# Analysis of gene expression in barley upon aphid attack

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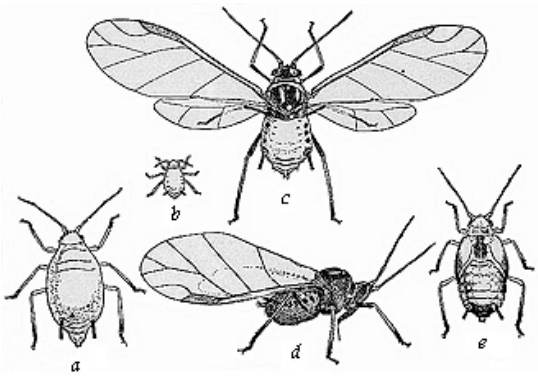


Aphids: a, wingless; b, newborn nymph; c and d, winged; e, nymph

## Content:



**Abbreviations:**

**BTH:** benzo(1,2,3)thiadiazole-7-carbothionic acid
**DCINA:** 2,6-dichloroisonicotinic acid
**DTT:** 1,4-dithiothreitol
**EDTA:** Ethylenediaminetetraacetic acid
**GSP:** Gene Specific Primer
**JM:** Jasmonic acid methyl
**PCR:** Polymerase Chain Reaction
**PR-proteins:** Pathogenesis-related proteins
**RACE:** Rapid amplification of cDNA ends
**RT-PCR:** reverse transcription-polymerase chain reaction
**SA:** salicylic acid
**sqf:** sufficient quantity for
**TBE:** Tris Borate EDTA

## Abstract:

Since plants can not escape their predators by walking, they use some other defense systems, like induction or repression of defense genes. A microarray experiment performed with barley attacked by the bird cherry-oat aphid (*Rhopalosiphum padi*), led to the hypothesis that contig 16360 (similar to ser/thr kinases) could be linked with the resistance of barley against *R. padi*, and contig 6519 (similar to WIR 1A) with the susceptibility. Time course experiments showed that contig16360 and AJ250283 (similar to BCI-4) are almost induced in the same way, each, by two different aphids (*R. padi* and *Metopolophium dirhodum*). Genomic PCR was used to test the hypothesis that when plants have the gene for contig 16360, they are more likely to be resistant against aphid attack, and when plants have the gene for contig 6519, they are more likely to be susceptible. This test was performed with 69 barley lines: wild, commercial or breeding lines. Results were that the presence of WIR 1A gene has no correlation with the susceptibility, while presence of ser/thr kinase seems to be correlated with resistance.

## Introduction:

When an insect attacks a plant for feeding, the plant induces various defence responses. In this study, the defence of barley against aphids was studied.

### *Various plant defense systems:*

Plants can answer to insect attack in various manners: unspecific and/or specific ways. Unspecific plant defense responses, are general answers against many insects. An example of unspecific defense systems is the use of different signaling pathways. As an example, calmodulin-binding protein can induce formation of $H_2O_2$ or reactive oxygen species (ROS). These molecules cause direct oxidative injury on aphids, and, in the case of ROS, act on midgut tissues. Calmodulin-binding protein is used by wheat against *Diuraphis noxia* and by *Arabidopsis* against *Myzus persicae*. Another example is the cell wall resistance, indirectly increased by methyl jasmonic acid which induces accumulation of phenolic polymers and ferulic acid. This method is used by barley and maize against several arthropods. As the last example for unspecific plant defense responses, tobacco, certain wheat species and sorghum plants have the ability to increase the production of chlorophyll or sap proteins, to shield themselves against the losses caused by aphids. Moreover many plants produce secondary metabolites like volatiles, which can prevent aphid settling by repugnance, by attracting some predators or parasites of aphids (review of Smith and Boyko, 2007), or reduce aphid fecundity like certain $C_6$ volatiles (*trans*-2-hexenal, *cis*-3-hexenal and hexanol) which are derived from lipids (review of Walling, 2000). Many specific defense responses are known, and are probably induced by gene-for gene recognition or by modification of plant chemistry during feeding which can induce general stress-related defense responses (Smith and Boyko, 2007). Pathogenesis-related proteins (PR proteins) may play an important role in plant defense. One group of these PR proteins are chitinases, which might be able to hydrolyse chitin in aphid gut. Another PR protein group are the β-1,3-glucanases which can, indirectly, get under way other plant defense reactions (Forslund et al. 2000). Unspecic defense responses may act jointly with specific responses, and so have a synergistic effect. Defense mechanisms act very quickly, about a few hours after infestation. It is important to know that many defense strategies developed by plants to fight against insects, can also damage the plants themselves. That is why plants have to find a balance in order to not commit suicide during defense (Smith and Boyko, 2007).

***Plant predators in insect kingdom:***

Aphids are insects, exclusively plant dependant for feeding. They can, with their four sharp stylets which make up a proboscis, sting the plant tissues and feed from the phloem transport tissue. A plant can react to aphid feeding, because aphids secrete saliva which can go in the plant tissues (hortiauray.com (a) and pbcr.hawaii.edu ). There are two different kinds of plant predators in insect kingdom: phloem-feeding insects and chewing insects. Interaction of phloem-feding insects with plants is very unlike that of chewing-insects. Phloem feeders have a longer interaction with plants than chewing-insects, lasting many hours, or even many weeks. So, plant responses are also longer lasting. Moreover, phloem feeding is not as detrimental as damage caused by chewing-insects because it is just picking in the leaf, while the second sort literally eat the leaf. Both of them secrete saliva during feeding. Saliva can induce activation of signaling pathways. When tomatoes are wounded by chewing-insects, important damages in tissue are created and then electrical signals are transducted and trigger the jasmonic acid-dependent wound signal transduction pathway. That leeds to many things: Signal transduction, generation of ROS or formation of deterrents to insects (Walling, 2000).

***Problems caused by aphids on plants:***

When the plant is attacked by a large population of aphids, it can lead to the weakening of the plant or growth repression, because aphids need the components of the sap, like sugar but also proteins which are present in less quantity than the sugar. The aphids suck a lot of sap to obtain the necessary quantity of protein they need, and as a consequence, deprive the plant for water and nutritive elements.

Aphids can also be the cause of indirect damages: the excretion of honeydew promoting fungal growth or the transmission of virus. Honeydew is a substance secreted by the aphids and it contains 6% to 11% of saccharose. It attracts ants and bees, and saprophytic fungi can develop on the honeydew. It can also prevent the plant respiration and the photosynthesis. Virus diseases are transmitted by the aphid during feeding, and the virus disease is incurable. A virus can induce a lower growth, a discolouration, and deformation of the plant (ext.vt.edu and hortiauray.com (b)). In the case of the bird cherry oat aphid (*Rhopalosiphum padi)*, the major virus disease transmitted is the barley yellow dwarf virus (Lucio-Zavaleta et al. 2001) in barley and winter wheat, *Triticum aestivum L.* (Riedell et al.1999). That is why aphids are serious pests in cereals.

***Aphids: Diuraphis noxia, Metopolophium dirhodum, Rhopalosiphum padi:***

The type and the severity of the symptoms caused by aphids can vary. Of the three aphids used in this study, the first, *Metopolophium dirhodum*, causes orange spots in the feeding area on barley. The second the bird-cherry oat aphid (*Rhopalosiphum padi)*, is the aphid which creates least symptoms, or at least no visible symptoms, and does not induce peroxidase (Forslund, 2000). *Diuraphis noxia*, causes severe symptoms, leading to the death of a barley culture, which is why it is forbidden to import *D. noxia* in Sweden. Because of these differences in symptoms caused by aphids, also what we define as resistance differs in different plant/aphid combinations. In the case of *R. padi*, a barley genotype is called resistant when aphids feeding on it have significantly lower weight than those on a reference line (here it is Lina), while there are no obvious differences in plant growth. Inger Åhman (SLU, Alnarp) has identified two quantitative trait loci (QTLs) which explain 20% and 37 % of the resistance in a wild barley, *Hordeum spontaneum 5.*

When the Russian Wheat aphid, *D. noxia*, eats on a susceptible wheat, affected leaves become streaked in white, yellow or purple, following streaks of the leaves. Wheat possesses a

gene, *Dnx,* which confers resistance to *D. noxia*: pTaDnx826, similar to 12-oxo-phytodienoic acid, used to create Jasmonic acid. pTaDnx660 is similar to transkelotases, wich have many functions like herbivores repellent, toxins or photosynthetic pigment. pTaDnx1167 is similar to calmodulin-binding protein, involved in $Ca^{2+}$-mediated defense. *D. noxia* has also many ways to block defense system of wheat, like pDnDnx872, similar to P450 monooxygenase from *Blattella germanica. It is* used for detoxification of wheat toxins pDnDnx872 and is maybe up-regulated by wheat jasmonate and terpenes. Wheat is able to inhibit aphid midgut enzymes, but *D. noxia* is able to up-regulated these enzymes to counteract the inhibition (Boyko et al., 2006).

***Background of the experiment and studied genes:***

To see differences in gene expression, induced by aphid attack in barley in response to *R. padi*, a microarray experiment was carried out using the Barley1 GeneChip (Affymetrix). For the microarray experiment, four lines differing in their aphid resistance were used: 2 resistant lines (*Hordeum spontaneum* 5 which is a wild barley and *5172-28:4,* a breeding line obtained by crossing *H. spontaneum* with the susceptible line Lina), and 2 susceptible lines (*Lina* and *Kara* wich are commercial lines). Analysis of the microarray data showed that several genes (up to 200) were up-regulated in their expression by aphid attack, and several genes (up to 100) were down-regulated (G. Delp, personal communication). There were differences in regulation between the lines, but also overlaps. Twenty-four sequences were upregulated in all 4 lines, 5 were upregulated only in the resistant and 17 only in the susceptible lines. Among the genes showing a distinct expression pattern were the following:

- Contig “16360” which has similarity to kinases. It was expressed and up-regulated in resistant lines and not in susceptible lines. Kinases are enzymes of the group of transferases, catalysing phosphorylation reactions (addition of phosphate groups) of three amino acids: serine, threonine and tyrosin. Contig “16360” shows similarity with receptor-like kinases, and is very similar to an already known Ser/Thr kinase induced by *Erwinia carotovora* in potato. This last has a catalytic domain which is highly homologous to protein kinases but its extracellular domain is different from the other kinases, which makes it a new class of plant kinase (RLK). It is induced during stress response by the kinases activation pathway. The mRNA of this gene quickly accumulates in roots when the plant is attacked (wound, insect) so it is tissue specific (Montesano et al. 2001).

- Contig “6519” was expressed and up-regulated in susceptible lines and not in resistant lines. It shows similarity to the gene WIR 1A from wheat, which is up-regulated by brown planthopper feeding. The protein encoded by WIR 1A could be involved in the cell wall resistance, in making bonds between polysaccharide molecules and is maybe involved in tissue repair (Yuan et al. 2004).

- AJ250283 represents a gene coding for a putative $Ca^{2+}$-binding EF-hand protein (BCI-4) from barley. BCI-4 is up-regulated by 2,6-dichloroisonicotinic acid (DCINA), benzo(1,2,3)thiadiazole-7-carbothionic acid (BTH), salicylic acid (SA) and jasmonic acid methyl ester (JM) (Beßer et al. 2000). There is a gene with high similarity in *Arabidopsis* (Beßer et al. 2000). This kind of protein can, by conformational changes upon calcium binding, mediate the regulatory effects of calcium (Ikura 1996). In the microarray experiment, AJ250283 was expressed and up-regulated in resistant lines and not present in susceptible lines.

- Contig “12753” has strongest similarity to ATP-binding cassette transporters, (ABC) which is found in *Arabidopsis thaliana.* This last is a member of a group of proteins involved in plant pleiotropic drug resistance (PDR). ABC transporters are used to transport substances across membranes. They are composed of a hydrophobic transmembrane-spanning domain (TMC) linked with an ABC cytosolic domain. The pattern TMC-ABC may be repeated to form a larger

protein. The ABC domain utilises ATP as energy source to perform the transport. ABC transporters can be used to respond to many factors: environmental, chemical and hormonal, and are "tools" for plants resistance to drugs. ABC transporters can be found in eukaryotic and prokaryotic cells, like in *Candida albicans* and *Saccharomyces cerevisae* (van den Brûle and Smart, 2002 and Jasiński et al. 2001).

**Aims:**

Results from a microarray experiment (G. Delp unpublished) indicated a correlation between resistance/susceptibility and contig 16360/contig 6519 in the four barley lines: *H.sp 5* (wild line), 5172-28:4 (breeding line), Lina (commercial line), Kara (commercial line). Only in the resistant lines was contig16360 expressed, whereas contig6519 had the opposite expression pattern. A preliminary genomic analysis with PCR with gene-specific primers indicated that the lines where the genes were not expressed lacked the genes.

The first aim of this project was to test the hypothesis that when plants have the gene for contig 16360, they are more likely to be resistant against aphid attack, and when plants have the gene for contig 6519, they are more likely to be susceptible. This test was performed by doing genomic PCR with a total of 69 barley lines: wild, commercial or breeding lines.

The second aim was to know how many time more the genes are induced when aphid are present in comparison of the expression when aphids are absent, and whether the gene is up-regulated with several aphids and not only with *R. padi*, and if yes compare these results.

A third aim was to have, by RACE, the 5' cDNA end missing in the ATP-binding cassette, and cloning the full-length of this cDNA.

## Materials and methods:

| Name | Sequence |
|---|---|
| Actin long forw | 5'- GAG AGG TAT CCT CAC GCT CAA GT -3' |
| Actin short forw | 5'- TTC TCG ACT CTG GTG ATG GTG T-3' |
| Actin rev | 5'- CAA GCT TCT CCT TGA TGT CCC -3' |
| 16360 forw1 | 5'- TTA GCA GTT CGT TCT TTT GGT TCA AC -3' |
| 16360 forw2 | 5'-GGA CCC ATC CAT GAA CAG TAG CTT-3' |
| 16360 rev | 5'-ACT CCA GGT ATC TTG TGC ATG CGT-3' |
| 6519 forw2 (F2) | 5'-TTG AGC ACA AGT TAG AGA CTCTGT TT-3' |
| 6519 forw3 (F3) | 5'-GCT CGA ATT ATA AAC TAG CAG CCT C-3' |
| 6519 rev1 (R1) | 5'-AAA CAG GGT CTT ACT TTA CTG TCA-3' |
| 6519 rev2 (R2) | 5'-GCA GGT GGC AAA CCC TGA TTA CAC-3' |
| BCI 4 forw | 5'-GAT ATA TTT CCC GAC CTT GTA TTC TC-3' |
| BCI 4 rev | 5'-ATT AGG TGG ATG AGC CTT ATG CTT-3' |
| 12753 forw | 5'- AGG AGC AGT CTA CAC TGC AT -3' |
| 12753 rev | 5'- CTG TATC CAT ATG TGT TTG CAC T-3' |
| GeneRacer™ 5' primer | 5'-CGA CTG GAG CAC GAG GAC ACT GA-3' |
| GeneRacer™ 5' nested primer | 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3' |
| Control primer A | 5'-GCT CAC CAT GGA TGA TGA TAT CGC-3' |
| Control primer B.1 | 5'-GAC CTG GCC GTC AGG CAG CTC G-3' |

***Figure 1:*** *List of used primers. Primers were ordered from MWG-Biotech AG, Ebersberg, GERMANY*

*Plant growth*

Lina, Barke and Kara are susceptible to *R. padi. Hordeum spontaneum* 5 and the breeding line *5172-28:4* are resistant to *R. padi* (Inger Åhman, personal communication). Seeds of these plants and their offsprings, lines 6652, 6653, 6654 and 6655 (fig.4), as well as some other lines were surface-sterilized for 2 minutes in 0,75% $H_2O_2$. Then 5 seeds of the same line were planted in wet vermiculite and placed in a growth chamber at 21°C and with a photoperiod of 18h light / 6h dark. Plants were 6 days old at harvest. It is necessary, for the DNA extraction, to harvest the basis of the plant between the seed and the middle of the primary leef. Powder was kept in a tube in liquid nitrogen or at - 86°C when not used immediately.

*DNA extraction from Barley:*

Barley DNA was extracted using the "Dneasy(R) Plant Mini Kit" for isolation of DNA from plant tissue, QIAGEN(R):, Hilden, GERMANY. The DNA was extracted from frozen barley tissues, using a mortar and pestle to grind the tissue to a fine powder. Liquid nitrogen kept the material frozen during this procedure to keep the DNA intact.

To 100 mg of the powder 400 µL of lysis buffer and 4 µL of RNase A (100 µg/mL) were added and incubated 10 min at 65°C. Precipitation buffer (130 µL) was added and incubated 5 min on ice and then centrifugated 5 min at 20000 x g (14000 rpm).

Lysate was applied to a QIAshredder Mini Spin Column and centrifugated 2 min at 20000 x g. Binding buffer (1,5 x volumes) was added to the cleared lysate. This mixture was applied to the DNeasy Mini Spin Column and centrifugated 1 min at 6000 x g. The column was washed twice by applying washing buffer (500 µL) and centrifugating 1 min at 6000 x g (first wash) and one time 2 min at 20000 x g (second wash). Elution: 100 µL of elution buffer (preheated at 65°C) was applied to the column, incubated at room temperature for 5 min and then centrifugated for 1 min at 6000 x g.

The concentration of the DNA was measured with NanoDrop®, using the elution buffer as blank.

*Genomic PCR:*

Genomic PCR was carried out with 20 ng DNA from each barley line as template. To detect the genes, the following primer pairs were used:

- Receptor-like protein kinase gene: 16360 forw & 16360 rev
- WIR 1A gene: 6519 forw3 (F3) & 6519 rev2 (R2)
- Transposon: 6519 forw2 (F2) & 6519 rev1 (R1)
- Presence of the Transposon in the WIR 1A gene: 6519 forw3 (F3) & 6519 rev1 (R1)
- As a control, PCR was performed with primers specific for Actin

The composition of the mix, for a total volume of 20 µL, was: 2µL of 10x Buffer (containing 500 mM of KCl and no $MgCl_2$), 1,5 mM $MgCl_2$ (final concentration), 0,2 mM dNTP (final concentration), 1u *Taq* DNApolymerase, 0,5 µM primer1 and primer2 (final concentration), 20 ng DNA, $H_2O$.

Cycling conditions were as follows: 94°C for 4 min; (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min) x32.

*Reverse Transcription PCR:*

Reverse Transcription PCR was performed as one-step RT-PCR with 30 ng total RNA as template. To detect the genes, these primer pairs were used:

- Kinase : 16360 forw & 16360 rev : size 400 bp
- BCI 4 gene: BCI 4 forw & BCI 4 rev : size 600 bp
- As a control, RT-PCR was performed with primers specific for Actin.

The kit used for this was "SuperScript™ III One-Step RT-PCR with Platinum® *Taq"* (INVITROGEN).

The composition of the mix, for a total volume of 25 µL, was: 12,5 µL of 2x reaction mix (buffer containing 0,4 mM of each dNTP and 3,2 mM $MgCl_2$), 0.4 µM (final concentration) Primer1, 0.4µM (final concentration) Primer2 , 1u/µL Taq Mix (1µL; containing both reverse transcriptase and Taq polymerase), 30 ng RNA , $H_20$.

Cycling conditions (40 cycles) were as follows: before amplification of the cDNA, a first step is necessary to create it, the reverse transcription: 47°C during 30 min, and 94°C for 5min to inactivate the reverse transcriptase, and then for the PCR: 94°C 30 sec (denaturation of the template), 55°C 30 sec (primer annealing), 72°C 1 min (primer elongation), and after 40 cycles, another step: 72°C for 5 min (final extension).

*Real time PCR*

Real-time PCR was performed with the kit "iScript™ One-Step RT-PCR kit with SYBR® Green" from BIORAD, and with these primer pairs:

- Kinase : 16360 forw & 16360 rev : size 400 bp
- BCI 4 gene: BCI 4 forw & BCI 4 rev: size 600 bp
- As a control, RT-PCR was performed with primers specific for Actin. Actin long forw & actin rev: size 400 bp.

The composition of the mix, for a total volume of 25 µL, was: 0,5µL iScript Reverse Transcriptase for One-Step RT-PCR, 12,5µL of 2x SYBR® Green RT-PCR reaction mix (buffer containing 0,4 mM of each dNTP, Magnesium Chloride (quantity not precised), i*Taq* DNA polymerase (quantity not precised), 20 mM fluorescein SYBR® Green I dye and stabilizers), 0,3mM (final concentration) of each primer 1 and 2, 50 ng of total RNA, Nuclease-free $H_20$.

Cycling conditions were as follows: 10 min at 50°C for cDNA synthesis, 5 min at 95°C for iScript Reverse transcriptase inactivation, 30x (10 sec at 95°C, 30 sec at 55°C) for PCR cycling, and detection. This PCR was followed by a melting curve analysis of the products.

*RACE:*

To obtain the 5' missing cDNA end of contig 12753, a potential ABC (ATP-binding cassette) transporter, with total RNA from the susceptible line Lina, which grew with presence of aphid during 3 days, RACE (Rapid Amplification of cDNA Ends) was performed. Steps in this procedure were dephosphorylating RNA, removing the mRNA cap structure, ligating the RNA oligo to decapped mRNA, reverse transcribing mRNA, amplifying cDNA ends and performing nested PCR. A positive control (included in the kit) was performed at the same time as the sample. All these steps were carried out according to the protocol called "GeneRacer™ Kit" for

full-length, RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) from INVITROGEN™.

After each step, It was necessary to precipitate RNA, adding mussel glycogen to increase the yield, so as to separate it from the previous reagents needed for the reaction.

Reverse transcription of mRNA, was performed using the SuperScript™III RT reaction, also supplied with the kit. Incubation temperature used for reverse transcription was 55°C for 45 min, because the primer used was a GSP (Gene specific primer): "12753 rev". For subsequent PCR, 1 µl of the RT reaction was used as template.

Amplification of cDNA 5' ends was performed by PCR, with Platinum™ *Pfx* DNA Polymerase. For the sample, reagents are as follows, for a total volume of 50 µL: 4,5 µL of GeneRacer™ 5' primer (10 µM), 1,5 µL of GSP (reverse GSPprimer 12753rev for ABC transporter, control primer B.1 for the positive control) (10 µM), 1 µL of RT template, 5 µL of 10x *Pfx* amplification buffer, 1,5 µL of dNTP solution (10 mM each), 0,5 µL of Platinum® Pfx DNA polymerase (2,5 U/µL), 2 µL of $MgSO_4$ (50 mM), 34 µL of sterile water. Cycling conditions were as follows: 95°C for 2 min, 5 x (95°C for 30 sec and 72°C for 3 min), 5 x (95°C for 30 sec and 70°C for 3 min), 25 x (95°C for 30 sec 60°C for 30 sec and 72°C for 3 min), 72°C for 10 min.

Cycling conditions for the positive control (HeLA) are as follows: 94°C for 2 min, 5 x (94°C for 30 sec and 72°C for 2 min), 5 x (94°C for 30 sec and 70°C for 2 min), 25 x (94°C for 30 sec 65°C for 30 sec and 68°C for 2 min), 68°C for 10 min, as specified in the protocol

Nested PCR was carried out as follows, for a total volume of 50 µL: 1 µL of GeneRacer™ 5' Nested (10 µM), 1 µL of reverse nested GSP (10 µM; control primer B.1 for control reaction, primer for ABC transporter), 1 µL of initial PCR (from amplification of cDNA 5' ends), 5 µL of 10x Buffer (containing 500 mM KCl and no $MgCl_2$), 1 µL of dNTP solution (10 mM each), 1,5 mM $MgCl_2$ (final concentration), 2u *Taq* DNApolymerase, sterile water.
Cycling conditions for the PCR are as follows: 47°C for 30 sec, 94°C for 2 min, 34 x (94°C for 30 sec, 65° for 30 sec and 68°C for 2 min), 72°C for 5 min.

After dephosphorylation, decapping, and ligation, the integrity of the RNA was tested by gel electrophoresis. PCR and nested PCR products were analysed by agarose gels.

*Gel electrophoresis of DNA and RNA*

To analyse the products of Reverse-Transcription PCR, genomic PCR and RACE, the DNA products were loaded on a 2% agarose gel, run with TBE buffer (0,5xTBE; for 1 litre: 0,54 g Tris-base, 2 mL of 0,5M EDTA (pH8), 2,75g Acid Boric). The DNA was visualized by adding Ethidium bromide: 0,001% (w/v) to the gel. As a size marker the "Massruler DNA ladder mix" (FERMENTAS) was used. Bands were visualised by UV light.

## Results:

**1 / Genes are up-regulated during aphid attack, and regulation is not specific for aphid species:**

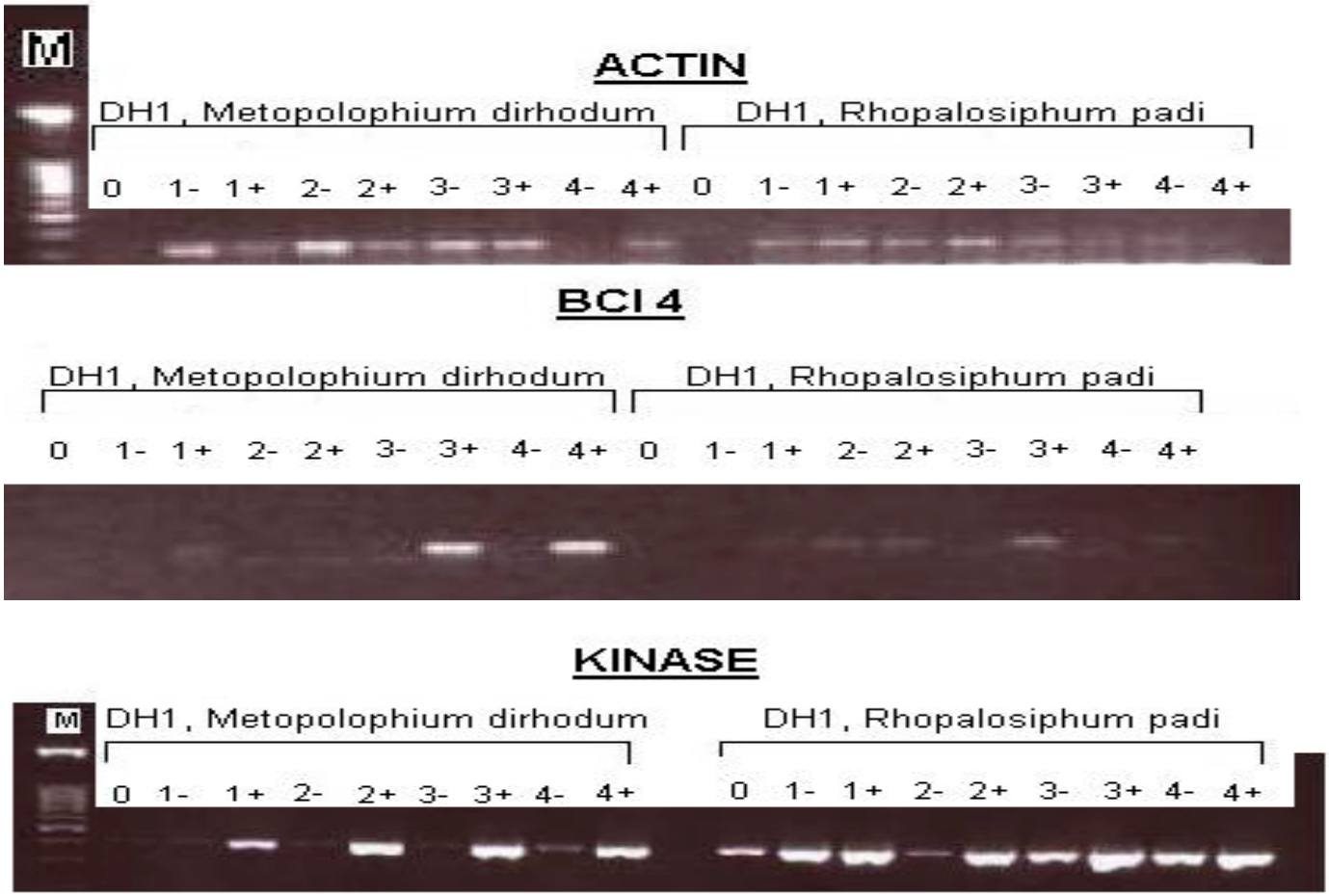


***Figure 2:*** *RT-PCR of BCI 4 and Ser/thr kinase genes, from a resistant barley line:* ***5172-28:4*** *(DH1). 18 samples of DH1 grown between 0 and 4 days (time course), with (+) or without (-) aphid: R. padi or M. dirhodum. RT-PCR was carried out with 30 ng of total RNA, and PCR products loaded on agarose gels and visualised by ethidium bromide staining.*

The microarray experiment was carried out with 4 barley lines: *Hordeum spontaneum 5*, 5172-28:4, Lina and Kara. Several genes were identified for which there was a clear difference in their expression depending on presence or absence of the aphid on the plant (G. Delp , personal communication). A sequence with similarity to WIR 1A was up-regulated when aphids were present, and only in susceptible barley lines (Kara and Lina). A sequence with similarity to a kinase showed the opposite expression pattern. It was up-regulated only in resistant lines (*H. spontaneum 5* and 5172-28:4).

In order to confirm these regulation differences in barley when aphids are present or absent and also to see if the aphid species has an influence on the expression of these genes, two time-courses were performed in the same barley strain: 5172-28:4 (DH1). But one series had the aphid *Metopolophium dirhodum* (*M. dirhodum*) and the second had *Rhopalosiphum padi* (*R. padi*) as pest.

RTPCR with gene-specific primers gave products of the expected sizes for all genes. Actin was used as a control, because actin is present in all cells, and is considered not to be regulated by aphid attack. Results are shown in Figure 2.

BCI 4 (AJ250283) shows a band at 600 bp and is up-regulated by aphid attack The bands are strongest at 3 days and 4 days with aphids, and stronger in *M. dirhodum*-infested samples than in plants attacked by *R. padi*

Kinase (contig 16360) was also induced each day between 0 and 4 days, but contrary to BCI 4, a clear up-regulation appears from the first day when aphids were present. The comparison between *M. dirhodum* and *R. padi* showed that in *M. dirhodum*-infested plants there was a clear up-regulation from day one of aphid attack. In the experiment with *R. padi*, the bands were stronger on all days with aphids than in the control samples or the sample at 0 days. But there was considerable variation in the expression of the gene in the control samples (i.e. 0, 1-, 2-, 3-, 4-).

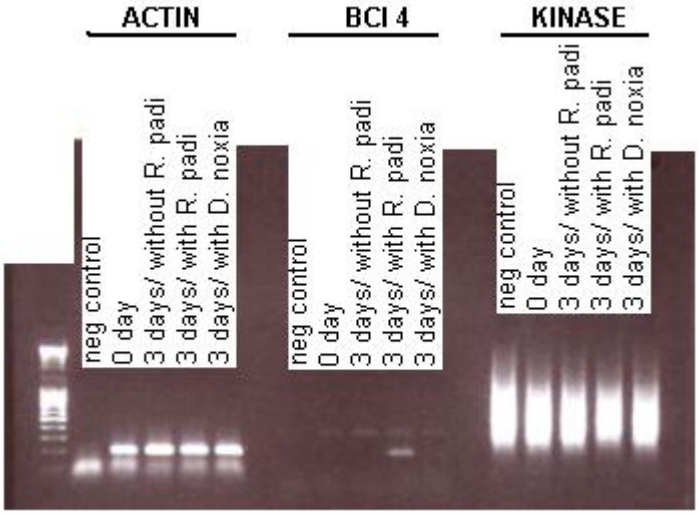


**Figure 3:** *RT-PCR of BCI 4 and Ser/thr kinase genes, from a susceptible barley line: Clipper. samples of Clipper grown between 0 and 3 days (time course), with or without aphid: R. padi or D. noxia. RT-PCR was carried out with 30 ng of total RNA, and PCR products loaded on agarose gels and visualised by ethidium bromide staining.*

Those genes were also tested with *Diuraphis noxia* on Clipper, a barley line susceptible against *D. noxia* and not known to be resistant against *R. padi* (fig. 3*)*. Actin was used as control. BCI 4 was found in Clipper which had been grown 3 days with *Rhopalosiphum padi* but not with *Diuraphis noxia*. For kinase, a smear appeared.

## 2/ Reverse Real-time PCR

Real-time RT-PCR was performed, but due to a failure in the protocol used for it no data were registered. No results are available.

## 3 / Correlation between kinase/WIR 1A and resistance/susceptibility respectively:

Based on previous experiments, the hypothesis was postulated that contig 6519 (WIR 1A) is maybe correlated with the susceptibility of barley against aphid attack and that the contig16360 (kinase) is maybe linked with the resistance of barley against aphid attack. To test this hypothesis, 69 barley lines that differed in their resistance to *R. padi* were used to increase the number of data. All these lines were obtained from Inger Åhman, who performed several back-crosses between the offspring of a cross between Lina and *H. spontaneum* and Lina or Barke, two barley lines currently used in agriculture. These two barley lines are 2-row barley for malting (beer and whiskey). They are susceptible to *R. padi*. First, Lina was crossed with a wild barley, *Hordeum spontaneum*, naturally resistant against aphids, to obtain as first generation the line called 660-6:8. Then 660-6:8 was back-crossed with Lina and from the offspring three lines with good resistance were selected: 5172-28:4, 5172-39:9, 5172-48-12. Finally, these 3 lines were

again crossed with Lina to obtain series 6652, 6653 and 6654 respectively. 660-6:8 was also crossed with Barke, the offspring was 5175-50:20, which, by back-cross with Barke gave the 6655 series (fig. 4).

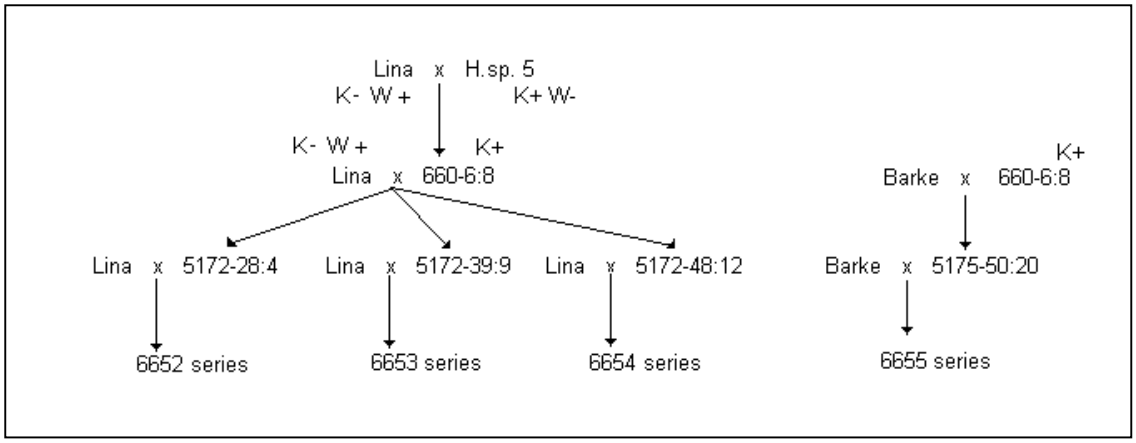


***Figure 4:*** *Crossing scheme leading to 46 barley lines segregating for aphid resistance, giving the 6652, 6653, 6654 and the 6655 series. The susceptible commercial line Lina was crossed with the most resistant wild type Hordeum spontaneum 5 to obtain 6652, 6653 and 6654 series. The susceptible commercial line Barke was crossed with 660-6:8 to obtain the 6655 series.*

In addition to these four series of related barley lines that segregated for aphid resistance, a number of commercial lines and several accessions of *H. spontaneum* with varying aphid resistance were used (see appendix B). DNA was extracted from young tissue of barley plants that were 6 days old.

Several genomic PCRs were performed to detect contig 6519 and contig 16360 in the extracted DNA of all barley lines (appendix A). When the sequence of contig 6519 from the Affymetrix database was analysed by BLAST search against nucleic acid databases (www.ncbi.nlm.nih.gov), it became clear that contig 6519 may contain a transposon between nucleotides 377 and 643. It is called “Islay” and it’s accession n° is AJ862550.1. The first primers (6519F2 and 6519R1) that were used for the PCR and most of the oligos used for detection of contig6519 on the Affymetrix chip were located in the transposon. So, in the microarray, it was not contig 6519 wich was shown to be expressed only and up-regulated in susceptible lines and not in resistant lines, but it was the transposon “Islay”. In order to really detect contig 6519, new primers were designed as shown in the figures 5 and 6. Two primers were created with the aim to only see the presence of contig 6519 gene thanks to the primers called F3 and R2, only the transposon “Islay” thanks to the primers F2 and R1, and finally the combination of F3 and R1 used to see the presence of “Islay” inside contig 6519 (fig. 5 and 6).

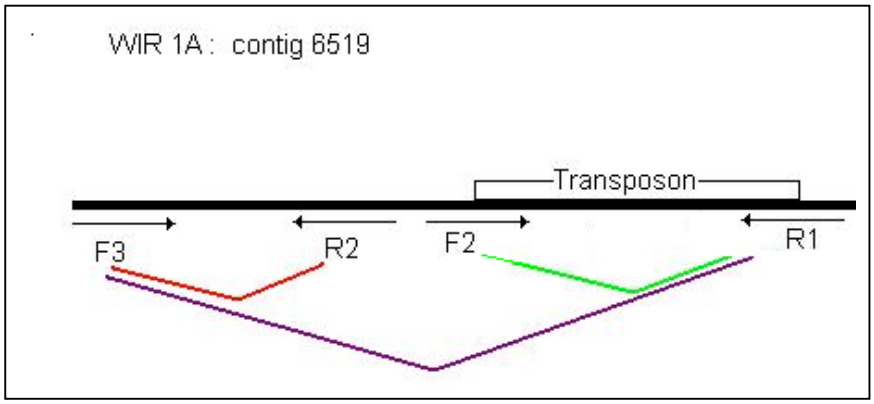


***Figure 5:*** *Three couples of primers Were used to see only the WIR 1A Gene (F3/R2), the Transposon (F2/R1) and the presence of the Transposon in the WIR 1A gene.*

BARLEY1: CONTIG 6519_AT


***Figure 6:*** *Location of the primers in WIR 1A. Written in bold and red : The transposon; In yellow: forward primers. In blue: reverse primers. Sequence from the Affymetrix website*

Expected sizes for the fragments were 400bp for contig 16360 (kinase), 300bp for the Transposon “Islay”, 390bp for contig 6519 (WIR 1A) and 600bp when the transposon was in contig 6519 (fig. 6). The results showed that the transposon was found in the genome of 36% of all barley lines, of which 52% are susceptible and 48% resistant (Appendix B).

To test if the new designed primers for contig 6519 worked well, a genomic PCR was run with the same conditions as the previous genomic PCR. In a first test, (fig. 7) ten lines differing in their aphid resistance were used, five susceptibles lines which are Lina, Kara, BCC38, CI 11506 and 6652-121, and five resistant lines which are H.sp.3, H.sp.5, 5175-50:20, 6652-16 and 5172-28:4. Actin primers were used as control, and a band of the expected size appeared in all samples. The intensity of the actin band varied, which was perhaps due to an uneven amount of template. This preliminary test for the WIR 1A primers showed that a band of 390 bp appeared in all barley lines when primers F3 and R2, which both are lying outside of the transposon, were used. This means that the Wir1a gene is present in all lines, both susceptible and resistant. Here, the transposon, revealed by the primer pair F3 and R1, appeared only with the five susceptible lines (at 300 bp). In all of them the transposon was detected in contig 6519 with primers F3/R1 (at 600 bp).

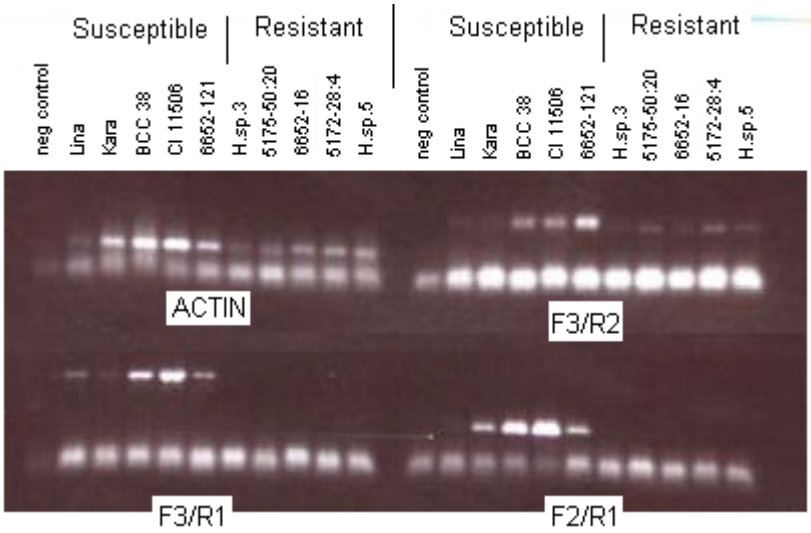


***figure 7:*** *genomic PCR with all primer pairs, used on DNA from some susceptible and some resistant barley lines. Actin was used as control. Primers F3 and R2 are both outside of the transposon, F3 and R1 are outside and inside respectively, F2 and R1 are both inside. The PCR products were visualised by ethidium bromide staining.*

Since the distinction between the contig 6519 and the transposon AJ862550.1 “Islay” is done, these three primers pairs were used for genomic PCR on all lines (appendix A), and the results are (appendix B)**:**

- contig 6519 (Wir1a): 93% of all barley lines have the gene for WIR 1A, and in these, 44% are susceptible and 56% are resistant. Moreother, 97% of resistant lines have the gene for contig 6519. 88% of susceptible lines have gene for contig 6519.

Only Haisa, H.sp.39759, 6655-90, 6655-135 and 6655-160 are negative for WIR 1A.

- AJ862550.1 (transposon): 36% of all barley lines have the transposon, and in these, 52% are susceptible and 48% are resistant. 31% of susceptible lines have “Islay”, and 30% of resistant lines have “Islay”. Each time “Islay” was present, it was inserted in the contig 6519, except for Haisa, where it is present but probably not in WIR 1A.

- contig 16360 (kinase): bands appeared at 399 bp and in both susceptible and resistant barley lines: 46% (32) of all 69 barley lines have the gene for kinase, and in these, 63% (20) are resistant and 38% (12) are susceptible. 54% (14) of the 37 resistant lines have the gene for contig 16360 and 38% (12) of the 32 susceptible lines have this gene. However, when only the four families of segregating lines (total of 46) were analyzed, 52% (24) had the gene. Of these, 58% (14) were resistant, 42% (10) were susceptible. Of the 23 resistant lines in this population, 61% (14) had the gene, while only 39% (9) of the susceptible had the kinase gene. There is one sample that gave an unexpected result: 5172 48:12 was negative for kinase, but the offspring of the cross of this line with Lina gave several lines in the 6654 series that were positive for kinase.

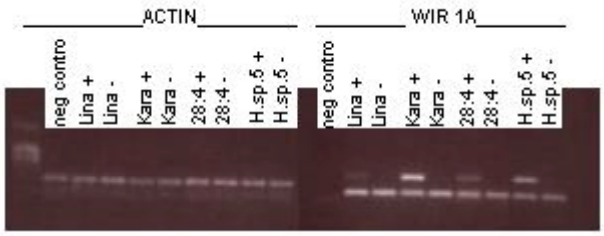


***Figure 8:*** *RT-PCR for WIR 1A gene with total RNA of resistant lines (5172-28:4 and H.sp.5), and in susceptible lines (Lina and Kara). The PCR products were visualised by ethidium bromide staining.*

To see if WIR 1A is only expressed in susceptible lines, RT-PCR was performed (fig. 8) with total RNA of 2 resistant lines (5172-28:4 and *H.sp.5*), and in 2 susceptible lines (Lina and Kara). Band was found in both resistant and susceptible lines, when aphid are present. No band when aphid are absent in both resistant and susceptible lines.

**3 / results of the Rapid amplification of the 5' cDNA end.**

The susceptible line Lina has the gene coding for contig 12753, which has similarity with the ATP-binding cassette transporters. But contig 12753 misses the 5' cDNA end, so a Rapid Amplification of the 5' cDNA end (RACE) was performed, following instructions in the kit supplied by INVITROGEN. The first three steps of the RACE were dephosphorylation, decapping and ligation, and after each of them, RNA was precipitated to isolate it from necessary reagents for the previous steps. In order to see if the RNA was still intact after these steps, 1µL of both sample and positive control RNA were kept at -20°C for gel analysis.

Three negative controls were performed during the amplification of cDNA 5' ends:
- one, containing all components as samples, except template to test for DNA contamination.
- one, containing all components as samples, except GSP to test for unspecific binding of primer GeneRacer$^{TM}$5' primer.
- one, containing all components as samples, except GeneRacer$^{TM}$ 5' primer to test for unspecific binding of GSP.

For all negative controls, nothing appeared, so the result could be accepted (fig. 9). After the first round of PCR, there was a smear visible on the gel. In this case a nested PCR is advised by the kit manufacturer.

When nested PCR was performed as it is described in the kit protocol, a strong band appeared through a little smear (it could be seen on the monitor, but not on the picture) (fig. 9).

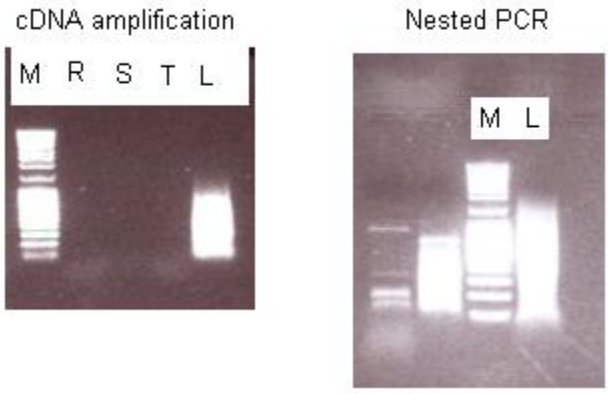


***Figure 9**: results of cDNA amplification and the nested PCR, from the Rapid Amplification of the 5' cDNA end of the ATP-binding cassette gene. M is the size ladder, R, S and T are negative controls. R is without GeneRacer$^{TM}$ 5' primer, S is without GSP, T is without sample. L is the sample, Lina.*

## Discussion

### 1 / Genes are up-regulated during aphid attack, and are not specific for aphid species:

RT-PCR (fig. 2) showed that actin, the control for equal amount of template, did not give the same intensity of product in each sample, although this was expected because the concentrations of all RNA samples were identical. The differences were maybe due to an error with the pipetting. Kinase was expressed even if aphids were absent, and it is up-regulated when aphids are present, whatever the aphid (*Metopolophium dirhodum* or *Rhopalosiphum padi*). In the controls for the experiment with *R. padi* there was strong expression in some samples. Like for actin, this could have been due to inconsistent pipetting when adding the template. But it could also be caused by some stress that the control plants did experience during the experiment. It could have been physical stress, but also pathogens that maybe attacked the roots or other parts of the plant although there were no visible symptoms on the plants. The kinase was induced rather quickly, one day was sufficient for the plant to react with the aphid and induce expression. That indicates that the kinase is up-regulated by aphid feeding and that the increase is done one day after invasion by aphids. In the case of BCI 4, the observation is the same, expressed weakly when aphids are absent, up-regulated when aphids are present. However, three days are necessary to reach similar expression levels as the kinase after one day in the presence of aphids. Also for BCI 4 there is some weak expression detectable. That indicates that BCI 4 is up-regulated by aphid feeding and that the increase is done three days after invasion by aphids. There is a stronger up-regulation caused by *M. dirhodum* than by *R. padi*. This correlates with *M. dirhodum* causing stronger damage to the plant than *R. padi*.

BCI 4 is a $Ca^{2+}$ binding protein that is induced by the plant signal substance SA, as well as by the synthetic SA-analogues BTH, DCINA and a little bit by JM, but not during infection with the fungal pathogen *Blumeria graminis v. hordei* (powdery mildew) (Beßer et al., 2000). SA is a defence response signal for plant during aphid feeding, it can induce, among others, PR proteins, production of ROS, $H_2O_2$, and induced plant arthropod defense allelochemicals ( Smith and Boyko, 2006). It is used as a marker for chemically-induced resistance in barley (Hückelhoven et al. 2001). BCI 4 is maybe corrrelated with both SA and JA signal transduction pathway.

These results confirm the results from the microarray experiments that indicated that kinase and BCI4 are induced by *R. padi* in barley after 2 days of aphid feeding.

Moreover, it is not sure that those gene induce themselves resistance of barley. It could be the case against *R. padi*, but it is not sure against *M. dirhodum*, because we do not know if 5172-28:4 is resistant against *M. dirhodum* feeding

### 2 / WIR 1A gene and transposon are not correlated with susceptibility:

Based on the microarray experiment and preliminary PCR analysis it was expected that only susceptible barley lines contain the gene coding for Wir1a. However, this was based on the use of primers that were located in a transposon present in Wir1A in some barley lines (transposon “Islay”, see fig.6). Thus these preliminary results were not valid for WIR 1A but the transposon.

This study showed the presence of the WIR 1A gene in all barley lines, both resistant and susceptible. So, it is clear that presence of the gene coding for WIR 1A is not specific to susceptible lines. Moreover, RT-PCR were performed (fig. 8) with the 4 lines of the microarray, and it appears that WIR 1A was expressed in both susceptible and resistant lines.

Another possibility could be that the transposon is present in Wir 1A only in susceptible lines and that this presence interrupts the reading frame of Wir 1A. Since Wir 1A genes have been

described to be induced during plant defense reactions, this could contribute to susceptibility to aphids. This study showed that the transposon is present in both susceptible and resistant lines and that not all susceptible lines have the transposon. Thus, the transposon AJ862550.1 “Islay” has correlation neither with susceptibility, nor with resistance.

A lot of technical problems were observed with genomic PCR, and in particular to detect the gene for contig16360 (kinase). At the beginning of the experiment, 40 cycles were used for the genomic PCR, and results were very weak bands detectable. Then little smears appeared, but did not interfere with results. Later the smears became stronger and were visible over the entire lenght of migration on the gels. To attempt to solve the problem, a fresh dilution of the 16360 primers were used and compared with the old ones, on *H.sp*.5 DNA, a line known to have the kinase gene. Smear was present in both of them, but a band was clearly detected through the smear. The smear appeared even in the negative control and so made results unusable. One hypothesis was that one of the components used for the master mix was corrupted. Each product were changed, as primers, $MgCl_2$, dNTP, 10x buffer, *Taq* polymerase and even sterile water, without changes in results. The concentration of the loading gel was also changed, it was at 2% of agarose, and was changed in 1,5%. Results stayed the same.

Sometimes, PCR products were not detectable because of the absence of any bands, even in the positive control (*H. sp.*5). Another hypothesis was that the number of PCR cycles was too high, and so too much DNA products migrated in the gel. The number of PCR cycles was reduced from 40 to 37, 35, 32 and finally 30. No change in either results (smears or sometimes nothing) was observed.

Another hypothesis was that the DNA templates were corrupted or damaged. Elution were changed, and tested with 32 and 37 cycles, and finally 40 cycles. Nothing or smear appeared.

Finally, other primers were used (16360 forw1 and 16360 rev, product size: 100 bp), but they did not get expected result for the positive control *H.sp*.5, and for the breeding lines 5172-28:4, which was positive for kinase with old primers.

Only a little more than 50% of total resistant barley lines have the contig 16360, and less than 40% of susceptible lines have it. There is not a very large difference between resistant and susceptible lines. This difference is increased when only the four groups of segregating lines, 6652, 6653, 6654 and 6655 lines were analysed (fig.4). 61% of resistant lines had the gene, while only 39 % of the susceptible had it. So, the balance leaned a little in favour of the starting hypothesis: When a barley line has the gene for contig 16360 (kinase), it is more likely to be resistant against aphid attack.

However, there is one sample that gave an unexpected result: 5172 48:12 was negative for kinase, but the offspring of the cross of this line with Lina gave several lines in the 6654 series that were positive for kinase. So, results must be taken with a certain reserve, because some other resistant lines could have the gene for contig 16360, but susceptible lines too.

An interesting result, is that in each cross with Lina as parent (Lina x 5172-28:4 ; Lina x 5172-39:9 ; Lina x 5172-48:12), the correlation between resistance and presence of the kinase gene is not very clear. Only the cross Barke x 5175-50:20 give some results in this way (5 of 6 resistant lines have kinase gene).

## 3 / RACE

A Product of the nested PCR was visible on the gel. However, it was not pure enough for cloning, so further optimisation of the PCR would be needed to really separate the full-lenght cDNA of the ATP-binding cassette.

## Buffers and mixes:

**SYBR® Green RT-PCR reaction mix:** 0,4 mM of each dNTP, Magnesium Chloride (not precised), i*Taq* DNA polymerase (not precised), 20 mM fluorescein SYBR® Green I dye and stabilizers),

**TBE:** 0,54 g Tris-base, 2 mL of 0,5M EDTA (pH8), 2,75g Boric Acid for 1 Litre

**2x reaction mix:** 0,4 mM of each dNTP and 3,2 mM $MgCl_2$

**10x Buffer:** 500 mM KCl and no $MgCl_2$

**SuperScript™III reverse transcriptase:** 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0,1 mM EDTA, 1 mM DTT, 0,01% Nonidet P-40, 50% glycerol.

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http://hortiauray.com/puceronsbiologie.htm (a)

http://hortiauray.com/puceronssymptomes.htm (b)

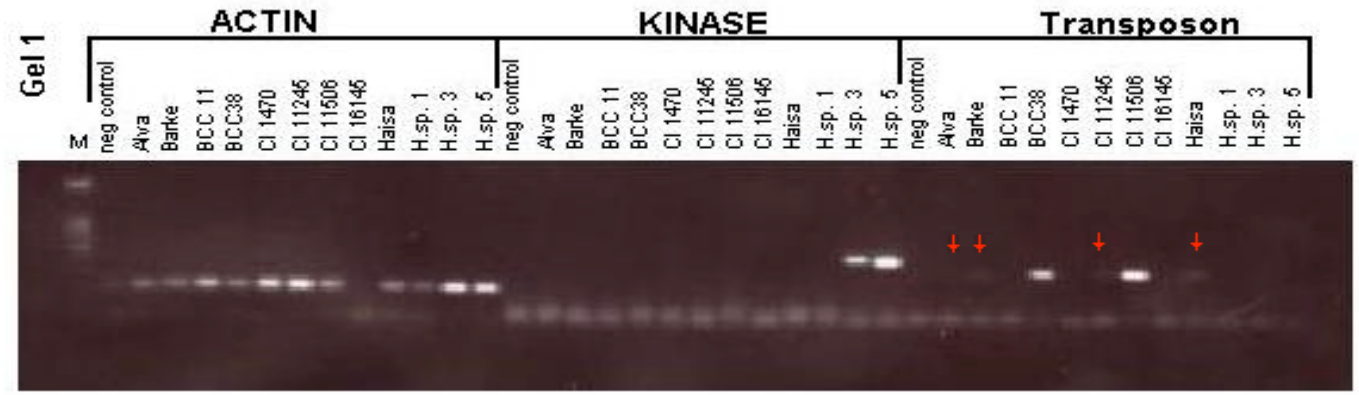
http://www.ext.vt.edu/departments/entomology/factsheets/gaphids.html

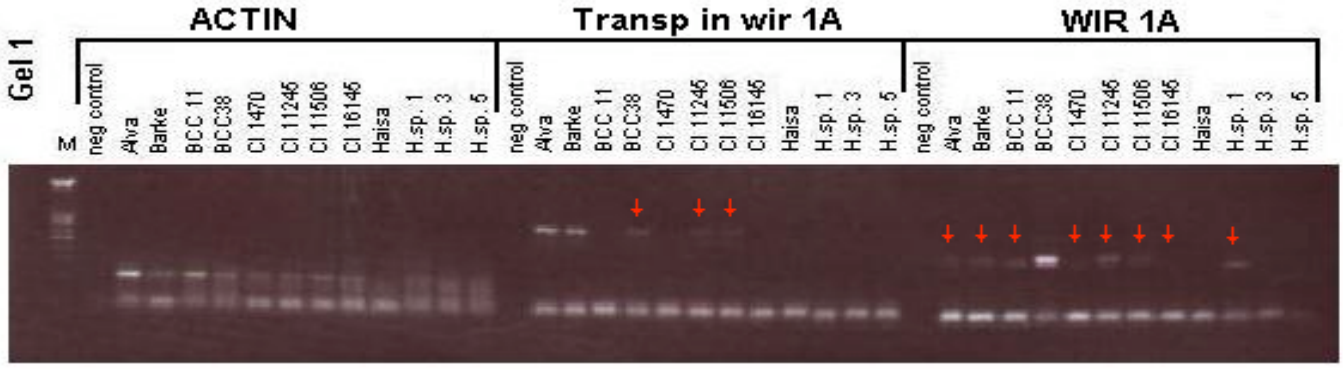
http://www.freefoto.com/preview.jsp?id=07-08-1&k=Barley

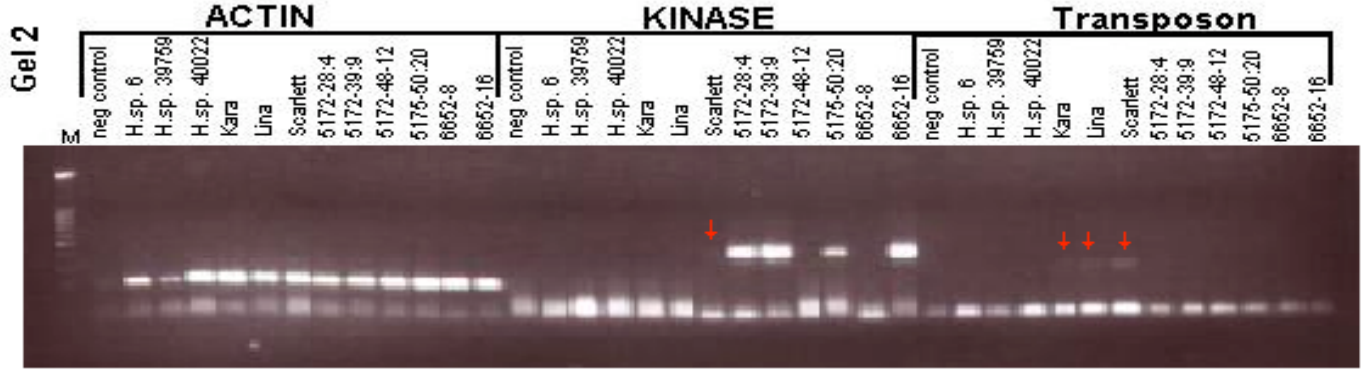
http://www.pbcr.hawaii.edu/microangela/aphid.htm

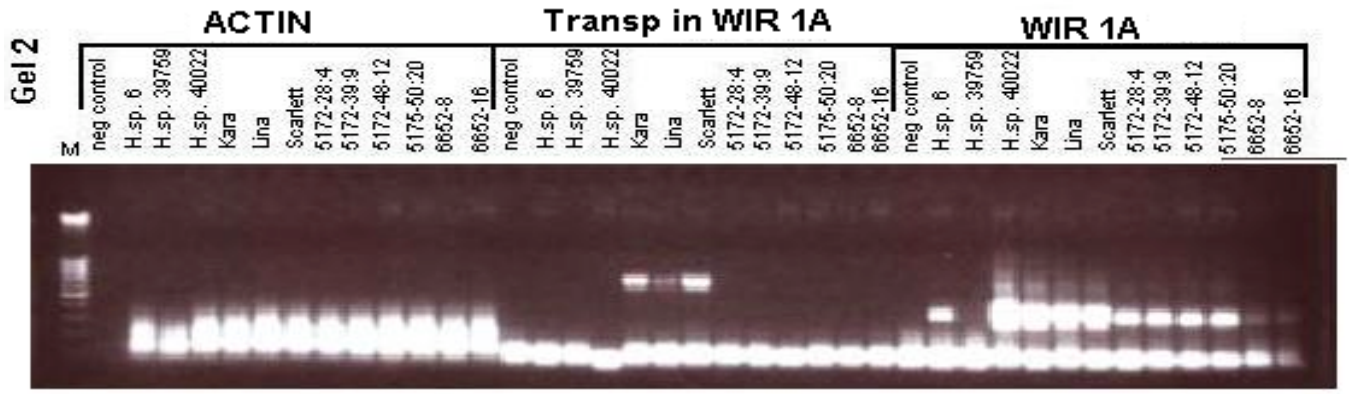
***Appendix A**: Genomic PCR loaded on agarose gel (2%), with primers for kinase and Transposon, for each of 69 barley lines.*

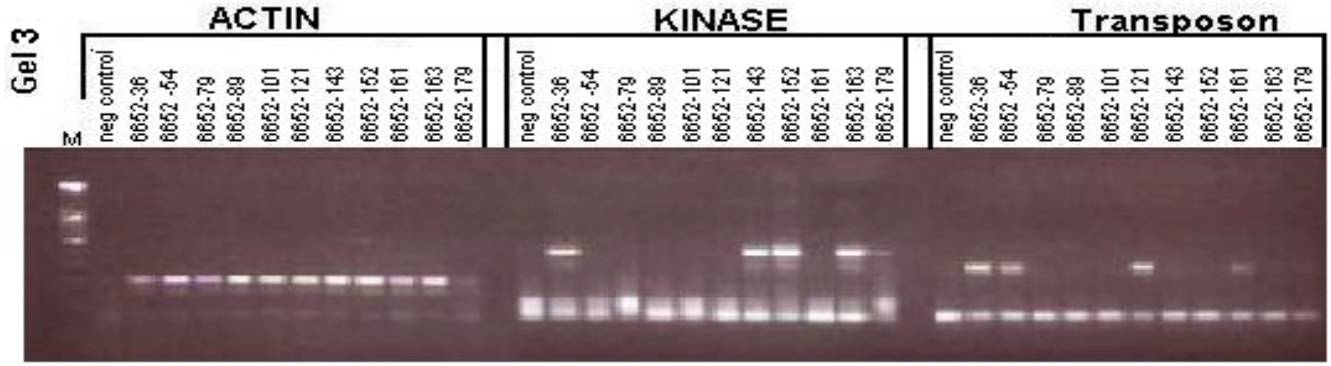
With kinase's primers, a band appears and its size is near 400 bp.
With Transposon's primers, there is only one band which appears at 300 bp.
With WIR 1A's primers, the band has a size of 390 bp
With the primers to find transposon in WIR 1A gene, the band appears at nearly 600 bp.
If spots on the sides of the picture appear lower than the others, it is because the spots were in the sides of the U.V. light.




Gel 2
ACTIN
Transp in WIR 1A
WIR 1A
M
neg control
H.sp. 6
H.sp. 39759
H.sp. 40022
Kara
Lina
Scarlett
5172-28:4
5172-39:9
5172-48-12
5175-50:20
6652-8
6652-16


Gel 3
ACTIN
KINASE
Transposon
M
neg control
6652-36
6652 -54
6652-79
6652-89
6652-101
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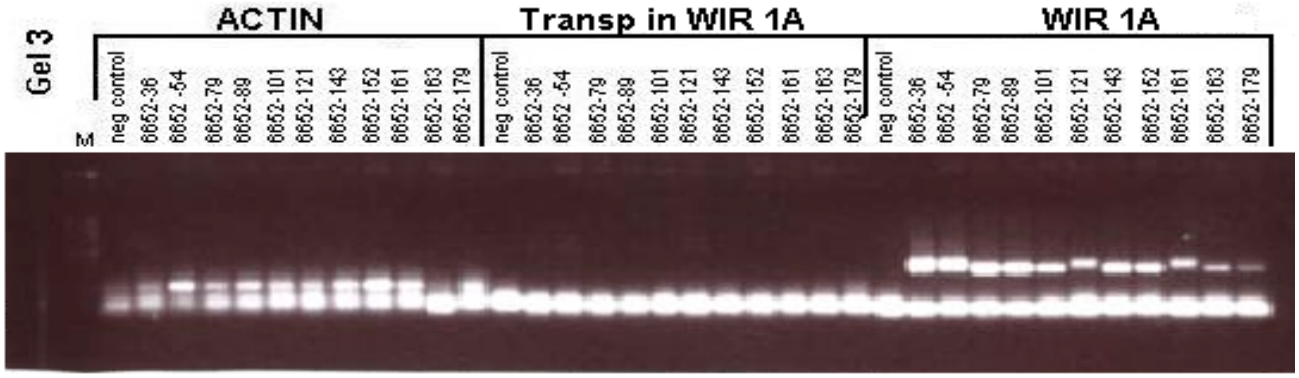
6652-143
6652-152
6652-161
6652-163
6652-179


Gel 3
ACTIN
Transp in WIR 1A
WIR 1A
M
neg control
6652-36
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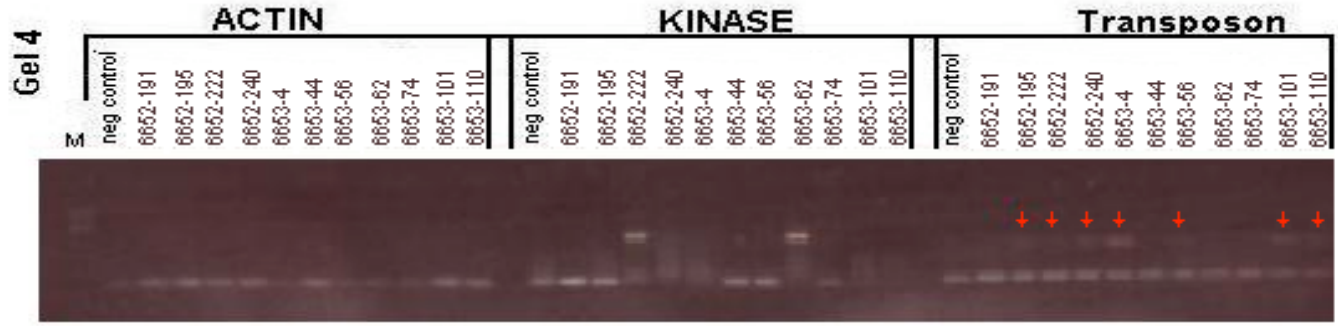
Gel 4
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KINASE
Transposon
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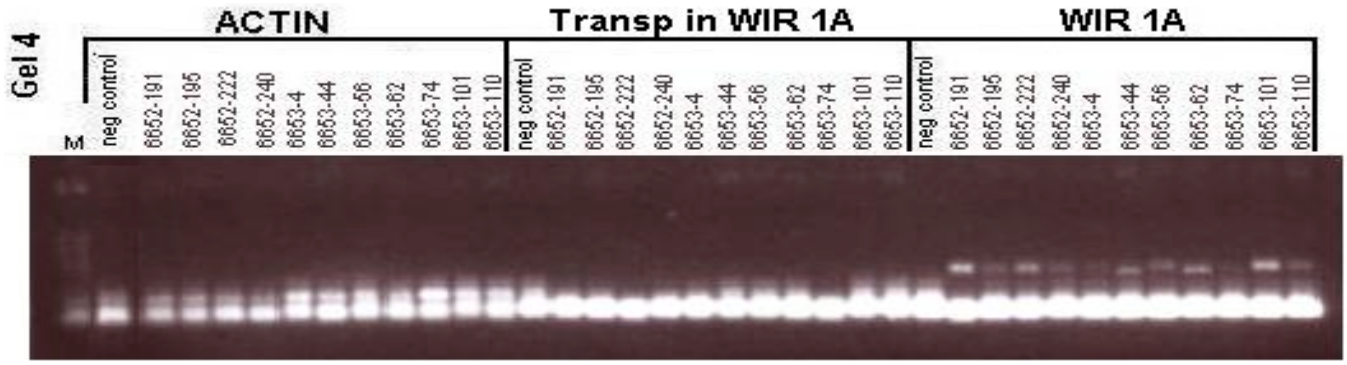
Gel 4
ACTIN
Transp in WIR 1A
WIR 1A
M
neg control
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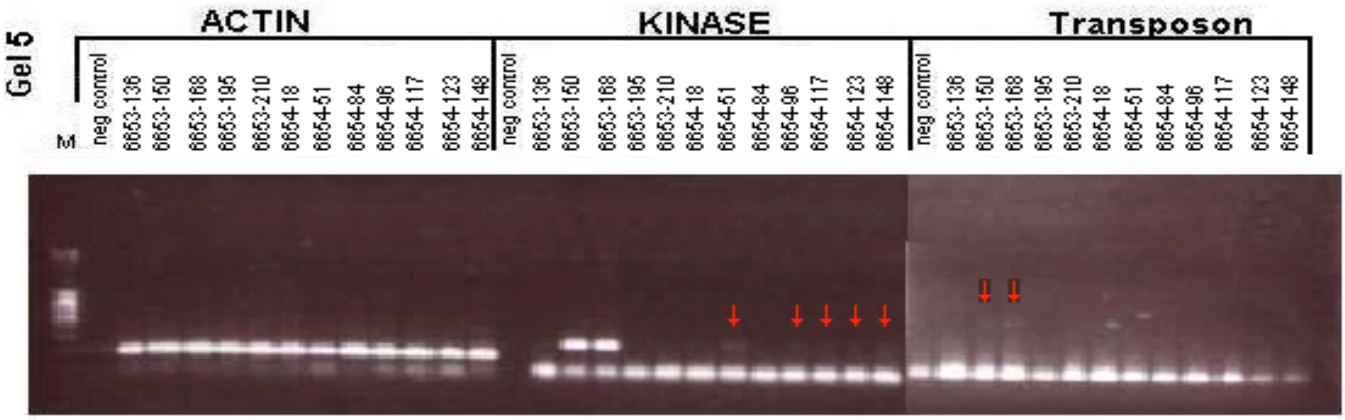
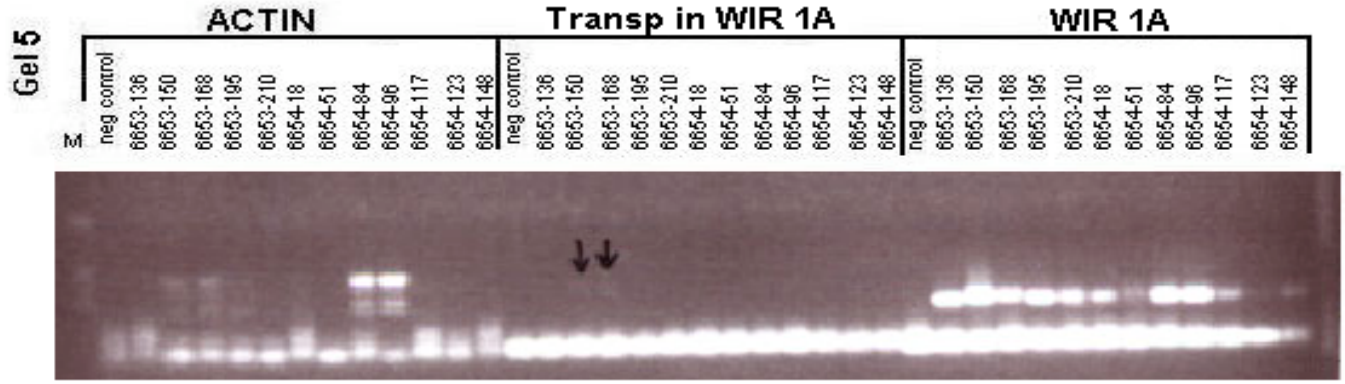
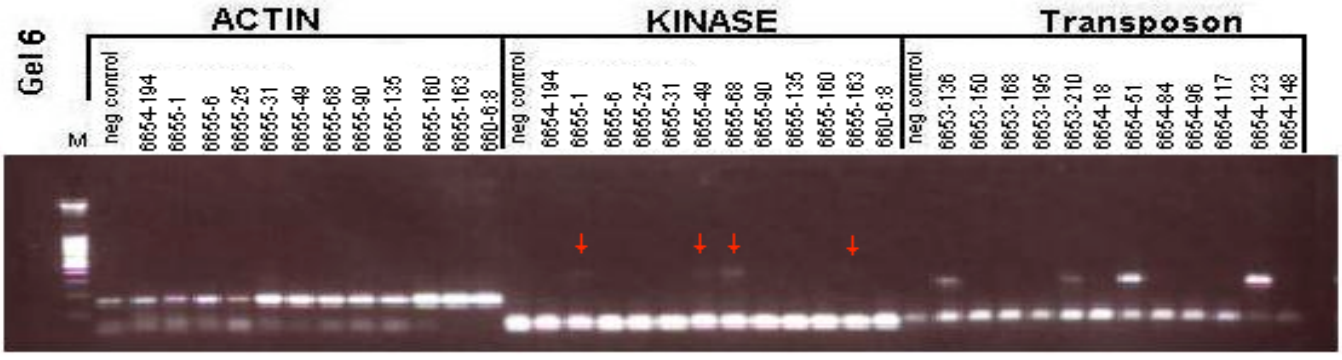
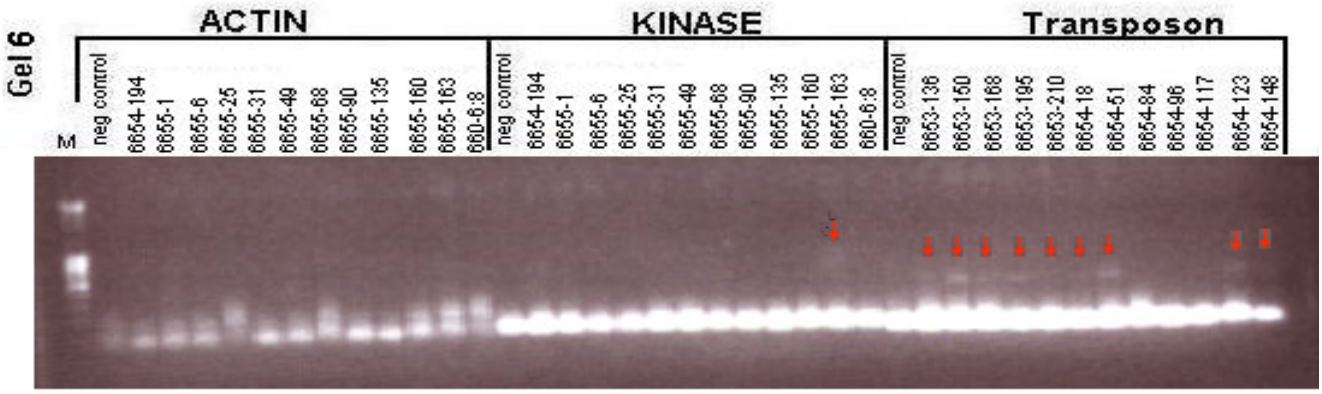
6653-56
6653-62
6653-74
6653-101
6653-110


Gel 5
ACTIN
KINASE
Transposon
M
neg control
6653-136
6653-150
6653-168
6653-195
6653-210
6654-18
6654-51
6654-84
6654-96
6654-117
6654-123
6654-148


Gel 5
ACTIN
Transp in WIR 1A
WIR 1A
M
neg control
6653-136
6653-150
6653-168
6653-195
6653-210
6654-18
6654-51
6654-84
6654-96
6654-117
6654-123
6654-148


Gel 6
ACTIN
KINASE
Transposon
M
neg control
6654-194
6655-1
6655-6
6655-25
6655-31
6655-49
6655-68
6655-90
6655-135
6655-160
6655-163
660-6:8
6653-136
6653-150
6653-168
6653-195
6653-210
6654-18
6654-51
6654-84
6654-96
6654-117
6654-123
6654-148


Gel 6
ACTIN
KINASE
Transposon
M
neg control
6654-194
6655-1
6655-6
6655-25
6655-31
6655-49
6655-68
6655-90
6655-135
6655-160
6655-163
660-6:8
neg control
6654-194
6655-1
6655-6
6655-25
6655-31
6655-49
6655-68
6655-90
6655-135
6655-160
6655-163
660-6:8
neg control
6653-136
6653-150
6653-168
6653-195
6653-210
6654-18
6654-51
6654-84
6654-96
6654-117
6654-123
6654-148

**APPENDIX B:** Table showing results of genomic PCRs, performed in 69 barley lines, for several genes: contig 16360 (kinase), contig 6519 (WIR 1A), AJ862550.1(Transposon), and AJ862550.1 in contig 6519.

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Alva | Sus | - | + | (+) | + |
| Barke | Sus | - | + | + | + |
| BCC 11 | Res | - | + | - | - |
| BCC 38 (2) | Res | - | + | + | + |
| CI 1470 (1) | Res | - | + | - | - |
| CI 11245 | Res | - | + | + | + |
| CI 11506 | Res | + | + | + | + |
| CI 16145 | Res | - | (+) | - | - |
| Haisa | Sus | - | - | + | - |
| Hsp 1 | Sus | ? | + | - | - |
| Hsp 3 | Sus | + | + | - | - |
| Hsp 5 | Res | + | + | - | - |
| Hsp 6 | Sus | ? | + | - | - |
| Hsp 39759 | Res | ? | - | - | - |
| Hsp 40022 | Res | ? | + | - | - |
| Kara | Sus | ? | + | (+) | + |
| Lina | Sus | ? | + | (+) | + |
| Scarlett | Sus | (+) | + | (+) | + |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Lina | Sus | - | + | (+) | + |
| Hsp 5 | Res | + | + | - | - |
| 660 - 6:8 | Res | + | (+) | | - |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Lina | Sus | - | + | (+) | + |
| 660 - 6:8 | Res | + | (+) | | - |
| 5172 28:4 | Res | + | + | - | - |
| 5172 39:9 | Res | + | + | - | - |
| 5172 48:12 | Res | ? | + | | - |
| 5175 50:20 | Res | + | + | - | - |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Lina | Sus | - | + | (+) | + |
| 5172 28:4 | Res | + | + | - | - |
| 6652 - 8 | Sus | ? | + | - | - |
| 6652 - 16 | Res | + | + | - | - |
| 6652 - 36 | Sus | + | + | + | (+) |
| 6652 - 54 | Sus | ? | + | + | (+) |
| 6652 - 79 | Sus | ? | + | - | - |
| 6652 - 89 | Sus | ? | + | - | - |
| 6652 - 101 | Res | ? | + | - | - |
| 6652 - 121 | Res | ? | + | + | (+) |
| 6652 - 143 | Sus | + | + | - | - |
| 6652 - 152 | Res | + | + | - | - |
| 6652 - 163 | Sus | + | + | - | - |
| 6652 - 179 | Res | + | + | - | - |
| 6652 - 191 | Sus | ? | + | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 6652 - 195 | Res | ? | (+) | + | - |
| 6652 - 222 | Res | + | + | + | (+) |
| 6652 - 240 | Res | ? | (+) | + | - |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Lina | Sus | - | + | (+) | + |
| 5172 39:9 | Res | + | + | - | - |
| 6653 - 4 | Sus | ? | (+) | + | - |
| 6653 - 44 | Sus | ? | + | - | - |
| 6653 - 56 | Sus | ? | + | + | - |
| 6653 - 62 | Res | + | + | - | - |
| 6653 - 74 | Res | ? | (+) | - | - |
| 6653 - 101 | Res | ? | + | + | - |
| 6653 - 110 | Sus | ? | (+) | + | - |
| 6653 - 136 | Res | + | + | - | - |
| 6653 - 150 | Sus | + | + | + | + |
| 6653 - 168 | Sus | + | + | + | + |
| 6653 - 195 | Res | + | + | - | - |
| 6653 - 210 | Res | - | + | - | - |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Lina | Sus | - | + | (+) | + |
| 5172 48:12 | Res | - | + | | - |
| 6654 - 18 | Sus | - | + | - | - |
| 6654 - 51 | Sus | + | (+) | - | - |
| 6654 - 84 | Res | - | + | - | - |
| 6654 - 96 | Sus | (+) | + | - | - |
| 6654 - 117 | Res | (+) | + | - | - |
| 6654 - 123 | Sus | (+) | (+) | - | - |
| 6654 - 148 | Res | (+) | (+) | - | - |
| 6654 - 194 | Res | - | (+) | + | - |

| SAMPLE | Res/Sus | Kinase | WIR 1A | Transposon | Transposon in WIR 1A |
|---|---|---|---|---|---|
| Barke | Sus | - | + | + | + |
| 5175 50:20 | Res | + | + | - | - |
| 6655 - 1 | Res | (+) | (+) | - | - |
| 6655 - 6 | Sus | - | (+) | - | - |
| 6655 - 25 | Sus | - | (+) | - | - |
| 6655 - 31 | Res | + | (+) | + | - |
| 6655 - 49 | Res | (+) | (+) | - | - |
| 6655 - 68 | Res | + | (+) | + | - |
| 6655 - 90 | Sus | - | - | - | - |
| 6655 - 135 | Sus | + | - | - | - |
| 6655 - 160 | Sus | + | - | - | - |
| 6655 - 163 | Res | (+) | (+) | + | + |