Molecular characterisation of differentially expressed genes in the interaction of barley and *Rhynchosporium secalis*

Submitted by

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chapter 5: Characterisation of d1057 clone

5.1 Introduction

The outermost cellular layer of plant organs is the epidermis, which is covered by a cuticle layer. It plays a dual role as a medium for the regulation of epidermal permeability as well as plant protection from the environment and other organisms. The cuticle prevents uncontrolled water loss (or uptake) through the epidermis (Popp *et al.*, 2005; Riederer and Schreiber, 2001), provides protection of the photosynthetic tissues from excess light by reflection (Brodersen *et al.*, 2007; Brodersen and Vogelmann, 2007), protects plants against herbivores (Eigenbrode *et al.*, 1996), pathogens (Gniwotta *et al.*, 2005; Jenks *et al.*, 1994; Kerstiens, 1996), UV radiation (Barnes *et al.*, 1996; Gonzalez *et al.*, 1996; Long *et al.*, 2003) mechanical damage (Baker and Hunt, 1986) and have important roles in normal plant developmental processes (Bird and Gray, 2003; Yephremov *et al.*, 1999). The outer walls of epidermal cells are thicker than other walls and its cuticle layer comprises cutin and waxes.

The cuticle of mature organs consists of a cuticle proper (Kolattukudy, 1981) that is embedded in intracuticular waxes and an outermost layer formed by epicuticular waxes. The intracuticular wax is defined as the amorphous mixture of lipids embedded in the cutin, which links the cuticle with the cell wall matrix, and epicuticular wax refers to the surface lipids forming the crystalloids or smooth film on the cuticle exterior (Post-Beittenmiller, 1996). Cuticular wax is commonly composed of homologous series of very-long-chain aliphatic molecules such as aldehydes, alkanes, alkanoic acids, alkanols, ketones and esters as well as cyclic compounds such as triterpenoids, sterols, flavonoids and phenylpropanoids (Kunst and Samuels, 2003; Post-Beittenmiller, 1996) with predominant chain lengths from 20 to 34 carbons. The precursors of wax biosynthesis are fatty acids that are derived from de novo synthesis in plastids and are discussed in the following sections.

Aliphatic components of cuticular wax are synthesized in the epidermal cells by fatty acid elongase (FAE) from C_{16} and C_{18} fatty acids, resulting in very long chain fatty acids (VLCFA) ranging from C_{20} to C_{36} in length. Synthesis of VLCFA wax precursors is a complex process that takes place in two stages in different cellular compartments. The first stage, the de novo fatty acid synthesis of C_{16} and C_{18} acyl chains, occurs in the stroma of

plastids by soluble enzymes forming the fatty acid synthase complex (FAS) (Ohlrogge and Browse, 1995). The long chain fatty acids (C_{16}-C_{18}) are released by the action of the acyl-ACP thioesterases, exported to the cytoplasm, and conjugate to CoenzymeA. Acyl-CoAs then are channelled to different pathways for wax and cutin monomer biosynthesis (Kunst and Samuels, 2005). The second stage of fatty acid elongation occurs in epidermal cells and involves the extension of the ubiquitous C_{16} and C_{18} fatty acids to VLCFA chains by membrane-bound FAE (von Wettstein-Knowles, 1982). Each FAE includes the condensing enzyme β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase, β(R)-hydroxyacyl-CoA dehydrase and (E)-2,3-enoyl-CoA reductase (Millar and Kunst, 1997). Two-carbon extension of the chain takes place in a series of four consecutive enzymatic reactions. CoA-esterified fatty acyl substrate is condensed with malonyl-CoA, followed by a β-keto reduction, dehydration and a final enoyl reduction (Fehling and Mukherjee, 1991). Chain lengths of aliphatic wax components are in the range of 20–34 carbons indicating multiple rounds of elongation. Sequential acyl chain extensions are catalysed by distinct elongases, each having unique substrate chain length specificities. Rate limiting step of the complex is KCS as increasing the level of a condensing enzyme component in transgenic plants results in higher levels of VLCFAs product (Millar and Kunst, 1997). The condensing enzyme is also believed to regulate the spatial distribution of VLCFA production in the plant (Millar *et al.*, 1998). Therefore, expression pattern of KCS genes not only determines where VLCFAs are produced, but also quantity and length of VLCFAs produced (Millar and Kunst, 1997). Thus, the expression of the KCS gene family is determines the composition of the cuticular waxes.

VLCFAs in the epidermal cells are converted through three biosynthetic pathways to all of the aliphatic wax components (von Wettstein-Knowles, 1982). These systems include the decarbonylation, acyl-reduction and β-ketoacyl-elongation, which are found in the epidermal tissue of most plants as distinct and parallel pathways. Each parallel pathway includes sequential elongation reactions that terminate with chain modifications or with the release of free elongated fatty acids. A summarised pathway for wax biosynthesis in plants is depicted in Figure 5.1.

Figure 5.1 Plant wax biosynthetic pathways. C_{16} and C_{18} fatty acids produced in plastids are utilised as precursors in biosynthesis of very long chain fatty acids. Fatty acids with chain length larger than C_{18} can enter one of the three wax biosynthesis pathways and result in biosynthesis of different components of epidermal wax (adopted from Post-Beittenmiller, 1996).

One of the roles of the cuticle is protection of the plants from other organisms by providing a physical barrier between the pathogen and host cell (Jenks *et al.*, 1994). Moreover, the chemical composition of epicuticular leaf waxes influences pre-penetration processes of fungi (Hwang and Kolattukudy, 1995; Niks and Rubiales, 2002; Rubiales and Niks, 1996; Tsuba *et al.*, 2002). For example, cuticle components of barley coleoptiles are needed for fungal penetration and protection of the appressoria against excessive surface moisture during attempted penetration (Iwamoto *et al.*, 2002). The long-chain alcohol fraction of avocado surface wax selectively induces germination and appressorium formation (AF) in *Colletotrichum gloeosporioides* (Podila *et al.*, 1993). Rice surface wax relieves self-inhibition of germination and AF by the conidia of *M. grisea* (Hegde and Kolattukudy, 1997). A study in barley (Tsuba *et al.*, 2002) showed that the epidermal surface and the wax of barley leaves induced high level of appressorium differentiation (AD) of *Blumeria graminis* mainly due to the C_{26} aldehyde fraction. In addition, chemically synthesized C_{26} aldehyde showed the potential to induce AD of *B. graminis* strongly. On the other hand, in wax of cabbage (a non-host plant), the amount of aldehydes was greater than that in wax of barley with the main aldehydes being of C_{28} and C_{30} chain lengths and less AD was observed. The structure of waxes also affects host-pathogen interaction. The success of *Erysiphe pisi* in infesting pea plants depends on the physical structure and the chemical composition of the epicuticular wax layer. Gniwotta and colleagues (2005) found that on adaxial surfaces of pea 80% of *Erysiphe pisi* spores germinated and 70% differentiated appressoria. In contrast, only 57% germination and 49% AF rates were found for abaxial surfaces. A subsequent study of the leaf revealed that wax crystals on the adaxial leaf surface were different from wax crystals on the abaxial surface.

In addition to developmental controls, cuticular lipids are synthesized in response to environmental signals such as humidity (Sutter, 1984), chilling (Nordby and McDonald, 1991), seasonal variation (Gulz and Muller, 1992), heat and salinity. Experiments in maize and oat have shown that wax deposition is often a response to water stress that occurs within a few days (Bengtson *et al.*, 1978; Premachandra *et al.*, 1991). Carnation plants grown *in vitro* in high humidity have decreased wax load relative to plants grown at lower humidity (Majada *et al.*, 2001). Under different environmental conditions, the ratio of different wax components can also vary without affecting the overall wax load. Kale and

swede plants grown indoors have a greater proportion of long chain esters in their epicuticular waxes, than similar plants grown outdoors (Shepherd *et al.*, 1995; Shepherd *et al.*, 1997). Plants exposed to higher temperatures during different periods of their light/dark cycle, also show differences in their wax composition, depending on when the plant is exposed to higher temperatures (Riederer and Schneider, 1990). Wax crystal morphology also changes in response to environmental conditions, such as water stress (Prior *et al.*, 1997). Plants such as peanut (*Arachis hypogaea*) and salt-sensitive jojoba deposit more wax upon exposure to salinity stress (Mills *et al.*, 2001; Rao *et al.*, 1981). This response appears to be primarily to water deficit caused by salinity. Wax deposition on leaves of salt-sensitive jojoba is also induced by application of exogenous abscisic acid (Mills *et al.*, 2001) a known plant growth regulator involved in drought stress response. The increase in wax loading caused by exposure of cabbage seedlings to moderate NaCl-induced water stress has been commercially exploited to improve drought resistance in transplanted plants and is a useful hardening technique (Fujiwara *et al.*, 2002). Exposure of plants to cold also affects wax layer. For instance, in maize a 7-day-long cold spell lowered cuticular wax quantity on the third leaf of four-leaf plants by 29%, resulting in increased wettability and increased herbicide retention (Gauvrit and Gaillardon, 1991).

Biosynthetic pathways and the role of VLCFAs in plants are well documented. However, genes encoding enzymes in FAE systems in non-model plants such as barley have not been described. Isolation of the full-length gene and analysis of the protein encoded by clone d1057 revealed its similarity to very long chain fatty acid elongases from other species and the characterised Arabidopsis *Fiddlehead*. In this chapter, molecular cloning of the barley *Fiddlehead* (*Fdh*) gene, functional analysis and its role in both abiotic and biotic stress tolerance is reported.

5.2 Materials and Methods

In this section materials and methods specifically used for the characterisation of barley *Fdh* is given. The general material and methods were described in Chapter 2.

5.2.1 Isolation of barley *Fiddlehead* cDNA and genomic DNA

EST databases were queried with d1057 clone sequence for overlapping ESTs to form a contagious sequence (Section 2.31). 3'- and 5'-RACE was performed by using primers designed based on contig sequence (Section 2.12). The sequences of RACE products were used to amend and extend the contig. Then, full length of the cDNA was amplified from Atlas 46 5'-RACE ready cDNA by *PfuUltra*HF and primer pair d1057F6/R following CP 14. Corresponding genomic region was amplified from Atlas 46 genomic DNA in a similar reaction to the full-length cDNA by CP16. The PCR products were cloned into pGEM-T Easy vector and sequenced.

5.2.2 Construction of vectors for expression in Arabidopsis

Two vectors were constructed for expression in Arabidopsis using Gateway multisite LR recombination. As a first step, two entry clones were produced. Entry clone pENTR5'-TOPOAtFdhP was assembled by ligating Arabidopsis *Fdh* promoter to vector pENTR5'-TOPO. The promoter region of *AtFdh* (-16 to -667 nucleotides) was amplified from Arabidopsis ecotype Columbia-0 genomic DNA by using *PfuUltra*HF polymerase and primer pair AtFdhF/R with CP17. To produce entry clone pENTR-DTOPO-HvFdh, the *HvFdh* (d1057) coding region was amplified by *PfuUltra*HF polymerase and d1057F14/R14 primer pair and cloned into pENTR-DTOPO. CP 18 was used for cycling. Ligations were performed according to manufacturer's instructions (Invitrogen). The orientation and accuracy of the inserts were confirmed by sequencing.

At the second step, expression vector pAtFdh::HvFdh (Appendix G) was assembled in a Gateway multisite LR reaction according to manufacturer's recommendations (Invitrogen) by recombining of a binary vector (pTOOL6), pENTR5'-TOPOAtFdh and pENTR-DTOPO-HvFdh vectors. A similar reaction was performed to recombine plasmid pPCR8GFP (obtained from Dr Andrew Jacob, ACPFG) with pENTR5'-TOPO-AtFdhP

and destination vector pTOOL6 to assemble expression vector pAtFdh::GFP. In the resultant vectors *HvFdh* ORF and *GFP* expression, respectively, is driven by the *AtFdh* promoter. The accuracy of recombination was checked by restriction digest of resultant vectors.

5.2.3 Transient transformation of pAtFdh::GFP

The vector pAtFdh::GFP was transformed into detached Arabidopsis leaves by bombardment (Section 2.24) and expression of GFP was studied under a fluorescent microscope (Section 2.25).

5.2.4 PCR based genotyping of *AtFdh* alleles

Extract-N-Amp Plant PCR kit (Sigma) was used for extraction of genomic DNA from Arabidopsis according to manufacturer's instructions. The genotype of segregating Arabidopsis *fiddlehead* mutant population and transformed lines was identified by setting up a multiplex PCR with primers AtFdhF3/R3/Fm3/R2 using Extract-N-Amp plant PCR kit and separating the product on 1.5% agarose gel. The reaction included 5 µl PCR mix, 2 µl DNA template, 0.5 µl of each primer AtFdhFm3 and AtFdhR2 as well as 0.2 µl of AtFdhF3/R3 (10 µM concentration) in a 10 µl reaction. CP 23 was used for amplification.

5.3 Results

5.3.1 Isolation and analysis of barley *Fiddlehead*

The first step in the characterisation of clone d1057 was the isolation of the gene sequence using public databases and RACE PCR. Nested 3'-RACE (Fig. 5.2) with d1057 specific primers resulted in amplification of homologous fragments. These fragments varied in the position of poly A^+ tails (Fig. 5.3). The sequences of the 5' end of the gene was identified by 5'-RACE (Fig. 5.4). Sequence data was used to isolate the full-length coding sequence of clone d1057 from cDNA and genomic DNA of Atlas 46 (Fig. 5.5). Sequencing and comparison of the genomic region with respective cDNA did not indicate any intron in its codon sequences. Full-length sequence of the gene is shown in Figure 5.6. The size of the band in Northern blot analysis was 1.9 kb which supports that the obtained sequence is full-length (Fig. 5.14).

Next, public databases were searched for identifying similar sequences and functional annotation. Search of the Conserved Domain Database (CDD) identified a chalcon and stilbene synthase domain (cd00831) belonging to polyketide synthases. A database search for proteins homologous to d1057 with the tBLASTx algorithm revealed several sequences exhibiting high levels of similarity with the query sequence. In particular, a putative hypothetical β-ketoacyl-CoA synthase protein from rice (Accession No. EAY88792) showed 94% identity. Furthermore, the most similar characterised homologue of the gene in higher plants was *Fiddlehead* (71% identity) from Arabidopsis (Yephremov *et al.*, 1999) and because of this homology *d1057* was named barley *Fiddlehead* gene, *HvFdh*. Protein sequences giving highest similarity belong to β-ketoacyl-CoA synthases whereas polyketide synthases showed a lower level of homology. Both synthases extend the initial chain in a carboxylative condensation reaction and the generated product depends on the nature of the starter molecule.

Following annotation, the nucleotide sequences of *HvFdh* cDNA was analysed for the presence of ORFs. It contains a single ORF and downstream of the first stop codon seven more in-frame stop codons were found in the 3'UTR. No putative polyadenylation site was present downstream of the translation stop codon. Conceptual translation of the 1620 bp nucleotide-long ORF present in the *Fdh* cDNA sequences gave rise to a putative

polypeptide of 540 amino acids with an apparent molecular mass of 60067 Da, and a pI of 9.26.

To investigate the genomic organisation of *Fdh*-like genes in barley, Southern blot hybridisation was performed using a 3' and a full-length probe (Fig. 5.7). DNA gel blot analysis with the 3' probe (Fig. 5.8A) hybridised to one band. On the contrary, the full-length probe (Fig. 5.8B) hybridised to one strong band and some weak bands. This hybridisation pattern indicates the specificity of the 3' probe for *HvFdh* detection in blots and existence of other genes with similar domains in barley.

5.3.2 Subcellular localisation

As it was outlined earlier, β-ketoacyl-CoA synthases (KCS) are membrane bound proteins and in agreement with its putative function, HvFdh was predicted to be a membrane protein by PSORT. Moreover, sequence analysis at the PredictProtein server predicted the presence of two transmembrane domains in the N-terminal region of the HvFdh polypeptide (Fig. 5.9). To confirm the localisation, GFP fusion was employed. The *HvFdhORFfu* fragment was amplified (Fig. 5.10) to construct vector pHvFdh:GFP. The vector was transiently expressed in barley leaves and targeting of the HvFDH:GFP fusion protein was observed in multiple independent bombardment experiments. GFP fluorescence was visible on the endoplasmic reticulum (Fig. 5.11).

5.3.3 Prediction of HvFdh secondary structure and post-translational modifications

To characterise the encoded protein, post-translational modifications of HvFdh and the secondary structure of the encoded protein were predicted using various programs at the EXPASY website (Section 2.32). Threonine 360 was predicted by OGPET to be O-glycosylated. In addition, several putative phosphorylation sites including kinase specific phosphorylation for serine 316 was identified. The secondary structure of the HvFdh protein was determined using PSIPRED. It shows that the protein mostly will form helices and coil structures (Fig. 5.9).

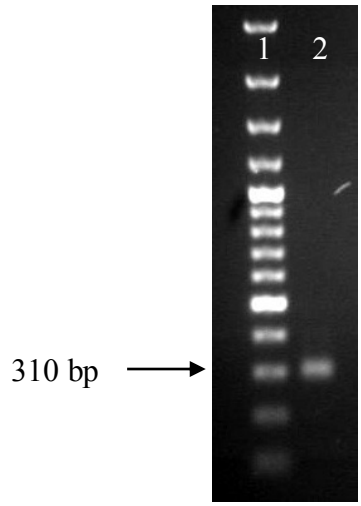

Figure 5.2 Barley *Fiddlehead* 3'-RACE. The *HvFdh* nested 3'-RACE product using primer pair d1057F6/CDSIIA was separated on 2% agarose gel and purified for sequencing. Lane 1: DNA size marker, Lane 2: 3'-RACE product.

```
                1                                                    50
HvFdha3'    (1) TGAGGCCTGACCGATCGAGACAAGCAGGCGCATGCACCATGCAGGTGCTA
HvFdhb3'    (1) TGAGGCCTGACCGATCGAGACAAGCAGGCGCATGCACCATGCAGGTGCTA
HvFdhc3'    (1) TGAGGCCTGACCGATCGAGACAAGCAGGCGCATGCACCATGCAGGTGCTA
               51                                                   100
HvFdha3'   (51) ATAATTAAGCGAGACACGTCGCCGCATCCGCGTGGGTAAGTGCAGGCTAA
HvFdhb3'   (51) ATAATTAAGCGAGACACGTCGCCGCATCCGCGTGGGTAAGTGCAGGCTAA
HvFdhc3'   (51) ATAATTAAGCGAGACACGTCGCCGCATCCGCGTGGGTAAGTGCAGGCTAA
              101                                                   150
HvFdha3'  (101) TGGATTTGCATGTAACTGCATGCAGTGCCGCTGGTTAAGGTTTGAGCTTT
HvFdhb3'  (101) TGGATTTGCATGTAACTGCATGCAGTGCCGCTGGTTAAGGTTTGAGCTTT
HvFdhc3'  (101) TGGATTTGCATGTAACTGCATGCAGTGCCGCTGGTTAAGGTTTGAGCTTT
              151                                                   200
HvFdha3'  (151) GGTTAATTTGTGCTGGTGTTGTAGCAGTTGTCATCATTATTAAGGGTTTT
HvFdhb3'  (151) GGTTAATTTGTGCTGGTGTTGTAGCAGTTGTCATCATTATTAAGGGTTTT
HvFdhc3'  (151) GGTTAATTTGTGCTGGTGTTGTAGCAGTTGTCATCATTATTAAGGGTTTT
              201                                                   250
HvFdha3'  (201) AATCATAGACTACGTACGTACGTACCATAATGCGCAAGTGTATGTGCGCG
HvFdhb3'  (201) AATCATAGACTACGTACGTACGTACCATAATGCGCAAGTGTATGTGCGCG
HvFdhc3'  (201) AATCATAGACTACGTACGTACGTACCATAATGCGCAAGTGTATGTGCGCG
              251                                                   300
HvFdha3'  (251) CTCTTGTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAA----------
HvFdhb3'  (251) CTCTTGTGTGAAACTTATGTCAACTTTGATTATCTACGTGCAATTTCAAA
HvFdhc3'  (251) CTCTTGTGTGAAACTTATGTCAACTTTGATTATCTACGTGCAATTTCATT
              301                                                345
HvFdha3'  (291) ------------------------------------------
HvFdhb3'  (301) AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA--------------
HvFdhc3'  (301) ACTTTGGACTTTGGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 5. 3 Variation in poly A^+ tail position of *HvFdh*. The variation (sequences in bold) was revealed by sequencing 3'-RACE fragments cloned in pGEM-T Easy vector.

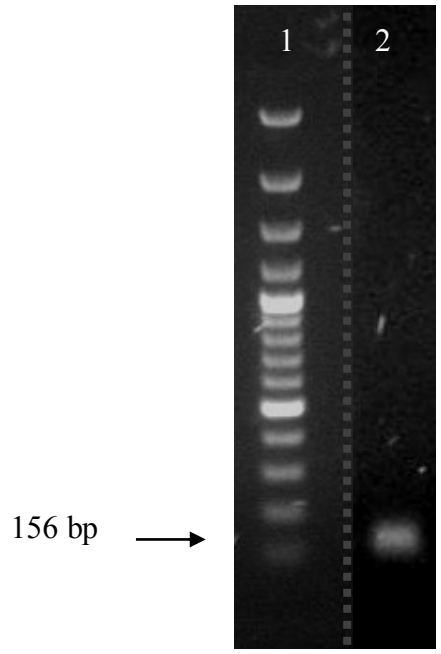


Figure 5.4 *HvFdh* 5'-RACE. Nested 5'-RACE product amplified by primer pair d1057R2/NUPA was separated on 2% gel and purified before cloning into vector. Lane 1: DNA size marker, Lane 2: 5'-RACE product. Vertical dashed line indicates that picture has been cut.

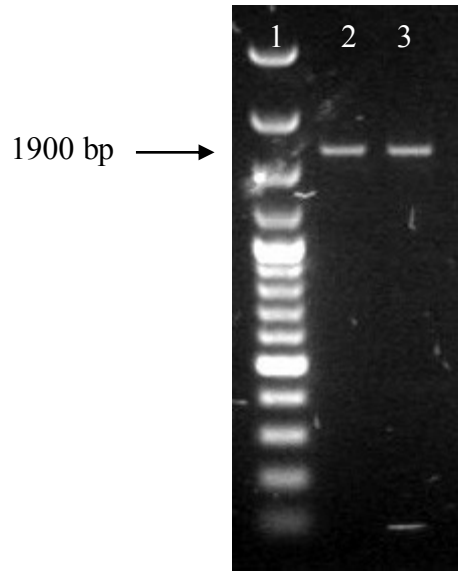


Figure 5.5 cDNA and genomic DNA fragment of *HvFdh*. PCR products amplified from cDNA (Lane 2) and genomic DNA (Lane 3) of Atlas 46 by using the primer pair d1057F6/R. Lane 1: marker DNA.

Figure 5.6 *HvFdh* sequence. *HvFdh* full-length sequence was obtained by combining 3'- and 5'-RACE products with fragment amplified by primer pair d1057F6/R. The sequences in red mark the primers and the direction of arrows indicates orientation of primers. For some primers only partial sequence is shown. d1057F7 and d1057R14 primers are in bold and italic characters to distinguish them from overlapping primers. The sequence used for RNAi construct (Section 5.3.8) is underlined.

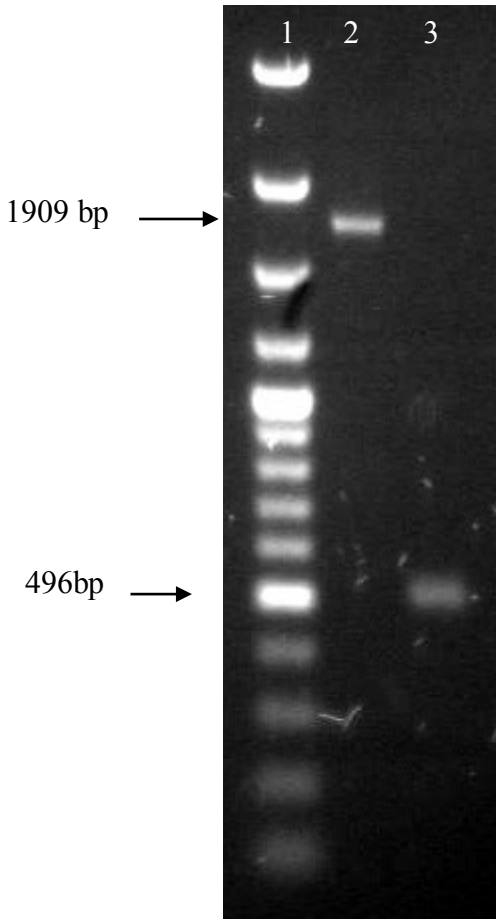


Figure 5.7 Amplified *HvFdh* probe templates. 3' probe was amplified by primer pair d1057F5(3')/R and full-length probe amplified with primers d1057F6/R from cDNA of *HvFdh*. Lane 1: DNA marker, Lane 2: full-length template and Lane 3: 3' template.

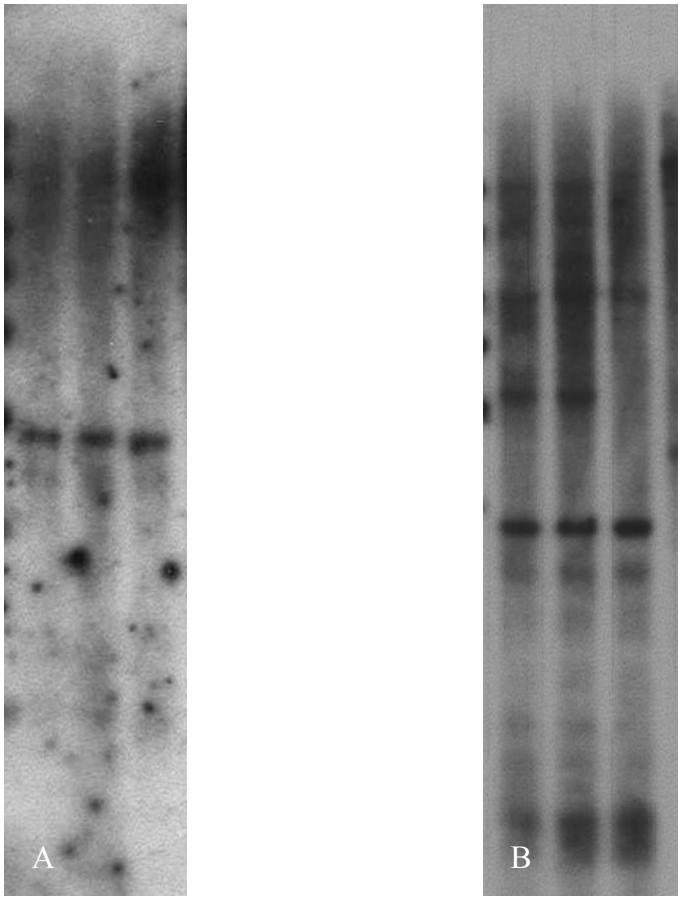


Figure 5.8 Southern blot analyses of barley cultivars with *HvFdh* probes. Genomic DNA was digested with DraI restriction enzyme and probed with 3' probe (A) and full-length probe (B) of *HvFdh*. Cultivars from left to right: Atlas, Atlas 46, and Golden Promise.

```
Conf: 966686788861776556778888616887516770444311110120028889999999
Pred: CCHHHHHHHHHCCCCCCCCCCCCCEEEEEECCCCCHHHHHCCCCEEECHHHHHHHHHHH
  AA: MAREELSTEIVNRGVEPSGPDAGSPTFSVRVRRRLPDFLQSVNLKYVRLGYHYLLSHGVY
              10        20        30        40        50        60

Conf: 999999999998887516989999999998677548999999999999975752899704
Pred: HHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCE
  AA: LATIPVIVLVCGAEVGSLSRDELWRKVWDEATYDLATVLAFLAVLAFTISVYIMSRPRPI
              70        80        90       100       110       120

Conf: 997862015883202388999999986167896679999999985054761352554456
Pred: EEEEEEEECCCHHHCCCHHHHHHHHHHCCCCCHHHHHHHHHHHHHHCCCCCEEECCCCCC
  AA: YLIDFATYKPADELKVSKAEFIDLARKSGKFDEESLAFQARLLAKSGIGDESYMPRCVFQ
             130       140       150       160       170       180

Conf: 788764468879999999999999999639888996689986799866852389999998
Pred: CCCCCCCHHHHHHHHHHHHHHHHHHHHCCCCHHHCCEEEEECCCCCCCCCCHHHHHHHH
  AA: PDANCATMKEGRAEASAAMFAALDELFDKCRVRPKDVGVLVVNCSLFNPTPSLSAMIVNH
             190       200       210       220       230       240

Conf: 2899872478513221889999999999998608998899997410125878886723 31
Pred: HCCCCCEEEEECCCCHHHHHHHHHHHHHHHHHCCCCCEEEEEEEECCCCCCCCCCCHHHH
  AA: YKMRGNILSYNLGGMGCSAGVISIDLARDMLQASGAGLAVVVSTEAVSFTWYAGKRRSML
             250       260       270       280       290       300

Conf: 1432221103688971582113332210112121246788510233203567777420 34
Pred: HHHHHHCCCEEEEEEECCCCCCCCCCCCHHHHHHHCCCCCCCCHHHHHCCCCCCCCCCEE
  AA: IPNAFFRAGAAAVLLSNRRRDFRRAKYQLEHVVRTHKGADDRAFRSVYQEEDEQRIKGLS
             310       320       330       340       350       360

Conf: 0431000156665677653063224411566877888999998888888778887524 65
Pred: ECCCHHHHHHHHHHHHHCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
  AA: ISRDLVEVGGHALKTNITTLGPLVLPFSEQLLFFAGVLFRHLYPSKTSTPPPPAANGDTS
             370       380       390       400       410       420

Conf: 3100243303322577977899899999999819997781136689987286167899 99
Pred: HHHCCCCCCHHHCCEEEECCCCHHHHHHHHHHCCCCHHHHHHHHHHHHHCCCHHHHHHH
  AA: AAAPYIPDFKRAFEHFCMHAASRDVLEHLQRNLGLRDADLEASRAALHRFGNTSSSSIWY
             430       440       450       460       470       480

Conf: 9999998388899988999963253114201325135678888898787886189988 98
Pred: HHHHHHHCCCCCCCCCEEEEEEECCCCCHHEEEEEECCCCCCCCCCCCCHHHHHHCCCCCCC
  AA: ELAYLEAKGRVRRGDRVWQLAFGSGFKCNSAVWRAVGRVRRPSRSPWLDCIDQYPARMDA
             490       500       510       520       530       540
```

Conf: Confidence (0=low, 9=high)
Pred: Predicted secondary structure (H=helix, E=strand, C=coil)
AA: Target sequence
Underlined amino acids indicates transmembrane region with bold italic characters implying higher probability.

Figure 5.9 Secondary structure of the HvFdh was predicted by PSIPRED. The prediction schematically depicts the helix, β-strand and coil structures. The numbers under sequence indicate the amino acids in the primary structure of the protein. Transmembrane regions were predicted by the PredictProtein server (underlined) and regions predicted by high probability are in italic bold characters.

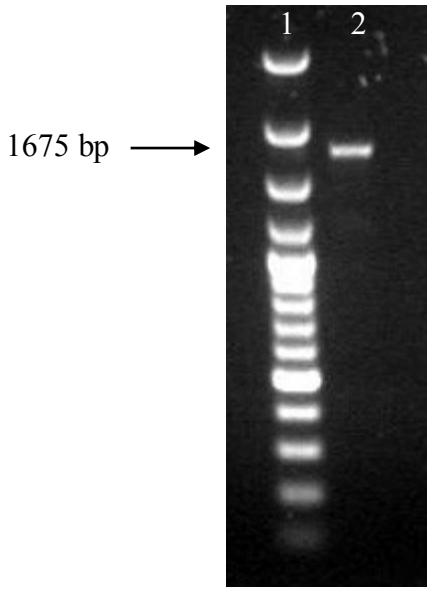


Figure 5.10 Amplification of the barley *FdhGFPFu* fragment. PCR fragment was amplified using the d1057F7/R12 primer set. Lane1: 100 bp DNA ladder, Lane 2: PCR product.

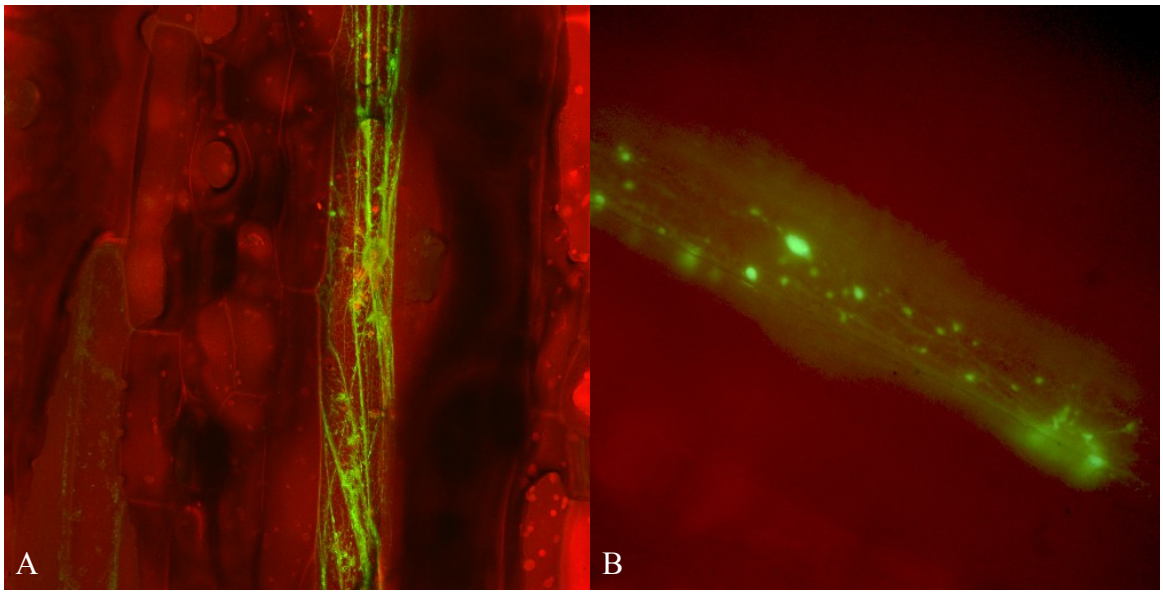


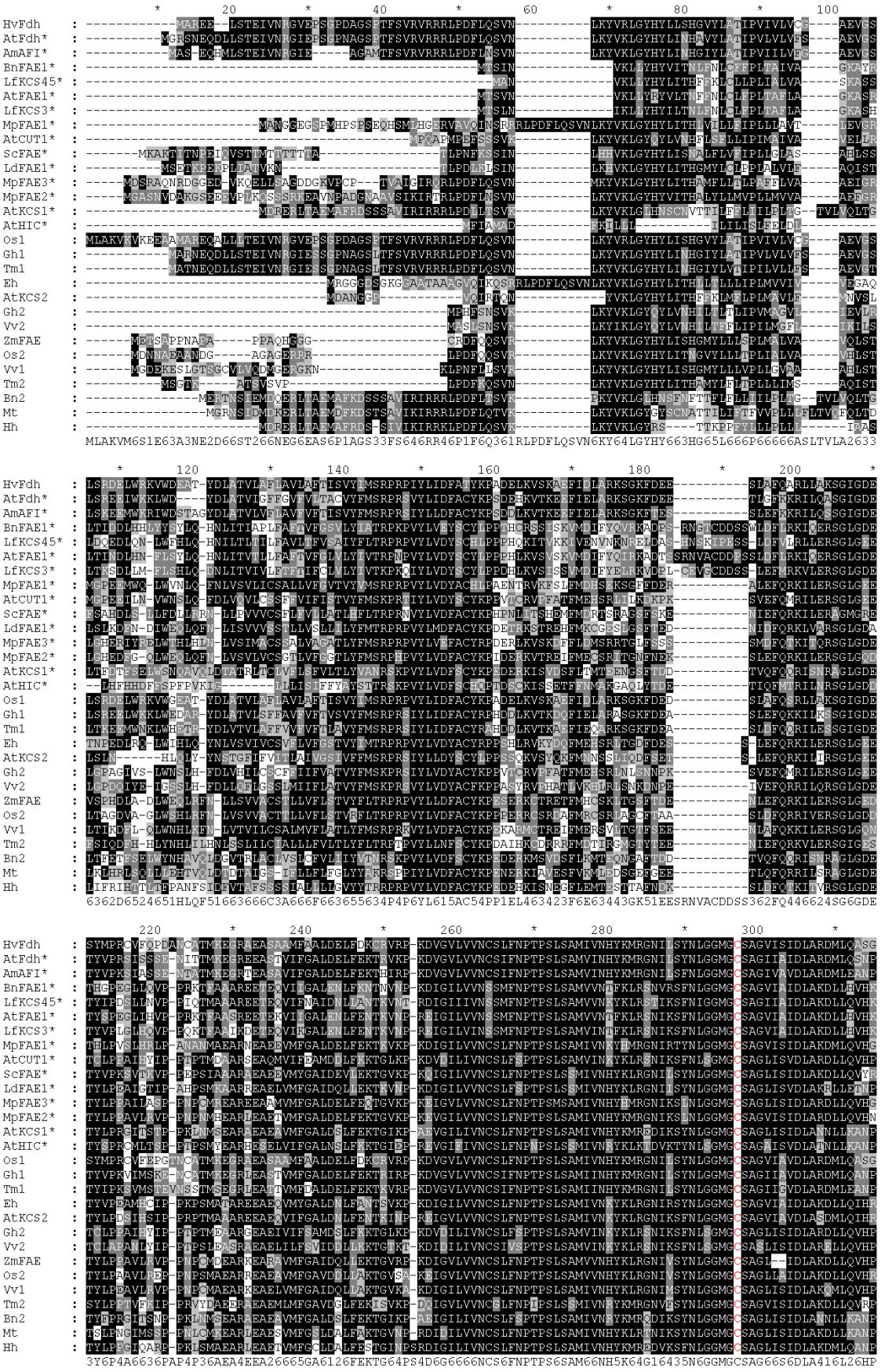
Figure 5.11 Localisation of HvFdh protein. Barley epidermal cells bombarded with pHvFdh:GFPFu construct showed endoplasmic reticulum localisation. (A) Combined shots of a cell expressing fusion protein taken by confocal laser scanning microscope. (B) A barley cell expressing fusion protein under fluorescence microscope.

5.3.4 Phylogenetic analysis

As a putative β-ketoacyl-CoA synthase function was established for the protein based on the analysis and different β-ketoacyl-CoA synthases have specific substrate preference, all entries in the Genbank non-redundant plant protein database were screened for proteins similar to HvFdh (Section 2.34) to predict its specific enzymatic function based on phylogenetic analysis. Twenty-one non-redundant proteins from dicotyledonous species, five from monocotyledonous species and three from liverworts (moss) were used for phylogenetic study. Some of the proteins have experimentally been shown to be β-ketoacyl-CoA synthases that are involved in VLCFA synthesis. All the identified proteins were aligned (Fig. 5.12) by the ClustalX program. Sequences of these proteins are much conserved among different species. An un-rooted phylogenetic analysis was performed on aligned amino acid sequences using neighbour-joining algorithm. Sequences were found to form four distinct groups (Fig. 5.13) independent of their botanic relationship.

5.3.5 Analysis of *HvFdh* expression under biotic and abiotic stresses

Previous investigations lead to the assumption of a FAE function for HvFdh. The products of FAE system are deposited on the cuticle that has been shown to affect plant-pathogen interaction and abiotic stress tolerance. Possible role of *HvFdh* in stress response was investigated by studying its expression under stress conditions using Northern blot hybridisations, Q-PCR and publically available Barley1 GeneChip data (*Fdh* is represented by probe set 4206_at on the Barley1 GeneChip). Temporal expression of *HvFdh* transcripts in leaf tissues inoculated with *R. secalis* showed up-regulation in response to the pathogen at 6-12 h post-inoculation (hpi) in Atlas (susceptible) and 12-48 hpi in Atlas 46 (resistant) cultivars in comparison to control plants (Fig. 5.14). In *Blumeria graminis*-inoculated plants the expression level of *HvFdh* in the incompatible interaction was generally lower than compatible interaction. In compatible interaction after an initial transient down-regulation the transcript reached levels as before the inoculation (Fig. 5.15). In resistance conferred by *mlo* alleles in both resistant and susceptible interactions the transcript level was decreased after inoculation (Fig. 5.16). Similarly, plants inoculated with *F. graminearum* showed down-regulation of the *Fdh* mRNA although a similar transcript trend was observed at different time points in inoculated and control plants (Fig. 5.17).



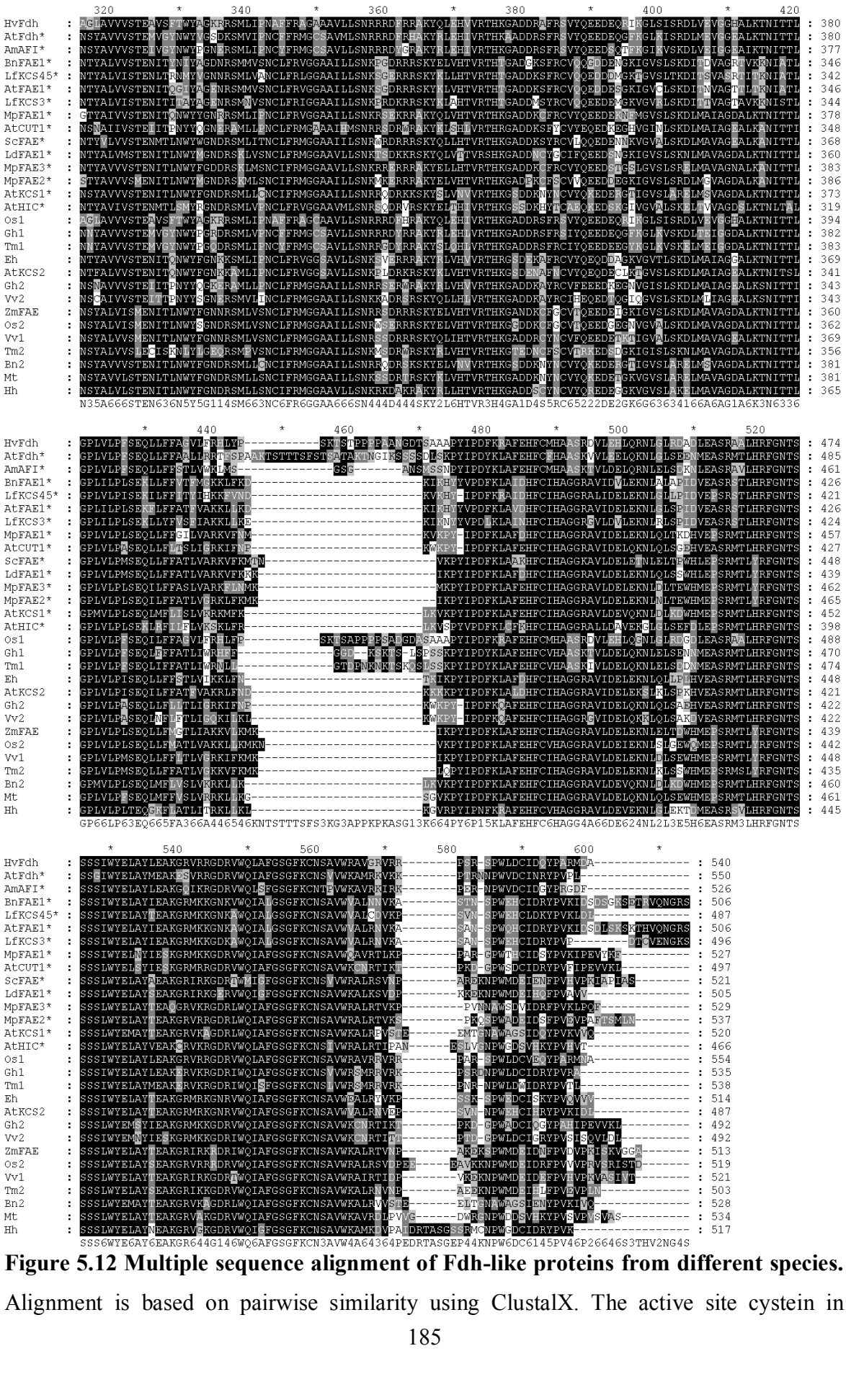


Figure 5.12 Multiple sequence alignment of Fdh-like proteins from different species.

Alignment is based on pairwise similarity using ClustalX. The active site cystein in

position 297 is in red. Dashes indicate introduced gaps for better alignment. Shading represents the degree of sequence identity at each residue position. Names followed with an * indicates that protein have experimentally been shown to be a FAE. The abbreviations and accession numbers are as following: Am: *Antirrhinum majus* (AmAFI: CAC84082), At: *Arabidopsis thaliana* (AtFAE1: AAA70154, AtCUT1: AAD37122, AtKCS1: AAC99312, AtKCS2: NP_195177, AtHIC: NP_182195, AtFdh: CAA09311), Bn: *Brassica napus* (BnFAE1: AAB72178, Bn2: AAT65207), Eh: *Eranthis hyemalis* (Eh: ABS18382), Gh: *Gossypium hirsutum* (Gh1: AAL67993, Gh2: ABA01490), Hh: *Hemerocallis hybrid* cultivar (Hh: AAC34858), Hv: *Hordeum vulgare*, Lf: *Lesquerella fendleri* (LfKCS3: AAK62348, LfKCS45: AAU05611), Ld: *Limnanthes douglasii* (LdFAE1: AAG28600), Mp: *Marchantia polymorpha* (MpFAE1: AAP74371, MpFAE2: AAO48425, MpFAE3: AAP74370), Mt: *Medicago truncatula* (ABD32702), Os: *Oryza sativa* (Os1: AAN65442, Os2: EAY84941), Sc: *Simmondsia chinensis* (ScFAE: AAC49186), Tm: *Tropaeolum majus* (Tm1: AAO47729, Tm2: ABD77097), Vv: *Vitis vinifera* (Vv1: CAO71273, Vv2: CAO63566), Zm: *Zea mays* (CAC01441).

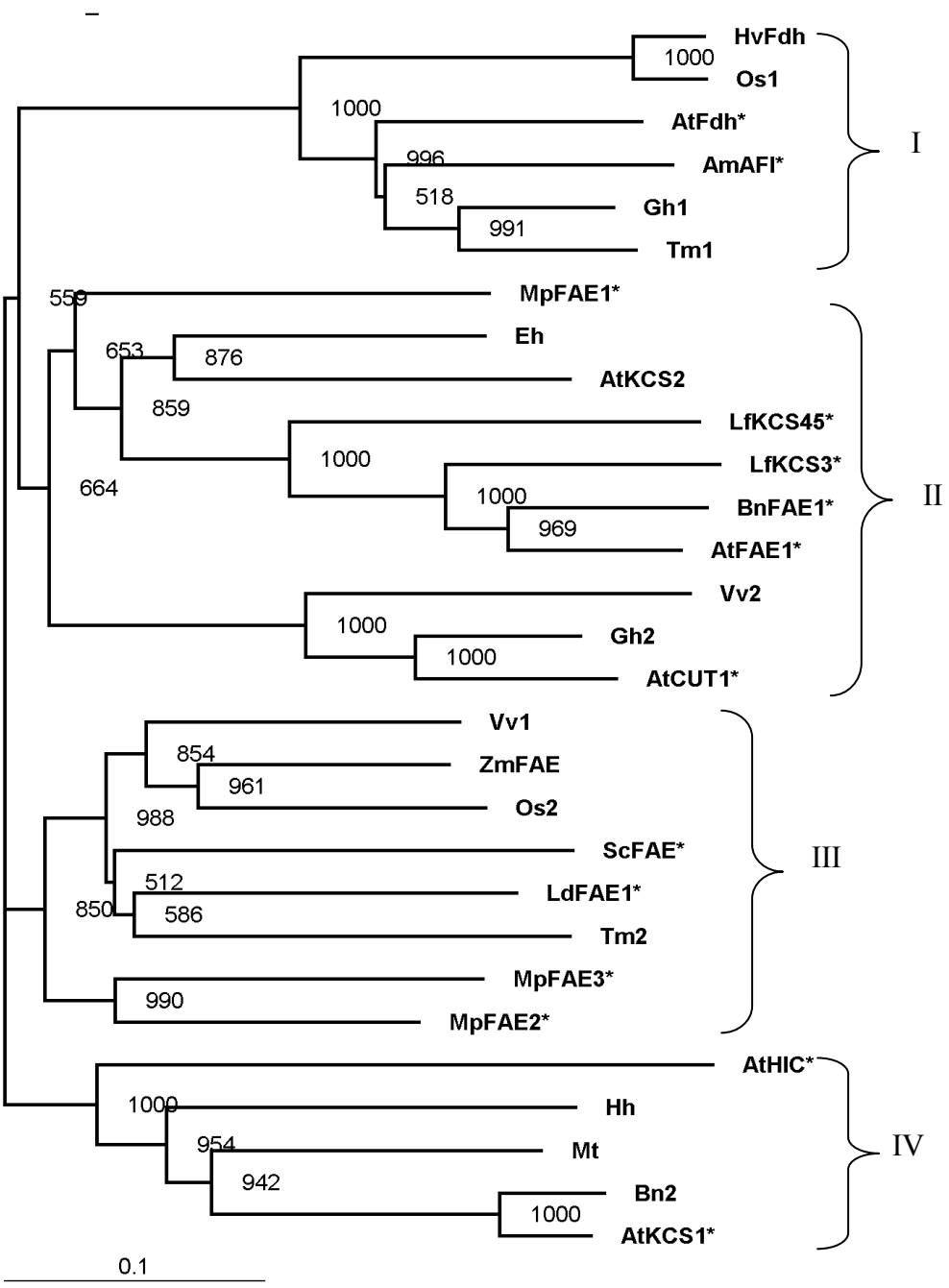


Figure 5.13 Phylogenetic tree of barley Fdh and its homologous proteins. The tree was constructed using neighbour-joining algorithm and displayed by TreeView software. The name and identification number of sequences are given in Figure 5.12 legend. Names followed with an * indicates that proteins have experimentally been shown to be a FAE. Branch length reflects the extent of sequence divergence. The reliability of the cluster analyses was tested by bootstrap confidence limits and indicated as success per 1000 bootstrap trials as presented on the nodes.

Fatty acid composition of cuticle can be affected by abiotic stresses. Assuming a FAE function the kinetics of *HvFdh* expression during frost, drought and salinity were studied. Before cold treatment, varying levels of transcripts were measured in different cultivars. Figure 5.18 shows that transcript levels during frost treatment were lower or equal in comparison with control plants with the exception of Galleon that had higher level at pre-frost 4ºC. Similarly, the response of *HvFdh* to drought was reduction in transcript levels. Messenger RNA levels were different among cultivars, but at any time point the level of transcript for each cultivar was higher in control plants than to drought-treated plants. When treated plants were rewatered, transcript levels increased (Fig. 5.19) and in Barque73 reached the control plants level. The effect of salt treatment on expression of the gene was dependent on cultivar and the time after treatment. One day after salt treatment all cultivars except CM72 showed lower level of transcript in comparison with control plants. Transcript level in control plants also varied depending on sampling time (Fig. 5.20). Measurement of tissue Na^+ concentration (Appendix C) at each time point showed that the expression level was not correlated with tissue Na^+ concentration. Publicly available data from various salinity experiments were also interrogated for expression of *HvFdh*. The expression levels were not greatly affected by salt, exposure time or JA pre-treatment followed by salt exposure (Figs. 5.21, 22 and 23).

Expression of the gene during barley development, generally showed a steady level in different tissues except radical, embryo during germination, seedling root, anthers and embryo 22 DAP tissues in which expression levels were lower with seedling roots showing the lowest levels (Fig. 5.24). In root tissues of plants in a salinity experiment the gene was not expressed (Fig. 5.22).

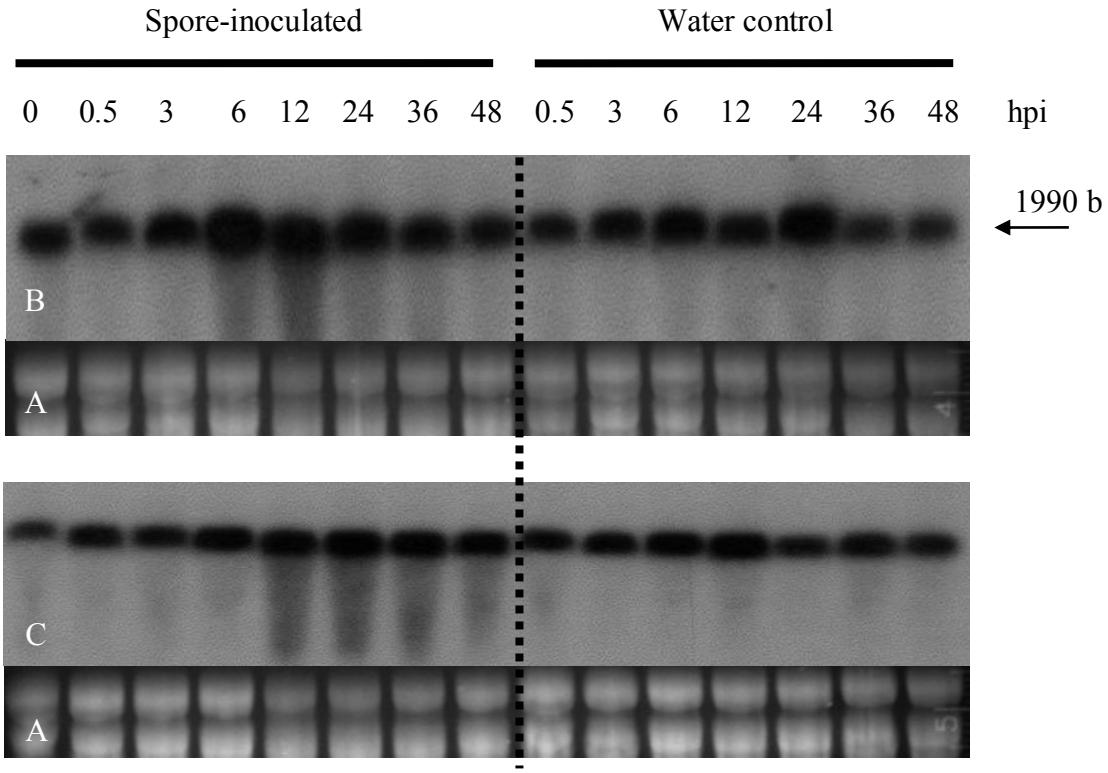


Figure 5.14 Temporal expression levels of *HvFdh* in leaves of barley plants inoculated with *R. secalis* strain SA385. (A) Denaturing agarose gel of total RNA (20 µg/lane) isolated from the leaves of barley. The tissue collection time after inoculation are indicated on top of lanes in hours post-inoculation. Northern blots were hybridised with the *HvFdh* 3' probe for susceptible cultivar Atlas (B) and resistant cultivar Atlas 46 RNA (C). Ribosomal 18S and 28S RNA were used as loading control.

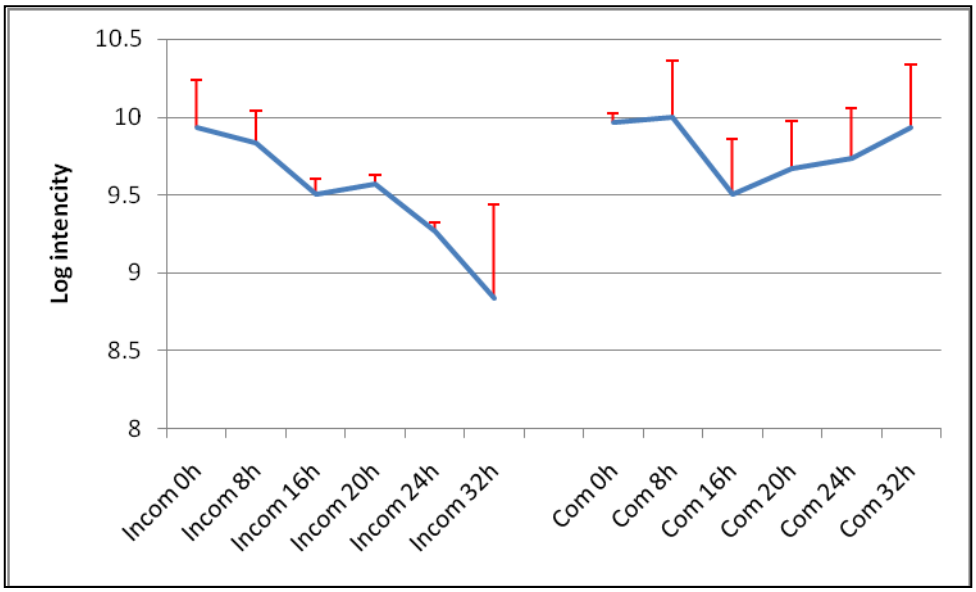


Figure 5.15 Expression of *Fdh* in response to *Blumeria graminis* f. sp *hordei (Bgh)*. Normalised average probe intensity levels of barley *Fdh* gene in incompatible interaction (Incom) between barely cultivar CI 16151 (Mla6) and *Bgh* 5874 (AvrMla6) and compatible interaction (Com) of the same barley cultivar with *Bgh* K1 (AvrMla13). Probe intensities were log of RMA normalised average of three biological replicates and error bars represent standard deviations.

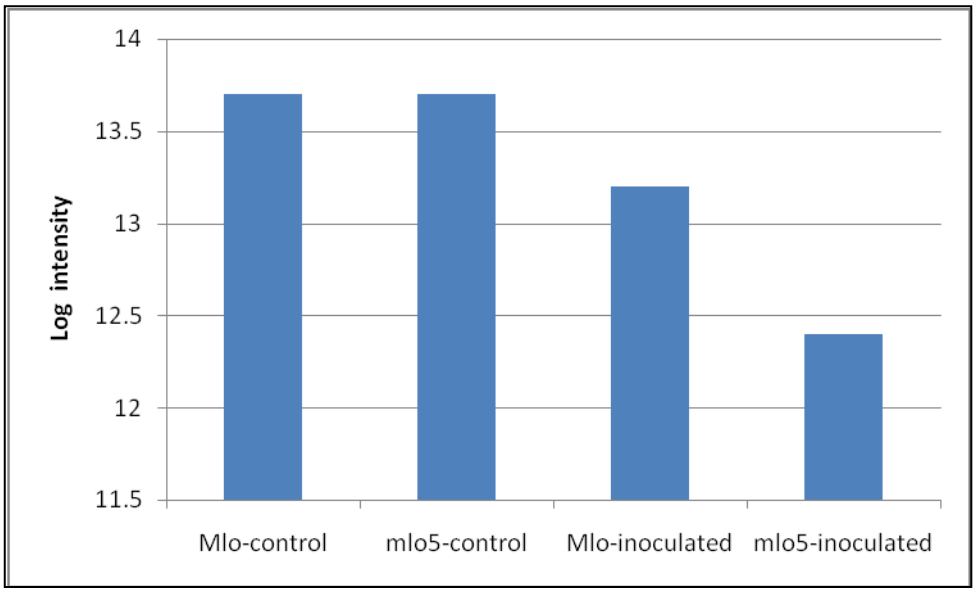


Figure 5.16 Expression of *HvFdh* in *Mlo* near isogenic lines. Normalised probe intensity levels of *HvFdh* genes in interaction between barley cultivars with *Mlo* and *mlo5* alleles inoculated with *Blumeria graminis* f. sp *hordei*. Probe intensities were log of RMA normalised data. The control plants were inoculated with water.

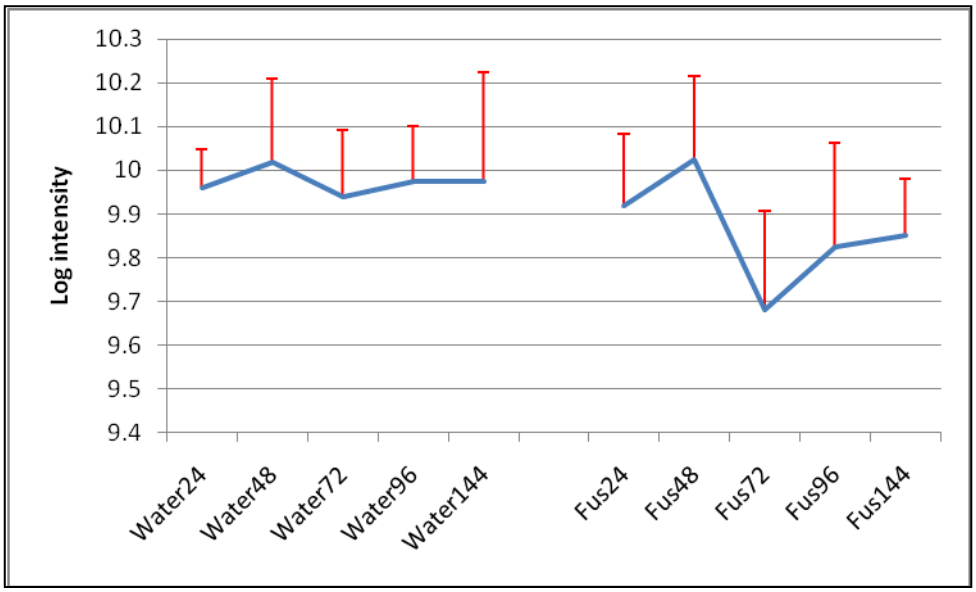


Figure 5.17 Transcript levels of *HvFdh* in interaction between barley Morex cultivar and *Fusarium graminearum*. The numbers following Water or Fus indicates the time (hours) after treatment with water or *F. graminearum* spores, respectively. Probe intensities were log RMA normalised average of four independent replications of the experiment and a fifth replication at 24 and 72 h after inoculation. Error bars represent standard deviations.

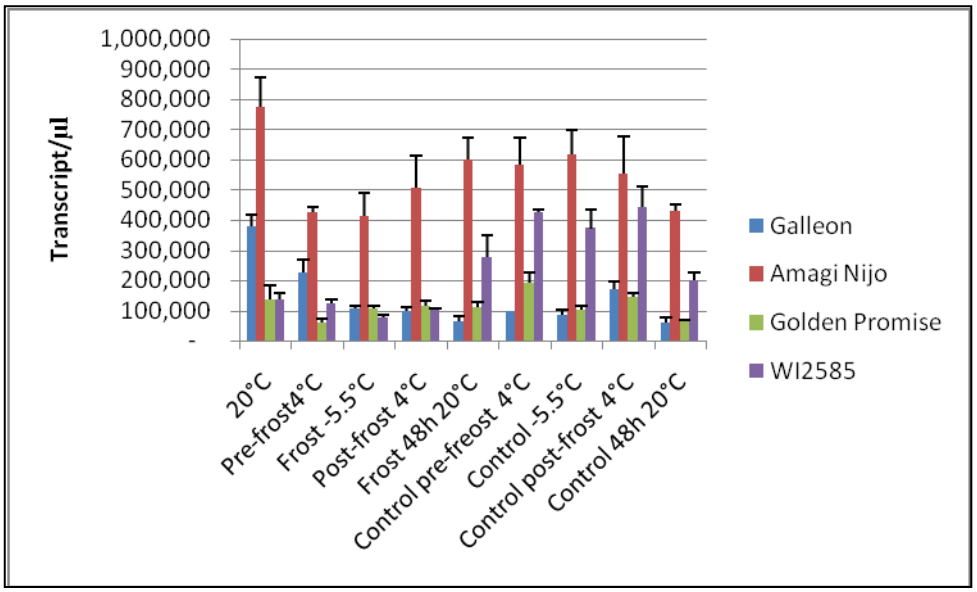


Figure 5.18 Effect of frost treatment on *HvFdh* expression. Transcript abundance was analysed in leaves at different times during frost treatment. Expression levels were calculated as copy number/µl of cDNA. Data were normalised against *GAPDH*, *Cyclophilin* and *α-Tubulin* levels. Data are mean values of triplicates in the PCR including standard deviations.

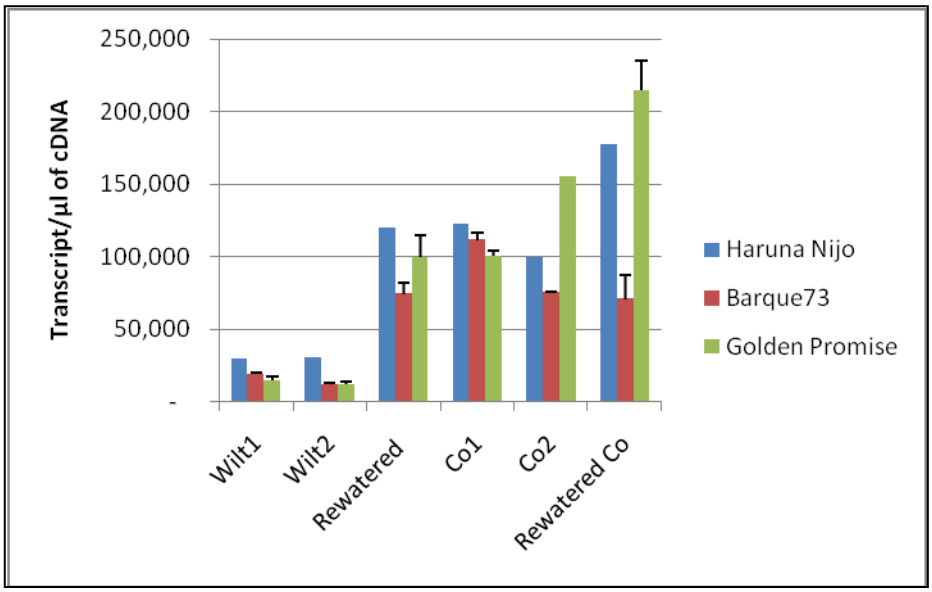


Figure 5.19 Response of barley *Fdh* to water stress in different barley cultivars. Two sets of plants were grown under the same conditions in a growth cabinet with different watering regime. Wilt1: wilting of leaves was observed, Wilt2: sampling 24 hours after Wilt1, rewatered: plants were watered to saturation after harvesting Wilt2 and samples taken 24 hours later, Co1, Co2 and rewatered Co, controls for coresponding treated plants. Data were normalised against *Cyclophilin*, *GAPDH* and *Heat Shock Protein 70* mRNA levels. Data are mean values of triplicates in the PCR including standard deviations.

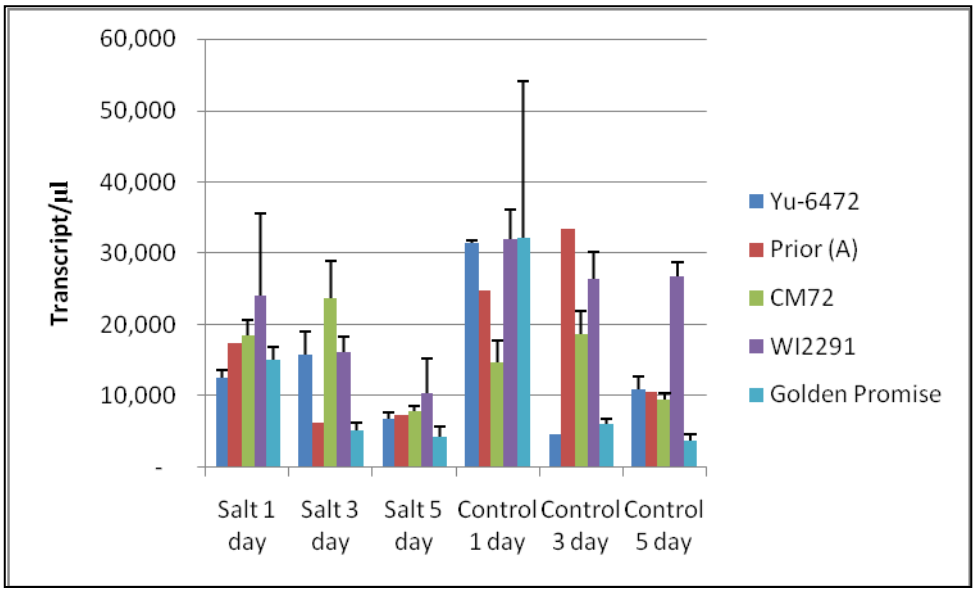


Figure 5.20 Quantitative RT-PCR analysis of the *HvFdh* in salt treated barley cultivars. Two sets of plants were grown under the same conditions in a hydroponic system until emergence of second leaf. For one set salt was added to a final concentration of 150 mM, with a 50 mM increase each 12 hours. Leaf samples were harvested one, three and five days after last salt addition. Data were normalised against *Cyclophilin*, *α-Tubulin* and *GAPDH* mRNA levels. Data are mean values of triplicates in the PCR including standard deviations.

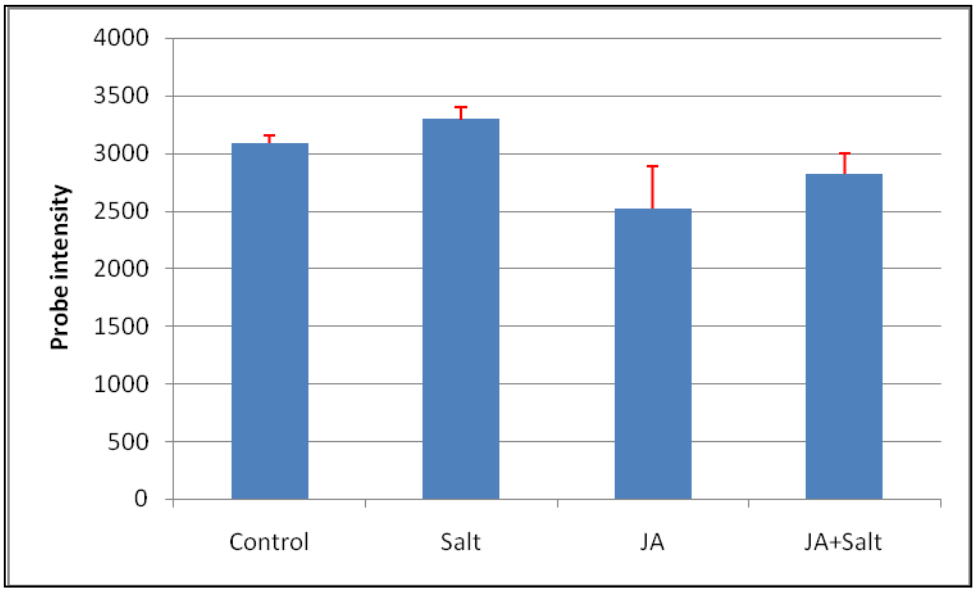


Figure 5.21 Effect of salinity stress and jasmonic acid (JA) on *HvFdh* expression. Transcript abundance of barley *Fdh* gene in response to salinity, JA application (12 µM) and pretreatment with JA followed by salinity treatment (JA+Salt) in cultivar Golden Promise. Salinity stress of ~ 18 dS m^{-1} was imposed gradually in four equal steps by adding NaCl to growth medium. Probe intensities were average of three independant replications of the experiment and error bars represent standard deviations.

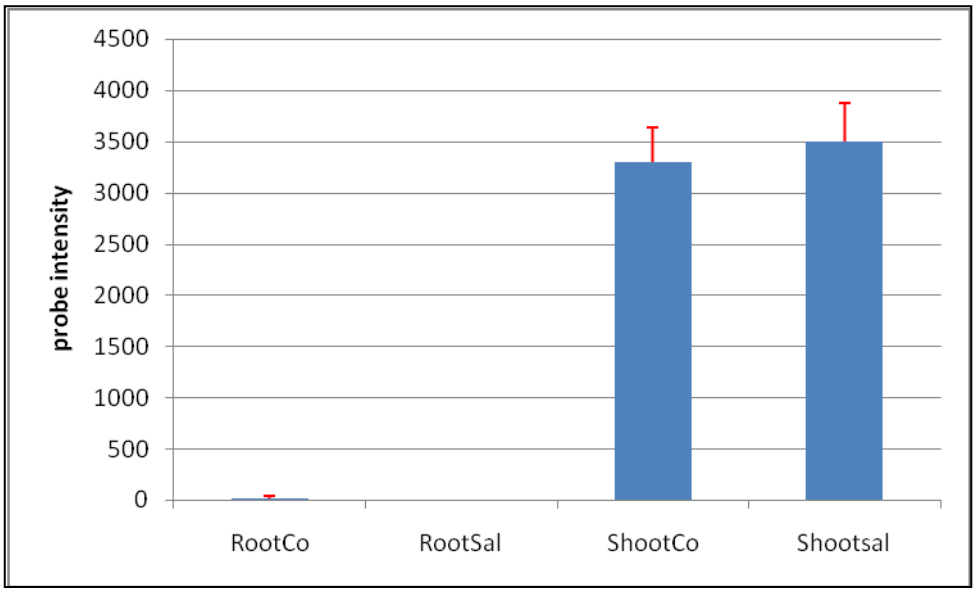


Figure 5.22 Normalised average probe intensity levels of *HvFdh* gene in response to salinity in shoot and root tissues in Golden Promise cultivar. Salinity stress was imposed over a period of five days in five equal steps to reach a final concentration of 150 mM NaCl and sampling was performed five days after last NaCl addition. Probe intensities were normalised average of three independent replications of the experiment and error bars represent standard deviations.

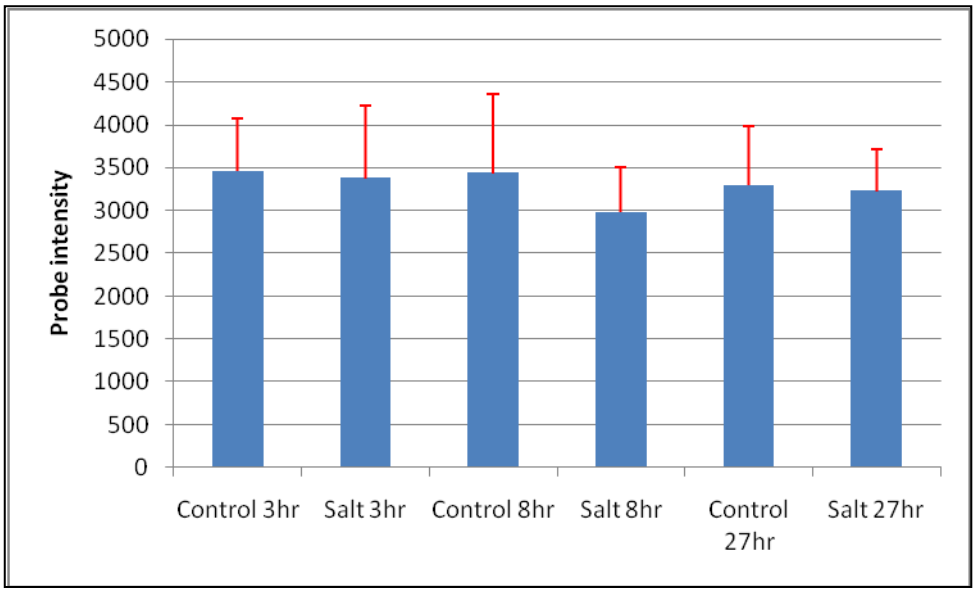


Figure 5.23 Expression levels of *HvFdh* gene in response to salinity in Morex at different times post addition of 100 mM NaCl. Gradual salt stress was imposed starting on day14 after transplanting until day 17. NaCl concentrations were brought up to 100 mM by increments of 25 mM NaCl per day. Probe intensities were normalised average of three independent replications of the experiment and error bars represent standard deviations.

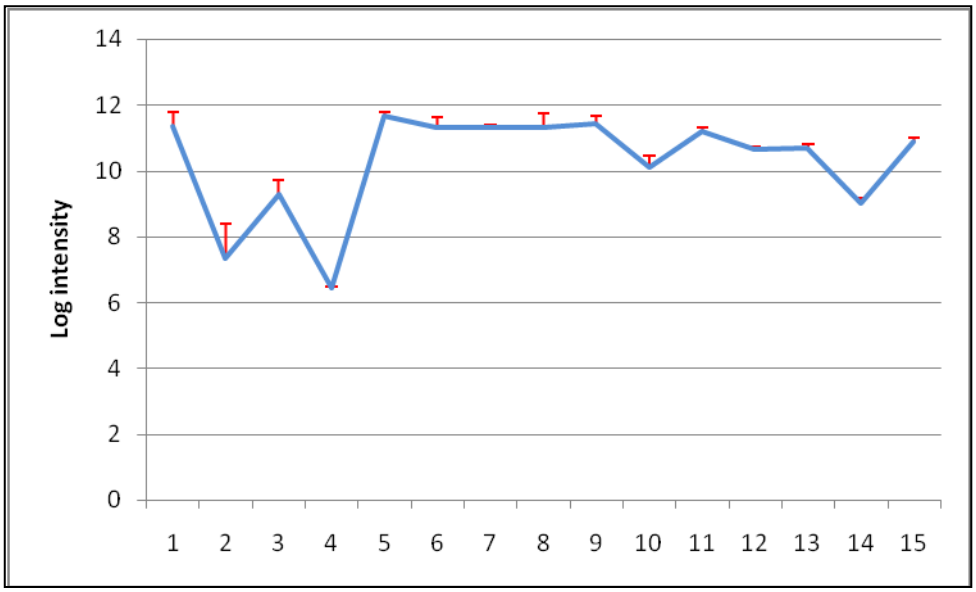


Figure 5.24 Expression levels of *HvFdh* during development in cultivar Morex. Probe intensities were log RMA normalised average of three biological replicates. The numbers on X-axis represent following tissue types: 1: coleoptyle, 2: radical, 3: embryo during germination, 4: seedling root, 5: seedling crown, 6: leaf, 7: immature inflorescence, 8: bracts, 9: pistil, 10: anthers, 11: caryopsis 5 day after pollination (DAP), 12: caryopsis 10 DAP, 13: caryopsis 16 DAP, 14: embryo 22 DAP, 15: endosperm 22 DAP. Error bars represent standard deviations.

5.3.6 Complementation of the Arabidopsis *fiddlehead* mutant

HvFdh encodes a protein showing high similarity (71% identity) to a protein characterised in the Arabidopsis mutant. The functional orthology of the genes was investigated by complementing the Arabidopsis mutant with the barley gene under control of the Arabidopsis *Fdh* promoter (Fig. 5.25). To test functionality of the cloned *AtFdh* promoter (Fig. 5.26) it was used to drive expression of GFP in Arabidopsis leaves by transient expression of vector pAtFdh::GFP. The expression of GFP in epidermal cells indicated functionality of the cloned promoter (Fig. 5.27). *HvFdh* ORF was amplified (Fig. 5.28) and used in constructing pAtFdh::HvFdh as complementation vector. Its correct assembly was verified by restriction digest (Fig. 5.29). Transformation and analysis of the transgene was performed at Dr Alexander Yephremov laboratory at the Max-Planck Institute in Cologne, Germany. The result reported to complement the Arabidopsis *fiddlehead* mutant.

To complement Arabidopsis *fdh* mutants, genotyping of plants was essential (Section 5.4.3). Since the mutant allele differs from the wild type allele by only two-nucleotide footprint of a transposon (Fig. 5.30), this difference was exploited to develop a multiplex PCR based genotyping technique. In this method, primers could amplify 2-3 different length fragments depending on the genomic DNA alleles. The success of the method was verified by excising the bands from gel and sequencing (Fig. 5.31).

5.3.7 Producing and analysis of transgenic barley lines

The possible role of HvFdh protein activity in drought tolerance and defence was examined by producing and characterising over-expression and knockdown transgenic barley lines. The coding region was amplified (Fig. 5.32) to generate over-expression (pHvFdhSE) and antisense (pHvFdhAS) constructs. To suppress endogenous *HvFdh* transcript levels antisense and RNAi approaches were used. To construct the RNAi vector a sense and an antisense fragment of a 3' region (Fig. 5.6) was amplified (Figs. 5.33, 5.34) to produce pHvFdhRNAi construct. The constructs were stably transformed into barley. Eight over-expression, seven antisense and two RNAi lines were regenerated and genomic integration of the transgene insert was verified by PCR amplification of the hygromycin resistance gene (Fig. 5.35). In over-expression lines transgene presence was verified, in

addition, by a primer pair consisting of a gene specific and a vector-anchored primer (Fig. 5.36).

Transcript level of *HvFdh* in transgenic lines was analysed by Q-PCR employing primer pair q1057F1/R1 (Fig. 5.37). Overexpression line G46-5 had the highest level of expression. The transcript level in suppressed lines was hardly detectable in whole leaf cDNA samples.

For disease resistance analysis eight plants from each line were evaluated for scald symptoms after inoculation with *R. secalis* race SA6 (Fig. 5.38). The t-test revealed significant reduction in symptoms in two antisense lines (59-6, 59-7) in comparison to non-transgenic progeny.

Drought tolerance of transgenic lines was evaluated as described in Section 2.29. The onset of wilting among pots varied by 19 days (Fig. 5.39), but no differences were observed among transgenic and non-transgenic progeny in the same pot. However, because of lack of non-transgenic progeny in some pots (because of the random selection of T_1 seeds) it was not possible to evaluate their drought tolerance. In transgenic over-expressers 46-4 and 46-5 the recovery rate of the transgenic lines was better than non-transgenic siblings evidenced by more growth after rewatering.

Among two of T_1 antisense lines grown to maturity three spikes in one inflorescence were observed (Fig. 5.40) and in some plants the inflorescence showed abnormal development. One of the T_0 RNAi lines stayed vegetative and did not produce any spike.

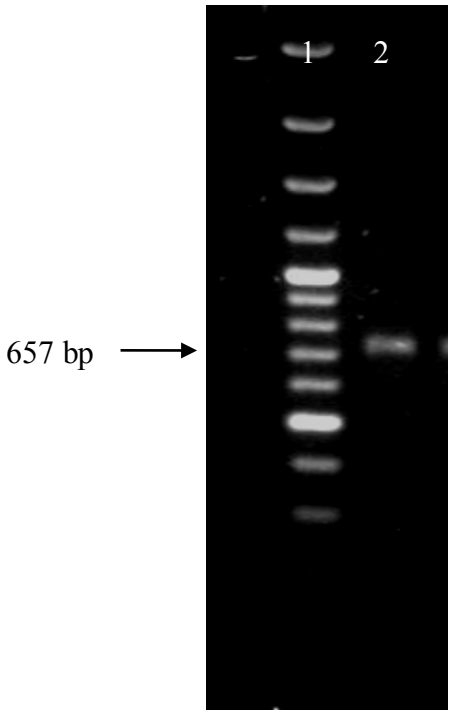


Figure 5.25 Amplification of the Arabidopsis *Fiddlehead* promoter. PCR fragment was amplified using the AtFdhPF/PR primer set. Lane1: 100 bp DNA ladder, Lane 2: PCR product.

```
  1    CCACTTTTTT TAGCTTAGAT ACAAACTCAA CTTTGTAAAA GATTTTCTTG
 51    TTGCATATTA AATTTTACAT CACAACAAAA AGAAAAAAAG AAATAAACAA
101    AGGAGATCAA ATAGGTTGTA CATAAATTAA GCAATCATAC AACATCAAAT
151    CAACAGCTAA AAAGTTCAGT ACAATCTTTA CTTCCGAATT TACTTCATTG
201    TCTTGACATC ATGTTCTAAA CAAAAACCAA ACCAAAGTTC TCCACAAACC
251    TGTCGGTTTT TAACCAAACC AAAGTTACTA GCTTAATTCA AAAAAAAAAA
301    AAAACAGAAC TGATTCAGAA ACCCCAAATT GACAAATTGT CAAATTGTCA
351    AAAAGTGGTC ATCTTTATCC TTTGACATCT GGTTAGTATA AAAAATTGTA
401    ATAAATGCAA ACATAAGATC CAAAACTACA CCTTATTAGA ACGGAGGAAT
451    TAAGAGTGAC ATAACTCACT CCAACTACGA CCAGTACAAG TAGTTGGCGA
501    CATTAACTAC CTCTCACCAA CCACCAAACC CAATCCCCAC AATATTACCA
551    TTACTCTCAT ATAACTACAC ATATTCATAT TTACATTTTT TGCCAACACA
601    ACTCCTTATA AGATATACAC TTCATCAACC TATAGATCTC ACTCACATAA
651    TCAACCTAC
```

Figure 5.26 Sequence of *AtFdh* promoter. DNA region from –16 to –667 was used to drive expression of marker *GFP* and *HvFdh* in transgenic Arabidopsis.

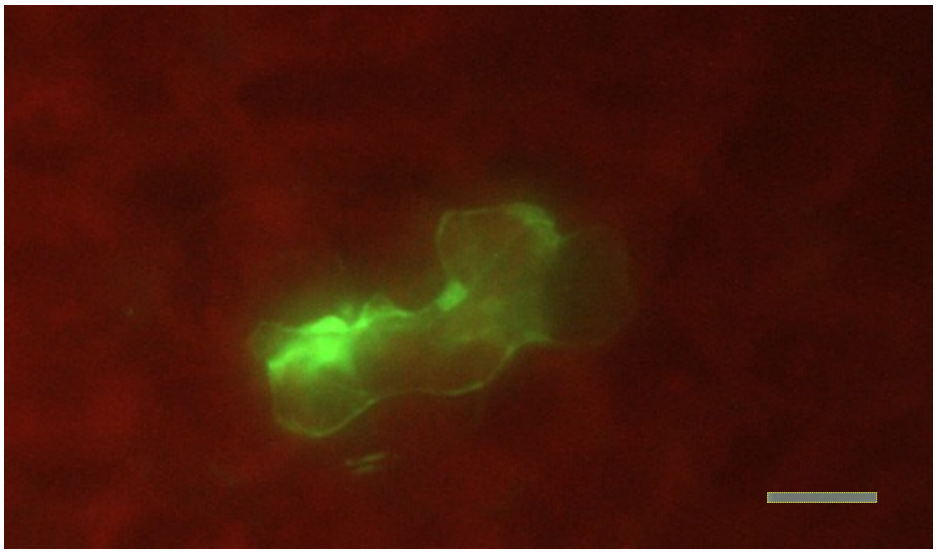


Figure 5.27 Validating AtFdh promoter activity. Arabidopsis epidermal cell bombarded with pAtFdh::GFP construct showed cytoplasmic localisation of GFP under fluorescent microscope and shows that promoter is functional. Bar= 100 µm.

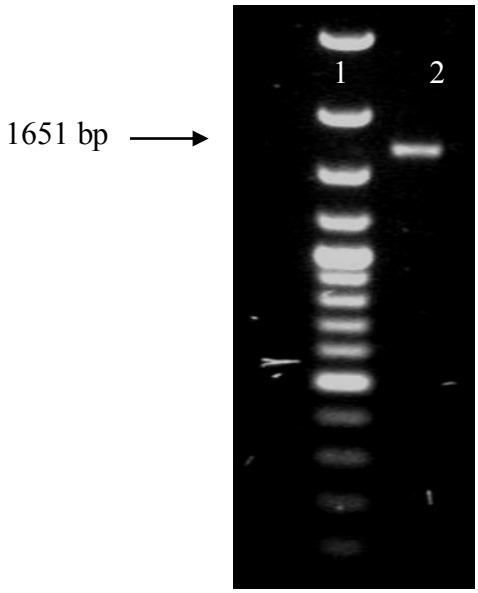


Figure 5.28 Amplification of the *HvFdh* coding region for complementing Arabidopsis *fiddlehead* mutant. PCR fragment was amplified using the d1057F14/R14 primer set and used in constructing complementation vector. Lane1: DNA ladder, Lane 2: PCR product.

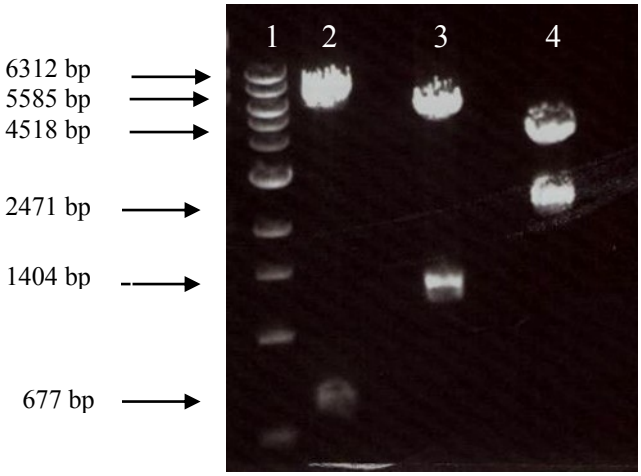


Figure 5.29 Restriction digest of pAtFdh::HvFdh to verify the correct assembly of construct. Lane 1: 1Kb ladder; Lane 2: EcoRI digest; Lane 3: SalI digest; Lane 4: PvuI digest. The digestions produced expected fragment sizes.

Figure 5.30 Region of *AtFdh* selected for genotyping. The nucleotides in pink are transposon footprints that have caused a frame shift. The nucleotides in red show location of outer primers and the arrows represent their orientation. The *italicised* region represents AtFdhFm3 and underlined region AtFdhR2. The mismatch nucleotides introduced into primers are not shown here.

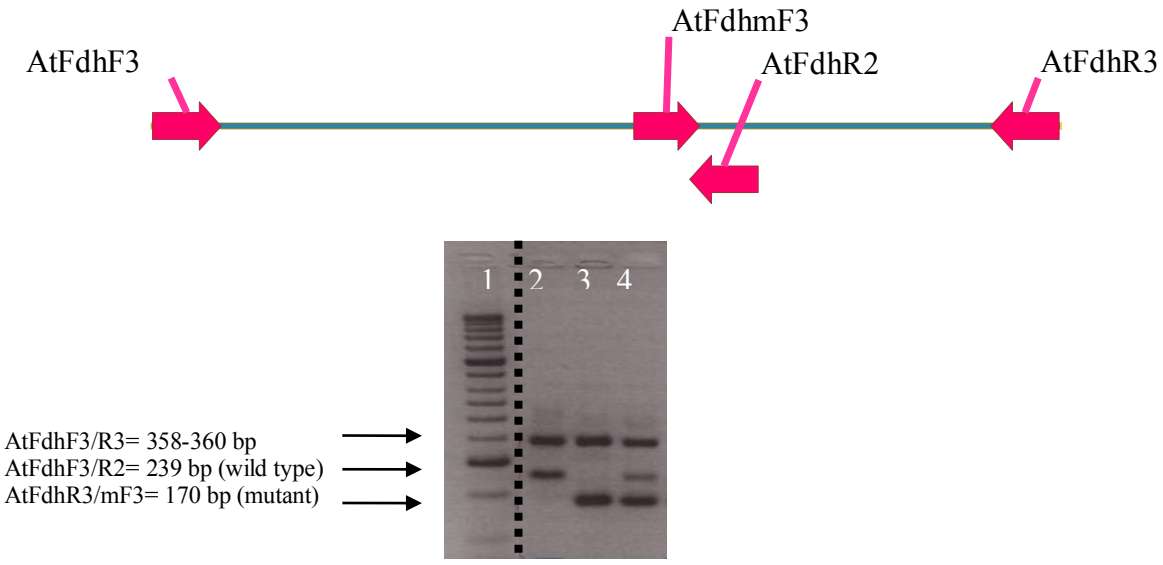


Figure 5.31 A schematic diagram of primer location for AtFdh genotyping. The gel picture shows the fragments and their size amplified by different primers. Lanes 1-4: marker, wild type, homozygous mutant, and heterozygous plants, respectively. Vertical dashed line indicates that picture has been cut.

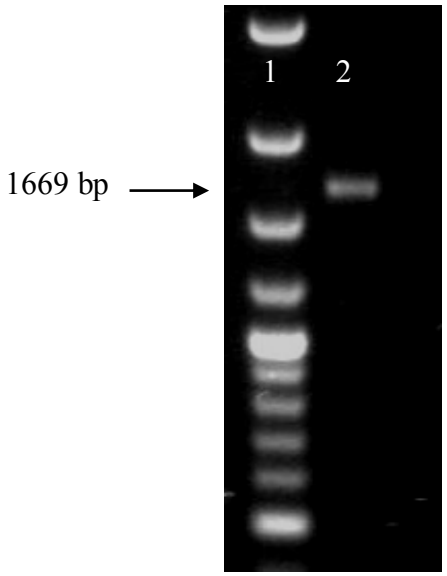


Figure 5.32 Amplification of barley *Fdh* ORF. Coding fragment of *HvFdh* incorporating BamHI restriction site at both ends was amplified by primer set d1057F7/R7. The sequence of full-length *HvFdh* is shown in Figure 5.6 and the location of primers are marked on the sequence. Lane1: DNA ladder, Lane 2: PCR product.

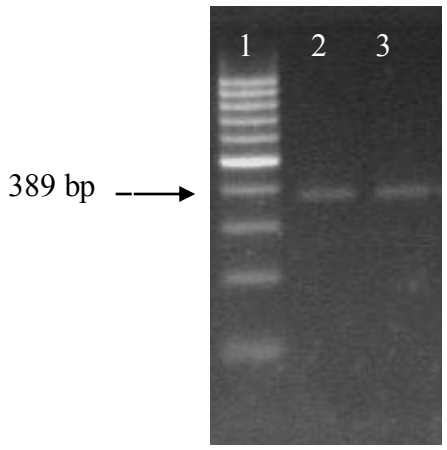


Figure 5. 33 Amplification of RNAi fragments. Sense and antisense fragments were amplified by primers d1057F10/R10 (sense) and d1057F11/R11 (antisense) for cloning into pHannibal vector. Lane 1: DNA marker, Lane 2: sense fragment; Lane 3: antisense fragment.

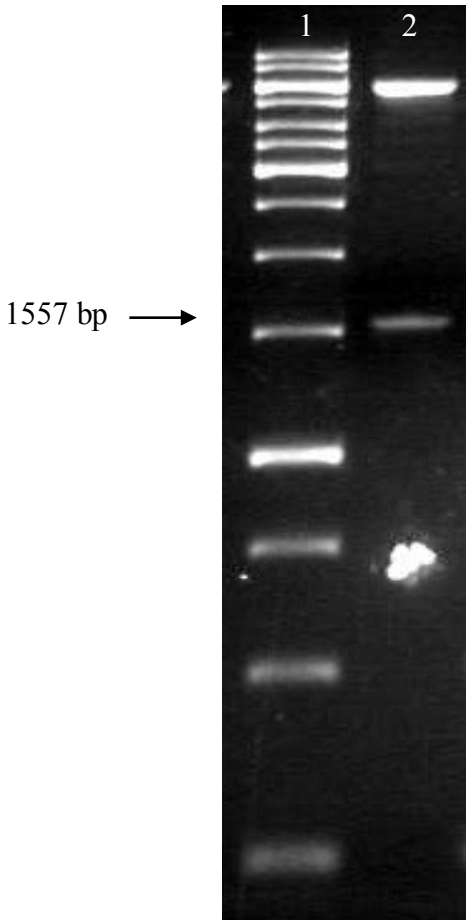


Figure 5.34 Restriction digest of pHannibal vector containing Fdh RNAi fragments. Plasmid region containing sense and anti-sense region of the *HvFdh* gene was cut from pHannibal by BamHI restriction and purified for cloning into pPZPUbi. Lane 1: marker, Lane 2: excised fragment containing sense and anti-sense fragment for RNAi (bottom band) and pHannibal vector backbone (top band).

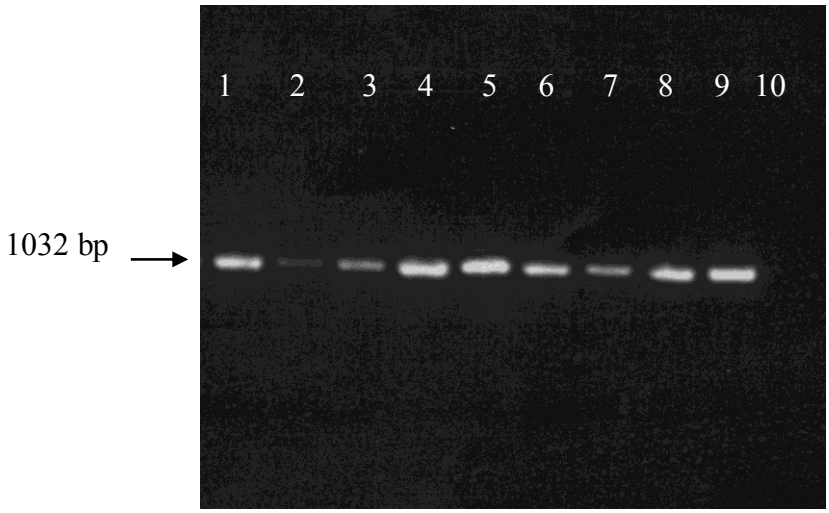


Figure 5.35 Validating *HvFdh* over-expression transgenic lines. Presence of transgene was verified by amplifying co-transformed hygromycin resistance gene by primer pair HygF/R. Lanes 1-8: HvFdh over-expression T_0 lines, Lane 9: pHvFdhSE, Lane 10: wild type plant.

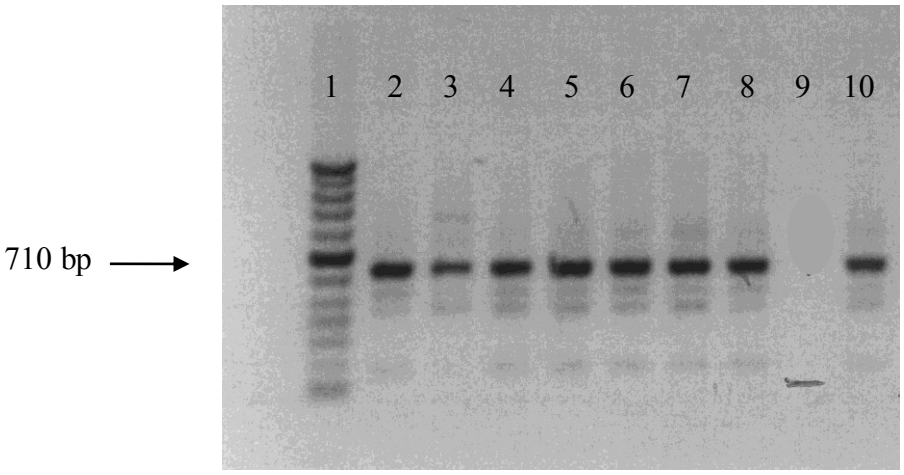


Figure 5.36 Vector anchored validation of *HvFdh* over-expression lines. The presence of pHvFdhSE in transgenic T_0 plants was verified by amplifying a 710 bp fragment by d1057F8 gene specific and vector derived PZPF primer combination. Lane 1: DNA marker, Lanes 2-8: transgenic plants, Lane 9: wild type plant, Lane 10: pHvFdhSE vector template.

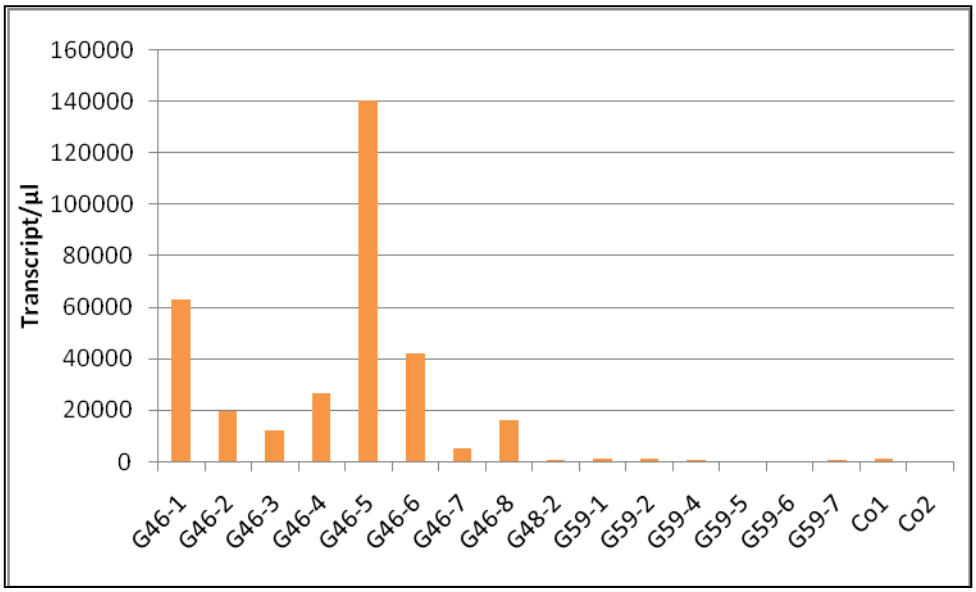


Figure 5.37 *HvFdh* transcript analysis in T_1 transgenic lines. Primer sets q1057F1/R1 was used to measure *HvFdh* transcripts in transgenic lines. The data were normalised against *SF400* and *SF427* mRNA levels. G46: over-expression, G48-2: RNAi, G59: antisense lines. Co1 and Co2 are nontransgenic progeny of G46-8 and G46-3 lines, respectively. Data are mean values of duplicates in the PCR.

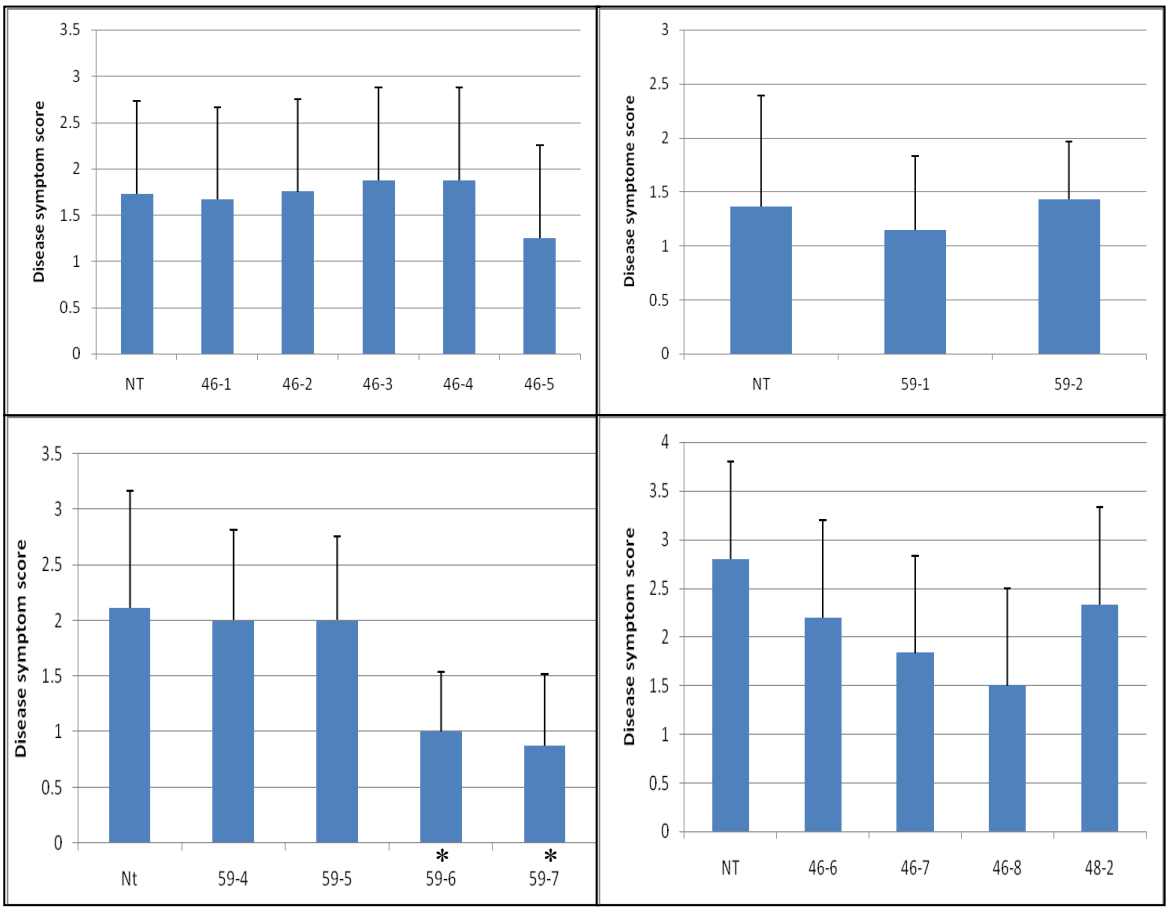


Figure 5.38 Scoring *HvFdh* transgenic lines for Scald symptoms. Eight T_1 plants from each line inoculated with *R. secalis* strain SA6 and scored for disease symptoms 14 days post-inoculation. The experiment was repeated twice. Controls are non-transgenic progeny of the lines (NT). Lines 46, 47 and 59 represent over-expression, RNAi and antisense lines, respectively. Student's t-test was used to determine significance of difference. Significant differences occurred in both experiments for the same line are reported. * indicates significant difference to control plants at 95% confidence.

Figure 5.39 Drought tolerance experiments of barley *Fiddlehead* transgenic lines. Eight T_1 plants from each line were grown in two separate pots (4 plants/pot) which were transgenic and non-transgenic progeny. Pots were randomly rotated during growth. (A) Variation in the onset of wilting among pots. (B) Variation in recovery after watering was observed among different pots and plants in the same pot.

Figure 5.40 Phenotype of *Fdh* antisense lines. Some T_1 plants transformed with pHvFdhAS construct had three heads emerging from one point in the inflorescence.

5.4 Discussion

It is well documented that, in addition to development, the plant cuticle composition affects the interaction of a plant with pathogens and its abiotic stress tolerance. In plants *Fiddlehead* (*Fdh*) encodes a putative FAE that takes part in the synthesis of VLCFA, the precursors of waxes covering the epidermis. In the study reported in this chapter, isolation and molecular characterisation of barley *Fdh* was undertaken and various functional genomics approaches were used to analyse its function and role in stress tolerance.

5.4.1 Analysis of the *Fdh* gene

Cloning genomic sequences showed that *HvFdh* lacks any intron in its coding region. In contrast, orthologs of *Fdh* in Arabidopsis and *Antirrhinum majus* each contain two introns of 900 and 200 bp, respectively (Efremova *et al.*, 2004). According to database annotations, HvFdh belongs to the β-ketoacyl-CoA synthase family of proteins which catalyse condensation reactions in the VLCFA synthesis pathway and its product(s) contribute to cuticular wax composition (Pruitt *et al.*, 2000; Yephremov *et al.*, 1999). Further investigation focused on to obtain supporting evidence for its FAE function.

5.4. 2 Evidence for function

The genomic organisation of *HvFdh* was determined by southern blot analysis. Multiple bands weakly hybridised to the full-length probe indicated the existence of multiple genes sharing some regions of similarity in their nucleotide sequence. The result is in agreement with the fact that sequential acyl chain extensions during VLCFA synthesis are carried out by several distinct elongases with unique substrate chain length specificities. Selectivity of the elongase condensing enzyme specifies the elongation reaction (Millar and Kunst, 1997). The conclusion is also supported by searching TIGR barley Transcript Assemblies (Section 2.31) which resulted in retrieving three contigs sharing a similar domain with the *Fdh* sequences (data not shown).

Several lines of evidence suggest fatty acid elongation occurs in the endoplasmic reticulum. When the GFP-tagged AtCer6 condensing enzyme was transiently expressed in the epidermis of *Nicotiana benthamiana*, the fluorescence appeared in an ER specific

pattern. In addition, in the *cer6* mutants, a proliferation of the smooth endoplasmic reticulum in epidermal cells suggested a build up of blocked substrate at this site (Kunst and Samuels, 2003). Elongation activity has also been demonstrated in the microsomal fractions of epidermal cells which carried ER marker enzymes (Lessire *et al.*, 1985) and in the microsomal fraction of cell extracts (Xu *et al.*, 2002). Moreover, proliferation of ER membranes was demonstrated when wax synthesis was switched on by light in sorghum cork cells (Jenks *et al.*, 1994). Other studies also support ER localization of elongase enzymes. For example, in *S. cerevisiae* the VLCFA enoyl reductase has been shown to reside in the ER membrane (Kohlwein *et al.*, 2001). ER localisation of β-ketoacyl reductase required for VLCFA production has also been suggested (Beaudoin *et al.*, 2002). In this study, the subcellular localisation of the *Fdh* gene product was determined using a GFP fusion construct transiently expressed in barley epidermal cells. The result obtained suggests that it is indeed an ER localised protein. The pattern of fluorescence is quite similar to that of ER localised TGBp3:GFP in tobacco leaves (Samuels *et al.*, 2007) and WCSP1:GFP (Nakaminami *et al.*, 2006) in wheat epidermal cells. The ER localisation supported the *in silico* prediction and is in agreement with the protein having two transmembrane regions predicted by the PredictProtein algorithm.

A FAE function was established for HvFdh. The specific activity of the protein was addressed by phylogenetic analysis, which can provide a mean to assign a putative function for newly cloned genes based on the function of experimentally characterised members in a clade. The collection, multiple alignment and phylogenetic analysis of Fdh-like and β-ketoacyl-CoA synthases from different species revealed a high level of identity. Sequence comparison indicated the presence of four distinct subgroups of *Fdh* related genes in plants independent of botanic classification. The barley Fdh analysed in this study is related to group I, which includes homologues from Arabidopsis, *Antirrhinum majus*, *Gossypium hirsutum*, *Tropaeolum majus* and *Oryza sativa*. Mis-expression of the *AtFdh* in Arabidopsis seeds under the FAE1 promoter increased C_{18} and C_{20} fatty acids in expense of C_{16} suggesting increasing elongation of C_{18} and C_{20} fatty acids (Efremova *et al.*, 2004). Because the gene was expressed in seeds that may lack the machinery for synthesising longer chain fatty acids, the epidermal specific function of the gene product still remains unknown. The protein encoded by *AtFdh* was shown to prevent contact mediated organ fusion in Arabidopsis (Yephremov *et al.*, 1999).

Some of the proteins in other subgroups have also been experimentally shown to be involved in VLCFA synthesis, although, their substrate, tissue specificity and products differ widely. For instance , AtFAE1, BnFAE and LdFAE1 from subgroup II and III catalyses C_{20} and C_{22} chain length VLCFA synthesis in Arabidopsis (Millar and Kunst, 1997), *B. napus* (Han *et al.,* 2001) and *L. douglasii* (Cahoon *et al.*, 2000) seeds, respectively. Epidermis-specific AtKCS1 (Todd *et al.*, 1999), AtCut1/Cer6 (Millar *et al.*, 1999) and root specific LfKCS45 (Moon *et al.*, 2004) from subgroup II and IV are required for the elongation of fatty acyl chains longer than C_{26}. Arabidopsis mutants with complete loss of KCS1 expression showed a decrease of 80% in levels of C_{26}- C_{30} wax alcohols and aldehydes. Contrary to Arabidopsis FAE1 and Cut1 mutants, *Fdh* knocked out plants did not show a severe glossy stem phenotype which is indicative of reduced surface wax load (Yephremov *et al.*, 1999). Two fatty acid elongases from group IV MpFAE2 (Kajikawa *et al.*, 2003b) and MpFAE3 (Kajikawa *et al.*, 2003a) are involved in elongating C_{18} to C_{20} and C_{22} fatty acids.

The phylogenetic analysis indicates that FAEs with different specificities can group together which makes it difficult to speculate on substrate specificity of uncharacterised members. However, the functional data for characterised homologous proteins makes it possible to speculate on enzyme activity for barley Fdh as a condensing enzyme. The active-site cystein of HvFdh at position 257 (Fig.5.8) is conserved in all the sequences. This cystein has been shown to be necessary for the condensing activity of AtKCS1 (Ghanevati and Jaworski, 2001).

Tissue series data from barley cultivar Morex (Druka *et al.*, 2006) were used to determine temporal expression pattern of the barley *Fdh* transcript accumulation during developmental stages. Developmentally regulated expression of *HvFdh* was found to be in agreement with its putative function as a VLCFA synthase. For instance, it was not expressed in roots a wax-free tissue (Fig. 5.22) and its mRNA level was also lower in anthers, embryo 22 DAP, embryo during seed germination and root tissues in comparison with other tissues. This pattern is consistent with the fact that these tissues do not have a waxy epidermis. Other FAE such as *Cer6* are highly transcribed exclusively in the epidermal cells of the Arabidopsis shoot throughout development (Hooker *et al.*, 2002). The only exception to the epidermal expression of *Cer6* was observed in the anthers nearing maturity similar to barley anthers.

Fdh in Arabidopsis and *A. majus* is also specifically expressed in epidermis tissue of the corresponding plant and when their promoter was exchanged to express a GUS/GFP reporter gene in the other species (Efremova *et al.*, 2004; Yephremov *et al.*, 1999) similar tissue specific expression were observed. The epidermis-specific *AtFdh* gene is implicated in the control of a barrier function of the epidermis (Lolle *et al.*, 1997; Pruitt *et al.*, 2000; Yephremov *et al.*, 1999). Suppression of this function in *fdh* mutants results in ectopic postgenital organ fusions, allows wild type pollen to germinate on the leaf epidermis of *fdh* mutants and changes cell wall and cuticular permeability (Lolle and Cheung, 1993; Lolle *et al.*, 1992). In this work barley *Fdh* expressed under the control of the Arabidopsis *Fdh* promoter was able to complement the Arabidopsis *fiddlehead* mutant, containing a two bp transposon footprint, indicating functional orthology. Wether *HvFdh* also shares a similar pattern of expression will require further investigation by *in situ* hybridization. Alternatively, isolation of the *HvFdh* promoter and expression of a reporter gene under its promoter in barley or Arabidopsis and *A. majus* transgenic plants may provide clues to the conservation of *Fdh* expression among plants.

5.4.3 Development of an *AtFdh* genotyping technique and promoter analysis

Genotyping the *Fdh* locus in Arabidopsis was an essential requirement for complementation for two reasons. Firstly, homozygous *fdh* is infertile (Pruitt *et al.*, 2000) which prevents transformation of those plants by the flower dipping method, so heterozygous plants need to be transformed. Secondly, homozygous mutant progeny transformed with the complementation construct need to be identified for phenotypic analysis. The difference between wild type and mutant allele is the existence of two extra nucleotides as a transposon footprint. The genotyping was previously done by amplifying the flanking region of the transposon footprint by PCR followed by analysing the product using DHPLC instrument (Efremova *et al.*, 2004). This method is expensive and time-consuming due to the use of additional post-amplification analysis. Here, a multiplex PCR-based genotyping method was devised that only requires post-amplification gel analysis for identifying the wild type and mutant alleles. This was achieved by exploiting the primer-template pairing dG and introducing suitable miss-match nucleotides in the

primer sequence at the flanking site of the footprint, which resulted in amplification of the specific allele with a defined length for scoring.

In this study, the expression of GFP by the *AtFdh* promoter region in transiently transformed Arabidopsis epidermal cells was demonstrated. Common transcriptional regulation of *Fdh*-like genes was shown in protodermis- and epidermis-specific activity of the *AtFdh* and *Antirrhinum majous Fdh* (AFI) promoters in reciprocally transformed Arabidopsis and *A. majous* plants (Efremova *et al.*, 2004). The promoter-GFP reporter construct produced in this study can be used to examine the activity of *AtFdh* promoter in barley or other plants.

5.4.4 Expression analysis

Temporal and spatial expression patterns of genes in response to a stress can provide insight to their possible role in stress tolerance. The transcripts of *Fdh* were up regulated in both compatible and incompatible interactions from 6-12 hours post inoculation with *R. secalis* in leaf tissues. In the susceptible cultivar, however, after 12 h the transcript level was returned to a similar level of control plants. *R. secalis* penetrates the cuticle 12 hours after inoculation (Ayesu-Offei and Clare, 1970) which indicates that expression might be suppressed by pathogen effectors in susceptible cultivar. Affymetrix GeneChip hybridisation data showed that in Mla6-mediated *B. graminis* resistance a transient down-regulation was followed by up-regulation at 8 and 16 hpi in resistant and susceptible interactions, respectively. However, at 16 hpi parallel to penetration and growth of the fungus inside plant tissue (Clark *et al.*, 1993), the transcript was accumulated in the compatible interaction whereas in the incompatible interaction it was down-regulated. In contrast, down-regulation of transcripts in response to *F. graminearum* and mlo-mediated resistance was observed. *F. graminearum* develops very slowly and increased fungal development and activity has been observed 48 hpi (Boddu *et al.*, 2006) which was concomitant with down-regulation of *Fdh* expression. Assuming similar function of *Fdh* in Arabidopsis and barley in increasing permeability of epidermis with down regulation of transcripts (Lolle *et al.*, 1997), it can be postulated that decreased transcripts of the *Fdh* is a positive defensive response to increase delivery of anti-microbial compounds to halt pathogen ingress. Alternatively, *Fdh* could be down regulated by the pathogen effectors to increase penetration rate and delivery of virulence factors. This reaction would be

analogue to germination and growth of pollens in epidermis of the Arabidopsis *fiddlehead* mutant due to increased permeability of epidermis (Lolle and Cheung, 1993; Lolle *et al.*, 1992). Further experiments to monitor the germ tube growth and penetration of fungus into leaves of transgenic plants with altered *Fdh* expression will help to test these hypotheses. Alternative to transgenic approach, temporal examination of chlorophyll leakage in both types of interactions in wild type plants would reveal if permeability of host cells changes during interaction.

As it was discussed in the introduction abiotic stresses can affect the composition and amount of waxes in the cuticle. The data from exposure of barley plants to salt in this work and also the microarray data revealed that the transcript levels of *Fdh* were not greatly influenced by salinity. The finding implies that barley plants exposed to salinity did not respond to decreases in external water availability by increasing their epidermal wax load through synthesis of more epicuticular waxes which has been shown to happen in order to reduce non-stomatal water loss in plants such as peanut and jojoba (Mills *et al.*, 2001; Rao *et al.*, 1981). The result found here is in accordance with Fricke and colleagues (2006) who found no difference in wax density in barley grown under control and saline conditions.

Frost and drought treatments effect on expression was a decrease in transcript levels in a cultivar-dependent manner that may be due to different physiological stages of the cultivars. However, rewatering plants reversed the decrease in transcript levels by drought treatment. This pattern could be due to growth arrest in drought treated plants and the resumption of their growth after rewatering which accompanies synthesis of VLCFA to cover the surface of newly formed cells. Among the drought treated cultivars transcript levels of Barque73 accumulated to control levels after rewatering. This cultivar is considered to have an improved drought tolerance and the expression pattern could be due to quick recovery of the cultivar from exposed stress.

5.4.5 Transgenic plants

The reverse genetics approach to over-express or suppress *Fdh* provided hints about the relevance of the encoded protein to the defence against pathogens. Q-PCR analysis showed that the over-expression lines had a high level of transgene expression whereas the

suppression lines showed a low level similar to wild type plants. However, as the expression level under normal conditions was low in whole leaf tissues, the expression analysis of suppressed lines under conditions causing elevated level of expression such as after inoculation may reveal if the gene is down regulated in suppression lines in comparison to wild type.

The role of *Fdh* in plant defence has not been investigated. Disruption of *Fdh* is thought to alter the composition of the cuticular waxes. Cuticle chemical composition and physical structure affects plant-pathogen interaction by either providing a barrier, controlling exchange of pathogen effectors or plant defence molecules or providing stimulation for germination and differentiation of pathogen structures. The analysis of scald symptoms demonstrated significantly less symptom development in two antisense lines out of six. Further analysis of wax composition of these lines will be helpful to propose a model for the observed phenotype. On the microscopic level, the study of germination and penetration of spores on epidermal cells in transgenic barley will be useful to investigate the effect of modified expression on *R. secalis*. Such studies may reveal approaches for modifying plant surface features that can confer novel forms of disease resistance. For example, *Botrytis-resistant 1* (*bre1*) is an Arabidopsis mutant that has a permeable cuticle resulting in resistance to *Botrytis cinerea*. Cloning *bre1* (Bessire *et al.*, 2007) revealed that it is the long-chain acyl-CoA synthase 2 (LACS2) shown to be involved in cuticle development and to be essential for cutin biosynthesis (Schnurr *et al.*, 2004). Comparison of *bre1/lacs2* with the other cuticle mutants such as *lacerate* (Wellesen *et al.*, 2001) and *hothead* (Krolikowski *et al.*, 2003; Kurdyukov *et al.*, 2006) revealed that an increased permeability of the cuticle facilitated perception of putative elicitors in potato dextrose broth, leading to the presence of antifungal compound(s) at the surface of Arabidopsis plants that confer resistance to *B.cinerea.*

Wxp1 (Wax production) from the model legume *Medicago truncatula* (Zhang *et al.*, 2005) have been shown to increase production of the C_{30} alcohol in leaves and its overexpression in transgenic alfalfa (*Medicago sativa*) activated wax production simultaneously to the induction of three *FAE* genes. Transgenic over-expression plants showed enhanced drought tolerance demonstrated by delayed wilting after cession of watering and quicker recovery when the dehydrated plants were rewatered. The drought tolerance of transgenic barley lines was evaluated by the time taken to wilt after cession of watering and recovery

after rewatering. There were significant differences (19 days) in the onset of wilting among pots exposed to drought. Such differences have been interpreted as indicator of drought tolerance in many publications where single plants or the plants of the same genotype were grown in each pot. In this study no difference was observed among the plants grown in the same pot. As some pots had non-transgenic progeny the differences cannot be attributed to drought tolerance. Those differences can be explained by possible environmental variation in the glasshouse and the differences in rate of plant growth as the slow growing plants extract less water from the soil and stay green for a longer period. However, the rate of recovery after resuming watering in two over-expression lines was faster than non-transgenic progeny.

Among antisense suppressed lines, developmental phenotypes such as emergence of three spikes from one inflorescence and abnormal inflorescence were observed. Whether this phenotype was caused by knockdown or is associated with the integration site resulting in modifying or inactivating other gene(s) will require further investigation. The antisense lines did not show a lower level of transcript in primary leaves. The overall transcript was low in whole leaves of wild type progeny, which makes detection of knockdown difficult. Quantifying gene expression in inflorescence tissues will be required to examine the efficacy of knockdown and its relevance to the phenotype.

In this study strong evidence for HvFdh function as a condensing enzyme involved in VLCFAS was shown. It can be postulated that if *Fdh* is directly or indirectly involved in FA metabolism, loss of its activity in down-regulated transgenic lines should result in an accumulation of its substrate and a decrease in its product, and vice versa in over-expression lines when compared to the wild type plants. Defects at specific points in the elongation by FAE are features of many of the *eceriforum* (Koornneef et al., 1989; McNevin et al., 1993) and *glassy* (Hannoufa *et al.*, 1993) mutants, resulting in accumulation of shorter products than found for wild type plants. For instance, the *kcs1-1* and *cer6* mutations affect each elongation step between C_{24} and C_{30}, with reduced levels of the corresponding C_{26}–C_{30} aldehydes and primary alcohols (*kcs1-1*, *cer6*) and C_{29} alkanes, secondary alcohols and ketones (*cer6*). Comparative fatty acid analysis of transgenic barley plants produced in the present study will be useful to identify the biochemical role of HvFdh in VLCFA and wax biosynthesis in barley. Other experiments that can be used to further characterise the transgenic lines produced in this study are

chlorophyll efflux analysis to reveal if the rate of chlorophyll extraction from leaves is altered in the transgenic plants, indicating altered epidermal permeability in transgenic leaves similar to Arabidopsis mutants (Yephremov *et al.*, 1999; Zhang *et al.*, 2005) and transgenic leaves over-expressing *Wxp1* which showed reduced water loss and chlorophyll leaching (Zhang *et al.*, 2005).

Chapter 6: Summary and future directions

6.1 Summary of experimental results

The control of disease and managing abiotic stresses in agricultural crops is a major challenge to primary producers worldwide. They cause significant losses every year in a broad range of crop species. Agricultural crops are particularly vulnerable to the spread of disease and unfavourable environmental conditions because they are usually grown as monocultures with little genetic variation. Although management practices may help prevent the onset or spread of disease through a crop and minimise damage due to abiotic stresses, the spread of soil and water-borne disease is often difficult to combat. Thus, there have been extensive studies on the plant's own compounds, both to elucidate their function and to examine their role towards plant protection. The overall aim of this thesis was to investigate the potential role of three differentially expressed barley candidate genes with regard to their role in the defence against pathogen attack and protection from damage through abiotic stresses based on their putative function. In addition, the results were expected to provide experimental evidence to evaluate candidate gene approaches based on expression profile and gene annotation.

Isolation and characterisation of candidate genes is an established approach to understand plant stress response and adaptation. This analysis started by selection of three partial length cDNA clones from a SSH library based on their putative function. The library was enriched for differentially expressed genes in the epidermis of resistant and susceptible near isogenic barley cultivars inoculated with *R. secalis*. Initially, attempts were focused on isolating full-length cDNA clones using bioinformatics and RACE techniques. Three cDNAs encoding pathogenesis-related proteins family members, two cDNAs representing galactinol synthase genes and one cDNA encoding a putative fatty acid elongase gene and their corresponding genomic sequences were isolated and characterised. Their putative functions were determined based on previously characterised homologues. Amino acid sequences deduced from the cDNAs matched those of PR-17 family genes, Galactinol synthase genes and the Fiddlehead and were therefore designated as the corresponding cDNA of *HvPR-17a1,c,d*, *HvGolS1,2* and *HvFdh.*

Southern blot hybridisation showed that the barley PR-17s is encoded by a small gene family. Three members were cloned and one member was found in the public domain. Two of the isolated genes are novel genes. The analysis also indicated existence of two

GolS genes in the barley genome and subsequently two *HvGolS* members were isolated. DNA gel blot analysis suggested a single gene corresponding to *HvFdh*. The presence of multiple genes for PR-17 and GolS in barley has raised important questions regarding their function such as, if the function of family members overlaps with one another or if they are expressed in a specific manner. These questions were addressed in part by the analysis of gene expression patterns in plants under specific stress conditions and during development.

Isolation and cloning of the genes were followed by detailed transcript analysis using Q-PCR, Northern blot and *in silico* analysis of publically available microarray data in order to establish a relationship between transcript levels, a particular stress and putative function. The results showed that representing members from each family (*PR-17, GolS*) were expressed earlier and with higher magnitude in the epidermis of the resistant cultivar than the susceptible one in *R. secalis* inoculated plants. This finding correlates higher expression of these genes with the resistant phenotype. Expression profiling was also used as an indicator of the involvement of these genes in abiotic stress responses of barley. Isolated genes exhibited diverse patterns of expression in response to a variety of abiotic stresses. Tissue-specific transcription of some genes during barley development suggested that the biochemical processes incorporating each *PR-17* and *GolS* gene was affected in a different manner and are indicative of each member having shared function in specific tissues. Moreover, the analysis revealed some patterns consistent with their putative function and results of other studies. Some expression profiles were not in agreement with previous findings in other plants indicating differential roles of the homologous proteins in different species and their stress adaptation mechanisms. Detailed transcript abundance data provided us with some useful clues in relation to the function of the isolated cDNA clones. Other methods were subsequently used to confirm the proposed roles.

Subcellular localisation of encoded proteins was investigated using transient expression of GFP fusion in epidermis cells followed by confocal laser scanning microscopy. The results obtained were in agreement with *in silico* predictions and their putative function. HvPR-17c, HvGolS1 and HvFdh were localised in the apoplast, cytoplasm and endoplasmic reticulum, respectively.

A comprehensive list of homologous genes from other species was compiled for each gene by using public databases. Analyses of phylogenetic relationships and multiple sequence

alignments of the homologous proteins provided a detailed profile of the copy number complexity of the genes in plants and other species. The analysis gave further clues to their function and conserved regions of the proteins based on functionally characterised members in each subgroup. An important finding of this work regarding PR-17 was the identification of similar sequences in plants, bacteria and fungi that raised questions about naming their conserved domain as Plant Basic Secretory proteins. One phylogenetic clade comprised sequences from plants, bacteria and fungi indicating that member proteins are ancient and arose either prior to, or early in the evolution of the orders.

Few of the putative functions ascribed to plant proteins have been verified experimentally. Mutant complementation and heterologous expression are most direct approaches towards the functional analysis of proteins. A construct in which the coding region of the barley *Fiddlehead* gene was driven by the *AtFdh* promoter, successfully complemented the Arabidopsis *fiddlehead* mutant indicating functional orthology between these genes from dicots and monocots. HvPR-17 anti-fungal properties were investigated by heterologous protein expression in *E. coli* and subsequent *in vitro* bioassays using purified protein under different pH conditions and with or without DDT against a number of plant pathogens. However, no anti-fungal activity was observed. The next approach was to investigate gene function via stable transformation of barley.

Currently the most promising tool for defining the function of unknown genes and the level of potential redundancy are those based upon transformation techniques and generation of an obvious phenotype. A frequently used approach includes loss- and gain-of-function approaches. In this project, constitutive expression, anti-sense and dsRNAi were used to target transcripts of the isolated cDNA clones. More than fifty transgenic barley lines were regenerated which over-express or suppress the genes. The analyses of the transgenic progeny exhibited some interesting developmental phenotypes such as variegation and albinism in PR-17 over-expressers and plants with altered reproductive morphology in HvFdh antisense lines. Among transgenic plants, lines with increased resistance toward scald were observed. Plants with a higher rate of recovery after water stress were seen in *HvFdh* over-expression transgenic lines. These lines are awaiting further experiments to investigate the effect of altered expression in conferring biotic and abiotic stress tolerance as well as metabolite analysis.

6.2 Future work

The functional analysis of the isolated genes was the focus of this project. Considerable progress was made in cloning and characterising the genes using different molecular and bioinformatics techniques. A definitive role for each gene is yet to be assigned.

Results presented from the heterologous expression of HvPR-17c protein did not link it to anti-fungal activity. Anti-microbial activity of PR-17 could be further investigated by *in vitro* assays against other plant pathogens. It may result in identifying specific pathogens, which are adversely affected by the protein.

Transgenic lines were investigated for macroscopic developmental phenotypes, scald resistance and drought tolerance but they present opportunities for further studies such as:

- Microscopic and genetic analysis of lines identified as having developmental phenotypes
- Inoculation of the transgenic lines with different barley pathogens followed by scoring symptom development and microscopic studies to identify the early stages of pathogen development on the host that might be affected.
- Frost, and salinity tolerance analysis on *HvFdh* and *HvGolS1* lines
- Chlorophyll and electrolyte leakage analysis on *HvFdh* and *HvGolS* transgenic tissues exposed to abiotic stress in order to assess a possible protection offered to cells
- Long chain fatty acid composition analysis on *HvFdh* lines to deduce the function of the gene
- Galactinol and RFO analysis on *HvGols1* lines

6.3 Concluding remarks

As the number of candidates identified through expression based methods can be high, the existence of fast and reliable functional analysis tools are highly desirable to quickly demonstrate their role in crop protection. A transient single cell assay system has been established (Nielsen *et al.*, 1999) for functional analysis of putative defence related genes against biotrophic pathogens such as powdery mildew and rusts. Development of such

system(s) would greatly accelerate the analysis of plant genes which are induced by necrotrophic pathogens. However, to date no such system has been established for necrotrophic fungi as they require intercellular growth and interact with multiple cells during infection.

Therefore, in this project, a small subset of genes from the subtraction library were selected for further analysis. The inclusion of the genes was based on their putative function and how they might affect plant-pathogen interaction. A gene encoding a novel pathogenesis-related protein which might exhibit antimicrobial activity, a putative fatty acid elongase with possible role in plant cuticle composition (the initial site of interaction) and a galactinol synthase which could alter osmotic properties of the cells and thereby affect pathogen nutrient acquisition were chosen for further analysis.

Among transgenic plants some lines showed reduced disease symptoms (two antisense lines of *Fdh*, one RNAi line of *HvPR-17c*) and some increased symptoms (two RNAi and one over-expression *Gol*S*1* lines). The GolS transgenic lines results are contradictory but these lines are interesting and will require biochemical analysis of raffinose family oligosaccharides to link the observed phenotype to the metabolite changes associated with altered gene expression. The transgenic lines evaluated for phenotype were T_1 generation and there is possibility that some phenotypes might be due to insertion site effect.

Two of the selected genes were also hypothesised to have a role in abiotic stress tolerance. *HvFdh* could affect drought tolerance as it would alter cuticle properties and thereby non-stomatal water loss. Although the drought tolerance of the transgenic lines were not different from non-transgenic progenies, the recovery rate of two over-expression lines after rewatering was faster. *HvGolS1* altered expression (assuming an alteration in RFO levels) did not influence drought tolerance although such a function has been shown in Arabidopsis lines over-expressing *AtGolS*.

AS plants often use multiple pathways to resist pathogen invasion and abiotic stress condition, the choice of three genes with different putative function seems well justified. The experiments in this study showed that altered expression of the genes in transgenic lines affected stress tolerance positively or in some instance negatively. If further experiments confirm that the observed phenotypes are a result of biochemical changes due

to the altered activity of the encoded proteins and not the insertion site(s), these genes can be utilised in breeding stress tolerant cultivars. Such aim can be achieved in different ways. Generation of transgenic plants with single or multiple transgenes including the genes discovered in this study or known from literature can be directly used to increase stress tolerance in elite cultivars. Naturally occuring accession lines that show differences in the expression of the gens can be used in conventional or molecular assisted breeding as a donor. Knock out lines for the genes identified in TILLING populations can also be used to introgress the desirable phenotypes observed in knock down transgenic lines.

As mentioned earlier, this project's scope was to define a role for the isolated genes in barley defence and abiotic stress tolerance as well as to investigate their biochemical function. The aims were achieved using multiple sequence alignments, transcript analysis under different conditions, protein subcellular localisation, *in silico* analysis, mutant - complementation, transgenic techniques and *in vitro* bioassay. The project also demonstrated strengths and weaknesses of both expression and annotation based candidate gene approaches. Interesting results were obtained through the analysis of transgenic lines and conclusions that are more definitive can be made by further analysis of over fifty transgenic lines made available through this project.

Appendices

Appendix A: Cycling programs

Cycling program 1

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 68°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 4 cycles

Step 6, 94°C for 30 sec

Step 7, 63°C for 30 sec

Step 8, 72°C for 1 min

Step 9, return to step 6 for 34 cycles

Step 10, 72°C for 10 min

Step 11, 12°C for ∞

Cycling program 2

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 60°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 19 cycles

Step 6, 94°C for 30 sec

Step 7, 58°C for 30 sec

Step 8, 72°C for 1 min

Step 9, return to step 6 for 14 cycles

Step 10, 72°C for 10 min

Step 11, 12°C for ∞

Cycling program 3

Step 1, 95°C for 1 min

Step 2, 94°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 39 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 4

Step 1, 95°C for 1 min

Step 2, 94°C for 30 sec

Step 3, 65°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 5

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 63°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 6

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 53°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 9 cycles

Step 6, 94°C for 30 sec

Step 7, 68°C for 30 sec

Step 8, 72°C for1 min

Step 9, return to step 6 for 24 cycles

Step 10, 72°C for 10 min

Step 11, 12°C for ∞

Cycling program 7

Step 1, 95°C for 5 min

Step 2, 95°C for 20 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1.20 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 14°C for ∞

Cycling program 8

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1.30 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 69°C for 30 sec

Step 8, 72°C for 1.30 min

Step 9, return to step 6 for 29 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program 9

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 60°C for 30 sec

Step 4, 72°C for 2.30 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 70°C for 30 sec

Step 8, 72°C for 2.30 min

Step 9, return to step 6 for 29 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program10

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 53°C for 30 sec

Step 4, 72°C for 1.30 min

Step 5, return to step 2 for 9 cycles

Step 6, 94°C for 30 sec

Step 7, 65°C for 30 sec

Step 8, 72°C for 1.30 min

Step 9, return to step 6 for 24 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program11

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 57°C for 30 sec

Step 4, 72°C for 2.30 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 66°C for 30 sec

Step 8, 72°C for 2.30 min

Step 9, return to step 6 for 25 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program 12

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1.1 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 70°C for 30 sec

Step 8, 72°C for 1.1 min

Step 9, return to step 6 for 24 cycles

Step 10, 72°C for 10 min

Step 11, 12°C for ∞

Cycling program 13

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 68°C for 30 sec

Step 4, 72°C for 1.30 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 14°C for ∞

Cycling program 14

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 68°C for 30 sec

Step 4, 72°C for 3 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 63°C for 30 sec

Step 8, 72°C for 3 min

Step 9, return to step 6 for 29 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program15

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 68°C for 30 sec

Step 4, 72°C for 2 min

Step 5, return to step 2 for 9 cycles

Step 6, 94°C for 30 sec

Step 7, 63°C for 30 sec

Step 8, 72°C for 2 min

Step 9, return to step 6 for 29 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program 16

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 68°C for 30 sec

Step 4, 72°C for 4 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 65°C for 30 sec

Step 8, 72°C for 4 min

Step 9, return to step 6 for 29 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program 17

Step 1, 95°C for 2 min

Step 2, 94°C for 30 sec

Step 3, 50°C for30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 18

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 64°C for30 sec

Step 4, 72°C for 1.5 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 19

Step 1, 95°C for 7 min

Step 2, 94°C for 30 sec

Step 3, 58°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 14°C for ∞

Cycling program 20

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 63°C for30 sec

Step 4, 72°C for 1.5 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 14°C for ∞

Cycling program 21

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 2 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 22

Step 1, 95°C for 2 min

Step 2, 95°C for 30 sec

Step 3, 62°C for 30 sec (54°C for pQE70)

Step 4, 72°C for 1.1 min

Step 5, return to step 2 for 9 cycles

Step 6, 95°C for 30 sec

Step 7, 70°C for 30 sec

Step 8, 72°C for 1.1 min

Step 9, return to step 6 for 24 cycles

Step 10, 72°C for 10 min

Step 11, 14°C for ∞

Cycling program 23

Step 1, 95°C for 3 min

Step 2, 94°C for 30 sec

Step 3, 62°C for 30 sec

Step 4, 72°C for 50 sec

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 24

Step 1, 95°C for 5 min

Step 2, 94°C for 30 sec

Step 3, 63°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 25

Step 1, 95°C for 7 min

Step 2, 94°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 26

Step 1, 95°C for 7 min

Step 2, 94°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 1.20 min

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Cycling program 27

Step 1, 95°C for 7 min

Step 2, 94°C for 30 sec

Step 3, 55°C for 30 sec

Step 4, 72°C for 30 sec

Step 5, return to step 2 for 34 cycles

Step 6, 72°C for 10 min

Step 7, 12°C for ∞

Appendix B: Hybridization probes' sequences

Sequence of *PR-17* 3' probe:

```
  1    GTGGCAGTGG AACGGGCAGG GCAAGGCGAA CGGCGGGCTC ATCGAGGGGA
 51    TCGCCGACTA CGTGCGGCTC AAGGCCGGGT TCGCGCCGGG GCACTGGGTG
101    AAGCCGGGGC AGGGCGACCG GTGGGATCAG GGGTACGACG TCACGGCGAG
151    GTTCCTCGAC TACTGCGACT CACTGAAGCC CGGGTTCGTC GCGCAGCTCA
201    ACGCCAAGAT GAAGAGTGGG TACACCGACG ACTTCTTCGC GCAGATTCTC
251    GGCAAGAACG TGCAGCAGCT GTGGCGGGAC TACAAATCCA AGTTTGGAGC
301    CTGAATACAC GATTAGCCTA CTTGACGGTG
```

Sequence of *PR-17* full-length probe:

```
  1    GCGACACAGG CACGTAGTAG CAACACCCAC TACAAAATGA AGCTTCAGGT
 51    AGCCACGGTC GCCTCCTTCC TCCTGGTGGC CTTGGCCGCG ACGGCCCAGG
101    CAGTGACGTT CGACGCGTCG AACAAGGCGT CGGGCACCTC CGGCGGCCGG
151    CGGTTCGAGC AGGCCGTCGG CCTCCCATAC TCCAAGAAGG TCCTCTCCGA
201    GGCCTCCGCC TTCATCTGGA AAACCTTCAA CCAGCGTGCC GTCGGCGACC
251    GCAAGCCTGT CAACGCAGTC ACCCTCGTCG TCGAGGACAT CAGCGGCGTC
301    GCCTTCACCA GCGCCAACGG CATCCACCTC AGCGCCCAGT ACGTCGCCAG
351    CATCTCCGGC GACGTCAAGA AGGAGGTGAC CGGCGTGCTG TACCACGAGG
401    CGACGCACGT GTGGCAGTGG AACGGGCAGG GCAAGGCGAA CGGCGGGCTC
451    ATCGAGGGGA TCGCCGACTA CGTGCGGCTC AAGGCCGGGT TCGCGCCGGG
501    GCACTGGGTG AAGCCGGGGC AGGGCGACCG GTGGGATCAG GGGTACGACG
551    TCACGGCGAG GTTCCTCGAC TACTGCGACT CACTGAAGCC CGGGTTCGTC
601    GCGCAGCTCA ACGCCAAGAT GAAGAGTGGG TACACCGACG ACTTCTTCGC
651    GCAGATTCTC GGCAAGAACG TGCAGCAGCT GTGGCGGGAC TACAAATCCA
701    AGTTTGGAGC CTGAATACAC GATTAGCCTA CTTGACGGTG TGATGGCATA
751    GCG
```

Sequence of *GolS1* 3' probe:

```
  1    CATCTACGAC GACGAGGGCC TCAACTACAA GCCTGCCGCC GACGAGGCCA
 51    CCGACCCGCT GCGTGCTGCC CTCGCCGAGG TCGTCGCCGT CAAGTCCTTC
101    CCGGCGCCCT CCGCCGCGTA GTCATCACGC GCCTGCCTTC CAGTTAGGCG
151    TATGTATTCT ACTAATAAAT ACTAGTAATA CGTAGGAAAG ATTTCGTCAG
201    TACTACGTAA TATCGTCGTC GATTTAGCAT CTACACGTTT TGGTATTGTA
251    GATCATCATC AATCGATCAT ATACAGTGTT CTTTTTCCGA TTGAGGTACG
301    TACGGCCACC ATAGTGTTTT TCGGTTTGTA TAAGGAAGGA GTATATTGTA
351    AACACGTACG TGCAAGCACA CGGTTGGTCC AAGTATGATT ATAGATCGAC
401    CTGCTACGTA CACGTATGCA TG
```

Sequence of *GolS1* full-length probe:

```
  1    CGCACAAGTC CACAGCACAC AGCAGACCCA TCCCATCCCA TCCATCCTTT
 51    GATTTGAAGC AAGACAAGAG GGACCGACCG AGCAAGCAAT GGCTCCCATG
101    CTCAAGCGGA TCGTGGAGGA CGAGCCCAAG AAGGCGGCGT ACGTGACCTT
151    CCTCGCCGGC TCCGGCGACT ACTGGAAGGG CGTGGTCGGC CTTGCCAAGG
201    GCCTCCGCGC CGTCAACTCC GCCTACCCGC TCGTGGTGGC CGTGCTCCCC
251    GACGTCCCCG AGGAGCACCG CCAGGAGCTG CTCAAGCAGG GCTGCGTCGT
301    CCGGGAGATC GTGCCCGTCT ACCCGCCGGA GAGCCAGACC CAGTTCGCCA
351    TGGCCTACTA CGTCATCAAC TACTCGAAGC TCCGCATCTG GGAGTTCGTG
401    GAGTACGAGA GGATGGTGTA CCTGGACGCG GACATCCAGG TGTACGACAA
```

```
 451    CATCGACCAC CTCTTCGACC TCGAGATGGG CAGCTTCTAC GCCGTCAAGG
 501    ACTGCTTCTG CGAGAAGACG TGGAGCCACA CCCGGCAGTA CGAGATCGGC
 551    TACTGCCAGC AGTGCCCCGA CAGGGTGGCG TGGCCGGAGC GCGAGCTGGG
 601    CGTGCCCCCG CCGCCGCTCT ACTTCAACGC CGGCATGTTC GTGCACGAGC
 651    CCAGCATGGC CACCGCCAAG GCCCTCCTCG ACAGGCTTGT CGTCACCGAC
 701    CCCACCCCGT TCGCCGAGCA GGACTTCCTC AACATGTTCT TCAGGGACGT
 751    GTACAAGCCC ATCCCGCCGG TGTACAACCT CGTGCTCGCC ATGCTCTGGA
 801    GGCACCCGGA GAACATCCAG CTCGGCGAGG TCAAGGTCGT CCACTACTGC
 851    GCCGCGGGTT CGAAGCCGTG GAGGTACACC GGCGAGGAGG CCAACATGGA
 901    CAGGGACGAC ATCAAGATGC TGGTGAAGAA ATGGTGGGCC ATCTACGACG
 951    ACGAGGGCCT CAACTACAAG CCTGCCGCCG ACGAGGCCAC CGACCCGCTG
1001    CGTGCTGCCC TCGCCGAGGT CGTCGCCGTC AAGTCCTTCC CGGCGCCCTC
1051    CGCCGCGTAG TCATCACGCG CCTGCCTTCC AGTTAGGCGT ATGTATTCTA
1101    CTAATAAATA CTAGTAATAC GTAGGAAAGA TTTCGTCAGT ACTACGTAAT
1151    ATCGTCGTCG ATTTAGCATC TACACGTTTT GGTATTGTAG ATCATCATCA
1201    ATCGATCATA TACAGTGTTC TTTTTCCGAT TGAGGTACGT ACGGCCACCA
1251    TAGTGTTTTT CGGTTTGTAT AAGGAAGGAG TATATTGTAA ACACGTACGT
1301    GCAAGCACAC GGTTGGTCCA AGTATGATTA TAGATCGACC TGCTACGTAC
1351    ACGTATGCAT G
```

Sequence of *Fdh* 3' probe:

```
   1    GCAACACCTC CAGCAGCAGC ATCTGGTACG AGCTGGCATA CCTGGAGGCC
  51    AAGGGTCGCG TCCGCCGCGG TGACCGCGTG TGGCAGCTCG CCTTCGGCTC
 101    CGGGTTCAAG TGCAACAGCG CCGTGTGGCG CGCCGTCGGA CGCGTGCGCC
 151    GCCCGTCCAG GAGCCCCTGG CTGGACTGCA TCGACCAGTA CCCGGCGCGC
 201    ATGGACGCCT GAGGCCTGAC CGATCGAGAC AAGCAGGCGC ATGCACCATG
 251    CAGGTGCTAA TAATTAAGCG AGACACGTCG CCGCATCCGC GTGGGTAAGT
 301    GCAGGCTAAT GGATTTGCAT GTAACTGCAT GCAGTGCCGC TGGTTAAGGT
 351    TTGAGCTTTG GTTAATTTGT GCTGGTGTTG TAGCAGTTGT CATCATTATT
 401    AAGGGTTTTA ATCATAGACT ACGTACGTAC GTACCATAAT GCGCAAGTGT
 451    ATGTGCGCGC TCTTGTGTGA
```

Sequence of *Fdh* full-length probe:

```
   1    CCAGGCCGCC ACCCAGCTGC GCCCAGCCAT GGCGCGGGAG GAGCTGTCGA
  51    CGGAGATCGT GAACCGCGGC GTGGAGCCGT CGGGCCCGGA CGCCGGGTCG
 101    CCGACCTTCT CGGTGCGCGT GCGCCGCCGG CTGCCGGACT TCCTGCAGTC
 151    GGTGAACCTC AAGTACGTGC GGCTCGGGTA CCACTACCTG CTGAGCCACG
 201    GCGTGTACCT GGCCACCATC CCGGTGATCG TGCTGGTGTG CGGCGCCGAG
 251    GTGGGCAGCC TCAGCCGCGA CGAGCTGTGG CGCAAGGTGT GGGACGAGGC
 301    TACCTACGAC CTCGCCACCG TGCTCGCCTT CCTCGCCGTC CTCGCCTTCA
 351    CCATCTCCGT CTACATCATG TCCCGCCCCA GGCCCATCTA CCTCATCGAC
 401    TTCGCCACTT ACAAGCCCGC CGACGAACTC AAGGTCTCCA AGGCGGAGTT
 451    CATCGATCTG GCGCGCAAGT CGGGCAAGTT CGACGAGGAG AGCCTTGCGT
 501    TCCAGGCGCG GCTGCTGGCC AAGTCCGGCA TCGGGGACGA GTCCTACATG
 551    CCGCGCTGCG TCTTCCAGCC CGACGCCAAC TGCGCCACCA TGAAGGAAGG
 601    CCGCGCCGAG GCCTCCGCCG CCATGTTCGC CGCGCTCGAC GAGCTCTTCG
 651    ACAAGTGCCG CGTCCGCCCC AAGGACGTCG GCGTCCTCGT CGTCAACTGC
 701    AGCCTCTTCA ACCCCACGCC CTCGCTCTCC GCCATGATCG TCAACCACTA
 751    CAAGATGCGC GGCAACATCC TCAGCTACAA CCTCGGCGGC ATGGGCTGCA
 801    GCGCCGGGGT CATCTCCATC GACCTCGCCC GCGACATGCT CCAGGCCAGC
 851    GGCGCCGGGC TCGCCGTCGT CGTCAGCACC GAGGCGGTGT CCTTCACCTG
 901    GTACGCCGGG AAGCGCCGCT CCATGCTCAT CCCCAACGCC TTCTTCCGCG
 951    CGGGCGCGGC CGCCGTGCTG CTGTCCAACC GCCGCAGGGA CTTCCGCCGC
1001    GCCAAGTACC AGCTGGAGCA CGTGGTGCGC ACGCACAAGG GCGCCGACGA
1051    CCGCGCCTTC CGGTCCGTGT ACCAGGAGGA GGACGAGCAG CGGATCAAGG
1101    GCCTGTCCAT CAGCCGCGAC CTGGTGGAGG TGGGCGGCCA CGCGCTCAAG
1151    ACCAACATCA CCACCCTGGG CCCGCTGGTG CTCCCCTTCT CGGAGCAACT
```

```
1201    GCTCTTCTTC GCCGGCGTGC TGTTCCGCCA CCTGTACCCG TCCAAGACGT
1251    CCACCCCGCC GCCGCCGGCC GCCAACGGGG ACACCTCGGC CGCCGCGCCC
1301    TACATCCCGG ACTTCAAGCG CGCGTTCGAG CACTTCTGCA TGCACGCGGC
1351    CAGCCGCGAC GTGCTGGAGC ACCTGCAGCG CAACCTGGGG CTCCGCGACG
1401    CCGACCTGGA GGCCTCCCGC GCCGCGCTGC ACCGCTTCGG CAACACCTCC
1451    AGCAGCAGCA TCTGGTACGA GCTGGCATAC CTGGAGGCCA AGGGTCGCGT
1501    CCGCCGCGGT GACCGCGTGT GGCAGCTCGC CTTCGGCTCC GGGTTCAAGT
1551    GCAACAGCGC CGTGTGGCGC GCCGTCGGAC GCGTGCGCCG CCCGTCCAGG
1601    AGCCCCTGGC TGGACTGCAT CGACCAGTAC CCGGCGCGCA TGGACGCCTG
1651    AGGCCTGACC GATCGAGACA AGCAGGCGCA TGCACCATGC AGGTGCTAAT
1701    AATTAAGCGA GACACGTCGC CGCATCCGCG TGGGTAAGTG CAGGCTAATG
1751    GATTTGCATG TAACTGCATG CAGTGCCGCT GGTTAAGGTT TGAGCTTTGG
1801    TTAATTTGTG CTGGTGTTGT AGCAGTTGTC ATCATTATTA AGGGTTTTAA
1851    TCATAGACTA CGTACGTACG TACCATAATG CGCAAGTGTA TGTGCGCGCT
1901    CTTGTGTGA
```

Hygromycin sequence used to analyse transgene:

```
   1    CAGATCCGGT CGGCATCTAC TCTATTTCTT TGCCCTCGGA CGAGTGCTGG
  51    GGCGTCGGTT TCCACTATCG GCGAGTACTT CTACACAGCC ATCGGTCCAG
 101    ACGGCCGCGC TTCTGCGGGC GATTTGTGTA CGCCCGACAG TCCCGGCTCC
 151    GGATCGGACG ATTGCGTCGC ATCGACCCTG CGCCCAAGCT GCATCATCGA
 201    AATTGCCGTC AACCAAGCTC TGATAGAGTT GGTCAAGACC AATGCGGAGC
 251    ATATACGCCC GGAGTCGTGG CGATCCTGCA AGCTCCGGAT GCCTCCGCTC
 301    GAAGTAGCGC GTCTGCTGCT CCATACAAGC CAACCACGGC CTCCAGAAGA
 351    AGATGTTGGC GACCTCGTAT TGGGAATCCC CGAACATCGC CTCGCTCCAG
 401    TCAATGACCG CTGTTATGCG GCCATTGTCC GTCAGGACAT TGTTGGAGCC
 451    GAAATCCGCG TGCACGAGGT GCCGGACTTC GGGGCAGTCC TCGGCCCAAA
 501    GCATCAGCTC ATCGAGAGCC TGCGCGACGG ACGCACTGAC GGTGTCGTCC
 551    ATCACAGTTT GCCAGTGATA CACATGGGGA TCAGCAATCG CGCATATGAA
 601    ATCACGCCAT GTAGTGTATT GACCGATTCC TTGCGGTCCG AATGGGCCGA
 651    ACCCGCTCGT CTGGCTAAGA TCGGCCGCAG CGATCGCATC CATAGCCTCC
 701    GCGACCGGTT GTAGAACAGC GGGCAGTTCG GTTTCAGGCA GGTCTTGCAA
 751    CGTGACACCC TGTGCACGGC GGGAGATGCA ATAGGTCAGG CTCTCGCTAA
 801    ACTCCCCAAT GTCAAGCACT TCCGGAATCG GGAGCGCGGC CGATGCAAAG
 851    TGCCGATAAA CATAACGATC TTTGTAGAAA CCATCGGCGC AGCTATTTAC
 901    CCGCAGGACA TATCCACGCC CTCCTACATC GAAGCTGAAA GCACGAGATT
 951    CTTCGCCCTC CGAGAGCTGC ATCAGGTCGG AGACGCTGTC GAACTTTTCG
1001    ATCAGAAACT TCTCGACAGA CGTCGCGGTG AG
```

Appendix C: Primer sequences

q134F1	CGACTACGTGCGGCTCAAGG
s134F3	GCGACACAGGCACGTAGTAGCAACACC
s134F5	CGCGGACTACGTGCGGCTCAAG
S134F6	TGGGATCAGGGGTACGACGTCA
s134F7	AATTATGGATCCACCATGAAGCTTCAGGTAGCCACG
s134F10	ATATCTCGAGGATCCGGTGACCGGCGTGCTGTA
s134F11	GCGCAGATCTCATCACACCGTCAAGTAGGCTAATC
s134F13	AAAGGATCCGCAGTGACGTTCGACGCGT
s134F14	AAAAGCATGCCAGTGACGTTCGACGCGT
s134F16	GCTCAACGCCAAGATGAAGAGT
s134F17	CTTAGCACGATGAAGATTGCCATTG
s134F18	CTTAGCACGATGAAGATTGCCATTG
s134R	TAGTCGAGGAACCTCGCCGTGACGTC
q134R1	GCCACAGCTGCTGCACGTTC
s134R2	TAGTCCGCGATCCCCTCGAAGATCC
s134R3	CGCTATGCCATCACACCGTCAAGTAGG
s134R7	ATCTCGGGATCCTGTATTCAGGCTCCAAACTTGG
s134R10	GCGCGGTACCCATCACACCGTCAAGTAGGCTAATC
s134R11	ATTATCTAGAGGATCCGGTGACCGGCGTGCTGTA
s134R12	ATACCTAGGCCTCCGGCTCCAAACTTGGATTTGTAG
s134R13	AGCAAAGCTTGGCTAATCGTGTATTCAGGCTCCA
s134R14	AGGGAGATCTGGCTCCAAACTTGGATTTGTAGT
s134R16	CATCACACCGTCAAGTAGGCTAATC
s134R17	GCTCCAGTCAATACAGCAACTGTGTTC
s134R18	GCTCCAGTCAATACAGCAACTGTGTTC
q194F1	AAGGTCGTCCACTACTGCGCCG
n194F3	CGCACAAGTCCACAGCACACAGCAG

n194F5	CATCTACGACGACGAGGGCCTCAACTA
n194F6	TGGGATCAGGGGTACGACGTCA
n194F7	TATATTGGATCCACCATGGCTCCCATGCTCAAGC
n194F10	ATATCTCGAGGATCCACGACATCAAGATGCTGGTGAA
n194F11	GCGCTAGATCTGGTGGCCGTACGTACCTCAA
n194F13	AAAGGATCCGCTCCCATGCTCAAGCGGAT
n194F14	AAAAGCATGCCGGCTCCCATGCTCAAGCGGAT
n194F16	CCTTCCAGTTAGGCGTATGTATTCTAC
n194R	CATGCATACGTGTACGTAGCAGGTCGATCTATAA
q194R1	GAAGGACTTGACGGCAACGACC
n194R3	TAACTGGAAGGCAGGCGCGTGATG
n194R7	ACGATAGGATCCAGGCGCGTGATGACTACG
n194R10	GCCTGGTACCGGTGGCCGTACGTACCTCAA
n194R11	ATTATCTAGAGGATCCACGACATCAAGATGCTGGTGAA
n194R12	ATACCTAGGCCTCCCGCGGCGGAGGGCGCC
n194R13	AGGGAAGCTTGTGATGACTACGCGGCGGA
n194R14	AAATAGATCTCGCGGCGGAGGGCGC
n194R16	GTAGCAGGTCGATCTATAATCATACTTG
Gols2F	GCACGAGCTCTCTAAATCCGACACA
Gols2R	GGTGGACTGTTTCGATCAAACCTCCTA
q1057F1	CACCTCCAGCAGCAGCATCTG
d1057F5	CCAGCTGCGCCCAGCCATG
d1057F5(3')	GCAACACCTCCAGCAGCAGCATCTG
d1057F6(3')	TGAGGCCTGACCGATCGAGACA
d1057F6	CCAGGCCGCCACCCAGCTG
d1057F7	ATATTAGGATCCTGCGCCCAGCCATGG
d1057F8	ACGAGCTCTTCGACAAGTGC
d1057F10	ATATCTCGAGGATCCAGCAGCAGCATCTGGTACGA
d1057F11	GCGCTAGATCTCAGCACAAATTAACCAAAGCTCAA

d1057F14	CACCAGCTGCGCCCAGCCATG
d1057F16	ATGCACCATGCAGGTGCTAATA
d1057R	TCACACAAGAGCGCGCACATACACTTG
q1057R1	ATGCAGTCCAGCCAGGGGCT
d1057R3	GAGGTTCACCGACTGCAGGAAGTC
d1057R4	ACGATCTCCGTCGACAGCTCCTC
d1057R7	ATATAAGGATCCAGGCCTCAGGCGTCCATG
D1057R10	GCGGGGTACCAGCACAAATTAACCAAAGCTCAA
d1057R11	ATTATCTAGAGGATCCAGCAGCAGCATCTGGTACGA
d1057R12	ATACCTAGGCCTCCGGCGTCCATGCGCGCCG
d1057R14	CGGTCAGGCCTCAGGCGTCCAT
d1057R16	CAAAGTTGACATAAGTTTCACACAAGAG
HygF	CAGATCCGGTCGGCATCTACTCTA
HygR	CTCACCGCGACGTCTGTCGA
3′-CDS	AAGCAGTGGTATCAACGCAGAGTAC
5′-CDS	5'-(T)25V N-3' (N = A, C, G, or T; V = A, G, or C)
CDSIIA	AAGCAGTGGTATCAACGCAGAGTACT(30)NN
BD smartII	AAGCAGTGGTATCAACGCAGAGTACGCGGG
SmNUPA	AAGCAGTGGTATCAACGCAGAGT
SmUPAL	CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
SmUPAS	CTAATACGACTCACTATAGGGC
AtFdhR	GTAGGTTGATTATGTGAGTGAGATCTA
AtFdhF	CCACTTTTTTTAGCTTAGATACAAACT
AtFdhFm3	CGGTTCTTGTGCTGGTTTTTAGTGAG
AtFdhR2	TCTGCTTAAACTCCCAACCTCAGGACT
AtFdhF3	GAGCAAGATCTGCTCTCTACCGAGATC
AtFdhR3	CAATAAGATAAACAGAGCGAGGACGAGAC
GFPfuF3	ATGGATCCCCTAGGAGGCAAGGGCGAG
GFPfuR3	CGCGCCAGATCTTCACTTGTAGAGTTCATCCA

Appendix D: Sequences of vector derived primers

Name	Sequence	Vector
PZPF	ACTGTTTCTTTTGTCGATGCTCAC	pPZPUbi
PZPR	CATCGCAAGACCGGCAAC	pPZPUbi
SP6	GATTTAGGTGACACTATAG	pGEM-T Easy
T7	GTAATACGACTCACTATAGGGC	pGEM-T Easy
qTnosR	TATATGATAATCATCGCAAGACCGGC	pPZPUbi
M13F	GTAAAACGACGGCCAGT	pGEM-T Easy
M13R	CAGGAAACAGCTATGAC	pGEM-T Easy
pQEF	CGGATAACAATTTCACACAG	pQE30, pQE70
pQER	GTTCTGAGGTCATTACTGG	QE30, pQE70
pHanF1	CCAACCACGTCTTCAAAGCA	pHannibal
pHanR1	CTTCGTCTTACACATCACTTGTCATA	pHannibal
pHanF2	GTCGAACATGAATAAACAAGGTAACA	pHannibal
pHanR2	ACGGGTGATATATTCATTAGAATGAA	pHannibal
pHanF3	CAAGGTAACATGATAGATCATGTC	pHannibal
pHanR3	GCAAATATCATGCGATCATAGGC	pHannibal
pHanF5	GTGACATCTCCACTGACGTAAGC	pHannibal
pHanR5	CATACTAATTAACATCACTTAAC	pHannibal
GW1	GTTGCAACAAATTGATGAGCAATGC	pENTR/D-TOPO, PCR8/GW/TOPO
GW2	GTTGCAACAAATTGATGAGCAATTA	pENTR/D-TOPO, PCR8/GW/TOPO

Appendix E: Abiotic stress general results

A: Na^+ and K^+ concentration and moisture content of salt experiment samples

Sample	Na^+ (μM/gFW)	K^+ (μM/gFW)	%Moisture
Golden Promise 1 day salt	198	202	89.9
Golden Promise 3 day salt	146	172	88.3
Golden Promise 5 day salt	181	156	91.3
Golden Promise 1 day control	160	210	87.5
Golden Promise 3 day control	74	207	85.7
Golden Promise 5 day control	41	193	89.2
CM72 1 day salt	144	182	93.7
CM72 3 day salt	186	170	86.9
CM72 5 day salt	187	145	88.1
CM72 1 day control	52	185	95.6
CM72 3 day control	35	180	91.9
CM72 5 day control	20	160	91.3
Yu6472 1 day salt	126	225	90.5
Yu6472 3 day salt	181	202	88.9
Yu6472 5 day salt	239	185	84
Yu6472 1 day control	55	213	91.3
Yu6472 3 day control	51	214	92
Yu6472 5 day control	24	239	90.9
Prior(A) 1 day salt	145	189	94.4
Prior(A) 3 day salt	182	162	89.3
Prior(A) 5 day salt	183	137	88.9
Prior(A) 1 day control	117	380	90.3
Prior(A) 3 day control	47	200	92.6
Prior(A) 5 day control	30	201	90.4
WI2291 1 day salt	135	189	90.5
WI2291 3 day salt	136	127	92.1
WI2291 5 day salt	199	131	89.9
WI2291 1 day control	59	163	90.5
WI2291 3 day control	24	192	92.7
WI2291 5 day control	35	208	90

B: Moisture content of samples in drought experiment

Sample	%Moisture
Haruna Nijo wilt1	89.47%
Haruna Nijo control 1	93.33%
Haruna Nijo wilt2	89.47%
Haruna Nijo control2	96.15%
Haruna Nijo rewatered	92.29%
Haruna Nijo rewatered control	93.33%
Barque73 wilt1	82.50%
Barque73 control 1	88.89%
Barque73 wilt2	83.33%
Barque73 control 2	89.58%
Barque73 rewatered	88.00%
Barque73 rewatered control	88.57%
Golden Promise wilt1	81.58%
Golden Promise control 1	92.50%
Golden Promise wilt2	78.95%
Golden Promise control 2	94.29%
Golden Promise rewatered	88.24%
Golden Promise rewatered control	90.91%

Appendix F: Control genes' values and normalisation factors

In all tables the values in control genes columns are transcript number/µl of cDNA.

1. Salt experiments:

Abbreviations are: Co: Control, S: Salt treated, d: Days after exposure.

Cultivar/treatment	*GAPDH*	*Cyclophilin*	*Tubulin*	Normalisation Factor
Golden Promise1dS	2,846,180	21,690,033	480,907	0.981
Golden Promise3dS	7,056,600	57,776,243	490,970	1.853
Golden Promise5dS	6,897,717	52,624,050	581,473	1.886
Golden Promise1dCo	2,086,560	23,937,257	464,860	0.904
Golden Promise3dCo	7,584,967	45,798,323	460,263	1.719
Golden Promise5dCo	5,747,447	40,055,047	363,630	1.386
CM72,1dS	2,163,187	12,891,423	344,230	0.673
CM72,3dS	3,229,210	27,112,540	623,137	1.202
CM72,5dS	2,821,253	17,884,360	209,513	0.695
CM72,1dCo	3,348,533	22,979,773	769,697	1.235
CM72,3dCo	2,330,250	17,613,260	1,282,140	1.187
CM72,5dCo	3,844,727	27,720,320	398,397	1.105
Yu-6472,1dS	3,387,220	26,690,470	543,740	1.161
Yu-6472,3dS	4,087,723	38,839,457	583,270	1.433
Yu-6472,5dS	3,404,647	30,715,057	342,920	1.045
Yu-6472,1dCo	2,333,485	23,368,267	461,487	0.928
Yu-6472,3dCo	4,048,163	18,464,850	338,675	0.930
Yu-6472,5dCo	4,319,717	12,791,257	252,277	0.762
Prior(A)1dS	3,455,030	13,271,997	297,570	0.757
Prior(A)3dS	5,511,207	29,275,017	521,990	1.389
Prior(A)5dS	4,138,473	13,648,357	381,657	0.882
Prior(A)1dCo	2,011,127	10,258,293	503,990	0.691
Prior(A)3dCo	3,692,390	13,024,863	311,593	0.781
Prior(A)5dCo	4,493,983	18,800,227	294,980	0.925
WI-2291,1dS	3,543,840	13,757,137	436,147	0.878
WI-2291,3dS	3,490,307	18,126,497	376,957	0.912
WI-2291,5dS	3,882,477	16,149,995	273,853	0.817
WI-2291,1dCo	1,660,313	7,445,653	538,507	0.596
WI-2291,3dCo	4,459,140	18,363,677	464,907	1.066
WI-2291,5dCo	3,973,160	12,029,260	202,670	0.675
M < 0.83	0.684	0.654	0.791	

2. Drought experiments:

Abbreviations are: HN:Haruna Nijo, GP: Golden Promise, D: Drought exposed, d: Days of exposure, Co: Control, 1: Wilt1, 2: Wilt2, 3:Rewatered.

Cultivar/treatment	*GAPDH*	*Cyclophilin*	*HSP70*	Normalisation factor
HND1d	1,619,847	3,124,603	88,850	1.014
HND2d	2,604,587	4,524,490	123,253	1.499
HND3d	799,643	1,570,513	68,213	0.583
HNCo1d	923,837	1,985,890	103,920	0.762
HNCo2d	874,980	1,663,813	33,010	0.481
HNCo3d	516,207	1,335,887	40,990	0.403
. Barque73D1d	3,790,230	4,014,887	165,097	1.799
Barque73D2d	3,961,093	3,857,073	187,407	1.879
Barque73D3d	2,736,703	4,963,877	87,513	1.402
Barque73Co1d	1,534,817	2,764,110	63,403	0.854
Barque73Co2d	1,406,550	2,932,200	94,333	0.966
Barque73Co3d	1,019,103	2,888,693	143,040	0.992
GPD1d	3,632,363	5,131,243	137,747	1.812
GPD2d	6,321,223	3,756,097	286,890	2.509
GPD3d	2,150,240	3,715,697	87,713	1.175
GPCo1d	1,716,137	2,335,320	94,835	0.959
GPCo2d	643,090	1,136,490	64,560	0.478
GPCo3d	1,027,803	2,448,920	89,055	0.804
M value	0.686	0.581	0.624	

3. Frost experiments:

Abbreviations are: AN: Amagi Nijo, HN: Haruna Nijo, GP: Golden Promise, Gal: Galleon, WI: WI2585, 0: Before exposure to frost at 20 ºC, +4B: Pre-frost 4ºC, +4A: Post-frost 4ºC -5.5: Frost -5.5ºC, 48: 48 hours at 20 ºC after exposure to -5 ºC, f: Frost treated, Co: Control.

Cultivar/treatment	*GAPDH*	*Cyclophilin*	*Tubulin*	Normalisation Factor
AN0	1,241,857	3,435,947	660,747	1.052
AN+4Af	773,827	3,439,650	634,593	0.887
AN-5.5f	809,867	5,048,627	656,177	1.035
AN+4Bf	860,947	4,793,840	587,413	1.001
AN48f	1,469,460	6,841,310	1,027,920	1.622
AN+4Aco	881,797	3,927,697	529,637	0.912
AN-5.5co	2,081,740	5,148,257	727,810	1.477
AN+4Bco	1,148,623	4,374,270	763,910	1.166
AN48co	1,461,690	7,292,337	752,540	1.491
HN0	1,232,580	3,584,387	684,857	1.077
HN+4Af	986,910	2,959,677	742,273	0.964
HN-5.5f	941,410	3,720,963	725,170	1.016
HN+4Bf	560,250	3,030,900	218,447	0.535
HN48f	1,354,255	4,940,607	840,673	1.325
HN+4Aco	847,383	3,044,947	603,820	0.863
HN-5.5co	2,183,057	4,282,193	876,303	1.501
HN+4Bco	549,700	4,949,843	53,357	0.391
HN48co	1,790,913	8,200,033	952,550	1.795
Gal0	1,739,000	5,021,940	540,895	1.250
Gal+4Af	1,454,207	5,638,643	575,433	1.249
Gal-5.5f	1,243,463	2,403,910	247,510	0.674
Gal+4Bf	1,250,990	2,862,310	361,535	0.812
Gal48f	1,185,057	3,380,670	540,957	0.964
Gal+4Aco	1,663,223	3,252,477	546,633	1.069
Gal-5.5co	2,118,617	2,720,670	385,930	0.972
Gal+4Bco	1,412,270	1,815,580	289,133	0.674
Gal48co	1,847,947	5,524,013	379,640	1.170
GP0	2,428,177	4,521,557	487,157	1.303
GP+4Af	2,227,930	2,886,167	287,840	0.914
GP-5.5f	677,793	1,487,917	171,905	0.415
GP+4Bf	1,119,063	3,704,890	214,370	0.716

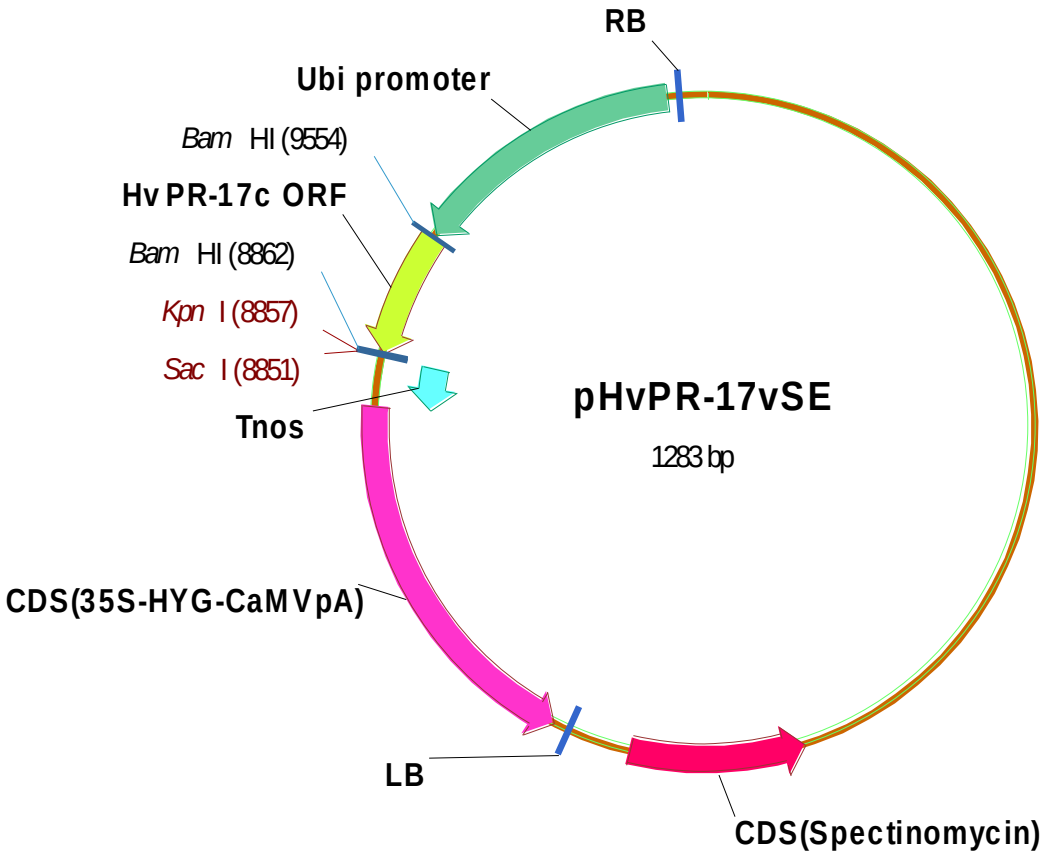
GP48f	1,297,610	5,257,873	498,443	1.120
GP+4Aco	578,160	1,316,160	475,387	0.531
GP-5.5co	967,617	2,291,453	842,393	0.917
GP+4Bco	959,717	1,765,453	514,140	0.711
GP48co	3,033,477	6,399,060	615,087	1.702
WI0	1,271,987	2,158,630	583,843	0.872
WI+4Af	714,560	2,072,560	466,550	0.658
WI-5.5f	743,077	1,695,980	429,280	0.607
WI+4Bf	1,199,100	3,436,280	533,500	0.968
WI48f	1,141,130	4,919,030	1,116,657	1.373
WI+4Aco	1,331,733	4,156,840	703,677	1.172
WI-5co	2,107,943	5,167,940	1,231,683	1.770
WI+4Bco	1,204,617	4,446,423	1,157,650	1.368
WI48co	1,680,610	5,523,870	1,874,433	1.930
M < 0.85	1	1	1	

4. *Rhynchosporium secalis* inoculation experiments:

Abbreviations are: At: Atlas, A46: Atlas 46, h0I: Before inoculation, h3I: 3 hours after inoculation, h24: 24 hours after inoculation, h24W: water inoculated plants after 24 hours.

Cultivar/treatment	*Cyclophilin*	*Tubulin*	*HSP70*	Normalisation Factor
Ath0I	5,643,247	441,920	34,147	1
Ath3I	3,112,390	222,080	73,283	1
Ath6I	1,131,377	137,117	38,493	0
Ath24I	2,823,617	288,003	121,610	1
Ath24W	2,986,540	97,393	135,400	1
A46h0I	6,706,430	426,833	50,533	1
A46h3I	6,083,413	269,347	104,557	1
A46h6I	5,411,927	167,247	93,763	1
A46h24I	2,453,950	18,787	17,023	0
A46h24W	1,575,313	38,847	16,857	0
M < 1.07	1	1	1	

Appendix G: Vector maps

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 101    CGCGACAGGC TGCCGCCCTG CCCTTTTCCT GGCGTTTTCT TGTCGCGTGT
 151    TTTAGTCGCA TAAAGTAGAA TACTTGCGAC TAGAACCGGA GACATTACGC
 201    CATGAACAAG AGCGCCGCCG CTGGCCTGCT GGGCTATGCC CGCGTCAGCA
 251    CCGACGACCA GGACTTGACC AACCAACGGG CCGAACTGCA CGCGGCCGGC
 301    TGCACCAAGC TGTTTTCCGA GAAGATCACC GGCACCAGGC GCGACCGCCC
 351    GGAGCTGGCC AGGATGCTTG ACCACCTACG CCCTGGCGAC GTTGTGACAG
 401    TGACCAGGCT AGACCGCCTG GCCCGCAGCA CCCGCGACCT ACTGGACATT
 451    GCCGAGCGCA TCCAGGAGGC CGGCGCGGGC CTGCGTAGCC TGGCAGAGCC
 501    GTGGGCCGAC ACCACCACGC CGGCCGGCCG CATGGTGTTG ACCGTGTTCG
 551    CCGGCATTGC CGAGTTCGAG CGTTCCCTAA TCATCGACCG CACCCGGAGC
 601    GGGCGCGAGG CCGCCAAGGC CCGAGGCGTG AAGTTTGGCC CCCGCCCTAC
 651    CCTCACCCCG GCACAGATCG CGCACGCCCG CGAGCTGATC GACCAGGAAG
 701    GCCGCACCGT GAAAGAGGCG GCTGCACTGC TTGGCGTGCA TCGCTCGACC
 751    CTGTACCGCG CACTTGAGCG CAGCGAGGAA GTGACGCCCA CCGAGGCCAG
 801    GCGGCGCGGT GCCTTCCGTG AGGACGCATT GACCGAGGCC GACGCCCTGG
 851    CGGCCGCCGA GAATGAACGC CAAGAGGAAC AAGCATGAAA CCGCACCAGG
 901    ACGGCCAGGA CGAACCGTTT TTCATTACCG AAGAGATCGA GGCGGAGATG
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1101    TGTGTATTTG AGTAAAACAG CTTGCGTCAT GCGGTCGCTG CGTATATGAT
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1751    CCCGAGGGCG ACGCTGCCCG CGAGGTCCAG GCGCTGGCCG CTGAAATTAA
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1851    AAACACGCTA AGTGCCGGCC GTCCGAGCGC ACGCAGCAGC AAGGCTGCAA
1901    CGTTGGCCAG CCTGGCAGAC ACGCCAGCCA TGAAGCGGGT CAACTTTCAG
1951    TTGCCGGCGG AGGATCACAC CAAGCTGAAG ATGTACGCGG TACGCCAAGG
2001    CAAGACCATT ACCGAGCTGC TATCTGAATA CATCGCGCAG CTACCAGAGT
2051    AAATGAGCAA ATGAATAAAT GAGTAGATGA ATTTTAGCGG CTAAAGGAGG
2101    CGGCATGGAA AATCAAGAAC AACCAGGCAC CGACGCCGTG GAATGCCCCA
2151    TGTGTGGAGG AACGGGCGGT TGGCCAGGCG TAAGCGGCTG GGTTGTCTGC
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2251    GTCGCAAACC ATCCGGCCCG GTACAAATCG GCGCGGCGCT GGGTGATGAC
2301    CTGGTGGAGA AGTTGAAGGC CGCGCAGGCC GCCCAGCGGC AACGCATCGA
2351    GGCAGAAGCA CGCCCCGGTG AATCGTGGCA AGCGGCCGCT GATCGAATCC
2401    GCAAAGAATC CCGGCAACCG CCGGCAGCCG GTGCGCCGTC GATTAGGAAG
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2501    CGTGGGCACC CGCGATAGTC GCAGCATCAT GGACGTGGCC GTTTTCCGTC
2551    TGTCGAAGCG TGACCGACGA GCTGGCGAGG TGATCCGCTA CGAGCTTCCA
2601    GACGGGCACG TAGAGGTTTC CGCAGGGCCG GCCGGCATGG CCAGTGTGTG
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2701    ACCGATACCG GGAAGGGAAG GGAGACAAGC CCGGCCGCGT GTTCCGTCCA
2751    CACGTTGCGG ACGTACTCAA GTTCTGCCGG CGAGCCGATG GCGGAAAGCA
2801    GAAAGACGAC CTGGTAGAAA CCTGCATTCG GTTAAACACC ACGCACGTTG
2851    CCATGCAGCG TACGAAGAAG GCCAAGAACG GCCGCCTGGT GACGGTATCC
2901    GAGGGTGAAG CCTTGATTAG CCGCTACAAG ATCGTAAAGA GCGAAACCGG
2951    GCGGCCGGAG TACATCGAGA TCGAGCTAGC TGATTGGATG TACCGCGAGA
3001    TCACAGAAGG CAAGAACCCG GACGTGCTGA CGGTTCACCC CGATTACTTT
3051    TTGATCGATC CCGGCATCGG CCGTTTTCTC TACCGCCTGG CACGCCGCGC
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3851    GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
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4401    CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC ATAGCTCACG
4451    CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG
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4551    CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC
4601    CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT
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4751    TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA
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4901    TTTGGTCATG CATGATATAT CTCCCAATTT GTGTAGGGCT TATTATGCAC
4951    GCTTAAAAAT AATAAAAGCA GACTTGACCT GATAGTTTGG CTGTGAGCAA
5001    TTATGTGCTT AGTGCATCTA ATCGCTTGAG TTAACGCCGG CGAAGCGGCG
                                    CDS(Spectinomycin)
                                     ~~~~~~~~~~~~~~~~~~~~~~~~
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5101    ATCTCGCCTT TCACGTAGTG GACAAATTCT TCCAACTGAT CTGCGCGCGA
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5201    TGACGGGCTG ATACTGGGCC GGCAGGCGCT CCATTGCCCA GTCGGCAGCG
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5451    TTCTCTTGCT TTTGTCAGCA AGATAGCCAG ATCAATGTCG ATCGTGGCTG
5501    GCTCGAAGAT ACCTGCAAGA ATGTCATTGC GCTGCCATTC TCCAAATTGC
5551    AGTTCGCGCT TAGCTGGATA ACGCCACGGA ATGATGTCGT CGTGCACAAC
5601    AATGGTGACT TCTACAGCGC GGAGAATCTC GCTCTCTCCA GGGGAAGCCG
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5751    GCCATCCACT GCGGAGCCGT ACAAATGTAC GGCCAGCAAC GTCGGTTCGA
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5901    CTGCTGCGTA ACATCGTTGC TGCTCCATAA CATCAAACAT CGACCCACGG
5951    CGTAACGCGC TTGCTGCTTG GATGCCCGAG GCATAGACTG TACCCCAAAA
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6301    CGGCTCTCCC GCTGACGCCG TCCCGGACTG ATGGGCTGCC TGTATCGAGT
6351    GGTGATTTTG TGCCGAGCTG CCGGTCGGGG AGCTGTTGGC TGGCTGGTGG
6401    CAGGATATAT TGTGGTGTAA ACAAATTGAC GCTTAGACAA CTTAATAACA
                                          CDS(35S-HYG-CaMVpA)
                                           ~~~~~~~~~~~
6451    CATTGCGGAC GTTTTTAATG TACTGAATTA ACGCCGAATT GAATTCACGC
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6701    TCGATCGACA GATCCGGTCG GCATCTACTC TATTTCTTTG CCCTCGGACG
6751    AGTGCTGGGG CGTCGGTTTC CACTATCGGC GAGTACTTCT ACACAGCCAT
6801    CGGTCCAGAC GGCCGCGCTT CTGCGGGCGA TTTGTGTACG CCCGACAGTC
6851    CCGGCTCCGG ATCGGACGAT TGCGTCGCAT CGACCCTGCG CCCAAGCTGC
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7101    CGCTCCAGTC AATGACCGCT GTTATGCGGC CATTGTCCGT CAGGACATTG
7151    TTGGAGCCGA AATCCGCGTG CACGAGGTGC CGGACTTCGG GGCAGTCCTC
7201    GGCCCAAAGC ATCAGCTCAT CGAGAGCCTG CGCGACGGAC GCACTGACGG
7251    TGTCGTCCAT CACAGTTTGC CAGTGATACA CATGGGGATC AGCAATCGCG
7301    CATATGAAAT CACGCCATGT AGTGTATTGA CCGATTCCTT GCGGTCCGAA
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7701    ACTTTTCGAT CAGAAACTTC TCGACAGACG TCGCGGTGAG TTCAGGCTTT
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7901    TCCCTTACGT CAGTGGAGAT ATCACATCAA TCCACTTGCT TTGAAGACGT
7951    GGTTGGAACG TCTTCTTTTT CCACGATGCT CCTCGTGGGT GGGGGTCCAT
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8051    TCGCAATGAT GGCATTTGTA GGTGCCACCT TCCTTTTCTA CTGTCCTTTT
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8151    TTACCCTTTG TTGAAAAGTC TCAATAGCCC TTTGGTCTTC TGAGACTGTA
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8351    GCATCTTGAA CGATAGCCTT TCCTTTATCG CAATGATGGC ATTTGTAGGT
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8451    AATGGAATCC GAGGAGGTTT CCCGATATTA CCCTTTGTTG AAAAGTCTCA
8501    ATAGCCCTTT GGTCTTCTGA GACTGTATCT TTGATATTCT TGGAGTAGAC
                                                  Tnos
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8551    GAGAGTGTCG TGCTCCACCA TGTTGAATTC CCGATCTAGT AACATAGATG
8601    ACACCGCGCG CGATAATTTA TCCTAGTTTG CGCGCTATAT TTTGTTTTCT
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8701    CATAAATAAC GTCATGCATT ACATGTTAAT TATTACATGC TTAACGTAAT
8751    TCAACAGAAA TTATATGATA ATCATCGCAA GACCGGCAAC AGGATTCAAT
                                                        MCS
                                                       ~~~~~

8801    CTTAAGAAAC TTTATTGCCA AATGTTTGAA CGATCGGGGA AATTCGAGCT
                                             Ubi1 promoter
                                          ~~~~~~~~~~~~~~~~~~~~~~~~~

8851    CGGTACCCGG GGATCCTCTA GAGTCGACCT GCAGAAGTAA CACCAAACAA
8901    CAGGGTGAGC ATCGACAAAA GAAACAGTAC CAAGCAAATA AATAGCGTAT
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9151    CGATCCCGTC TGCGGAACGG CTAGAGCCAT CCCAGGATTC CCCAAAGAGA
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9401    TGAAGCGGAG GTGCCGACGG GTGGATTTGG GGGAGATCTG GTTGTGTGTG
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 9651    GCGGAGCAAG TCCAACGGTG GAGCGGAACT CTCGAGAGGG GTCCAGAGGC
 9701    AGCGACAGAG ATGCCGTGCC GTCTGCTTCG CTTGGCCCGA CGCGACGCTG
 9751    CTGGTTCGCT GGTTGGTGTC CGTTAGACTC GTCGATCGAC GGCGTTTAAC
 9801    AGGCTGGCAT TATCTACTCG AAACAAGAAA AATGTTTCCT TAGTTTTTTT
 9851    AATTTCTTAA AGGGTATTTG TTTAATTTTT AGTCACTTTA TTTTATTCTA
 9901    TTTTATATCT AAATTATTAA ATAAAAAAAC TAAAATAGAG TTTTAGTTTT
 9951    CTTAATTTAG AGGCTAAAAT AGAATAAAAT AGATGTACTA AAAAAATTAG
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10101    TGCACACTAA AAAGATAAAA CTGTAGAGTC CTGTTGTCAA AATACTCAAT
10151    TGTCCTTTAG ACCATGTCTA ACTGTTCATT TATATGATTC TCTAAAACAC
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10251    ATATATGTAT AAAGATAGAT AAACTGCACT TCAAACAAGT GTGACAAAAA
10301    AAATATGTGG TAATTTTTTA TAACTTAGAC ATGCAATGCT CATTATCTCT
10351    AGAGAGGGGC ACGACCGGGT CACGCTGCAC TGCAGGCATG CAAGCTTAGC
10401    TTGAGCTTGG ATCAGATTGT CGTTTCCCGC CTTCAGTTTA AACTATCAGT
                  RB

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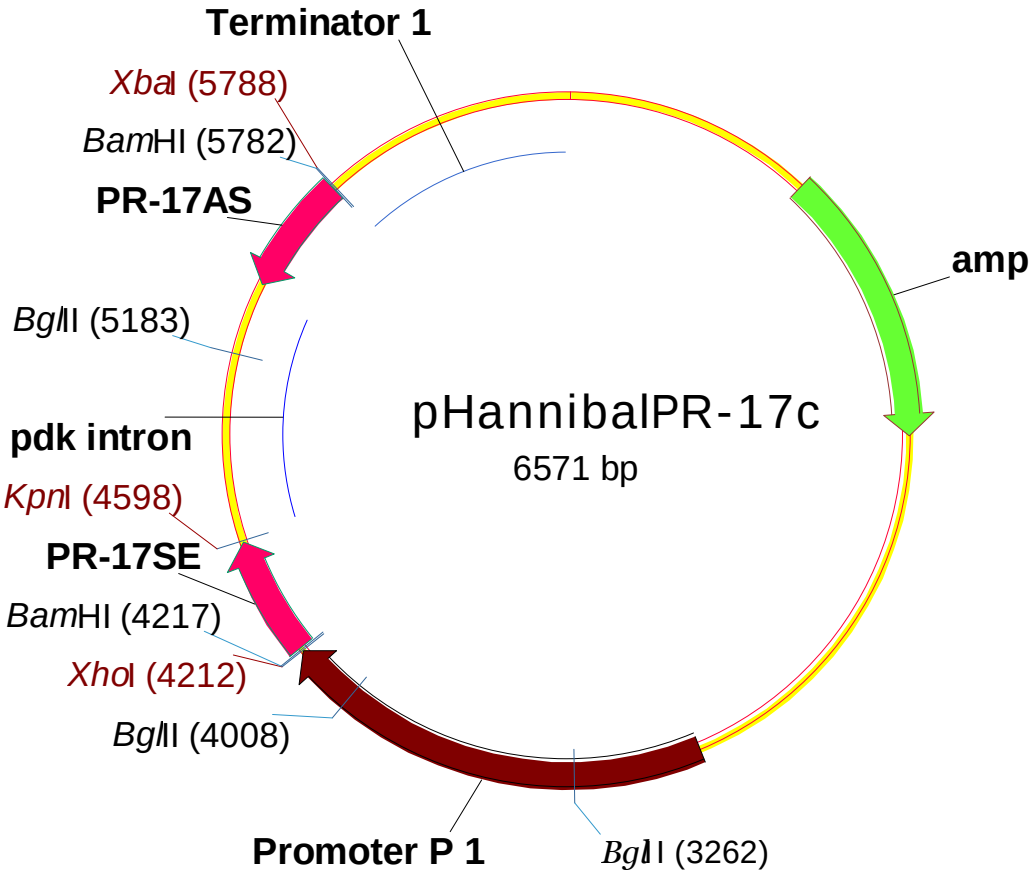
10451    GTTTGACAGG ATATATTGGC GGGTAAACCT AAGAGAAAAG AGCGTTTATT
10501    AGAATAACGG ATATTTAAAA GGGCGTGAAA AGGTTTATCC GTTCGTCCAT
10551    TTGTATGTGC ATGCCAACCA CAGGGTTCCC CTCGGGATCA A
```

pPZPUbi.cas backbone sequence and map of pHvPR-17cSE construct. For other genes, the *PR-17* ORF was replaced with their ORF. The same ORF ligated in reverse orientation served as antisense construct. The start and end sequences of the main features of the construct has been italicised and underlined. The features name is given above the sequence at the end of the feature. The sequences are in anti-sense orientation.

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 101    TACGTGCGGC TCAAGGCCGG GTTCGCGCCG GGGCACTGGG TGAAGCCGGG
 151    GCAGGGCGAC CGGTGGGATC AGGGGTACGA CGTCACGGCG AGGTTCCTCG
 201    ACTACTGCGA CTCACTGAAG CCCGGGTTCG TCGCGCAGCT CAACGCCAAG
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1051    ATGGATTGAC TATTAATTAA ATGAATTAGT CGAACATGAA TAAACAAGGT
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1151    ATTACAGTTG GGAAATTGGG TTCGAAATCG ATAAGCTTGG ATCTCATCAC
1201    ACCGTCAAGT AGGCTAATCG TGTATTCAGG CTCCAAACTT GGATTTGTAG
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1251    TCCCGCCACA GCTGCTGCAC GTTCTTGCCG AGAATCTGCG CGAAGAAGTC
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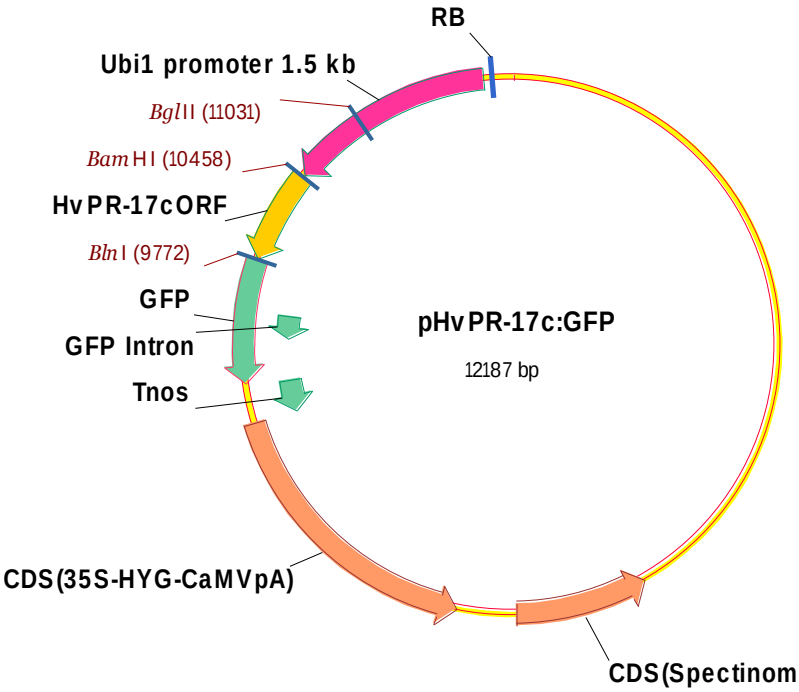
1351    GCTTCAGTGA GTCGCAGTAG TCGAGGAACC TCGCCGTGAC GTCGTACCCC
1401    TGATCCCACC GGTCGCCCTG CCCCGGCTTC ACCCAGTGCC CCGGCGCGAA
1451    CCCGGCCTTG AGCCGCACGT AGTCGGCGAT CCCCTCGATG AGCCCGCCGT
1501    TCGCCTTGCC CTGCCCGTTC CACTGCCACA CGTGCGTCGC CTCGTGGTAC
1551    AGCACGCCGG TCACCGGATC C
```

pHannibalPR-17c construct map and sequence of the fragment containing sense and antisense sequence of the *PR-17* gene. The fragment was released by BamHI restriction and ligated into pPZPUbi to construct the RNAi vector. Start and end sequence of the sense and anti-sense fragments are underlined and shown in bold and italic fonts, respectively.

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 101    CGGGCACCTC CGGCGGCCGG CGGTTCGAGC AGGCCGTCGG CCTCCCATAC
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 701    GCGAGGAACT GTTCACTGGC GTGGTCCCAA TCCTGGTGGA ACTGGATGGT
 751    GATGTGAACG GGCACAAGTT CTCCGTCAGC GGAGAGGGTG AAGGTGATGC
 801    CACCTACGGA AAGCTCACCC TGAAGTTCAT CTGCACTACC GGAAAGCTCC
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1351    AAGAACGGCA TCAAGGTGAA CTTCAAGATC AGGCACAACA TCGAAGATGG
1401    AAGCGTGCAA CTGGCGGACC ACTACCAGCA GAACACGCCC ATCGGCGATG
1451    GCCCTGTCCT GCTGCCGGAC AACCATTACC TGTCCACGCA ATCTGCCCTC
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1551    GACGGCTGCT GGGATCACGC ATCGCATGGA TGAACTCTAC AAGTGA
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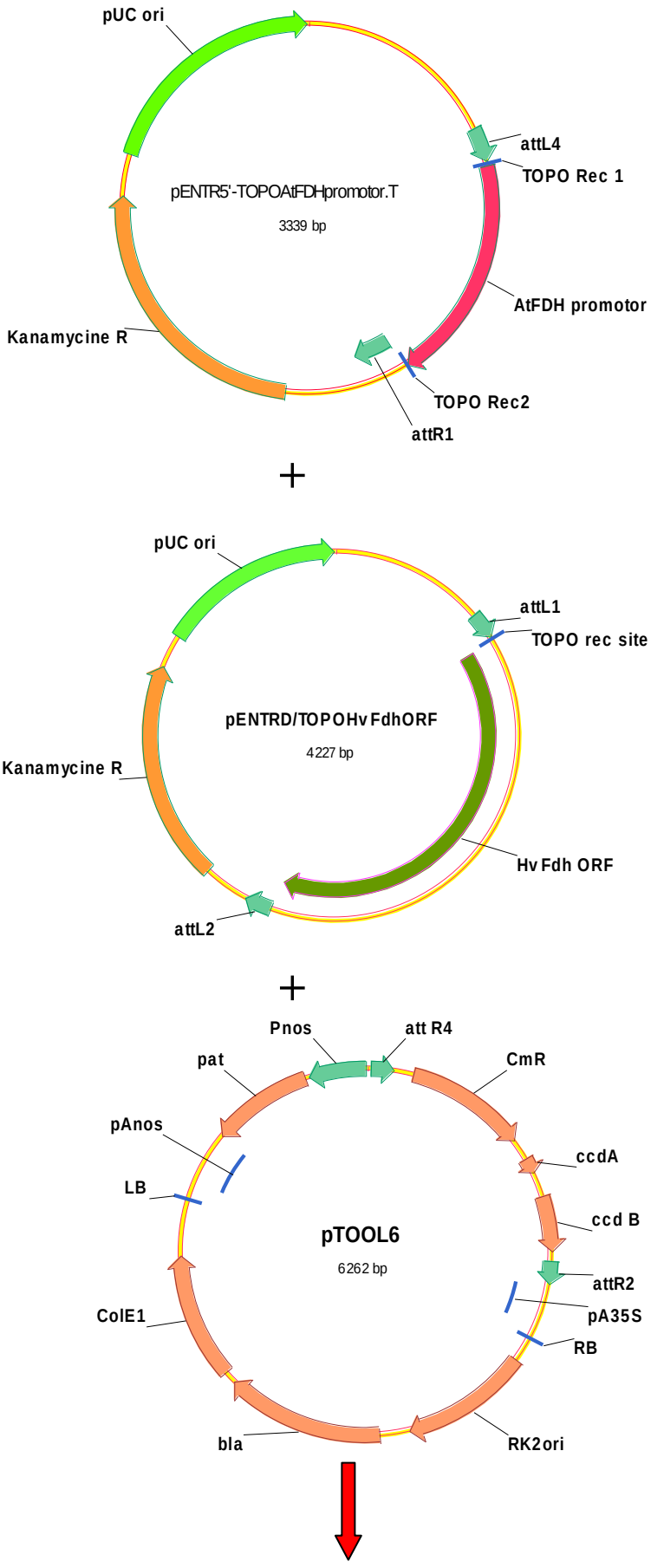
Map of pHvPR-17:GFP vector and nucleotide sequence of the fragment coding for fusion protein. The extra nucleotides added between the gene and GFP coding sequences including BlnI site to code for additional neutral amino acids as spacer is underlined. BlnI site used to fuse ORFs to pGFPfu is bold and underlined. The spacer sequences encodes for Gly Gly Leu Gly Gly.

pQEX expression plasmid vectors for N-terminal and C-terminal His6-tag constructs. The vectors contained a T5 promoter driving the expression of the target gene cloned into the multiple cloning site (MSC). The synthetic ribosomal binding site (RBS) increases the rate of translation. The β-lactomase gene (bla) confer resistance to ampicillin. Col E1 is the origin of replication. Lac O: lac repressor, which regulates recombinant protein expression, *: indicates three different reading frames for pQE vectors. Source: http://www1.qiagen.com/default.aspx?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TOPO Rec 1
pENTR5'-TOPOAtFDHpromotor.T
3339 bp
AtFDH promotor
Kanamycine R
TOPO Rec2
attR1
+
pUC ori
attL1
TOPO rec site
pENTRD/TOPOHv FdhORF
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Kanamycine R
Hv Fdh ORF
attL2
+
Pnos
att R4
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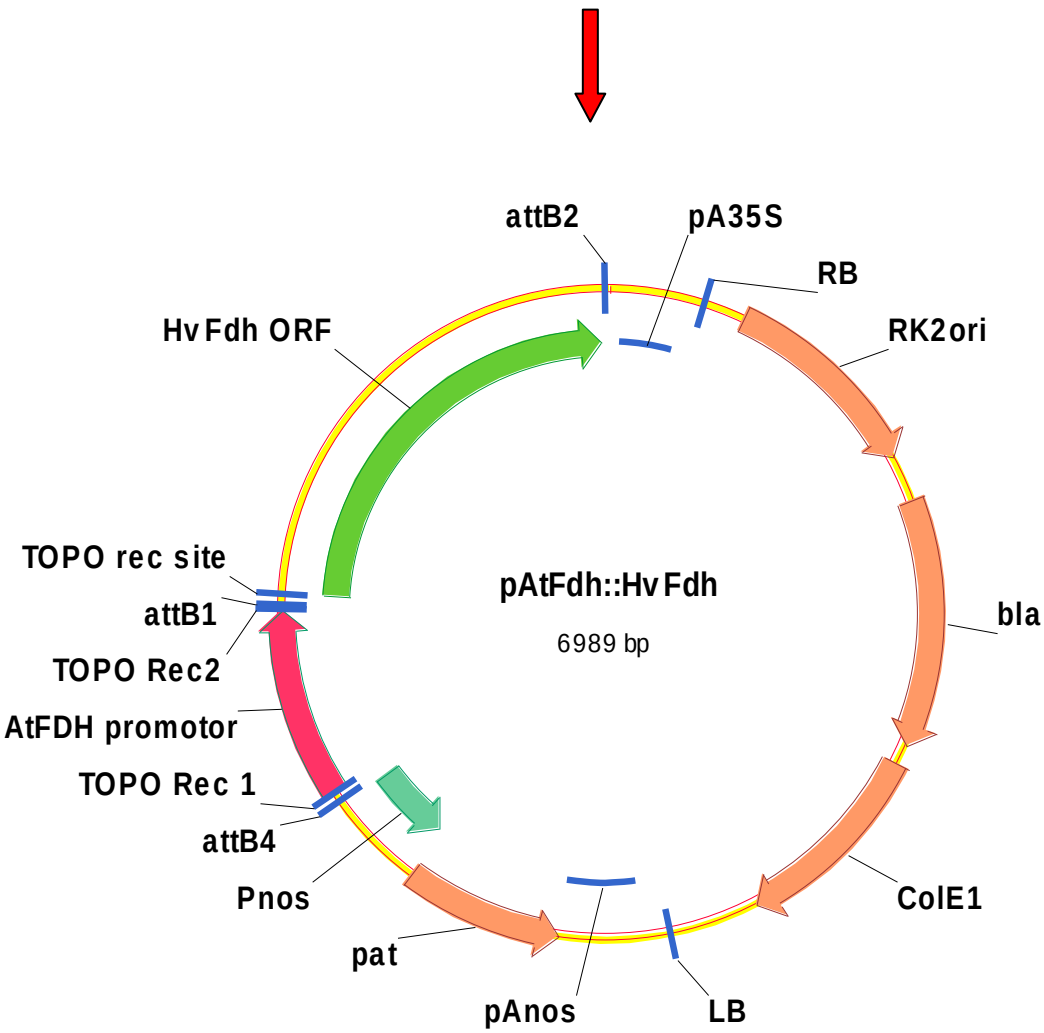

Maps of Gateway enabled plasmids used to assemble *Fdh* complementation construct (pAtFdh::HvFdh) by recombination, which used to complement Arabidopsis *fiddlehead* mutant.

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