Barley *Nec1* gene is a homologue of the *Arabidopsis Hlm1* encoding the cyclic nucleotide-gated ion channel 4

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SUMMARY
Plant necrotic or disease lesion mimic mutants belong to a class of phenotypic mutants that express hypersensitive response (HR), a form of programmed cell death, in absence of infection. Necrotic mutants may have deficient components of plant defence pathways and are therefore important for understanding disease resistance mechanisms. A number of genes conferring plant necrotic phenotype are known and several of them have been cloned. Here we describe cloning of the first barley necrotic mutant, *nec1,* based on homology to the *Arabidopsis* mutant *hlm1*. The barley *Nec1* encodes cyclic nucleotide-gated ion channel 4, a putative ortholog of Arabidopsis Hlm1. Four different barley *nec1* alleles were characterized. Two novel fast neutron (FN) induced mutants had extensive deletions in the gene. Two previously described *nec1* alleles had either a STOP codon in exon 1 (*nec1a* in Carlsberg II) or a MITE insertion in the intron 2 (*nec1* in Parkland) which caused alternative splicing resulting in a frameshift and production of predicted non-functional protein. The MITE insertion is consistent with the reported spontaneous origin of the *nec1* Parkland allele. The expression of two pathogenesis related (PR) genes, *HvPR-1a* and β-1,3-glucanase gene, was elevated in the two FN necrotic lines.

BARLEY NECROTIC MUTANTS
Three *nec1* alleles have been reported previously:
1. *nec1a* (GSHO989) - diethyl sulfate and gamma ray induced mutation in Carlsberg II (Jensen 1971);
2. *nec1c* (GSHO1284) - spontaneous mutation in Parkland (Fedak et al. 1972).
3. Possible *nec1* diethyl sulfate-induced mutant in Morex (CIho 15773) reported by Ramage and Eckhoff (1985). Seed of this mutant was not available.
nec1c was backcrossed to Bowman to create genetic stock in a uniform genetic background - GSHO2052 (Franckowiak et al. 2000)
Several new fast neutron-induced (FN) necrotic mutants have been identified in Steptoe and Morex (Rostoks et al. 2003). We show that two of these lines, FN085 (Steptoe background) and FN338 (Morex background) are allelic to the *nec1*.

BARLEY HOMOLOGUE OF THE *ARABIDOPSIS Hlm1* IS *Nec1*
Blast searches were used to isolate barley ESTs homologous to the *Arabidopsis* necrotic mutants *hlm1* (Balague et al. 2003) and *dnd1* (Clough et al. 2000). Barley ESTs were then used as probes in Southern blot analysis of the panel of barley necrotic mutants (Rostoks et al. 2003).

Hybridization pattern of the barley EST BF630384 (cDNA insert sequence AY273923, matching HarvEST contig 26333) with the highest homology to *Hlm1* showed that the major RFLP band was deleted in the two of the necrotic lines suggesting that deletion of the *Hlm1* homologue was responsible for the necrotic phenotype in barley .

Linkage mapping in the Steptoe x Morex mapping population was performed by RFLP using EST BF630384 as a probe which was shown to be linked to the previously mapped barley *nec1* mutant phenotype.

F1 and F2 progeny of the test cross of the FN338 and *nec1a* allele in Carlsberg II showed necrotic phenotype and confirmed that both mutations are allelic.

CLONING OF THE BARLEY *Nec1*
EST BF630384 was used to probe the Morex BAC library by Southern blot analysis. A positive BAC clone 746H18 was identified and shotgun-subcloned in a pUC19/*Bam*HI vector. A 10 kb clone NRG098 was identified as homologous to the EST BF630384 and completely sequenced.
The structure of the *Nec1* gene was determined by comparison to the cDNA AY273923, RT-PCR and 5' RACE.

SEQUENCE ANALYSIS OF THE *NEC1*ALLELES
A set of PCR primers was designed to cover the complete coding sequence of the *Nec1* gene and the gene was amplified from all the available *nec1* alleles and Bowman backcross.
Only two primer combinations amplified discrete fragments from the two FN alleles confirming extensive deletion / rearrangement within the gene.
nec1a allele in Carlsberg II showed a non-sense mutation in exon 1 that would cause truncated protein product.
nec1c allele in Parkland showed an insertion in the intron between exons 2 and 3. Sequence inspection suggested that the insertion represented a novel miniature inverted-repeat transposable element (MITE). The insertion did not directly change the coding sequence, however, it affected the splicing of the mutant gene causing insertion of extraneous amino acids, a frameshift and a truncated predicted protein. The insertion of the transposable element is consistent with the reported spontaneous origin of the mutant in Parkland (Fedak et al. 1972).
The Bowman backcross line contained the *nec1c* allele from cv. Parkland.

ELEVATED EXPRESSION OF PR GENES IN NECROTIC MUTANTS
Arabidopsis hlm1 mutant, in addition to necrotic phenotype, also displayed increased resistance to several strains of *Pseudomonas syringae* pv. *tomato* and enhanced expression of pathogenesis-related protein genes (Balague et al. 2003). The FN mutants were tested for reaction to the stem rust *Puccinia graminis* f.sp. *tritici* pathotype MCC. In both cases the mutants behaved the same as the parents, i.e., FN085 in the susceptible cv. Steptoe background was susceptible and FN338 in the resistant cv. Morex background was resistant. The expression of the barley *HvPR-1a* (GenBank X74939) and β-1,3-glucanase (GenBank AY239039) genes was quantified in the two FN mutant lines and parent cultivars using real-time PCR. *HvPR-1a* has been previously shown to be induced by powdery mildew infection in barley and *Puccinia graminis* f.sp. *avenae* infection in oat. Strong induction of both genes was found compared to the parent cultivars. Interestingly, Steptoe expressed both genes at higher level than Morex.

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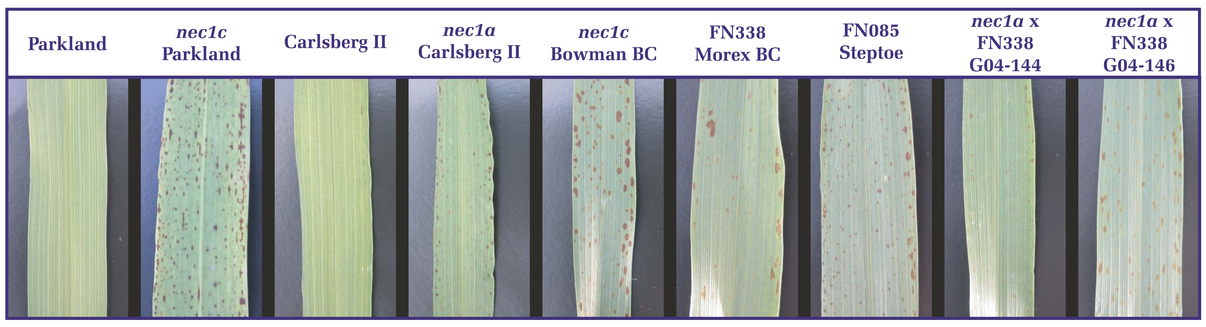
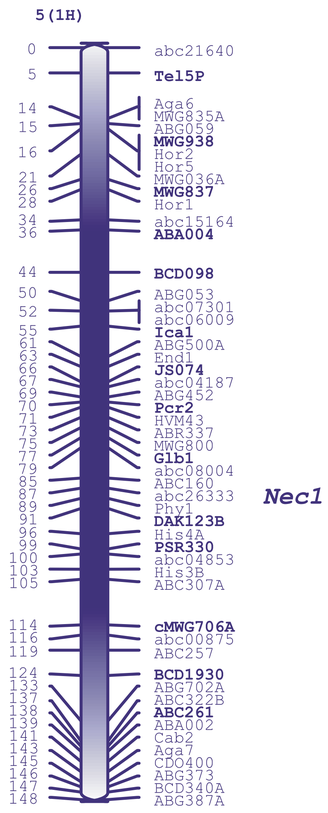
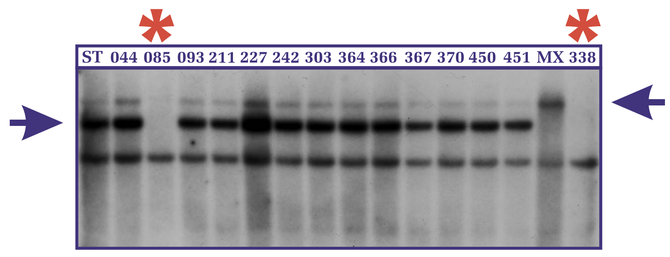
Phenotype of the different *nec1* alleles, parents and progeny of the allelism test of *nec1a* and FN338



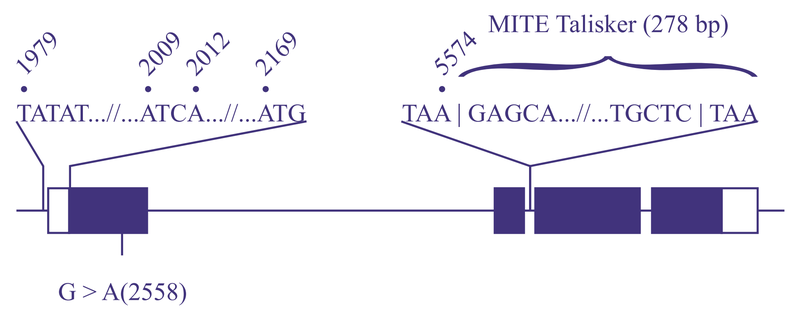
Genetic mapping of the barley homologue of the *Arabidopsis Hlm1* (HarvEST contig 26333) to the chromosome 5(1H) *nec1* region



Barley homologue of the *Arabidopsis Hlm1* is deleted in necrotic lines FN085 and FN338. Arrows indicate the RFLP band that disappeared in the mutant lines. The minor bands are caused by cross-hybridization to other members of the barley cyclic nucleotide-gated ion channel gene family.



Structure of the barley *Nec1* gene and the encoded cyclic nucleotide-gated ion channel. Exons are shown as solid squares, UTRs as open squares. Non-sense mutation in the exon 1 in the *nec1a* allele and MITE insertion in the intron between exons 2 and 3 in the *nec1c* allele are indicated. Putative TATA box, 2 transcription start sites and the ATG are shown at the start of gene.
Predicted structure of the Nec1 polypeptide with 6 transmembrane domains (S1-S6), cyclic nucleotide binding domain (CNB) and calmodulin binding domain (CaMB).



Expression of two pathogenesis-related (PR) genes in barley *nec1*mutants.
The expression levels were quantified using SYBR--Green real-time PCR and mRNA abundance was expressed as percentage of the housekeeping reference gene *GAPDH*. Vertical bars indicate standard error.
Expression of both *HvPR-1a* and β-1,3-glucanase genes was elevated in mutant lines compared to parent. FN085 - *nec1* allele in Steptoe background; FN338 - *nec1* allele in Morex background.

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