–synteny between *ABC10220* and *ABC06454* containing region in barley 5H and rice genome (modified from Mayer et al. 2011).

According to GenomeZipper (Mayer et al. 2011) *ABC10220* is located in syntenic position to rice gene *OsLOL2* (Os12g0611000). Although barley 5H region comprising *ABC06454* is syntenic to rice chromosome 3 containing *OsLOL3* (Os03g0639600) both genes are not located in syntenic position (Figure 38). Chromosome region comprising *CBC04043* (1H bin09-11) is syntenic to rice chromosome 5 which does not comprise any known rice *LSD1* homologue. This suggests that synteny of regions comprising *LSD1* homologues in barley and rice are conserved only partially.

3.4.3. Structure of barley LSD1 homologues

Pairwise alignment of cDNA and gDNA sequences identified the exon–intron structure of barley *LSD1* homologues (Figure 39). Genes *ABC10220* and *ABC06454* showed highly conserved exon-intron organization each comprising six exons of conserved size and sequence. Exon sequence and size of *ABC10220* and *ABC06454* resembles that of *AtLSD1*. Homology-based annotation of genomic sequences indicated presence of repetitive sequences within *ABC10220* and *ABC06454*. BLASTN search against TREP *Triticeae* Repeat database (Agricultural Research Service of USDA 2008 b) 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). Presence of repetitive sequences likely explains increased size of 1st and 2nd introns of barley homologues compared to *AtLSD1* (Figure 13).

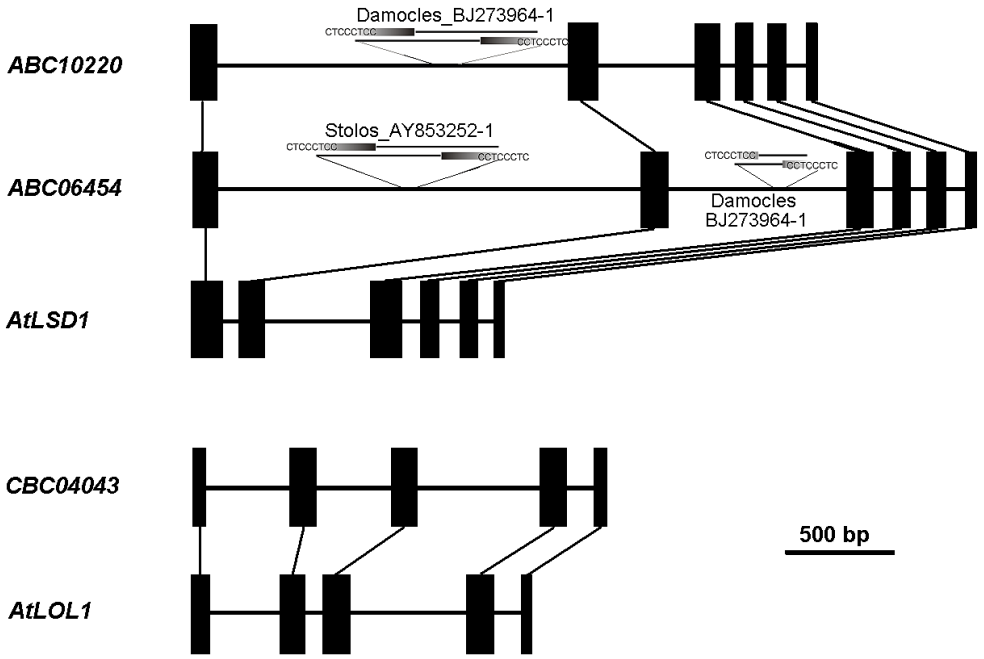


Figure 39. Exon-intron structure of the coding sequence of *AtLSD1, AtLOL1* and corresponding barley homologues. Exons are depicted as black boxes. Positions of MITE insertions are indicated. Lines connecting diagrams of different genes represent homology between exon sequences.

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, coding sequence of *CBC04043* consisted of five exons with Zn-finger domains positioned in the 2nd, 3rd and 4th exons. Such exon-intron sequence strongly resembles structure of *AtLOL1* supporting higher homology of *CBC04043* with *AtLOL1* rather than *AtLSD1*.

3.4.4. 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 homologous sequences in other *Poaceae* species. Retrieved amino acid sequences of the closest homologues were aligned and the alignment was used to reconstruct phylogenetic relationships among the barley LSD1 homologues and related protein sequences in other *Poaceae* species. Identified sequences clustered into three distinct groups each including a single barley gene (Figure 40). Cluster represented by barley CBC04043 sequence showed high degree of sequence conservation.

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 and used to reconstruct phylogeny of barley, *Arabidopsis* and rice *LSD1*-like genes (Figure 41). Both – *ABC10220* and *ABC06454* clustered together with *AtLSD1* whereas *CBC04043* grouped with homologues of *AtLOL1*. Interestingly – *ABC10220* and *ABC06454* clustered with *OsLOL2* and *OsLOL3* respectively – rice genes which are located in syntenic regions.

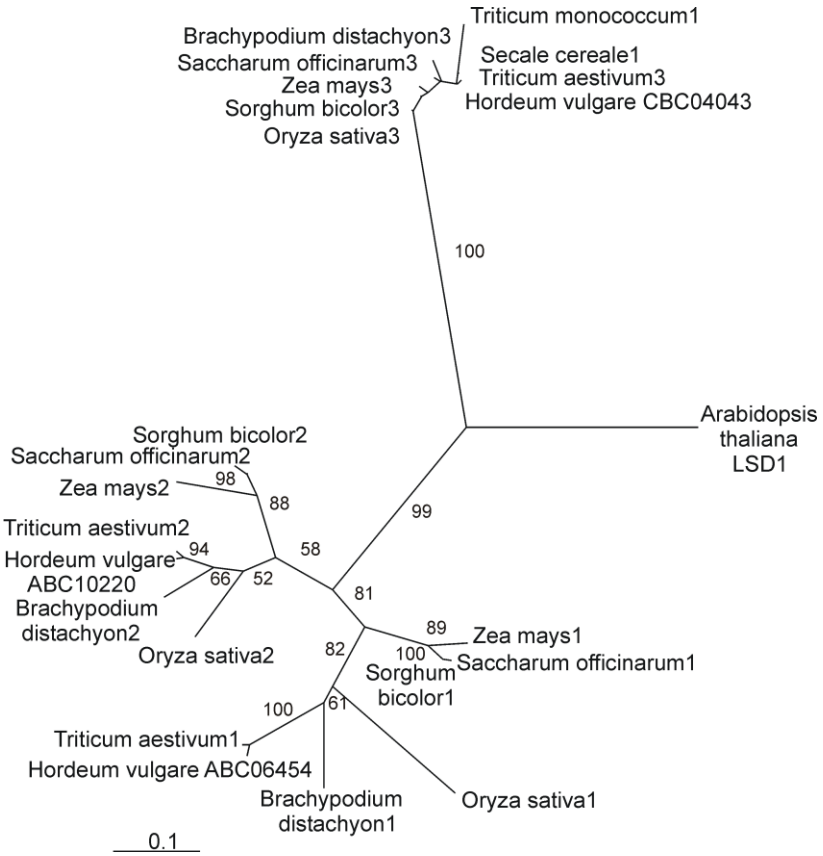


Figure 40. Phylogenetic relationships among the barley LSD1 homologues, related genes in other *Poaceae* species and AtLSD1. Phylogenetic tree is based on distances reconstructed with Maximum Likelihood method. Bootstrap confidence levels (calculated from 100 iterations) higher than 50 are shown.

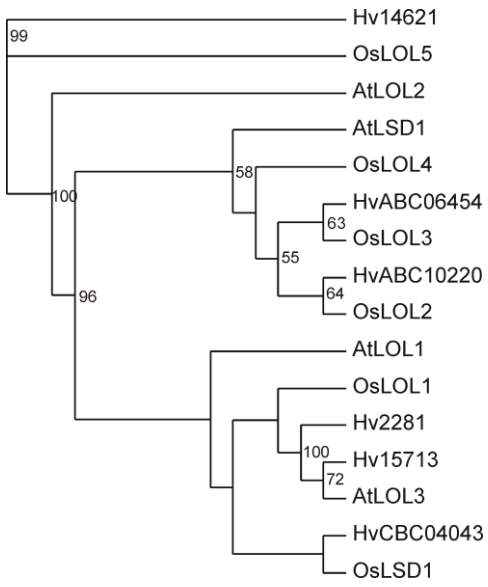


Figure 41. Phylogenetic relationships among known *LSD1*-like genes and barley genes comprising *LSD1*-like Zn-finger domains. Phylogenetic tree was reconstructed with Maximum Likelihood method. Bootstrap confidence levels (calculated from 100 iterations) higher than 50 are shown. Barley genes are designated according to the corresponding HarvEST assembly 21 unigene number.

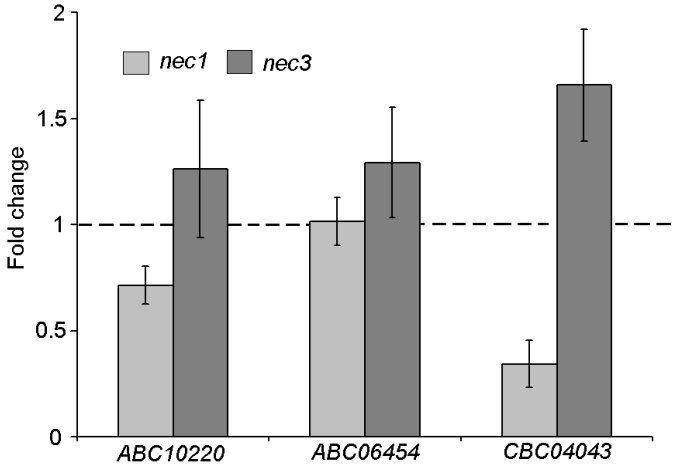


Figure 42. Expression of *AtLSD1* barley homologues – *ABC10220, ABC06454* and *CBC04043* in barley lesion mimic mutants *nec1* and *nec3*. Expression was assessed in leaves of two week old barley plants using real-time qRT-PCR. Gene expression levels are expressed as fold change compared to parental line (interrupted line). Error bars represent standard deviations. Three biological replicates each containing tissue from three plants were analyzed in each datapoint. Expression was assessed in two independent experiments with similar results.

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

Taking into account the role of *LSD1* in cell death regulation, expression of barley homologues of the *LSD1* was studied in leaves of two week old plants of two barley lesion mimic mutants *nec1* and *nec3* (Figure 42). Two fold decrease (p=0.0003) in transcript

abundance of *CBC04043* was observed in *nec1* mutant, while in *nec3 CBC04043* expression showed slight, but statistically significant (p=0.015) increase. Although no remarkable difference in transcript abundance of *ABC10220* was detected between lesion mimic mutants and respective parents, gene was slightly repressed in *nec1* (p=0.003). Observed differences in expression of *ABC06454* were not statistically significant.

3.4.6. Effect of abiotic and biotic factors on transcript abundance of barley homologues of AtLSD1 and AtLOL1

In order to assess the effect of light cycle on regulation of barley *LSD1* homologues expression of analyzed genes was examined using qRT-PCR (Figure 43). Tissue samples were collected every 2-6 h during 16 h/ 8 h day-night cycle. *CBC04043* expression was induced upon onset of light and continued to increase during whole light period. The same pattern of regulation was observed for *AtLOL1*. Upon onset of dark period both genes were down-regulated and expression remained invariably low during night. Opposite regulation was observed for *ABC10220*, *ABC06454* and *AtLSD1*, which reached the highest peak of expression at the first hours of dark period.

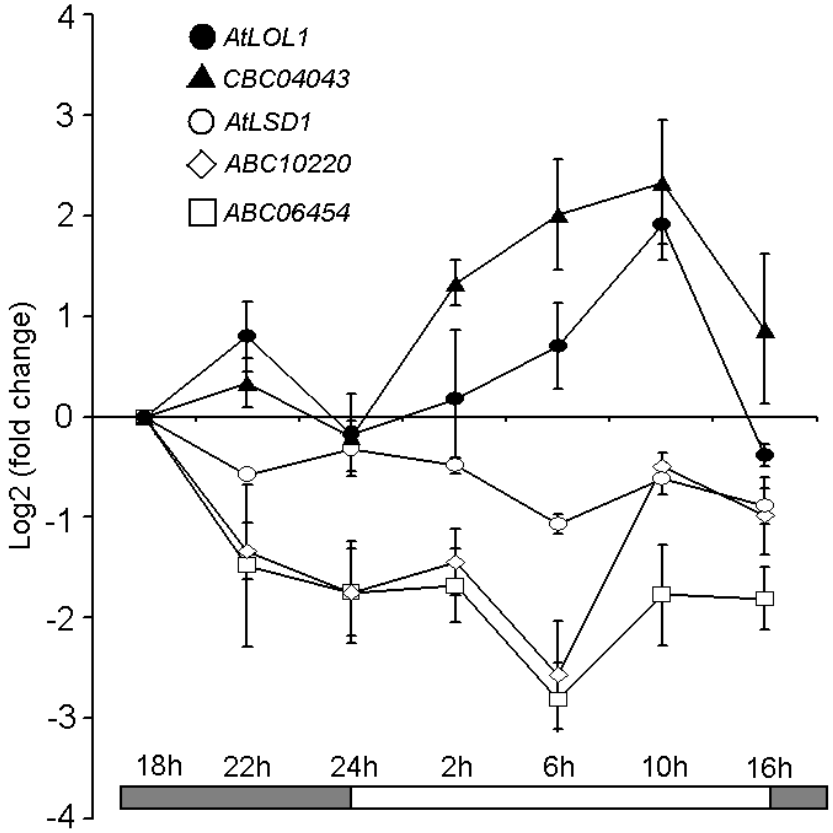


Figure 43. Effect of light cycle on transcript abundance of *AtLSD1*, *AtLOL1* and corresponding barley homologues. Expression was assessed in leaves of two week old cv Parkland and Columbia plants using real-time qRT-PCR. Gene expression levels are represented as logarithmic value of fold change compared to 18th hour (first data point after onset of dark) of light cycle. Bar beneath the figure represents course of day/night cycle. Error bars represent standard deviations. Figure represents data from one of two independent experiments with similar results.

Expression of barley *LSD1* homologues was also assessed in response to biotic stimulus – infection of virulent *Blumeria graminis* f.sp. *hordei* (Figure 44). *Bgh* infection slightly but statistically significantly suppressed *ABC10220* (p=0.01), *ABC06454* (p=0.02) and *CBC04043* (p=0.0006).

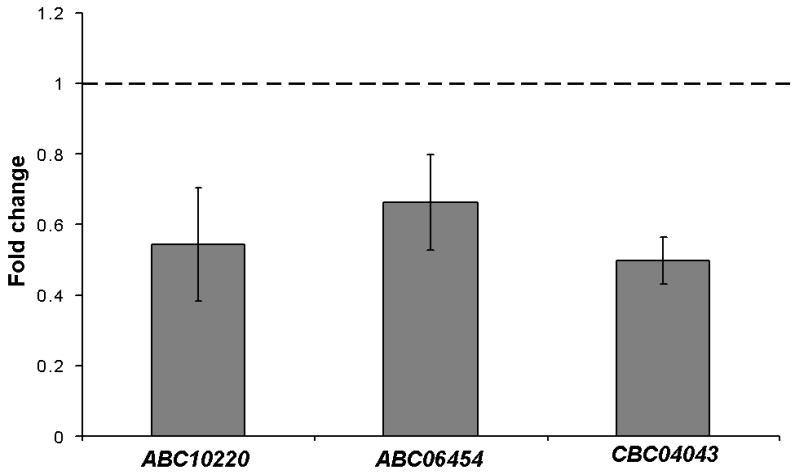


Figure 44. Change in mRNA abundance of barley *LSD1* homologues in response to *Blumeria graminis* f.sp. *hordei* infection. Expression was assessed in leaves of two week old cv Morex plants infected with mixed virulent population of *Blumeria graminis* f.sp. *hordei* using real-time qRT-PCR. Value represents difference between infected and uninfected plants 12 hpi. Interrupted line denotes value of uninfected plants. Mean values from 4 biological replicates each containing tissues from three plants are represented. Error bars represent standard deviations.

3.4.7. Identification of barley LSD1 mutants using reverse genetics approach - TILLING population screening

Identification of two barley genes *ABC10220* and *ABC06454* equally homologous to *AtLSD1* raised a problem of discrimination of functional homologue of *AtLSD1* in barley. It might be expected that disruption of functional homologue of *LSD1* in barley would result in lesion mimic phenotype allowing for identification of barley *HvLSD1*. Screening of barley TILLING populations was chosen as most appropriate approach for identification of barley mutants comprising mutations in either *ABC10220* or *ABC06454*. Two barley TILLING populations were screened – barley population of University of Silesia (Poland) in cv. Sebastian and TILLMore – population developed at University of Bologna (Italy) in cv. Morex (Talamé et al. 2008). Silesian population was screened by author of the thesis whereas TILLMore screening was performed at University of Bologna by Dr. V. Talamé.

Region with the highest probability of functionally deleterious mutations was identified and verified for specificity in barley genome (Figure 45). Since the designed primers yielded satisfactory results, labeled primers (5’ end-labeling with IrDye700 for forward and IrDye800 for reverse primer) were tested. Specific and strong amplification with labeled primers in University of Silesia TILLING population was obtained only for gene *ABC10220*, therefore

the further analysis of this population was carried-out only for *ABC10220*. TILLMore population (University of Bologna mutant barley population) was screened for both genes.

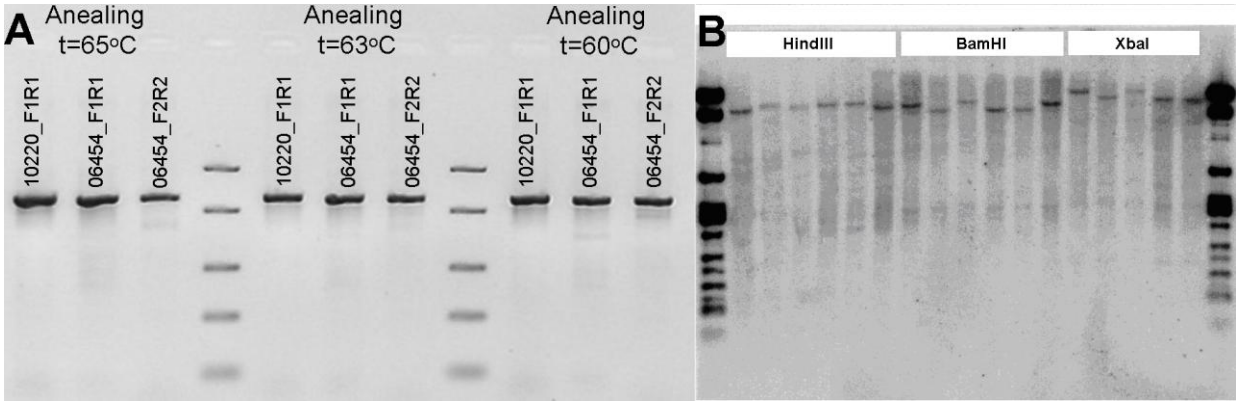


Figure 45. Fragments of *ABC10220* and *ABC06454* used for TILLING screening. **A** - PCR products amplified using primers designed for TILLING screen. Stability of amplification at optimal temperature range is shown. Analyzed fragments are 1kb long; **B** - Southern blot of *ABC10220* gene fragment chosen for TILLING screen. Blot represents DNA from six barley cultivars (Steptoe, Morex, Barke, Optic, Parkland, Bowman) digested with three different restriction enzymes confirming the specificity of the probe detecting a single copy gene in the barley genome.

In total 3072 mutant plants from Silesia barley TILLING population (Figure 46) and 4906 mutant plants from TILLMore population have been screened for *ABC10220* and *ABC06454*. In Silesia population 11 putative mutants were identified based on TILLING screen. In order to confirm and identify type of mutations analyzed gene fragments were amplified from the identified mutants and the obtained PCR products were sequenced. Obtained sequences were translated *in silico* to identify the type of mutation. For *ABC10220* in total six mutants were confirmed in Silesia population and five mutants were identified in TILLMore population (Table 27). Five remaining SNPs which were initially identified in Silesia population were natural variation present in parental cv. 'Sebastian' rather than true mutations induced by mutagen. For *ABC06454* in total six mutations were identified in TILLMore population. Majority of mutations detected were transitions (A to G or C to T in equal proportions) (Figure 47). Only one missense mutation was identified in *ABC10220* causing amino acid substitution G163E. The average density of identified mutations was one mutation per 430kb screened and one per 481kb in Silesia and TILLMore populations respectively (correction for effective screening window has been applied). Density of mutations identified in this study is comparable to average mutation density of the studied TILLING populations which is one mutation per 235kb screened and one per 374kb in Silesia (Kurowska et al. 2011) and TillMORE populations respectively (Talamé et al. 2008).

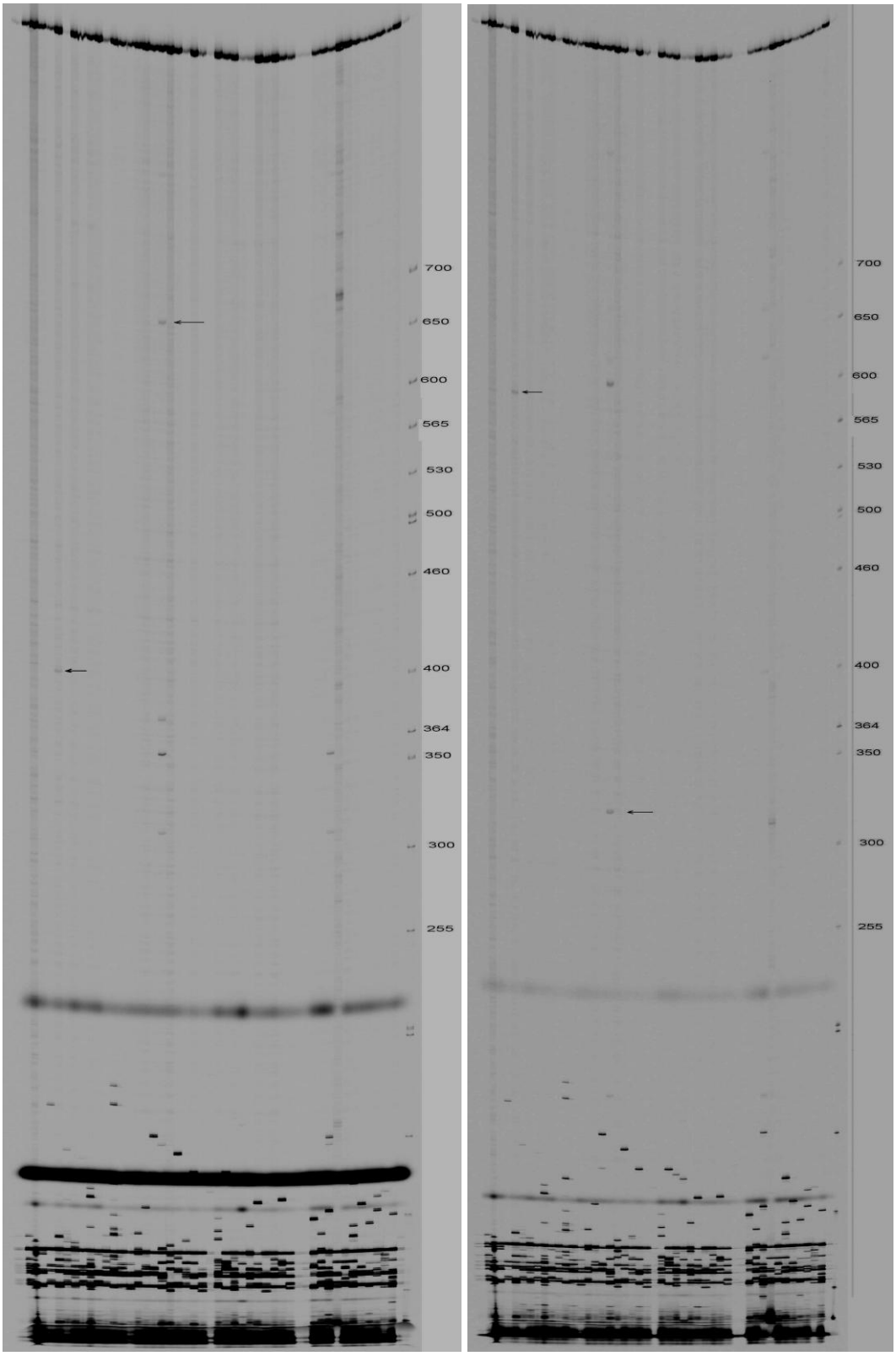


Figure 46. Identification of two mutations in *ABC10220*. Two figures represent complementary images from polyacrylamide gel loaded with *ABC10220* fragment amplified from subset of pooled DNA samples from barley TILLING population with IrDye700 labeled forward and IrDye800 labeled reverse primer run on Li-Cor analyzer. Two mutations are indicated with arrows.

Table 27. Types of mutations in *ABC10220* and *ABC06454* genes identified in the two barley TILLING populations.

TILLING population	Gene	Truncations	Missense	Silent (intron/ exon)*
University of Silesia	*ABC10220*	0	0	4/2
TILLMore (University of Bologne)	*ABC10220*	0	1 (G163E)	3/1
	ABC06454	0	0	6/0

*-site of silent mutation (either mutation has occurred in coding or non-coding sequence)

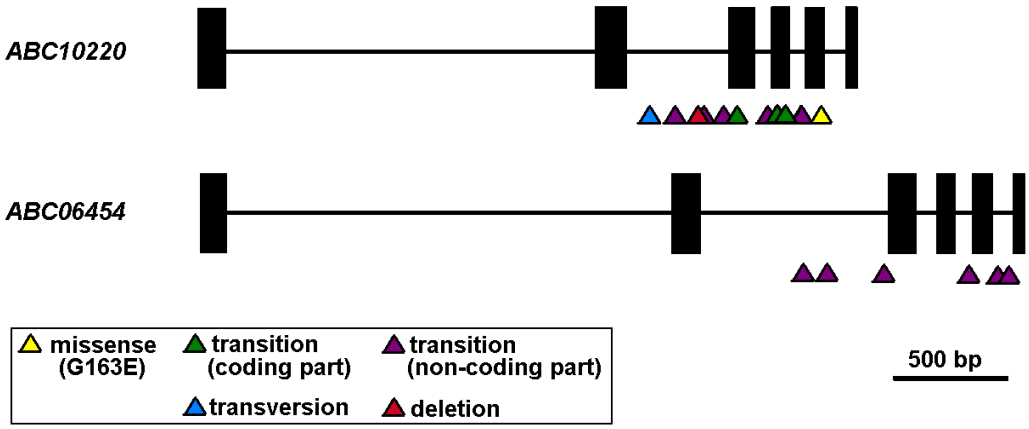


Figure 47. Sites of mutations detected in *ABC10220* and *ABC06454* by screening of TILLING populations. Different colors indicate type of nucleotide substitution or deletion.

3.4.8. Characterization of identified TILLING mutations

To determine the effect of identified mutations on mRNA splicing full sequences of *ABC10220* and *ABC06454* were analyzed using NetGene2 splice site prediction program (Hebsgaard et al. 1996). Based on *in silico* prediction none of the analyzed mutations identified from Silesia mutant population disrupted junction sites or branch points suggesting that altered mRNA splicing in identified mutants is unlikely. *In silico* analysis of mutations identified in TILLMore population identified one missense mutation causing G163E substitution and singled out two mutations in *ABC10220* and one mutation in *ABC06454* which might have affected mRNA splicing (Table 28). PCR amplification did not confirm altered mRNA splicing prediction for any of these mutations (Figure 48). Phenotypic characterization of the identified mutants cannot yet be performed since mutants are likely to other mutations induced by mutagen treatment. Mutants are being backcrossed to Morex in order to ensure that background mutations are eliminated and that lesion mimic phenotype is caused by mutations in *ABC10220*.

Table 28. Identified mutations from TillMORE barley TILLING population potentially affecting mRNA splicing or amino acid sequence of barley *LSD1* homologues.

Gene	Mutation *	Plant ID in TILLMore	Characteristics of mutation
ABC10220	T1951A	2127	Disrupts palindromic sequence TATCT(T/A)TTTTCTAT
	G2290A	776	affects branch point at 2290 TCTCCACTT(G/A)ACGAATTCTA
	G2748A	2097	Missense mutation G163E
ABC06454	C3474T	1265	Close to splice site at 3467 GGGTAAACTG::GTAAGGCC(T/C)CCGTTT

* - Nr represents bp from START codon of the gene,

::- denotes exon/intron splice site

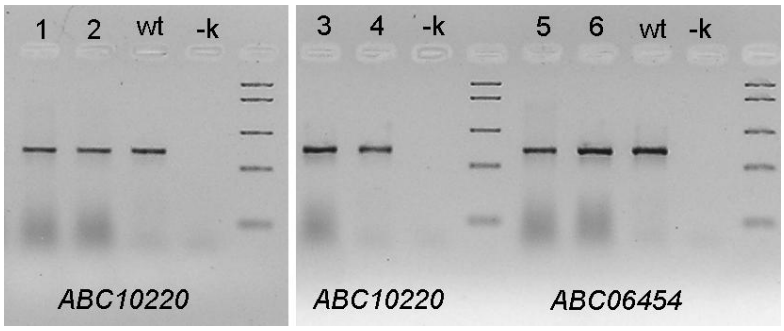


Figure 48. Full length coding part of *ABC10220* and *ABC06454* amplified from cDNA of identified TILLING barley mutants. 1 – TillMore2097, 2 – TillMore2127, 3 and 4 – TillMore 776, 5 and 6 – TillMore 1265. wt denotes sample from parental cv. 'Morex', -k denotes negative (cDNA minus) control. Presence of mutation in analyzed plants has been confirmed by sequencing.

Due to significantly different electrochemical properties of Glycine and Glutamic acid it might be expected that G163E mutation of ABC10220 can affect functional properties of the protein. *In silico* analysis of identified ABC10220 G163E mutation using PredictProtein server (Rost et al. 2004) suggests that the mutation could potentially affect low complexity region and eliminate sequence of non-regular secondary structure at the C-terminal part of the protein (Figure 49 A). However, *in silico* prediction of low complexity regions is challenging and often of low expected accuracy (Deng et al. 2012). In order to confirm PredictProtein results the effect of G163E mutation on low complexity regions of ABC10220 was assessed with two other programs – DISOPRED (Ward et al. 2004) and PrDOS (Ishida and Kinoshita 2007). Results obtained by these programs did not confirm the potential effect of G163E on presence of low-complexity regions in ABC10220 amino acid sequence.

Interestingly, G163E in ABC10220 is located close to the position of AtLSD1 P167L mutation (mutant *chs4*-3) (Huang et al. 2010 b). Both - G163E in ABC10220 and P167L in AtLSD1 - alter highly conserved C-terminal end of the protein (Figure 49 B).

Figure 49. Properties of predicted amino acid sequence of wt and mutated ABC10220 protein. A - Predicted low complexity region of ABC10220 in wt and mutant Tillmore2097 as predicted by NORSp program at PredictProtein server (Rost et al. 2004). Low complexity region denoted by x. TILLMore 2097 mutation is indicated in red. **B** – Predicted amino acid sequence alignment of C-terminal part of identified mutant TILLMore 2097, AtLSD1 mutant chs4-3 (Huang et al. 2010 b) and wt proteins AtLSD1, ABC10220. Amino acid substitutions in mutants are indicated in red. Amino acid conservation between *H. vulgare* and *A. thaliana* is denoted below alignment.

3.4.9. Complementation of A. thaliana lesion mimic mutant lsd1-1 with barley homologues ABC10220 and ABC06454

Two barley genes - *ABC10220* and *ABC06454* can be assigned as most probable candidate genes, based on phylogenetic analysis and expression pattern characterization. The third homologue, *CBC04043,* although comprises high sequence homology to *AtLSD1,* is more likely functional homologue of *AtLOL1,* as indicated by diurnal expression pattern and phylogenetic analysis. In order to find out if *ABC10220* and/or *ABC06454* are functional barley orthologue of *AtLSD1,* coding sequence of both genes was used to complement *A. thaliana lsd1-1* mutant. Although T-DNA constructs, containing coding part of either *ABC10220* or *ABC06454,* were successfully transformed into *lsd1-1* (as assessed by genomic DNA PCR), expression of introduced barley gene was achieved only for *ABC06454,* whereas *ABC10220* expression was not detected in transgenic plants (Figure 50). Transgenic *lsd1-1* plants, containing *ABC10220,* did not differ phenotypically from *lsd1-1* mutant (Figure 51), confirming negative results of transgene expression.

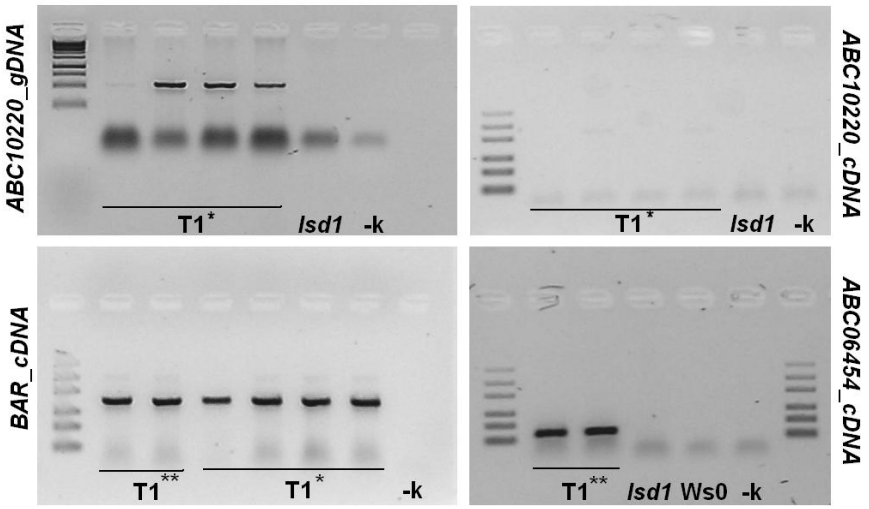


Figure 50. Transgene presence and expression in *A. thaliana* mutant *lsd1-1* transformed with barley *LSD1* homologues *ABC10220* or *ABC06454* under control of natural *AtLSD1* promoter. Title of each figure denotes either gDNA or cDNA has been used as a PCR template. T1* - primary transformants of *ABC10220* containing *lsd1-1*; T1** - primary transformants of *ABC06454* containing *lsd1-1*; -k – no template control; *lsd1* – non-transformed *lsd1-1* plants.

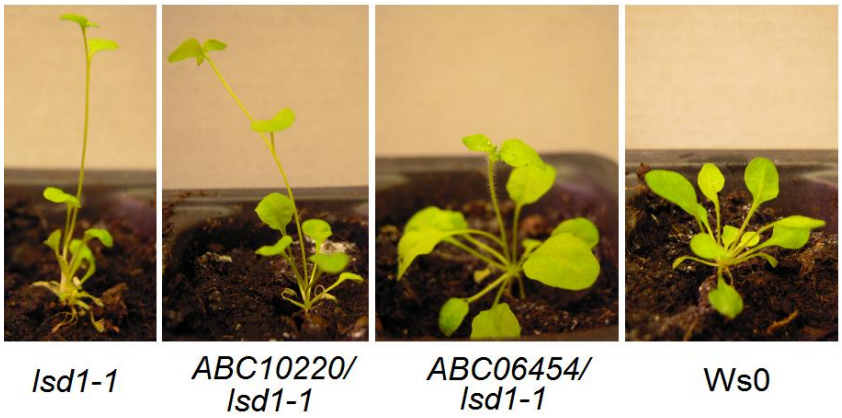


Figure 51. Phenotype of *A.thaliana lsd1-1* plants transformed with *ABC10220* or *ABC06454*. Photos of wt Ws0 and *lsd1-1* mutant are added for comparison.

To characterize *ABC06454* ability to complement *lsd1-1* mutant several physiological experiments were performed. First, *lsd1-1/ABC06454* plants were characterized for tolerance to long-day light conditions. *lsd1-1* mutation induces run-away cell death if subjected to long-day or high light conditions (Jabs et al. 1996). Therefore, tolerance of *lsd1-1/ABC06454* plants to long-day light conditions could serve as evidence for *ABC06454* ability to complement *lsd1-1*. *lsd1-1/ABC06454* plants were hypersensitive to long-day conditions - similarly to *lsd1-1* mutant necrotic spots on leaves of transgenic plants formed as early as few days after transfer to long-day growth conditions and continued to spread until engulfed whole leaf (Figure 52).

lsd1-1 mutants are known to exhibit run-away cell death phenotype in response to exogenously applied SA (Rusterucci et al. 2001). *lsd1-1/ABC06454* plants were characterized for induction of cell death upon SA treatment. No difference between non-transformed *lsd1-1* and transgenic *lsd1-1* containing *ABC06454* with regard to cell death induction and progression was observed (Figure 53).

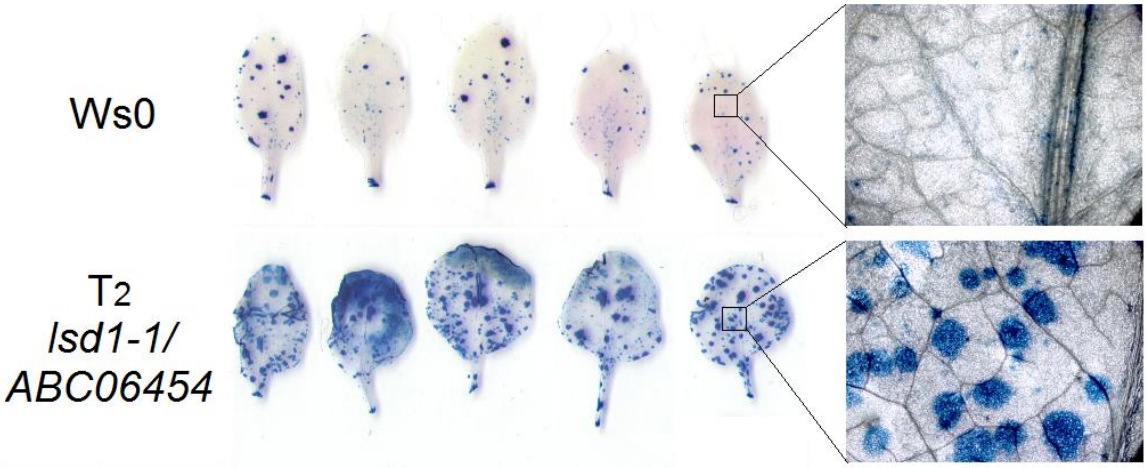


Figure 52. Cell death in leaves of wt Ws0 and transgenic *ABC06454/lsd1-1 A.thaliana* in response to long day light conditions. T_2 *lsd1/ABC06454* and wt plants were subjected to long day (16h/8h light/dark cycle) conditions for three days. Detached leaves were stained with trypan blue for cell death visualization.

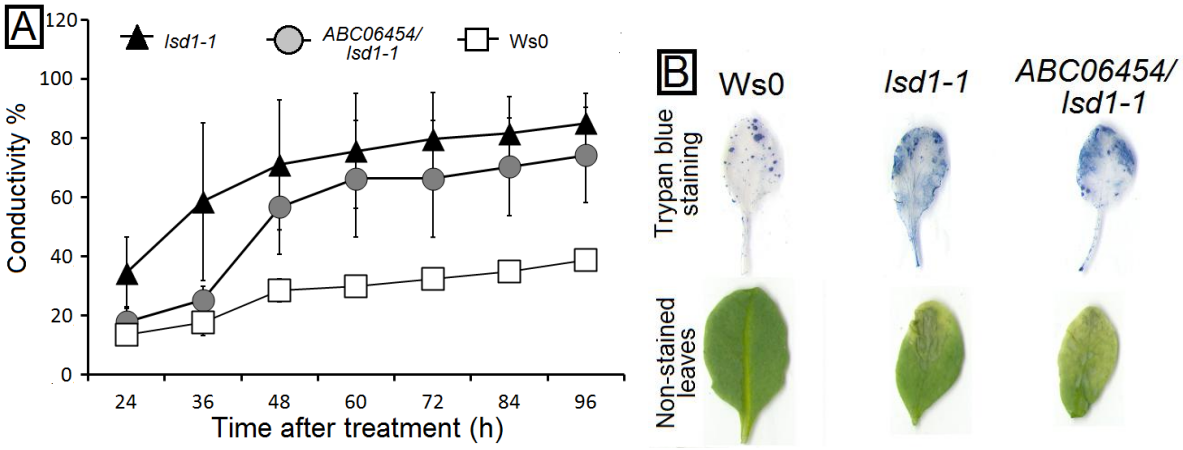


Figure 53. Run-away cell death phenotype of transgenic *ABC06454/lsd1-1 A.thaliana* in response to exogenously applied salicylic acid. A – ion conductivity of wt, *lsd1-1* and *ABC06454/lsd1-1* after treatment with 0.5 mM SA. Each datapoint represents average value of at least six biological replicates. Error bars represent standard deviation. **B** - representative leaves stained with trypan blue for cell death visualization.

4. Discussion

4.1. Characterization of barley lesion mimic mutant *nec1*

4.1.1. Role of NEC1 in barley disease resistance

Despite the fact, that ion fluxes are known to play an important role in early signaling events during plant-pathogen interaction (McDowell and Dangl 2000., Dangl and Jones 2001, Nurnberg and Scheele 2001), to date only several plant ion channels are shown to participate in plant disease resistance or plant-pathogen interaction signal transduction. CNGC (cyclic nucleotide gated ion channel) gene family is one of the best represented among the disease resistance related channels. *CNGC* mutants - *dnd1* (*AtCNGC2*), *dnd2* and *hlm1* (*AtCNGC4*) and *cpr22* (*AtCNGC11/12*) - exhibit wide range of pathogen resistance (Jurkowski et al. 2004, Balagué et al. 2003, Yoshioka et al. 2006, Clough et al. 2000). Mutations affecting *AtCNGC4* enhance resistance of *Arabidopsis thaliana* against certain pathotypes of *Pseudomonas syringae a*nd *Botrytis cinerea* (Jurkowski et al. 2004, Balagué et al. 2003, Genger et al. 2008). Although the effect of *CNGC* mutations on resistance to bacterial and oomycete pathogens is well studied in *Arabidopsis*, little is known about the role of these genes in non-host resistance and also about the functions of CNGCs in disease resistance of economically important monocot plant species, such as rice or barley. The present study analyzed the effect of *HvCNGC4* mutation *nec1* on barley resistance against *Pseudomonas syringae* pv. *tomato* and *Blumeria graminis* f. sp. *hordei*.

Results showed, that, similarly to *dnd2* in *A. thaliana* (Jurkowski et al. 2004), *nec1* in barley activates constitutive over-accumulation of SA. High level of SA contributes to enhanced disease resistance of *dnd2* to virulent *Pseudomonas syringae* pv. *tomato* (Jurkowski et al. 2004, Genger et al. 2008), and this resistance requires functional *PAD4* (Jirage et al. 2001), which is one of the central genes in SA-mediated effector-triggered immunity (ETI) (Wiermer et al. 2005) and SAR (Durrant and Dong 2004). Although disease resistance pathways seem to be largely conserved among monocots and dicots (Chern et al. 2001, Cooper et al. 2003, Qiu et al. 2007, Humphry et al. 2010), the position of SA in monocot immunity is ambiguous. Some monocots, such as rice, contain high endogenous SA levels (Chen et al. 1997) and SA is not required for *PR*-gene induction in rice upon infection (Yang et al. 2004). Ineffectiveness of externally applied SA on induction of *PR*-genes has also been observed in barley (Vallelian–Bindschedler et al. 1998) and wheat (Molina et al. 1999). However, inoculation with non-host bacteria *Pseudomonas syringae* triggers SA accumulation in barley (Vallelian – Bindschedler et al. 1998). Taking into account, that such differences occur in the SA mediated resistance signaling among monocots and dicots, it is interesting to see, whether mutation, affecting SA mediated disease resistance in *A. thaliana,* is also involved in barley disease resistance.

HR related cell death is suggested to serve in plant immunity as a factor triggering activation of SAR (Heath 2000, Kombrink and Schmelzer 2001). Spontaneous cell death might elicit constitutive activation of SAR related signaling pathway in *nec1*. Previously *nec1*

has been shown to constitutively overexpress *PR-1a* and *β-1,3-glucanase* (Rostoks et al. 2006) - molecular markers of SAR. This study confirmed the constitutive activation of SA-related signaling pathways in *nec1* mutants, since significant over-accumulation of H_2O_2 and SA in *nec1* plants was detected. Mutation in *NEC1* gene affected barley non-host resistance against *Pseudomonas syringae* pv. *tomato*. Bacterial growth in *nec1* plants was delayed at the initial phase of infection, if plants were inoculated with bacteria at high inoculum density. At the same time the increased electrolyte leakage suggested somewhat enhanced cell death, even though the conductivity values were much lower than reported for typical HR. Thus, electrolyte leakage data in *nec1* were generally in agreement with the expected "defense, no death" phenotype characteristic of *hlm1*/*dnd2* mutants, although differences between *nec1* and *hlm1*/*dnd2* mutants may exist in this respect. In *Arabidopsis thaliana*, non-host resistance against some types of pathogens involves SA signaling (Yun et al. 2003, van Wees et al. 2003, Zimmerli et al. 2004). In barley, a substantial increase in SA levels has been observed after infection with *Pseudomonas syringae* pv. *syringae*, but not after inoculation with non-host fungus *Blumeria (Erysiphe) graminis* f. sp. *tritici* (Vallelian – Bindschedler et al. 1998) or host pathogen *Bgh* (Hückelhoven et al. 1999), suggesting a differential role of SA in barley resistance against different pathogens. However, the cause for SA over-accumulation needs further investigation, and it remains to be determined, if SA-independent pathways are activated in *nec1* mutant, similarly to *Arabidopsis hlm1*/*dnd2* mutant.

Non-host resistance is predicted to share common defense responses with host resistance - either basal (PAMP-triggered immunity, PTI) or ETI (Mysore and Ryu 2004, Jones and Dangl 2006). The choice, of which layer of immunity is activated upon a particular interaction with nonhost pathogen, seems to be case specific (Niks and Marcel 2009, Schweizer 2007). Therefore, molecular mechanisms leading to changes in non-host resistance of *nec1* to *P. syringae* pv. *tomato* might have also had an effect on interaction with host pathogens. This prompted the assessment of the role of *nec1* mutation in resistance to powdery mildew, caused by the fungal pathogen *Blumeria graminis* f. sp. *hordei*. *nec1* restricted *Bgh* microcolony formation, while not affecting *Bgh* penetration or *mlo*-5 triggered resistance to *Bgh*. Interestingly, despite the fact, that *nec1* did not impede *mlo*-5 mediated race non-specific resistance to *Bgh*, *MLO* and *BI-1* mRNA abundance was significantly increased in barley *nec1* plants. Loss of functional *MLO* protein renders barley almost fully resistant against *Bgh*, whereas *BI-1* over-expression in *mlo* mutants leads to restoration of susceptibility against *Bgh* (Ihara-Ohori et al. 2007), and, in fact, *BI-1* is required for full susceptibility of barley to powdery mildew (Eichmann et al. 2010). Furthermore, overexpression of *MLO* in wild type plants leads to super susceptibility against *Bgh* (Kim et al. 2002). Taking into account, that significant induction of *MLO* and *BI-1* in *nec1* did not affect *mlo-5* mediated *Bgh* resistance of the mutant, significant over-expression of *MLO* and *BI-1* might result from general activation of cell death-related signaling pathways and systemic immunity responses, rather than from activation of particular powdery mildew resistance. Plant CNGCs are hypothesized to participate in plant immunity through effect on Ca^{2+} signal transduction (Ma et al. 2009 b, Chin et al. 2009). Ca^{2+} signal transduction has also been implicated in *MLO* triggered powdery mildew susceptibility. Mutations, affecting calmodulin (CaM) and MLO binding, interfere with MLO ability to trigger barley susceptibility against powdery mildew (Kim et al. 2002). Besides, inhibition of Ca^{2+} influx

reduce *OsMLO* mRNA abundance in rice (Kim et al. 2002), suggesting an effect of Ca^{2+} on transcriptional regulation of *MLO*. *Arabidopsis* AtBI-1 has been shown to interact with CaM in Ca^{2+} dependent manner, and over-expression of *AtBI-1* in tobacco cells alleviated Ca^{2+} rise (Ihara-Ohori et al. 2007). Interestingly, AtBI-1 and CaM interaction has recently been shown to affect also regulation of cell death during *Pseudomonas syringae* elicited HR (Kawai-Yamada et al. 2009). Constitutive induction of *MLO* and *BI-1* in *nec1* may be caused by constitutive activation of Ca^{2+} signaling, rather than by disruption of Ca^{2+} signal. In *Arabidopsis,* mutation in related *CNGC2* renders gene expression profile similar to that observed for WT plants at high Ca^{2+} conditions (Chan et al. 2008). Therefore it can be hypothesized, that altered expression of *Bgh* susceptibility pathway related genes in *nec1* might be attributed to possible constitutive activation of Ca^{2+} signaling.

The precise molecular mechanisms linking CNGC4 and SA signaling are unknown yet. Role of Ca^{2+} as a secondary messenger in SA signaling has been envisaged for long. Recently, a direct link between SA signaling, Ca^{2+} and calmodulin has been established by discovering two Ca^{2+}/calmodulin binding proteins directly participating in SA signaling - AtSR1 (Du et al. 2009) and CBP60g (Wang et al. 2009). Ca^{2+} influx preceding SA accumulation is most likely ensured by ion channel (Kawano et al. 2004), thus, rising an issue of identification of Ca^{2+} permeable ion channel, enabling cytosolic Ca^{2+} influx, required for SA signaling during plant pathogen response. Previously, two-pore channel TPC1 was hypothesized as a main candidate for Ca^{2+} permeable plant defense response related ion channel (Kawano et al. 2004, Kadota et al. 2004). However, the role of TPC1 in plant defense response was recently disproved (Ranf et al. 2008). CNGCs have been suggested as a missing link between SA and Ca^{2+} influx, required for defense signaling (Ma et al. 2009 b). Although only a few CNGCs have directly been proved to conduct Ca^{2+}, CNGCs comprise Ca^{2+}/calmodulin binding domain and are predicted to be Ca^{2+} permeable (Talke et al. 2003, Kaplan et al. 2007). Therefore CNGC4 could serve as a likely candidate for ion channel linking Ca^{2+} fluxes and SA mediated defense signaling.

In summary, the results suggest that *nec1* mutation most likely affects PTI and nonhost resistance related responses, and it is not associated with rapid localized defense responses, required to prevent fungal penetration. Constitutive activation of the SA-related defense pathway may contribute to differential resistance of *nec1* mutant against non-host bacteria *Pst* and virulent host pathogen *Bgh*. *nec1* effect on barley immunity significantly differs from effect of *dnd2* homologous mutation in *A. thaliana,* triggering broad-spectrum disease-resistance. Thus, information gained from studies of *A. thaliana,* can be useful to set the initial experimental framework for barley research, highlighting most relevant scientific targets and questions, however, direct application of *A. thaliana*-based knowledge to barley should be performed with certain precaution.

4.1.2. Constitutive induction of SAR markers in nec1 might be related to disturbed auxin signaling

Although the role of auxin as a plant disease resistance affecting agent has been suspected for almost two decades, molecular mechanisms linking auxin signaling and plant immunity are largely unknown (Kazan and Manners 2009). Antagonistic interaction with SA

mediated signaling is one of the proposed mechanisms, explaining the negative effect of auxin on plant disease resistance (Chen et al. 2007, Wang et al. 2007, Iglesias et al. 2011). Interestingly, several SA over-accumulating mutants are also impaired in auxin signal perception and *vice versa* - mutations impairing auxin related signaling also affect disease resistance. For example *A. thaliana* SA over-accumulating mutants *cpr6* and *snc1* produce less IAA, display reduced apical dominance and are less sensitive to exogenous auxin (Wang et al. 2007). Besides, over-expression of auxin related genes *GH3* and *WRKY31* enhanced disease resistance of rice to *Xanthomonas oryzae* and *Magnaporthe grisea* respectively (Ding et al. 2008, Zhang et al. 2009 a). *CNGC4* has previously been shown to affect SA mediated disease resistance in *A. thaliana* (Balagué et al. 2003, Jurkowski et al. 2004) and, according to results described above – also in barley. *CNGC4* mutant of *A. thaliana dnd2* exhibits reduced stature and altered branching, suggesting that impaired CNGC4 might affect auxin signaling in *A. thaliana* (Sherman and Fromm 2009). Therefore SAR marker induction in *nec1* might be associated with altered auxin signaling in the mutant. Results of this study show, that SA over-accumulating barley mutant *nec1,* comprising non-functional *CNGC4,* displays physiological responses, resembling altered auxin signaling.

nec1 showed altered sensitivity to exogenous auxin as suggested by coleoptile elongation and root growth in response to externally applied auxin. Physiological indicators representing effects of endogenous auxin did not reveal any significant difference between *nec1* and wt plants. *nec1* exhibited somewhat smaller size of epidermal cells and did not show any change in root gravitropic response. Due to uncertainty, introduced by physiological data, expression of auxin biosynthesis related genes was analyzed in *nec1*. *YUC1*, *VT2* and *NIT2* were chosen as genes representing different auxin biosynthesis pathways. *YUCCA* genes encode a family of flavin monooxygenases, that are rate – limiting enzymes in the auxin biosynthesis pathway through tryptamine in *A. thaliana* (Cheng et al. 2006). In addition, expression of *YUCCA* genes has been correlated with endogenous auxin levels in *A. thaliana* and barley (Sakata et al. 2010). *VT2* gene in maize is an orthologue of *A. thaliana* genes belonging to *TAA* gene family. *ZmVT2* was shown to be involved in auxin biosynthesis through indole-3-pyruvic acid (IPA) pathway (Phillips et al. 2011). *NIT2* gene has been implicated in conversion of indole-3-acetonitrile (IAN) to IAA in the indole-3-acetaldoxime (IAOx) auxin biosynthesis pathway in maize (Kriechbaumer et al. 2007). Expression analysis of auxin related genes in *nec1* revealed significant repression of two genes encoding key-enzymes of auxin biosynthesis - *VT2* (*AtTAA1* homologue) and *YUC1*. Although previously *TAAs* and *YUCCAs* have been suggested to represent two different auxin biosynthesis pathways (IPA and TAM pathway, respectively) (McSteen 2010), recently it has been shown that both – *TAAs* and *YUCCAs* are involved in IPA auxin biosynthesis pathway (Won et al. 2011, Mashiguchi et al. 2011). Therefore, significant repression of both *YUC1* and *VT2* genes in *nec1* suggests, that IPA pathway of auxin biosynthesis is likely repressed in *nec1* mutant. Low endogenous auxin concentration could explain altered *nec1* sensitivity to low dose exogenous auxin application and slightly but statistically significantly decreased cell size of *nec1*; therefore we analyzed IAA content of *nec1*. Surprisingly, HPLC analysis identified significantly increased IAA content in *nec1*. In view of contradictory results from gene expression and HPLC analyses, further experiments, elucidating the exact effect of *nec1* mutation on auxin biosynthesis in plants, will be needed.

Although it still remains to be elucidated, how the lack of cyclic nucleotide-gated ion channel 4 in *nec1* leads to increased level of SA and IAA, it might be anticipated that CNGC4 contributes to both pathways through Ca^{2+}/calmodulin regulatory mechanisms. Although Ca^{2+} permeability of CNGC4 has not been clearly demonstrated (Balagué et al. 2003), a related ion channel CNGC2 has been shown to ensure rise of cytosolic Ca^{2+} in response to PAMPs (Ma et al. 2009 a). Plant CNGCs are negatively regulated by CaM (Kaplan et al., 2007), and HvCNGC4 comprises calmodulin (CaM) binding domain (Rostoks et al., 2006), suggesting that CNGC4 might be involved in Ca^{2+}/CaM related signaling. Ca^{2+}/CaM have been implicated as secondary messengers in auxin (Yang and Poovaiah 2000), as well as SA signal transduction (Du et al. 2009). Interestingly, another Ca^{2+} permeable channel, *calcium exchanger 1 (CAX1)*, has also been shown to affect auxin related signaling, since *cax1* mutants exhibit reduced apical dominance, inhibited lateral root development and show reduced sensitivity to exogenous auxin treatment (Cheng et al. 2003). Moreover, mutations in the barley *HvCAX1* gene provide resistance to barley stem rust (Zhang et al. 2009b). Taking into account above mentioned evidence, *nec1* may serve as a model system, revealing role of SA-auxin-Ca^{2+} crosstalk in plant disease resistance.

4.1.3. Future perspectives of nec1 studies

nec1 studies can be continued in several directions. First, taking into account, that Ca^{2+} conductivity of plant CNGC4 has not been proved, it would be required to find out, if molecular functions of *CNGC4* are related to Ca^{2+} signaling. There are several methodological approaches, which can be taken, to study relationship between CNGC4 and Ca^{2+} mediated signaling. Patch clamp technique can be applied to gain direct proof for Ca^{2+} conductivity of CNGC4. Previously reported patch clamp analysis of AtCNGC4 in *Xenopus* oocytes proved to be problematic, and author suggested, that other expression system should be used (Balagué et al. 2003). Electrochemical characterization of CNGC2 has successfully been achieved using *A. thaliana* and *Vicia faba* guard cell protoplasts (Ali et al. 2007), suggesting that these systems might be appropriate also for CNGC4 studies.

Alternatively, other methods indirectly characterizing *CNGC4* role in Ca^{2+} signaling can be applied. CNGC4 can be used to complement Ca^{2+} transport deficient yeast mutants. However, such approach requires deletion of CaM binding domain of CNGC, since yeast calmodulin is able to bind and down-regulate plant CNGCs (Ali et al. 2007). Role of CNGC4 in Ca^{2+} signaling might also be assessed using techniques allowing visualization of Ca^{2+} in plant cells. Numerous techniques, based on either fluorescent proteins or synthetic dyes, can be applied for Ca^{2+} imaging (Russell 2011). It would be relevant to find out, if mutations impairing *CNGC4* affect intracellular Ca^{2+} level during plant response to different stress factors (PAMPs, exogenously applied phytohormones). Therefore, the method chosen for visualization of Ca^{2+} in *CNGC4* deficient plants needs to allow for long term monitoring, preferably using the same plant. Introduction of fluorescent protein-based Ca^{2+} indicator into *CNGC4* mutant plants might be a solution.

Second, it is relevant to continue *nec1* disease resistance studies. This study has revealed, that *nec1* mutation does not affect penetration resistance against *Bgh* and also does not impair *mlo-5* mediated *Bgh* resistance. However, taking into account that *nec1* plants

restricted growth of non-host pathogen *P. syringae* and also supported formation of less *Bgh* microcolonies, it might be expected, that certain mechanisms of PTI and non-host resistance (which is lately viewed as extreme version of PTI) might be enhanced by *nec1* mutation. Characterization of *nec1* resistance to non-host powdery mildew *Blumeria graminis* f.sp. *triticea* might yield more information on the role of *nec1* in non-host resistance. Besides, it still remains to be clarified, if *nec1* affects race-specific *Bgh* resistance of barley. In order, to find out the effect of *nec1* on race-specific *Bgh* resistance, it would first be required to introduce *nec1* into genetic background, containing *Mla* allele. This can be done either by traditional crossing or by silencing *NEC1* in background, containing *Mla*. Unfortunately, none of the lines, in which *nec1* is available, contains any *Mla* alleles, therefore such experimental approach would require series of backcrossing, and therefore would be time-consuming. Other approach, using *NEC1* silencing in *Mla* containing line, might also be challenging, taking into account difficulties related to genetic transformation of barley. Besides, since different *Mla* alleles employ different molecular pathways involving distinct genes, barley lines bearing different *Mla* alleles would be required to test for *nec1* effect.

Third, since this study has revealed possible link between *nec1* and auxin signaling in barley, it would be important, to find out, if the effect of *nec1* on barley disease resistance depends on altered auxin signaling. Induction of SAR markers in *nec1* indicates, that *nec1* shares certain characteristics of *A. thaliana CNGC4* mutants. At the same time *nec1* did not display broad-spectrum disease resistance characteristic to *dnd2* in *A. thaliana*. Taking into account different role and influence on disease resistance of SA in monocots and dicots discussed above, it might be expected that *nec1* effect on auxin signaling overcomes *nec1* effect on SA signaling in barley. To characterize the crosstalk between auxin and SA signaling with regard to disease resistance in *nec1*, auxin signaling could be visualized using reporter gene expression under auxin sensitive promoter. *nec1* comprising auxin induced reporter gene might be either directly infected with pathogens or treated with elicitors and effectors, to monitor change in auxin concentration and spatial distribution. Alternatively, temporal silencing of auxin signaling in *nec1*, using auxin repressors under chemically inducible promoters, would help to reveal the effect of auxin on *nec1* disease resistance related cellular responses (production of ROS, expression of pathogenesis related genes, reorganization of organelles or cytoskeleton). Although permanent or long-lasting silencing of auxin signaling in plants is not possible, auxin biosynthesis or signaling genes might be silenced using transfection of *nec1* protoplasts - this would allow monitoring of short term disease resistance responses.

4.2. Towards molecular cloning of *NEC3* gene of barley

Due to phenotypical resemblance with HR related cell death, lesion mimic mutants have been proposed to serve as a useful tool to study molecular mechanisms of HR in plants (Lorrain et al. 2003). Although *lmm* are well represented in barley mutant populations, only several genes responsible for lesion mimic phenotype in barley have been identified. *nec3* mutation triggers formation of lesion mimic phenotype in barley, therefore identification of *NEC3* gene might reveal new aspects of HR and also disease resistance of barley.

Different strategies have previously been applied in identification of genes, causing necrotic phenotype in barley. Identification of *HvCNGC4* responsible for *nec1* phenotype was achieved, based on information of homologous *A. thaliana* gene *HLM1* and its mutants (Rostoks et al. 2006), whereas discovery of barley gene, causing *necS* phenotype, was achieved by transcript based cloning (TBC) (Zhang et al. 2009 b). Identification of *NEC3* candidate genes in this study was based on two forward genetics approaches – TBC and positional cloning.

4.2.1. Identification of nec3 candidate genes using TBC

Significant reduction of mRNA abundance of a specific gene in a transcriptome of several allelic mutants in comparison to parental line allows identification of a candidate gene for the analyzed mutation (Zakhrabekova et al. 2002). Assuming, that the correct tissue at the correct developmental stage are analyzed, transcript based cloning (TBC) can be more straightforward and less laborious technique for gene identification than map - based cloning (MBC). Although SNPs can also affect mRNA abundance of the mutated gene through mechanism known as nonsense mediated decay (Gadijeva et al. 2004), use of fast neutron mutants, containing large deletions encompassing partial or entire gene, might be more robust for gene cloning using microarray hybridization (Bruce et al. 2009). TBC has successfully been applied for identification of candidate genes for several mutations in barley (Zakhrabekova et al. 2002; Mitra et al. 2004; Zhang et al. 2006 a; Zhang et al. 2009 b; Xi et al. 2009). Two fast neutron mutants FN362 and FN363, displaying tan or light brown necrotic leaf spots, both allelic to *nec3*, were chosen for Affymetrix Barley1 GeneChip experiment, to identify the *NEC3* candidate gene. Taking into account, that *nec3* lesion phenotype occurs at late developmental stage, two independent Affymetrix experiments, differing in sampled plant age (either 10 day or 7 week old plants), were performed. Although numerous genes were down-regulated in studied mutants in both experiments, only very few of these genes were overlapping between both experiments. Unfortunately, none of down-regulated genes of the analyzed mutants appeared to be deleted from either of the *nec3* mutants at either developmental stage. Failure to identify a candidate *NEC3* gene by TBC may result from several reasons, e.g., (a) the probe sets for *NEC3* gene are not present on the Barley1 GeneChip; (b) the expression level of wt gene is below the detection threshold of the GeneChip; (c) cv. Steptoe allele of the *NEC3* gene is not reliably detected by the GeneChip; (d) the expression of wt *NEC3* gene requires either specific environmental conditions or developmental stage. While the Affymetrix Barley1 GeneChip contains 22792 probe sets (Close et al. 2004), it only represents a fraction of the total transcribed portion of the barley genome. As the genomes of *Arabidopsis*, rice and maize contain an estimated number of 25498 (*Arabidopsis* Genome Initiative 2000), 37544 (International Rice Genome Sequencing Project 2005) and 32000 (Schnable et al. 2009) genes, respectively, and the number of genes in barley genome is likely to be similar to rice and maize, it is possible, that the Affymetrix Barley1 GeneChip does not contain probe sets for the *NEC3* gene. In addition, because the Barley1 GeneChip was designed primarily from the EST data, it only contains probesets for genes, that were expressed in the tissue and at the developmental stages, sampled during EST sequencing. The EST based unigenes, that were used for Barley1 GeneChip design were

designed from multiple barley accessions; however, the cv. Steptoe was not sampled. Natural allelic diversity, in cases of sequence mismatch between cRNA target and oligonucleotide probe, may result in artificially lower mRNA hybridization signal, a phenomenon, that has been used for single feature polymorphism discovery in barley (Rostoks et al. 2005 a) and yeast (Ronald et al. 2005) transcriptomes. Thus, it is possible, that the cv. Steptoe allele of the *NEC3* gene may not be detected using Barley1 GeneChip, even though, the probe sets for *NEC3* are present on the microarray, resulting in a undistinguishable expression level between cv. Steptoe and the mutants. There is also the possibility, that the fast neutron induced mutation is a small deletion or SNP, that was not detected by our analyses. Such fast neutron mutations are rare, but not unknown as exemplified by one of the *nec1* mutant alleles (Rostoks et al. 2006). The main disadvantage of TBC might be a requirement for an above-threshold expression of the target gene in the parental line. Since many genes require specific conditions to be induced, it significantly restricts range of genes, which can be identified using TBC (Bruce et al. 2009). If *NEC3* expression is developmentally or environmentally controlled, the observed failure to identify *NEC3* gene may be caused by the lack of its expression under our experimental setup.

4.2.2. Transcriptome analysis of the barley nec3 mutants FN362 and FN363

Studies of mutants displaying specific phenotype or altered response to abiotic or biotic stimulus help in identification of genes, critical for plant adaptation to adverse conditions (Svensson et al. 2006). Whole genome transcript analysis can be used to reveal major signaling pathways, activated in response to a particular stress factor or as a result of a mutation (Hoth et al. 2002, Ozturk et al. 2002). In general, different stressors elicit stress specific signaling pathways, with only a minor part of induced genes overlapping between various treatments (Kreps et al. 2002). The analysis of mutants with altered stress response can often help in unraveling molecular mechanisms of stress response, since mutations disrupting certain signaling pathway can mimic the effect of stress treatment (Bohnert et al. 2006). Analysis of *nec3* transcriptome aided in a better understanding of molecular mechanisms underlying missregulation of cell death in barley and a probable link between regulation of cell death and other physiological processes.

Due to the phenotypic similarity, it is tempting to associate missregulated cell death of lesion mimic mutants with hypersensitive response and disease resistance. Numerous lesion mimic mutants display enhanced resistance to certain pathogens (Lorrain et al. 2003, Mur et al. 2008, Wu et al. 2008, Zhang et al. 2009 b). However, several studies demonstrate enhanced or impaired abiotic stress resistance of lesion mimic mutants (Jambunathan et al. 2001, Yamanouchi et al. 2002, Mateo et al. 2004, Mühlenbock et al. 2007), suggesting that necrotic phenotype of lesion mimic mutants does not necessary result from alterations of disease resistance pathways, but can also be linked to abiotic stress response. Therefore, by focusing exceptionally on pathogenesis related factors in characterization of *lmm* one risks to overlook other important and maybe even primary characteristics of the mutant phenotype.

Characterization of differentially expressed gene set of *nec3* revealed several key aspects of *nec3* transcriptome. First, *nec3* triggers induction of catabolic processes, as indicated by over-representation of proteolytic and lipolytic enzymes and protein turnover

related genes in differentially expressed gene set of *nec3*. Second, *nec3* mutation induces genes involved in lipid transport and long-chain fatty acid biosynthesis. Third, *nec3* differentially regulated gene set comprises considerable number of secondary metabolism related genes, particularly those related to phenylpropanoid pathway. Fourth, *nec3* activates cell-wall related gene subset.

Induction of catabolic enzymes in *nec3* might be indicator of early senescence, caused by this mutation. Annotation of genes differentially regulated in *nec3* revealed, that at both developmental stages *nec3* metabolism is shifted towards carbohydrate production and protein degradation, increasing C:N ration. Increased C:N ratio is one of the main characteristics of senescence associated metabolism in plants in general (Buchanan-Wollaston et al. 2003) and in barley particularly (Parrott et al. 2010). Up-regulation of proteolytic enzymes is characteristic feature of senescing barley transcriptome, and it is required for N remobilization from senescing leaves (Parrott et al. 2010). Apart from proteolytic enzyme induction, *nec3* also over-expressed ubiquitin ligase genes, confirming onset of protein degradation in mutant. In addition, induction of numerous nucleolytic enzyme genes, amino-acid transporters and chlorophyll degrading enzymes in *nec3* confirms activation of N remobilization in mutant plants. Among the set of catabolic enzymes, induced in *nec3*, are also some genes, considered to be marker-genes of early senescence in barley. Particularly, *glutamate dehydrogenase* and *pheophorbide a oxygenase*, that were induced in *nec3*, have previously been shown to serve as senescence marker genes (Masclaux-Daubresse et al. 2000, Pružinská et al. 2003).

Remobilization of nitrogen is energetically costly process – it requires not only successful degradation of existing protein molecules, but also biosynthesis of new amino acids for N transportation from senescing organs (Hörtensteiner and Feller 2002). Therefore, in conditions, when photosynthesis is inhibited, due to degradation of photosynthetic machinery and due to over-accumulation of photosynthetic end products, plants need to find alternative source of energy and C skeletons for amino acid biosynthesis. Induction of glyoxylate pathway enzymes – isocitrate lyase and malate synthase can serve as an alternative source of malate for amino acid synthesis in Krebs cycle (Chen et al. 2000). Affymetrix chip analysis revealed highly significant induction of isocitrate lyase and malate synthase in *nec3* mutant, confirming senescence induction in mutant plants.

Apart from induction of N remobilization, *nec3* mutants significantly over-expressed fructan synthesis related genes. Over-accumulation of hexoses and other soluble sugars is another metabolic marker of plant senescence (Wingler et al. 2006). Increased accumulation of fructans and glucose is characteristic for early onset of senescence in barley (Parrott et al. 2007).

Thus, gene set, required for senescence related metabolic rearrangement of cell, is induced in *nec3*. Senescence in plants is highly controlled process, requiring activation of specific transcription factors, leading to induction of metabolic reorganization of cell, preparing it for the ultimate stage of senescence - the cell death (Buchanan-Wollaston et al. 2003). *nec3* transcriptome comprised significant number of induced transcription factors from different gene families. However, information on the role of specific barley transcription factors in senescence or cell death induction in barley is scarce. Due to high sequence homology within transcription factor families, it is difficult to distinguish any particular

transcription factor, which have previously been implicated in senescence or cell death induction in either rice or *A. thaliana*.

Surprisingly, *nec3* transcriptome did not show induction of any autophagy related genes, which is usually considered to be an integral part of plant senescence. While protein ubiquitination machinery is involved specifically in degradation of soluble proteins, autophagy ensures degradation of wide range of cytoplasmic components (Kim et al. 2012). Studies, analyzing senescence associated transcriptome change in *A. thaliana,* report induction of autophagy related genes as one of the characteristic features of *A. thaliana* senescence (Buchanan-Wollaston et al. 2005, van der Graaff et al. 2006, Breeze et al. 2011). There might be several reasons for lack of autophagy related gene up-regulation in *nec3*. First, induction of autophagy might not be characteristic for barley senescence as opposed to *A. thaliana*. However, this is unlikely explanation, since senescence-related transcriptomes of previously analyzed monocots (wheat and rice) have not significantly differed from *A. thaliana* senescence, including induction of autophagy related genes (Gregersen and Bach-Holm 2007, Liu et al. 2008). Second explanation for *nec3* failure to detect induction of autophagy-related genes during senescence might be improper functioning of autophagy in the mutant. Despite the lack of autophagy-related gene induction, *nec3* transcriptome showed several other features ubiquitous for senescing plant transcriptome. Induction of senescence marker genes from SAG family and presence of AtMC9 metacaspase in *nec3* transcriptome suggests, that early senescence in *nec3* and induction of cell death phenotype in mutant is not a stochastic process, but rather controlled by genetic and metabolic cellular senescence mechanisms.

Thus, induction of catabolic enzymes, up-regulation of glucose synthesis and over-expression of glyoxylate pathway enzymes and over-expression of SAG and cell death related genes in *nec3* indicate induction of early senescence in mutant plants.

Second characteristic of *nec3* transcriptome was induction of lipid transport and lipid metabolism related genes. Similarly to protein degradation and remobilization, lipid turnover is an important characteristic of plant senescence (Yang and Ohlrogge 2009). Lipid catabolism does not only result from disintegration of cellular membranes, but also serves as a source of energy and carbohydrate synthesis during senescence (Buchanan-Wollaston et al. 2003). Another important aspect of lipid turnover during senescence is jasmonic acid related metabolism. JA is considered to be one of the main hormonal regulators of plant senescence (He et al. 2002). Up-regulation of JA-related genes has been observed in transcriptome of senescing *A. thaliana* (van der Graaff et al. 2006) and wheat (Gregersen and Bach-Holm 2007). Induction of long-chain fatty acid CoA ligase, allene oxide cyclase and acyl-CoA oxidases indicates, that lipid metabolism gene induction in *nec3* might also be associated with up-regulation of JA signaling. Up-regulation of several genes annotated as jasmonate-induced protein in *nec3* supports this idea.

Another group of genes, induced in *nec3*, were genes related to secondary metabolite biosynthesis – particularly those related to phenylpropanoid biosynthesis. Most likely, induced synthesis of phenylpropanoids in *nec3* is required for adaptation to light stress, resulting from improper functioning of photosynthetic machinery, caused by degradation of the main photosynthesis proteins. For example, up-regulation of hydroxylcinnamoyl-

CoA:quinate transferase observed in *nec3* is one of the indicators for photoprotective phenylpropanoid synthesis in plants (Grace and Logan 2000).

Induction of secondary metabolism is closely related to fourth characteristic of *nec3* transcriptome – differential regulation of cell wall related genes. Transcripts of secondary metabolism genes, involved in cell wall fortification - lignin and suberin biosynthesis - were up-regulated in *nec3* transcriptome. Development of secondary cell wall, involving synthesis of suberin and lignification, is a common characteristic of late developmental stage and senescence of plants (Passardi et al. 2004). At the same time *nec3* also displayed induction of cell wall degrading enzymes such as xyloglucan transglycosylase, polygalacturonase and β-glucosidases. Degradation of cell wall carbohydrates serves as an additional carbohydrate and energy source during senescence in *A. thaliana* (Breeze et al. 2011, Lee et al. 2007).

Summarizing above described data, *nec3* mutation triggers induction of early senescence by enhanced activation of lipid hydrolyzing enzymes and subsequently also proteolytic enzymes. Enhanced cell wall lignification and synthesis of secondary metabolites might indicate, that *nec3* affects also stress related responses. Such assumption does not contradict with early senescence hypothesis, since certain abiotic factors (such as effect of drought, salinity, prolonged exposure to dark, phosphor and carbohydrate starvation) as well as biotic stressors (such as wounding and herbivory) can induce senescence metabolism. However, taking into account that GoSlim analysis did not single out stress related gene group as overrepresented in *nec3* transcriptome, identification of *nec3* should primarily be focused among senescence related genes or genes related to plant secondary metabolite synthesis.

Cluster analysis of *nec3* differentially expressed gene set and data from publicly available barley GeneChip experiments revealed certain overlap not only with barley transcriptome undergoing senescence, but also with abiotically stressed barley transcriptomes. The fact, that *nec3* also shared significant number of differentially regulated genes with those induced or repressed by drought, freezing or chilling, suggests that *nec3* mutation might also interfere with signaling pathways, required for abiotic stress response in barley. Characteristic features of *nec3* transcriptome, previously discussed with regard to senescence, could also be viewed in the light of abiotic stress tolerance. For example, grasses synthesize and accumulate fructans as short-term storage carbohydrates (Vijn and Smeekens 1999), but fructans also serve for cold and drought acclimation through membrane stabilization (Hincha et al. 2000; Hincha et al. 2002; Valluru and van den Ende 2008). Besides, overrepresentation of lipid biosynthesis and transport related proteins in the *nec3* transcriptome could be associated with abiotic stress response. Changes in lipid membrane composition and induction of genes, involved in lipid biosynthesis, are known to occur upon abiotic stress treatment (Blein et al. 2002; Gigon et al. 2004; Svensson et al. 2006). Induction of phenylpropanoids as photoprotective agents might not be required due to early onset of senescence, but rather due to down-regulation of photosynthetic capacity by adverse abiotic conditions (Grace and Long 2000).

Therefore, clustering of *nec3* transcriptome with both - abiotically triggered stress response and senescence related response initially seems contradictory and confusing. However, these seemingly different hypotheses, established based on cluster analysis of *nec3* transcriptome, can be merged, if *nec3* is unlikely associated with a particular stress response,

but rather participates in molecular processes, activated during suppression of primary metabolism – either as a consequence of non-optimal growth conditions (as salinity or drought) or senescence. It has previously been shown, that drought can cause metabolic responses similar to natural senescence (Munne-Bosch and Alegre 2004). Similarly to drought, chilling has also previously been associated with senescence related metabolic processes (Masclaux-Daubresse et al. 2007). However, senescence associated metabolism is down-regulated by chilling, allowing to maintain vital metabolic processes during acclimation (Masclaux-Daubresse et al. 2007). Interestingly, although transcriptome analysis revealed a differentially regulated gene set overlapping between 10d *nec3* transcriptome and abiotically stressed barley, majority of these genes were down regulated in response to abiotic stress and up-regulated in *nec3*. This observation might suggest, that similarity between *nec3* and abiotically stressed barley should rather be viewed in light of molecular mechanisms causing early senescence, rather than mechanisms directly mediating abiotic stress response in barley. Thus transcriptome analysis of *nec3* suggests, that *NEC3* is involved in molecular mechanisms controlling barley metabolism during senescence.

Several experiments were performed to test the effect of *nec3* mutation on barley senescence, in order to gain physiological evidence supporting conclusions derived from transcriptome analysis. Plant senescence can be triggered by artificial means – either by subjecting plant to nitrogen starvation or by prolonged incubation in dark triggering carbohydrate starvation. Although the effect of starvation is not identical to naturally occurring senescence, both types of senescence share high degree of similarity (Buchanan-Wollaston et al. 2005). Decline of photosynthetic activity and chlorophyll degradation is one of the early metabolic processes of natural as well as induced senescence (van der Graaff et al. 2006, Breeze et al. 2011), therefore monitoring of *nec3* chlorophyll content in response to prolonged darkness was used to characterize senescence dynamics of the mutant. Decrease in chlorophyll content of *nec3* plants was observed earlier and decline was more rapid than in wild type plants. Similar results were obtained, when *nec3* was subjected to nitrogen starvation – root growth in *nec3* plants was considerably slowed-down by nitrogen deprived conditions, compared to response of wild type plants. These results confirm conclusions derived from *nec3* transcriptome analysis, and suggest increased sensitivity of *nec3* to conditions artificially triggering senescence.

4.2.3. Towards map based cloning of nec3

Although transcript based cloning of *nec3* failed to identify *nec3* candidate gene, conclusions derived from analysis of *nec3* differentially expressed gene set can help in identification of *nec3* by other means. Map-based cloning (MBC) is one of the most often applied approaches of forward genetics. Majority of genes responsible for lesion mimic phenotype development in plants have been identified by means of MBC, e.g. - *mlo* (Büschges et al. 1997), *lsd1* (Dietrich et al. 1997), *dnd1* (Clough et al. 2000), *dnd2* (Jurkowski et al. 2004).

However, despite the wide application, MBC has several major disadvantages. First, fine-scale mapping of the mutation requires large mapping population, to ensure that at least some recombinants between the mutation and closely located markers are obtained. Second,

MBC can be successfully applied only if polymorphic molecular markers, densely covering target region, are available. Due to the low number of polymorphic markers available for the target region *nec3,* mutation was only roughly mapped to a 13 cM region on 6HS chromosome. However, fast development of comparative genomics can help to overcome disadvantages of MBC by using information on syntenic region in related species. Recently a comparison of barley genome with genomes of previously sequenced monocots - rice, *Sorghum* and *Brachypodium* has been published (Mayer et al. 2011). Based on this study, rice chromosome region syntenic to *nec3* target region in barley comprises 55 genes (Mayer et al. 2011).

Based on conclusions, derived from transcriptome analysis of *nec3,* syntenic rice region was screened for genes potentially related to either senescence and cell death or stress response. Screened gene set included serveral genes directly associated with programmed cell death in plants - rice homologue of autophagy gene *AtATG5,* proteasome activating nucleotidase and ubiquitin protein ligase *UPL5*. Previously reported autophagy mutants in *A. thaliana* and rice display early senescence, hypersensitivity to nutrient starvation and to adverse abiotic conditions (Shin et al. 2009, Yoshimoto et al. 2009). As suggested by the Affymetrix Barley GeneChip1 analysis, *nec3* transcriptome comprised up-regulated senescence related genes and shared certain common characteristics with barley under abiotic stress conditions. Moreover, some autophagy related genes have been implicated in restricting uncontrolled spread of HR related cell death (Liu et al. 2005, Patel and Dinesh-Kumar 2008). Another putative senescence related protein Os02g0120600 – is a representative of ubiquitin-protein ligase (UPL) family. Some plant UPLs have previously been associated with senescence related processes, e.g., UPL5 is responsible for senescence associated transcription factor ubiquitination and knock-out *upl5* plants display accelerated senescence (Miao and Zentgraf 2010). Taking into account the role of *ATG5* and *UPLs* in plant senescence, either of these genes could potentially be a *nec3* candidate gene.

nec3 10d differentially expressed gene set shared common characteristics with barley transcriptome under abiotic stress conditions, therefore genes involved in stress response could also be viewed as *nec3* candidate genes. Syntenic rice region contained several stress related genes - homologue of *HSP70, SAP18, cyclophilin 2* and syntaxin protein. *HSP* (heat shock protein) family genes are known to be ivolved in wide range of stress responses in plants, whereas *SAP18* is primary involved in salt stress response, although it might be related also to other stress factors in *A. thaliana* (Song and Galbraith 2006). Cyclophilins ensure proper protein folding and assembly, and mutations impairing certain cyclophilins have been shown to render plants more sensitive to adverse abiotic conditions, such as increased concentration of salts in growth medium or high light conditions (Dominguez-Solis et al. 2008). Syntaxin proteins have been ascribed various functions in plants - some of which also involve stress related responses (Zhu et al. 2002).

Rice gene set syntenic to *nec3* target region also contained gene encoding *protocholorophyllide a reductase*. Several plant necrotic mutants have been shown to comprise mutations in chlorophill synthesis and metabolism related genes. For example, *flu* mutant in *A. thaliana* and *tigrina* mutant in barley over-accumulate protochlorophyllide, resulting in light/dark shift triggered necrotic phenotype (Lee et al. 2003). This observation

suggest, that mutation affecting gene encoding *protochlorophyllide reductase* might trigger cell death in barley and initiate development of *nec3* phenotype.

List of *nec3* candidate genes also included isoprenoid biosynthesis enzyme, since secondary metabolite related pathways were significantly over-represented in *nec3* transcriptome.

Screen of candidate genes, selected based on synteny with *nec3* target region in rice, did not detect any large deletions or mRNA splicing variants in screened genes, suggesting, that these genes are very unlikely candidates for *NEC3*. However, these candidate genes can serve as additional markers for fine-mapping *nec3*. The development of molecular markers for these genes based on resequencing of PCR amplicons from parental varieties is underway.

In conclusion, although applied methods have not yet allowed identification of *nec3* mutation, integrated approach involving transcriptomics, map-based cloning, candidate gene screen and physiological characterization of mutant plants have significantly narrowed down candidate-gene list and will help in focusing on further search of *nec3*.

4.2.4. Future perspectives of nec3 studies

Although transcript based cloning and map-based cloning has not given satisfactory results regarding *NEC3* identification, both methods has not yet completed all options. One of the potential reasons for failure of TBC to identify *NEC3* might be absence of the gene from Affymetrix barley genome array. Several of *NEC3* candidate genes, identified by map - based cloning, were not represented on Affy microarray. This example shows, that incomplete representation of barley genome on Affy microarray might indeed be one of the main reasons for unsuccessful TBC of *NEC3*. Affymetrix barley genome array contains ~22,500 probes while 44K Agilent barley gene expression microarray represents 43,803 probes. The estimated gene number represented on Agilent 44K barley microarray is 38 000, whereas on Affymetrix Barley1 GeneChip – 18 000 (Druka et al. 2010). Therefore, application of alternative barley genome array for hybridization might help in identification of *nec3*. Alternatively, taking into account fast development and increasing availability of next-generation sequencing technologies, *nec3* mutation could be detected by high-throughput sequencing of mRNAs from wt and *nec3* plants.

Map-based cloning of *nec3* was restricted by low polymorphism of target region in parental lines of applied mapping populations. Development of larger mapping population, based on very distinct and well characterized barley lines comprising large number of markers, will allow fine-scale mapping of *nec3*. *nec3* mutation is available in different genetic backgrounds, including barley cultivars widely used for marker development and mapping such as cv. Steptoe. Therefore, development of new *nec3* mapping population, theoretically, could be straight forward.

4.3. Barley homologues of *AtLSD1*

4.3.1. Identification and characterization of barley homologues of AtLSD1

AtLSD1 is one of the central regulators of cell death in *A. thaliana* (Coll et al. 2010). Presence of homologous proteins in diverse plant species, including gymnosperms, mosses, dicots and monocots, suggests important functional role and early evolutionary origin of *LSD1*-like gene family (Liu and Xue 2007). *LSD1* homologues have been reported to control disease resistance of *A. thaliana* (Rustérucci et al.2001), *O. sativa* (Xu and He 2007), *Bambusa oldhamii* (Yeh et al. 2011) and also *H. vulgare* (Spies et al. 2012), suggesting that *LSD1* is a potential target gene for crop stress tolerance improvement. Taking into account the role of *LSD1* in broad spectrum stress tolerance, it was important to identify functional homologue of *AtLSD1* in barley.

Three barley homologues of *Arabidopsis thaliana LSD1* were identified, based on sequence homology searches. All three genes encoded three Zn-finger-*LSD1* domains and showed almost equal 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, *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* homologues from other *Poaceae* species partitioned sequences into three clusters, each comprising homologues from all analyzed grass species, including barley (Figure 40). 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 and Xue 2007).

Monocots and dicots diverged about 140–150 million years ago (Chaw et al. 2004). Since then, *Arabidopsis* 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 lineage leading to barley (Stein et al. 2007). Large proportion (48%) of barley genome is estimated to be generated by major gene duplications in ancestral species (Mayer et al. 2011). 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 may acquire a novel function. Thus gene and genome duplications may complicate identification of functional orthologues in distant taxonomic groups, based on sequence homology alone.

The phylogenetic analysis of more distant *LSD1*-like gene homologues from different monocots gave a deeper insight into the putative origin and divergence time of the three barley homologues. Analogy of rice and barley *LSD1*-like gene phylogeny allowed for

assumption, that the comparison with rice *LSD1*-like genes may suggest putative functional characteristics of studied barley homologues. *LSD1* and *LOL1* genes act as antagonists to regulate cell death in *A. thaliana* (Epple et al. 2003), therefore clear distinction between *LSD1* and *LOL1* homologues might help in assigning putative functions to analyzed barley homologues. According to the phylogenetic analysis *OsLOL2* and *OsLOL3* are the closest rice homologues of *ABC10220* and *ABC06454* respectively, whereas the closest *Arabidopsis* homologue of these genes was *AtLSD1* (Figure 41). Similar discrepancies between *Arabidopsis* and rice homology with barley were also observed in case of *CBC04043*, which clustered with *OsLSD1*, whereas the closest *Arabidopsis* homologue was *AtLOL1*. Based on gene exon-intron organization, distribution of Zn-finger domains and sequence homology *ABC10220* and *ABC06454* were suggested to be closer to *LSD1* than to *LOL1*. However, phylogenetic analysis, grouping rice *LOL* genes with *ABC10220* and *ABC06454* and rice *LSD1* gene with *CBC04043*, questioned previous assumption, that *ABC10220* and *ABC06454* might be functional homologues of *LSD1* rather than *LOL1*.

Thus, discrepancies in clustering of barley homologues with *Arabidopsis* or rice *LSD1*-like genes prevented from an unambiguous designation of the *ABC10220* and *ABC06454* to a certain functional group of *LSD1*-like genes. On one hand, 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 *Arabidopsis thaliana* in response to hypoxia (Mühlenbock et al. 2007). Since growth habits of rice might be associated with higher stress of hypoxia compared to *Arabidopsis* or barley, putative functional divergence of *LSD1*-like genes in these species could be anticipated. Considering the 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. On the other hand, discrepancies in barley *LSD1*-like gene homology with rice and *Arabidopsis* could be caused by the fact, that nomenclature of rice *LSD1* family genes in certain cases has not been based on functional analysis. For example, according to Wang et al. 2005, rice cDNA AY525368 has been designated *OsLSD1* (despite higher sequence homology and gene exon-intron structure similarity to *AtLOL1*), based on the fact, that this protein does not comprise N-terminal glycosylation site and C-terminal phosphorylation site present in AtLOL1. However, Wang et al. 2005 has not taken into account highly conservative C-terminal end sequence of AtLSD1, which is not present in either AtLOL1 or OsLSD1. This sequence is present in OsLOL2 and OsLOL3 as well as in predicted AA sequence of both barley homologues ABC10220 and ABC06454. Besides, *OsLOL2* and *AtLSD1* share common characteristics of resistance to bacterial pathogens - both genes are required to enhance disease resistance in either *O. sativa* or *A. thaliana*. Overexpression of *OsLOL2* in rice and tobacco has been reported to enhance resistance to bacterial pathogens (Xu and He 2007, Bhatti et al. 2008, Bhatti et al. 2011), whereas repression of *AtLSD1* caused increased susceptibility to bacterial pathogens, carrying certain *avr* genes (Rustérucci et al. 2001). In addition, *ABC10220* mapping results showed, that the gene is positioned syntenically to *OsLOL2*, confirming results of phylogenetic analysis. Thus, based on sequence homology, gene exon-intron organization, presence and distribution of highly conserved sequence domains, phylogenetic analysis and mapping positions *ABC10220*

and *ABC06454* are more similar to *LSD1*, than to *LOL1,* despite some discrepancies with gene nomenclature of rice *LSD1* gene family.

Taking into account, that phylogenetic analysis did not allow unambiguous identification of functional homologue of *AtLSD1* in barley, further experiments were needed. *AtLSD1* and *AtLOL1* has antagonistic role in cell death regulation (Epple et al. 2003), therefore it was presumed, that expression pattern of analyzed barley homologues in cell death mutants *nec1* and *nec3* might help to characterize barley *LSD1* homologues. The expression analysis of the identified *LSD1* homologues in barley *lmm* showed, that all three genes have opposite regulation pattern in the *nec1* and *nec3* – genes were either repressed or unaffected in *nec1*, whereas expression was induced or unaltered in *nec3*. Such opposite effect might be explained by distinct nature of cell death mechanisms involved in establishment of necrotic leaf spots in *nec1* and *nec3*. Macroscopically visible cell death occurs in *nec1* at first few weeks after germination, whereas phenotype characteristic for *nec3* establishes only at heading stage at 5-7 weeks after germination. In addition, appearance of leaf spots is very distinct in both mutants – *nec1* comprises small dark brown spots, which rarely coalesce, whereas leaf spots on *nec3* are comparatively larger, light brown or orange and coalesce, especially at later developmental stages. Thus, most likely, different signaling pathways are involved in establishment of leaf spots in *nec1* and *nec3,* which would explain differential regulation of *LSD1* barley homologues in both mutants. The most notable change in gene expression was observed for *CBC04043*. *CBC04043* expression was significantly decreased in the *nec1* genetic background and slightly increased in *nec3* background. In case of *AtLOL1*, reduction of transcript abundance to 25-60% from wild type level was enough, to increase susceptibility to virulent pathogen and alter HR elicitor treatment response (Epple et al. 2003). Therefore, two fold decrease in transcript abundance of *CBC04043* (putative *HvLOL1*) in *nec1* could serve as indication for possible 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. Alternatively, formation of necrotic spots with clear-cut edge in *nec1* suggests strong induction of anti-apoptotic mechanisms in cells surrounding the necrotic spots, which might explain requirement for repression of pro-apoptotic *CBC04043*. Such opposing regulation in cell death is also characteristic for naturally occurring HR – it has been shown, that plants induce autophagy in cells surrounding HR site, to restrict spread of HR related cell death (Liu et al. 2005, Patel and Dinesh-Kumar 2008, Yoshimoto et al. 2009). Therefore repression of *CBC04043* in HR mutant *nec1* does not contradict to suggestion, that *CBC04043* might be functional homologue of pro-apoptotic *AtLOL1*.

Since characterization of expression of analyzed genes in barley necrotic mutants did not give a clear evidence, allowing assigning of *ABC10220* and *ABC06454* to either *LSD1* or *LOL1,* additional experiment was performed. AtLSD1 has been implicated in regulation of response to abiotic conditions, such as unfavorable light conditions (Mateo et al. 2004), hypoxia (Mühlenbock et al. 2007), cold treatment (Huang et al. 2010 b) as well as in regulation of biotic interactions (Aviv et al. 2002). AtLSD1 has been reported to participate in light acclimation of *A. thaliana* (Mateo et al. 2004). *lsd1-1* mutant phenotype is enhanced by long-day high light conditions (Mateo et al. 2004). In addition, *OsLSD1* expression is strongly affected by light/dark cycle – it has been shown, that the gene is induced by light and

repressed by dark (Wang et al. 2005). Therefore, expression of all three barley homologues and also *AtLSD1* and *AtLOL1* was assessed during diurnal cycle. Both – *ABC10220* and *ABC06454* were induced during early night hours and gradually repressed with onset of light. Such expression pattern clearly mimicked that of *AtLSD1*. On contrary to *AtLSD1*, *AtLOL1* reached the highest level of expression during light (with gradual increase in expression starting with onset of light) and the lowest expression at dark period. *CBC04043* expression pattern was nearly identical to *AtLOL1*. Thus, similarly to *OsLSD1, AtLOL1* and *CBC04043* are induced by light and suppressed by dark, suggesting that these genes are likely functional homologues. Similarity between diurnal expression pattern of *AtLSD1* and *ABC10220* and *ABC06454* serves as an addition evidence for functional homology of these genes to *LSD1*.

In conclusion, three barley *LSD1* homologues have been identified. Based on the sequence homology, phylogenetic analysis and expression pattern one of the identified genes, *CBC04043*, can be designated as barley *LOL1*. Sequence and gene expression analyses indicate, that *ABC10220* and *ABC06454* genes represent the best candidates for barley *LSD1*.

4.3.2. Barley TILLING population screening for identification of mutations in ABC10220 and ABC06454

Full sequence information, results of phylogenetic analysis and gene diurnal expression pattern study did not yield unambiguous results regarding the functional barley *LSD1* homologue. Therefore, it was decided to take a reverse genetics approach, to find out, what phenotypical effect would have mutations disrupting either of the studied barley genes. Mutations affecting *AtLSD1* have explicit phenotypical consequences – run-away cell death in response to high-light or long-day growth conditions, as well as increased sensitivity to low temperature treatment and enhanced susceptibility to certain pathogens (Mateo et al. 2004, Epple et al. 2003, Huang et al. 2010 b, Rustérucci et al. 2001). Identification of barley mutants bearing non-functional proteins ABC10220 or ABC06454 would allow assessment of the role of the studied genes in regulation of barley cell death, and, probably, help to identify differences of both genes with regard to barley stress tolerance.

Screening of two barley TILLING populations identified no mis-sense mutations for ABC06454 and only one mis-sense mutation for ABC10220, although the overall mutation frequency was comparable to average mutation density of the screened populations. Proportion of mis-sense mutations, identified in this study, is considerably lower, than the average proportion of mis-sense mutations in analyzed populations. Choice of 1kb region, used for TILLING screening in both genes, was based on information about probability of occurrence of functionally deleterious mutations. Therefore, low proportion of mis-sense mutations among identified mutants is unlikely attributable to unfavorable choice of screening window. Low number of mutations altering amino acid sequence of the studied proteins might be related to functional role of ABC10220 and ABC06454. Mutations of *AtLSD1* so far identified in *A. thaliana* have severe phenotype of run-away cell death, which can considerably lower plant viability at certain environmental conditions. Similarly, only mutations weakly affecting *AtLOL1* expression have been identified so far, and failure to identify mis-sense mutations in screening of TILLING population for *AtLOL1* (Epple et al. 2003), suggests that *AtLOL1* mutations, significantly affecting gene functioning might be

lethal. Taking into account phenotypical severity of *AtLSD1* mutations and the fact, that TILLING procedure usually involves high background frequency of mutations, it might be expected, that mutations affecting functional barley homologue of *LSD1*, would significantly affect plant viability, thus reducing chance of maintenance of such mutants in TILLING population.

The only miss-sense mutation identified in this study G163E in ABC10220 is predicted to alter highly conserved C-terminal end of the protein. Identified mutation did not alter *ABC10220* expression and plants comprising mutation G163E did not exhibit any visible phenotype, when grown at optimal growth conditions. Despite high sequence conservation of C-terminal end of LSD1 (and also ABC10220 and ABC06454), no protein functional domain corresponds to this sequence, making it difficult to predict functional effect of G163E mutation. AtLSD1 is known to form dimers (Walter et al. 2004) and to interact also with other proteins - LOL1 (Epple et al. 2003), AtMC1 (Coll et al. 2010) and GILP (He et al. 2011). Although none of these interactions have so far been shown to require C-terminal end of LSD1, it cannot be excluded that C-terminal end sequence is required for binding of other, yet undiscovered LSD1 interacting protein. *In silico* analysis of predicted amino acid sequence of G163E mutant revealed, that the mutation might disrupt low complexity region, located in the C-terminal end of the protein. It has previously been reported, that low complexity regions can be required for proper functioning of the protein, ensuring formation of stable structure upon binding to the protein target (Dyson and Wright 2005), therefore identified mutation , affecting conservative C-terminal end, might be functionally important for ABC10220. The identified mis-sense mutation G163E in ABC10220 is located close to the position of AtLSD1 P167L mutation (mutant *chs4*-3) (Huang et al. 2010 b). According to Huang et al. 2010 b, *chs4-3* also cannot be distinguished from wt plants at optimal growth conditions, whereas low temperature treatment triggers run-away cell death in *A. thaliana* mutant. Preliminary observations suggest, that G163E mutation of ABC10220 does not trigger increased barley sensitivity to low temperature treatment. However, taking into account that TILLING mutants usually comprise large number of background mutations, any phenotypical characterization at early generations can be biased. Therefore, these observations have to be experimentally confirmed after series of backcrossing allowing elimination of background mutations.

4.3.3. Complementation of A. thaliana lsd1-1 mutant with barley LSD1 homologues ABC10220 and ABC06454

Due to available molecular tools, full genome sequence and significant amount of scientific knowledge accumulated, *A. thaliana* is usually the first choice plant for new gene discovery and characterization. Therefore, gene discovery in other plant species can benefit from information, gained from *A. thaliana* studies. Complementation of *A. thaliana* mutants with homologous genes from other species can be applied, to functionally characterize newly discovered plant genes from less studied species.

Difficulties in identification of *ABC06454* barley TILLING mutants precluded use of mutant barley phenotype comparison, to distinguish between function of *ABC10220* and *ABC06454*. Therefore, it was decided to use *A. thaliana lsd1-1* complementation with

ABC10220 and *ABC06454,* to see if either of barley homologues is able to substitute *AtLSD1* and thereby rescue *lsd1-1*. ABC10220 and ABC06454 shares 54% and 55% AA identity with AtLSD1. AA sequence identity as low as 50% has been sufficient for partial complementation of *A. thaliana* mutant *ein3,* using orthologous rice gene *OsEIL1* (Mao et al. 2006). Similarly, only 48% AA identity between maize gene *Si1* and *A. thaliana AP3* ensured partial complementation of *A. thaliana ap3* mutant (Whipple et al. 2004). Thus, high sequence homology is not crucial, although successful complementation may require higher transgene expression, compared to native wt gene (Whipple et al. 2004). Hence, comparatively low AA identity between AtLSD1 and corresponding barley homologues, theoretically, could be sufficient for *lsd1-1* complementation.

Promoters ensuring strong transcription, such as CaMV p35S for dicots (Cauliflower mosaic virus 35S promoter) (Odell et al. 1985) and ZmUbi1 (ubiquitin gene promoter from *Zea mays*) (Christensen et al. 1992) for monocots, are often used to drive high level of transgene expression. However, high transgene expression by p35S can also have undesirable effects, as it may increase chance of transgene DNA rearrangements (Porsch et al. 1998), likely caused by recombination hotspot present in minimal CaMV35S promoter (Kohli et al. 1999). To avoid negative side effects, associated with application of CaMVp35S, native *AtLSD1* promoter was used for *ABC10220* and *ABC06454* expression in *lsd1*-1. In order to achieve higher transgene expression, 5'UTR region of *A. thaliana LSD1* was placed before start codon of *ABC10220* and *ABC06454.* Positive effect of 5'UTR introns on gene expression (so called intron mediated enhancement) has been reported in dicots as well as in monocots, and is likely attributed to sequence signals, conserved among wide range of species (Parra et al. 2011). Despite precautions taken in construct design (use of native promoter and inclusion of 5'UTR), transgene expression in *lsd1-1* was achieved only for *ABC06454* and not for *ABC10220*. There are several factors, which might explain failure to detect *ABC10220* mRNA: 1) transcriptional gene silencing due to epigenetic modifications at promoter region, 2) posttranscriptional transgene silencing due to instability and accelerated turnover of *ABC10220* mRNA, 3) transgene insertion site specific silencing.

So called position effect (difference in transgene expression, caused by differences in flanking DNA or chromosomal integration site) in early transgene silencing studies has been viewed as one of the main factors potentially affecting transgene expression (Matzke and Matzke 1998). However, more recent studies have revealed, that neither the chromosomal location of insert nor epigenetic regulation of flanking DNA does not comprise a crucial role in transgene expression (De Buck et al. 2004, Schubert et al. 2004). *lsd1-1* transformation yielded four independent transgenic lines harboring *ABC10220,* all of which failed to produce expression of the barley gene. Repression of transgene expression in independent transgenic lines also questions validity of assumption about insertion site effect on transgene expression. Besides, expression of marker gene *BAR* was strong in all transformants, ruling out possibility of insertion site specific transgene expression repression in case of *ABC10220*.

Homology dependent transgene silencing, involving either transcriptional (TGS) or posttranscriptional silencing (PTGS) is a common phenomenon in plants (Kooter et al. 1999). TGS and PTGS can be triggered by homology between promoter sequence or coding part of transgene respectively and any other native sequence (Kooter et al. 1999). Although it was previously thought, that TGS and PTGS are regulated by distinct molecular mechanisms –

TGS involving epigenetic modifications (methylation and changes in chromatin configuration) of promoter sequence and PTGS involving siRNA dependent mechanisms - lately it is becoming evident that both - PTGS as well as TGS involve siRNA regulation (Mlotshwa et al. 2008, Mlotshwa et al. 2010). HDGS in plants has been shown to be affected by environmental factors, the most important being light (Kotakis et al. 2010) and temperature (Meza et al. 2001). Homology dependent PTGS might explain failure to detect *ABC10220* expression in transgenic plants. Both constructs (either *ABC10220* or *ABC06454* harboring) were based on the same binary vector pMOA36 and both contained *AtLSD1* native promoter and 5'UTR, the only difference between constructs being barley gene cDNA. Therefore, it might be expected that differences in transgene expression might be attributed to intrinsic characteristics of *ABC10220* coding sequence.

On contrary to *ABC10220, ABC06454* was successfully expressed in *lsd1-1*. *ABC06454* was not able to complement run-away cell death phenotype of *lsd1-1* – transgenic plants were equally sensitive to long day light conditions and to exogenously applied SA. Therefore, *ABC06454* is very unlikely to be functional barley *LSD1,* despite sequence homology to *AtLSD1*. Although methodological constraints precluded assessment of *ABC10220* ability to complement *lsd1-1*, failure of *ABC06454* to complement *lsd1-1* does not exclude possibility, that *ABC10220* is the functional *HvLSD1*. Such hypothesis requires assumption, that *ABC10220* and *ABC06454* are functionally divergent. Functional divergence of *ABC10220* and *ABC06454* is confirmed by the fact, that silencing of *ABC1022*0 in barley significantly affects basal *Bgh* resistance (Schweizer 2012), whereas *ABC06454* is estimated to have only minor effect on *Bgh* resistance (Spies et al. 2012). Functional divergence of closely homologous genes is a common fate of genes, developed by gene duplication. For example, expression analysis of sorghum genes, involved in resistance response to *Bipolaris sorghicola*, revealed differential expression of duplicated genes, suggesting divergent function of close homologues (Mizuno et al. 2012). Similarly, two barley paralogues *Nbs1-Rdga2a* and *Nbs2-Rdga2a*, comprising high sequence homology, conferred differential resistance to hemi-biotrophic *Pyrenophora graminea*, when transformed into susceptible barley cultivar (Bulgarelli et al. 2010). It has been proposed, that MITE insertion in promoter region of *Nbs2-Rdga2* has contributed to functional diversification of both paralogues (Bulgarelli et al. 2010). Retrotansposon triggered divergence has also been observed in rice gene *Pit,* conferring resistance to rice blast fungus (Hayashi and Yoshida 2009). Interestingly, *ABC10220* and *ABC06454* sequence and gene exon-intron organization is highly homologous, except that *ABC06454* comprises additional MITE insertion between 2st and 3nd exon (Figure 39). Therefore, *ABC10220* and *ABC06454* could potentially be closely related, but functionally divergent genes, that likely arised from gene duplication and subsequent diversification possibly triggered by MITE insertion event.

4.3.4. Future perspectives of HvLSD1 homologue studies

It was expected, that either phylogenetic analysis, TILLING screening or complementation tests using *A. thaliana lsd1-1* mutant would reveal functional barley homologue of *LSD1*. Phylogenetic analysis and expression pattern characterization revealed, that *CBC04043* is more related to *AtLOL1* and therefore can be excluded from the barley

LSD1 candidate gene list. *ABC06454* did not complement *A. thaliana* mutant *lsd1-1* and therefore is unlikely to be the functional barley *LSD1*. However, neither of these experiments yielded unequivocal and convincing results regarding *ABC10220.* Therefore, it is important to take different methodological approach, to test, whether *ABC10220* is barley *LSD1*. Characterization of ABC10220 protein-protein interactions would reveal, if ABC10220 in barley functions similarly to LSD1 in *A. thaliana*. However, this approach would require prior knowledge of barley homologues of AtZIP10, AtMC1, AtLOL1 and AtGILP - *A. thaliana* LSD1 interacting proteins. None of these proteins have previously been characterized in barley, therefore such approach would be challenging and might yield unexpected and unequivocal results. Alternatively, it might be expected, that functional barley LSD1 is able to interact with *A. thaliana* LSD1 interactors. Since *ABC10220* expression in *A. thaliana* appears to be difficult to achieve, application of methods based on characterization of protein-protein interactions *in vivo*, within *A. thaliana* plant (as plant cell based co-immunoprecipitation or co-localization with bimolecular fluorescence complementation (BiFC)) might be challenging. To avoid problems related to *ABC10220* expression in *A. thaliana*, ABC10220 protein-protein interactions might be characterized using yeast two hybrid system or yeast-based co-immunoprecipitation. Yeast does not comprise *LSD1* homologues, therefore it is unlikely, that results might be biased due to interference with native yeast proteins. Yeast two hybrid system has successfully been applied to characterize AtLSD1 interaction with AtMC1 (Coll et al. 2010), which proves suitability of this method for LSD1 type protein interaction studies.

Recent studies of Dr. Patric Schweizer research group at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) (Germany) have revealed, that *ABC10220* might be required for barley susceptibility to powdery mildew. This discovery means, that mutations impairing *ABC10220* might improve basal resistance of barley to powdery mildew. Artificial silencing of *ABC10220* using RNAi technology is used by Dr. Patric Schweizer to proof the role of *ABC10220* in barley response to powdery mildew. Although *ABC10220* down-regulation mutants obtained by RNAi method are useful for scientific purposes, due to restrictions imposed on GMOs it is unlikely that these mutants could be used as breeding material, to improve disease resistance of barley cultivars. In contrast, there are no legal restrictions, precluding use of *ABC10220* mutant Tillmore2097 described in this study, for barley breeding for conventional farming. Therefore, it is planned to assess the effect of G163E mutation of *ABC10220* on barley basal resistance to powdery mildew, after backcrossing eliminating background mutations is accomplished.

In addition, differential powdery mildew resistance of highly homologous *ABC10220* and *ABC06454* offers opportunity to use these two genes as a model for studies investigating functional divergence of resistance related genes. As proposed in the previous chapter, MITE insertion could have contributed to functional divergence of *ABC10220* and *ABC06454* rendering both genes an interesting example also for studies considering retrotansposon role in plant gene evolution. Characterization of natural variation in both loci or use of chimeric vector constructs, comprising different combinations of *ABC10220* and *ABC06454* fragments, to complement mutant plants could reveal sequence characteristics underlying functional divergence of both genes.

This study has revealed only several aspects of barley homologues of *AtLSD1*, however there are still numerous questions remaining to be answered. Sequencing of *ABC10220* and *ABC06454* has revealed, that both genes comprise highly conserved sequence at C-terminal end. Sequence alignment of analyzed barley genes with *LSD1* homologues from other plant species representing wide range of genera has confirmed high sequence conservation of C-terminal sequence across plant kingdom. C-terminal end sequence is not recognized as previously characterized protein functional domain in pfam database, however highly conservative nature of the sequence suggests, that it might be functionally essential for LSD1. Interestingly, presence of C-terminal end sequence distinguishes anti-apoptotic LSD1 from related pro-apoptotic protein LOL1. Previously characterized LSD1 interactions with other proteins have been reported to require Zn-finger domains positioned at N-terminal half of protein, however the functional role of C-terminal end remains unknown. Unfortunately, crystal-structure of LSD1 protein has not yet been reported precluding *in silico* predictions of functional role of C-terminal end. Taking into account highly conservative nature of LSD1 C-terminal end, functional characterization of this sequence might yield information of molecular mechanisms allowing LSD1 function as anti-apoptotic protein.

5. Conclusions

1. *nec1* mutation in barley triggers induction of systemic acquired resistance related markers and differentially affects barley disease resistance.

2. *nec1* mutation alters barley response to exogenously applied auxin and affects endogenous auxin concentration in barley.

3. *nec3* mutation triggers early senescence in barley as assessed by molecular and physiological characteristics of the mutant.

4. Combined application of forward genetics approach of map-based cloning and transcriptome analysis significantly narrows down candidate gene list and therefore facilitates identification of mutation underlying *nec3* lesion mimic phenotype.

5. *ABC10220* is the most likely candidate gene for barley *LSD1*.

6. TILLING is efficient tool for identification of mutations in barley genes, related to hypersensitive response and disease resistance.

6. Theses for defense

Application of barley lesion mimic mutants as a model system for identification and characterization of molecular components of barley immunity is a valid approach allowing for discovery of previously unknown aspects of barley disease resistance.

Successful identification and characterization of HR-related genes in barley requires integrated approach involving application of forward (positional cloning, transcript-based cloning) and reverse (TILLING population screening, complementation of model-organism *A. thaliana*) genetics as well as physiology and plant pathology – based experimental methods.

Although information gained from studies of *A. thaliana* can be useful to set the initial experimental framework for barley research highlighting most relevant scientific targets and questions, direct application of *A. thaliana*-based knowledge to barley should be performed with certain precaution.

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Approbation of results

Publications

Keisa A., Nakurte I., Kunga L., Kale L., Rostoks N. 2012. Increased auxin content and altered auxin response in barley necrotic mutant *nec1*. In: Guoping Z., Chengdao L., Xu L. (eds.) *Advance in barley sciences*. Springer and Zheijiang University press, pp 140–148.

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Oral presentation

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