Doctoral Thesis

Pleiotropic effects of antifungal transgenes under different environmental conditions: endogenous gene expression and flavonoid metabolome

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Publication Date:
2005

Permanent Link:
https://doi.org/10.3929/ethz-a-005063471

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PLEIOTROPIC EFFECTS OF ANTIFUNGAL TRANSGENES UNDER DIFFERENT ENVIRONMENTAL CONDITIONS: ENDOGENOUS GENE EXPRESSION AND FLAVONOID METABOLOME.

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Sciences

presented by
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accepted on the recommendation of
PD Dr. Christof Sautter, examiner
Prof. Bruce McDonald, co-examiner

2005
Summary

Genetic modifications might cause pleiotropic effects, unexpectedly changing the expression pattern of endogenous genes. This could result in altered phenotypes of e.g. metabolic pathways. Thus, pleiotropic effects are an important issue in biosafety of transgenic crop plants. We have studied such putative expression modulation after introduction of an anti-fungal transgene in wheat. Three different spring wheat varieties containing different anti-fungal transgenes were analysed and compared to the non-transgenic plants of the same variety. We analysed the transcriptional level using the Affymetrix Barley1 GeneChip. On the metabolic level we studied the flavonoid content, because of its wide functional spectrum including pathogen defence. An extraction method has been optimized to analyse small quantities of wheat leaves. Phenolic compounds have been identified by liquid chromatography, mass spectrography and nuclear magnetic resonance.

Natural changes of the expression pattern due to different growing conditions have been choosen as a baseline for comparison. Significant increase of the concentration of certain flavonoids and considerable changes in the gene expression patterns have been observed when plants were grown in the field compared to the greenhouse.

In contrast, the transcription analysis revealed very small differences between wild type and transgenic plants of the same variety. These differences were considerably smaller than the background variability among varieties. The changes in the flavonoid pattern induced by the transgenes, although detectable, were much smaller then the natural variation between wild type plants of different varieties.

The results show that pleiotropic modulation of expression patterns of endogenous genes after introducing chitinase/glucanase or KP4 via genetic engineering techniques, although detectable, proved to be much smaller than those occurring naturally due to the environmental changes. The level of these changes may depend on the conditions under which the plants are grown. This again points out the importance of field testing of new transgenic plant lines, since the potentially unwanted effects of a modification might be detectable only under natural outdoors conditions.
**Riassunto**

Modificazioni genetiche possono causare effetti pleiotropici risultanti in alterazioni indesiderate dell'espressione di geni endogeni. Tali modificazioni porterebbero alla comparsa di fenotipi riguardanti, per esempio, vie metaboliche. Gli effetti pleiotropici costituiscono quindi un importante aspetto riguardante la sicurezza degli organismi geneticamente modificati e in particolare per le piante coltivate. In questo lavoro abbiamo studiato tali effetti pleiotropici in seguito ad introduzione di un transgene codificante per un fattore con proprietà anti-fungine in frumento. Sono state utilizzate tre differenti varietà di frumento contenenti transgeni codificanti per fattori anti-fungini e, come riferimento, le stesse varietà non transgeniche. Il profilo dell'espressione genica è stato analizzato utilizzando i Genechip per orzo prodotti da Affymetrix. Per un'analisi a livello metabolico abbiamo misurato il contenuto di flavonoidi, metaboliti secondari coinvolti in un ampio spettro di reazioni tra le quali quelle di difesa contro l'attacco di patogeni. Nel corso del lavoro è stato ottimizzato un metodo d'estrazione dei flavonoidi a partire da piccole quantità di tessuto fogliare di frumento. I composti fenolici sono stati identificati utilizzando tecniche di cromatografia, di spettrometria di massa e di risonanza magnetica nucleare.

Variazioni naturali dell'espressione genica dovute alle diverse condizioni di crescita sono state scelte come riferimento per le successive comparazioni. Incrementi rilevanti della concentrazione di alcuni flavonoidi e considerevoli variazioni del profilo di espressione genica sono stati osservati quando le piante erano cresciute in campo rispetto alla crescita in serra. Al contrario, l'analisi del profilo d'espressione ha mostrato differenze molto piccole tra wild type e piante transgeniche appartenenti alla stessa varietà. Tali differenze sono risultate considerevolmente minori rispetto a quelle riscontrate tra varietà diverse. La stessa osservazione è stata fatta riguardo alla concentrazione di flavonoidi. Questi risultati indicano che le variazioni d'espressione di geni endogeni in piante transgeniche per i geni chinasi/glucanasi e KP4 sono minori di quelle osservate naturalmente sotto l'effetto di diversi condizionamenti ambientali. L'ampiezza di tali variazioni può dipendere dalle condizioni di crescita delle piante. Questo evidenzia l'importanza di studiare ogni nuova pianta transgenica con esperimenti in campo, dove le condizioni naturali di crescita possono rivelare possibili effetti indesiderati del transgene non riscontrabili nelle condizioni di coltura utilizzate in laboratorio.
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Abbreviations and units used in the text

CH$_3$CN - acetonitrile
DAD - diode array detector
Fr - variety Frisal
Frisal A5 - variety Frisal chitinase line
Frisal B12 - variety Frisal RIP line
Go - variety Golin
Gr - variety Greina
HPLC - high pressure liquid chromatography
LC - liquid chromatography
LN$_2$ - liquid nitrogen
MeOH - methanol
[M+H]$^+$ - protonised molecular ion
MS/MS - full mass scan consisted of fragmentation of the compound followed by the fragmentation and second scan of the most abundant ion
MS$^2$ - second mass scan of fragmented molecular ion
m.u. - mass units
$m/z$ - ratio mass to charge (in mass spectrometry)
NMR - nuclear magnetic resonance
RT - room temperature
SPE - solid phase extraction
TFA - trifluoroacetic acid
UV - ultra violet
wt - wild type
Chapter 1: Introduction

1.1 Fungal pathogens of wheat

Wheat (*Triticum aestivum*) is one of the most important crop plants hosting fungal pathogens (Oerke *et al.* 1994) and, conversely, fungal diseases cause the most devastating losses in wheat worldwide (Murray and Brennan 1998, Wiese 1991). Fungi, being heterotrophic, absorb nutrients from either living or dead host tissue rather than produce them by themselves. Most of fungal plant pathogens are facultative saprophytes, other are obligate parasites (Wiese 1991). Infection by fungal pathogens depends on several factors: free water on the host plant surface is usually required, the susceptibility of the host, the density of inoculum, and ambient temperature, as well as other environmental factors. Fungi are dispersed in many ways: they may be seed borne or soil borne, or they may be spread by way of wind, water (rain, irrigation water), insects, animals, and man. While some fungi attack only one or a few host species, others attack many. A small selection of the most important wheat fungal diseases are presented in Table 1 (Bonde *et al.* 1997; Chester *et al.* 1946; Doling, 1966; Holton *et al.* 1941; King *et al.* 1983; Leukel, 1937; Mesterhazy, 1985; Prescott *et al.* 1986; Roelfs *et al.* 1995; Samborski *et al.* 1968; Shipton *et al.* 1971; Sutton, 1982; Wiese 1991).

Table 1: Most important wheat diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Occurrence</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf rust (<em>Puccinia recondita</em>)</td>
<td>The disease is found wherever temperate cereals are grown.</td>
<td>Infections can cause up to 25% yield losses, mainly by reducing the number of kernels per spike, test weights, and kernel quality.</td>
</tr>
</tbody>
</table>
## Table 1: Most important wheat diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Occurrence</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stem rust</strong> <em>(Puccinia graminis f.sp. tritici)</em></td>
<td>The disease is found wherever temperate cereals are grown. The disease develops from wind-borne urediospores that may have travelled long distances.</td>
<td>If infection occurs during the early crop stages, the effects can be very severe: reductions in tillering and losses in grain weight and quality. Under favourable conditions, it can affect most of the wheat crop in the area.</td>
</tr>
<tr>
<td><strong>Powdery Mildew</strong> <em>(Blumeria graminis f. sp. tritici)</em></td>
<td>The fungus has a high degree of host specificity. Powdery mildew occurs worldwide in cool, humid, and semiarid areas where cereals are grown.</td>
<td>Powdery mildew can cause up to 45% yield losses if infection occurs early in the crop cycle and conditions remain favourable for development so that high infection levels are reached before heading.</td>
</tr>
<tr>
<td><strong>Septoria Diseases</strong></td>
<td>The diseases are limited to temperate wheat-growing areas where cool and moist conditions prevail.</td>
<td>Major losses can occur, through seed shrivelling and lower test weights, if these diseases reach severe levels prior to harvest. Septoria destroys nearly 2% of world’s wheat annually.</td>
</tr>
<tr>
<td>- <strong>Septoria Triticum Blotch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <strong>Septoria Nodorum Blotch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <strong>Septoria Avenae Blotch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Scab (Head Blight)</strong> <em>(Fusarium sp.)</em></td>
<td>Infection is favoured by warm and humid weather during and after heading. Temperatures between 10 and 28°C are required for infection. The disease can spread from floret to floret by mycelial growth through the spike structure. <em>Fusarium</em> sp. are present in nearly all soils and crop residues.</td>
<td>Disease can cause yield losses of more than 50% and significant reductions in grain quality. Kernels from diseased spikes are often shrivelled. Harvested grain containing more than 5% infected kernels can contain enough toxic metabolites to be harmful to humans and animals.</td>
</tr>
</tbody>
</table>
Table 1: Most important wheat diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Occurrence</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Take-all</td>
<td>Disease is the most important in temperate, humid climate. Widely distributed in Australia, Europe, South Africa, Japan, Brazil, Chile, Argentina, China and North America</td>
<td>Severe yield losses, up to more than half of the seed count of healthy plants.</td>
</tr>
<tr>
<td>(Gaeumannomyces graminis var. tritici)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stinking smut</td>
<td>Infection is favoured by cool temperatures during germination. The disease can occur worldwide, but it's limited to temperate climates.</td>
<td>Considerable yield losses can occur in susceptible cultivars. Chemical seed treatments is necessary to prevent the disease.</td>
</tr>
<tr>
<td>(Tilletia caries)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karnal bunt</td>
<td>The disease is endemic in the Asian Subcontinent as well as Mexico, USA and Canada.</td>
<td>Actual losses in yield are minimal, but the disease is on the quarantine lists of many countries and therefore of importance in world grain trade.</td>
</tr>
<tr>
<td>(Neovossia indica)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loose smut</td>
<td>Infection and disease development are favoured by cool, humid conditions, which prolong the flowering period of the host plant. The disease can occur wherever wheat is grown.</td>
<td>Yield losses depend on the number of spikes affected by the disease. Incidence is usually less than one percent and rarely exceeds thirty percent of the spikes in any given location. Grain from infected field must be quarantined.</td>
</tr>
<tr>
<td>(Ustilago tritici)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 provides just a selection of most important fungal pathogens. Among other major pathogens one can list: *Aspergillus* and *Penicillum* ssp. (storage molds) and *Helminthosporium* ssp. (Kernel Smudge).

Smut and bunt species belong to a group of seed transmitted pathogens meaning, that they produce their spores in the kernels of the host plant (Prescott et al. 1986). If not controlled, they can accumulate, if a farmer uses repeatedly part of his own harvest for sowing. They also cause a major threat to countries and regions from which they are presently absent (McIntosh 1998) since shipping of infected seeds spreads the
disease. Severe outbreaks of seed transmitted diseases usually lead to quarantine measures, which by preventing farmers from selling their harvest outside of the epidemic territory cause strong economical impact. In addition, all harvesting machines have to be sterilized, in order to prevent further spreading of the disease (Royer 2005). The methods of fighting smuts and bunts are known since the work of Prévost (1807) who discovered that exposure of the infected wheat seeds to copper salts, especially copper sulphate, prevents the germination and kills the spores of Tilletia ssp. Singh and Hooda (1994) showed that solar heat treatment might be effective against Ustilago tritici. Nowadays the use of chemical treatment is widely used. Even though seed chemical treatment uses very low quantities of chemicals and is considered as ecologically safe, it may not be always desirable. Jacobsen and Backman (1993) among main dissadvantages of chemical treatment point out the public concerns about chemical residues creating food safety problems and the safety of farm workers applying pesticides, high costs of development and registration of new chemicals and finally competition in the organic food market. Moreover, the chemical treatment sometimes might not be possible. For example, according to Hoffmann (1982) in the region of West Asia and North Africa less than 40% of seeds sown is treated. Also Mamluk (1998) and Rubiales et al. (2001) point out that the continued prevalence of smuts and bunts in that region might be connected to relaxed seed treatment or use of ineffective chemicals as well as low abundance of smut-resistant cultivars grown. Therefore alternative or supplementary approaches, using genetic transformation are studied to reduce the chemical control to the host plant itself. However most of these approaches use broad spectrum fungicidal proteins like chitinases, glucanases or ribosomal inhibiting proteins (Bliffeld et al. 1999, Bieri et al. 2003, Broglie et al. 1991, Jach et al. 1995, Lin et al. 1995, Swords et al. 1997). In approaches with broad spectrum anti-fungal transgenes, side effects are reduced, but useful fungi like mycorrhiza still might be affected as well.

1.2 Pathogen control by use of transgenic plants

**Virus coat proteins**

The resistance to plant viral diseases can be achieved by expressing virus coat protein genes in the transgenic plants. Such modification confers the resistance not only to the
particular virus, but also to the related ones (Abel et al. 1986). This approach have been proved effective for different groups of viruses, like: potato virus A (Savenkov and Valkonen 2001), potato virus Y (Han et al. 1999), cucumber mosaic virus (Nakajima et al. 1993), tobacco streak virus (van Dun et al. 1988) and others. The approach have been commercialized in the case of papaya ringspot virus resistant plants (Fitch et al. 1992, Gonsalves 1998). Stark and Beachy (1989) proved that the coat protein-mediated protection can be extended beyond one gene/one virus protection phenomenon. In their experiments expression of a single coat protein gene in transgenic tobacco plants resulted in a protection against several different viruses. However they suggest that this kind of protection requires both structural and sequence homology between the protecting and challenge virus coat proteins.

**β-glucanases and chitinases**

Chitinases and glucanases have hydrolytic activities against structural components of fungal cell walls and may exhibit strong anti-fungal activities in vitro (Leah et al. 1991, Schlumbaum et al. 1986). In vivo, chitin oligomers released from fungal cell walls function in addition as elicitors that stimulate a general resistance response (Cote and Hahn 1994). The induction of resistance responses by chitin-derived oligosaccharides has also been described for wheat (Barber et al. 1989).

Transgenic dicot species and rice plants constitutively expressing chitinases, alone or in combination with glucanases or RIPs, have shown enhanced resistance to certain pathogens (Broglie et al. 1991, Jach et al. 1995, Lin et al. 1995, Swords et al. 1997, Vierheilig et al. 1993). Transgenic wheat plants overexpressing a barley seed chitinase gene under the control of the constitutive maize ubiquitin 1 promoter exhibited increased resistance to infection by the powdery mildew-causing fungus B. graminis (Bieri et al. 2003, Bliffeld et al. 1999).

**Ribosome inactivating proteins (RIPs)**

Ribosome-inactivating proteins (RIPs) are specific N-glycosidases that remove a conserved adenine residue from the large rRNA of the large ribosomal subunit. This inactivates the ribosome and blocks translation elongation. RIPs have been found in a wide range of plant families and in different plant organs (Barbieri et al. 1993, Girbes
Even though RIPs are thought to be defense proteins, they may also have other functions. Some of the strongest plant toxins, like ricin, belong to this group of compounds (Lord and Roberts 1996). They are known to have anti-fungal and anti-viral activities (Leah et al. 1991, Roberts and Selitrennikoff 1986, Turner et al. 1997 and 1998).

Most RIPs are active on a wide variety of ribosomes, including conspecific ones. Self-protection of RIP-producing plant cells is achieved by compartmentalization to the extracellular space or the vacuole, so that RIPs are not able to reach the ribosome target in their own cytoplasm (Svinth et al. 1998).

Transgenic tobacco plants expressing the barley seed RIP under a wound-inducible promoter have shown certain level of protection against *Rhizoctonia solani* (Logemann et al. 1992). Other experiments with transgenic tobacco have shown anti-fungal activity of the barley seed RIP also under a constitutive promoter (Jach et al. 1995). Also transgenic wheat expressing barley seed RIP (RIP30) under constitutive promoter has been tested and shown an anti-fungal effect against *Blumeria graminis* (Bieri et al. 2000 and 2003).

**KP4**

As an alternative or supplementary approach, an inter-strain inhibition system of so-called "killer proteins" (KP) from *Ustilago maydis* (Corn smut) viruses (Bruenn 2002, Puhalla 1968) for increasing resistance against smut fungi in wheat has been explored in our laboratory. Three non-homologous genes from three different viruses are known that encode proteins KP1, KP4, and KP6 with anti-fungal properties (Koltin and Day 1975). About 5% of naturally occurring strains of *Ustilago maydis* contain one of those three viruses (Koltin and Day 1976) and produce virally encoded toxin (KP) which is secreted into the apoplast of the plant host where it inhibits growth of fungal hyphae of the order *Ustilaginales*, which do not contain this virus (Koltin 1988). The KP system act specifically only against members of the order *Ustilaginales*, which contains exclusively plant pathogenic fungi.

In our laboratory, KP4 has been transferred into the two Swiss spring wheat varieties Golin and Greina, which are particularly susceptible to stinking smut (Winter et al. 1995), and showed specific anti-fungal activity due to the *kp4* gene product in the
transformed lines *in vitro* and *in planta* in the greenhouse (Clausen *et al.* 2000). Both KP4 lines, in the greenhouse, exhibited an approximately 30% reduction in fungal disease symptoms compared with wild-type controls or null segregants (Clausen *et al.* 2000, Sautter *et al.* 2000).

### 1.3 Pleiotropic effects of a genetic modification


Pleiotropic effects are one of the concerns about genetically modified organisms (GMO) (Miraglia *et al.* 1998). This includes undesired side effects of the transgene itself or its insertion site on other endogenous genes and their regulation. This concern is particularly conceivable in case it might influence accumulation of secondary metabolites, among them phytoalexins, which are important for disease and pest resistance (Jeandet *et al.* 2002). Moreover, some crop species such as potato contain secondary metabolites that can even be toxic to humans if present in too large amounts (Rhodes *et al.* 1994, Valkonen *et al.* 1996).

Changes in the accumulation of secondary metabolites might not only reflect pleiotropic effects caused by transgenes, but are also, very likely, due to external stimuli such as biotic or abiotic stress factors. In addition, genetically determined differences in secondary metabolite accumulation are expected between different varieties of a given species.

### 1.4 Flavonoids

According to literature and to preliminary HPLC/UV and HPLC/MS experiments, flavonoid derivatives are among the most important secondary metabolites found in wheat, although beside of these compounds wheat accumulates further polyphenolic derivatives (coumarines and phenylpropanoids), gibberellins (plant growth regulators), alkaloids and sterols.
Flavonoids are a class of plant secondary metabolites of phenolic nature which play a number of important roles in the interaction of plants with their environment. They possess a 15 carbon skeleton with a chromane ring bearing a second aromatic ring B in position 2,3 or 4 (Fig. 1).

According to the substitution of the ring C, flavonoids are divided into various subgroups. This classification is also dependent on the oxidation state of heterocyclic ring C and the position of ring B. 6 major subgroups of flavonoids are presented in the Table 2.

**Table 2: Structures of the major groups of flavonoids**

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalcones</td>
<td><img src="http://www.friedli.com/herbs/phytochem/flavonoids.html" alt="Structure" /></td>
</tr>
<tr>
<td>Flavones</td>
<td><img src="http://www.friedli.com/herbs/phytochem/flavonoids.html" alt="Structure" /></td>
</tr>
<tr>
<td>Flavonols</td>
<td><img src="http://www.friedli.com/herbs/phytochem/flavonoids.html" alt="Structure" /></td>
</tr>
<tr>
<td>Flavanones</td>
<td><img src="http://www.friedli.com/herbs/phytochem/flavonoids.html" alt="Structure" /></td>
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Biosynthesis of flavonoids and regulation of the pathway

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</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td><img src="structure1.png" alt="Anthocyanins" /></td>
</tr>
<tr>
<td>Isoflavonoids</td>
<td><img src="structure2.png" alt="Isoflavonoids" /></td>
</tr>
<tr>
<td>Neoflavonoids</td>
<td><img src="structure3.png" alt="Neoflavonoids" /></td>
</tr>
</tbody>
</table>

The concentration of flavonoids and other phenolic compounds varies among organs, tissues and developmental stages, and is influenced by environmental factors. Among such factors are temperature, UV and visible radiation, nutrient and water availabilities (Bohm 1987).

Flavonoids contribute to the disease resistance, either as constitutive anti-fungal agents or as phytoalexins.

1.5 Biosynthesis of flavonoids and regulation of the pathway

In different plant species or even varieties, synthesis of flavonoids in response to the environmental factors or pathogens varies significantly. Depending on the genotype, a strong increase of the flavonoid synthesis or even changes on the flavonoid composition can be observed. In order to explain those differences, the flavonoid biosynthesis pathway and its regulation has to be presented.

Flavonoids as all other phenylpropanoids are predominantly derived from the aromatic amino acid phenylalanine. The initial steps in the synthesis of all phenylpropanoids is designated the common phenylpropanoid pathway (Fig. 2). Through a series of
conversions, the aromatic amino acid phenylalanine is converted to highly energetic hydroxycinnamoyl coenzyme A (CoA) esters (Dixon et al. 2002). These esters represent a branching point in phenylpropanoid biosynthesis. The CoA esters can either be converted to lignin precursors, cell wall bound hydroxycinnamoyl esters or soluble glucosides. Alternatively, the CoA esters can be converted to a diversity of flavonoid derivatives via the flavonoid biosynthetic pathway (Winkel-Shirley, 2001) (Fig. 3).
Fig. 2. Biosynthetic pathways leading to phenylpropanoid products in plants. The core reactions are shown in larger type. Abbreviations: BA, benzoic acid; BA2H, benzoic acid 2-hydroxylase; t-CA, trans-cinnamic acid; 4-CA, 4-coumaric acid; CA2H, cinnamate 2-hydroxylase; Calc, coniferyl alcohol; Cald, coniferaldehyde; CalCoA, caffeoyl CoA; 4-CCoA, 4-coumaroyl CoA; CGA, chlorogenic acid; C3H, coumarate (coumaroyl quinate/ shikimate) 3-hydroxylase; C4H, cinnamate 4-hydroxylase; ChA, chorismic acid; i-ChA, isochorismic acid; 4-CL, 4-coumarate:CoA ligase; CHR, chalcone reductase; CHS, chalcone synthase; COMT, caffeic acid O-methyltransferase; Csh, 4-coumaroyl shikimate; Daid, daidzein; FerA, ferulic acid; FerCoA, feruloyl CoA; Gen, genistein; 5-HCald, 5-hydroxyconiferaldehyde; HQT, hydroxycinnamoyl-CoA:quinase hydroxycinnamoyltransferase; ICS, isochorismate synthase; IFR, isoflavone reductase; IFS, isoflavone synthase; II, isoliquiritigenin; IOMT, isoflavone O-methyltransferase; Liq, liquiritigenin; MCoA, malonyl CoA; Med, medicarpin; Nar, naringenin; Nc, naringenin chalcone, PAL, l-phenylalanine ammonia-lyase; l-phe, l-phenylalanine; PL, pyruvate lyase; SA, salicylic acid; Salc, sinapyl alcohol; Said, sinapaldehyde; ShA, shikimic acid; Van, vanillin; VR, vestitone reductase. Reactions not designated with an enzyme name may be catalysed by more than one enzyme (from: Dixon et al. 2002).
Common phenylpropanoid pathway

L-phenylalanine ammonia lyase (PAL) mediates the conversion of phenylalanine to cinnamic acid, the first committed step in the biosynthesis of phenylpropanoids, and, as such, PAL is well suited to play a regulatory role in controlling biosynthesis of all phenylpropanoid compounds. An activity analogous to PAL that deaminates tyrosine (tyrosine ammonia-lyase - TAL) to form 4-coumarate has been detected in grasses (Neish 1961, Gupta and Acton 1979). When wheat leaves are inoculated with the non-host pathogenic fungus Botrytis cinerea PAL activity increases locally in and around lignifying tissue and the increase is very rapid and precedes the development of highly lignified HR cells (Maule and Ride 1976). A rapid increase in PAL activity occurs also after inoculation of barley with powdery mildew (Blumeria graminis). The timing of the accumulation of PAL exhibited a two peak pattern consistent with the development and attempted penetration of the leaf by mildew, suggesting that PAL activation is closely regulated by pathogen derived factors (Clark et al. 1994, Shiraishi et al. 1995).

Feedback inhibition of PAL has also been suggested to explain the rapid enhancement (within minutes) of PAL activity, and subsequent accumulation of flavonoids in the leaf epidermis, seen in rye (Secale cereale) and several other species in response to illumination with UV light. The PAL enzyme produces cinnamic acid with trans conformation. When rye leaves are irradiated with UV light, a partial conversion of trans-cinnamic acids to the cis isomers is induced, and, at the same time, there is a rapid reduction in the inhibition of PAL activity. This observation may be explained by a feedback inhibition model in which trans-cinnamic acid inhibits PAL activity under normal conditions. After UV light illumination, when the trans form of cinnamic acid is converted to the cis form, this inhibition is removed, and an increased flow through the pathway is initiated. This model also implies that trans-phenylpropanoids can be regarded as photoreceptors and regulators of the phenylpropanoid pathway during UV-B (280-320 nm) irradiation (Braun and Tevini 1993).

The next step of the pathway, conversion of cinnamic acid to 4-coumaric acid, is catalysed by cinnamate 4-hydroxylase (C4H) - a cytochrome P450-type of enzyme. Infection of wheat with Botrytis cinerea cause a rapid, local increase in the activity of C4H (Maule and Ride 1983). Also in alfalfa C4H appears to be regulated at the transcriptional level in the defence response (Fahrendorf and Dixon 1993).

The conversion of 4-coumaric acid to 4-coumaroyl-CoA is mediated by 4-
Coumarate-CoA ligase (4CL). After inoculation of wheat with an incompatible isolate of *Botrytis cinerea*, 4CL activity increases prior to the appearance of the HR (hypersensitive response). As has been found with all tested wheat enzymes in the common phenylpropanoid pathway, the increased in 4CL activity was localized to an area of the leaf closely surrounding the site of infection (Maule and Ride 1983). In maize, higher levels of 4CL activity were detected in a cultivar resistant to inoculation with *Helminthosporium maydis* than in a cultivar susceptible to this fungus (Dickerson *et al.* 1984).

**Flavonoid biosynthetic pathway**

Fig. 3 Scheme of the major branch pathways of flavonoid biosynthesis. P450 hydroxylases that may function as membrane anchors for multienzyme assemblies are indicated in red. Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl-CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSIi), flavonoid 3' hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), isoflavone O-methyltransferase (OMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), O-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR) (from: Winkel-Shirley 2001).

Chalcone synthase (CHS) is the first committed enzyme in the flavonoid biosynthesis catalysing the condensing of 3 malonyl-CoA and 4-coumaroyl-CoA to form a chalcone
The role of flavonoids in UV-B protection

intermediate (naringenin chalcone). CHS activity has been described in many plants and the enzymology and expression pattern in defence and other forms of stress have been studied in several systems (Dangl et al. 1989). CHS activity and transcripts are known to accumulate in response to pathogen attack and abiotic elicitation in many species (Hahlbrock and Scheel 1989, Rhodes 1994). In rye, as in other plants, the accumulation of CHS activity is tightly linked to the accumulation of flavonoids, and it has been proposed that CHS is the limiting step in the biosynthesis of flavonoids (Knogge et al. 1986). In sorghum, an increase in CHS activity can be seen 3 to 6 hours before the increase in phytoalexin accumulation in an incompatible interaction with Collettrichum graminicola (Lue et al. 1989).

The conversion of a chalcone into a flavanone is catalysed by the chalcone isomerase (CHI), which catalyses the closure of the C ring in chalcones to form the corresponding flavanones. However in barley, CHI activity is constitutively present and does not accumulate in response to UV-light. This is despite an enhanced biosynthesis of flavonoids following UV-light illumination (Liu and McClure 1995). Hence, CHI does not seem to be a limiting step in flavonoid biosynthesis, at least in barley.

At the level of the flavanone, the flavonoid biosynthesis extends into different branches. Firstly, flavanones can be converted to flavones via the action of flavone synthase (FS). Secondly, dihydroflavonols can be synthesized from flavanones by the enzyme flavanone 3' hydroxylase (F3'H). Dihydroflavonols are the precursors for the production of the flavonols and the pigmented anthocyanins. Thirdly, in a reaction catalysed by isoflavonone synthase (IFS) flavanones can be converted into isoflavonones.

Several hundred flavonoids have been described and the flavonoid backbone can be modified in many ways and at several positions of the molecule (Heller and Forkman 1993). The modification of flavonoids includes: reduction, hydroxylation, methylation and substitutions with various sugar groups. Although many enzymes mediating modifications of flavonoids have been described, few of the enzymatic activities involved in such modifications are well characterized.

1.6 The role of flavonoids in UV-B protection

Ultraviolet radiation is divided into 3 bands, each with different energy and with different ecological significance (Harborne and Williams 2000). Of these UV-B (280-315 nm) is
the band of lowest wavelength and highest energy being a potential damage to the plant life. Resistance to UV-B might take many forms but, one type of it could lie in the flavonoid pigments which absorb generally in the 280-315nm region and thus are capable of acting like a UV-filter protecting the underlying photosynthetic tissues (Harborne and Williams 2000). Several experiments have provided convincing evidence supporting this theory. Arabidopsis mutants lacking epidermal flavonoids became very sensitive to artificial UV-B radiation (Ormrod et al. 1995). In maize, the purple leaved variety (producing anthocyanin) suffered less DNA damages than the green leaved cultivar after exposure to UV-B radiation (Stapleton and Walbot 1994). Another cereal in which mutant with affected flavonoid synthesis exists is barley. Here the mutant has been produced, which contains only 7% of the flavonoids of the wild type. UV-B treatment of this mutant decreased the yield of photosynthesis. In contrast, the wild type plants photosynthesised normally and, at the same time, increased the concentration of saponarin by 30% and the concentration of lutonarin by 500% (Reuber et al. 1996).

Another way to study plants with lack of flavonoid synthesis is to treat them with an inhibitor of phenylpropanoid synthesis pathway. This can be done with 2-amino-indan-2-phosphonic acid (AIP) at 50μM (Harborne and Williams 2000). Gitz et al. (1998) treated red cabbage seedlings with AIP, which completely blocked anthocyanin synthesis. Treated plants were twice as sensitive to UV-B damage as non treated controls.

UV-B radiation often changes the ratios of flavonol or flavone compositions in UV-B tolerant plants. For example UV-B tolerant cultivar of rice produced increased amounts of three isoorientin glucosides with lower amounts of isovitexin glucosides, while the susceptible cultivar failed to synthesise these flavonoids (Markham et al. 1998). The 3',4'-dihydroxyflavonoids (like isoorientin) are capable of free radical scavenging and this might be a very important response to UV-damage in plants.

1.7 Antimicrobial properties of flavonoids

The role of flavonoids in protecting plants against a microbial attack does not only involve their constitutive presence but also their accumulation as phytoalexins in response to microbial attack (Grayer and Harborne 1994, Harborne 1999). The
Antimicrobial properties of flavonoids

Isoflavonoid 3-hydroxy-8,9-methylenedioxypterocarpan (maackiain) has been identified as a constitutive anti-fungal agent in heartwood of legume trees and as an inducible phytoalexin in herbaceous legumes like *Pisum sativum* (Harborne and Williams 2000). It can have also both functions like in *Cicer bijugum* (wild relative to chickpea) (Stevenson and Haware 1999). The majority of flavonoids recognised as anti-fungal agents in plants are either isoflavonoids, flavans or flavanones (Harborne and Williams 2000).

In rice, the flavonoid sakuranetin is a major phytoalexin. This methylated flavanone accumulates in leaves in response to inoculation with the rice blast fungus *Magnaporthe grisea* (anam. *Pyricularia oryzae*) and irradiation with UV-light. The concentration of sakuranetin starts to elevate in rice leaves 32-40h following inoculation in a resistant cultivar whereas, in a susceptible cultivar, the flavanone could not be detected 48 h following inoculation (Kodama et al. 1992).

The flavonoid-derived isoflavonoids are the best described phytoalexins, and major parts of the current knowledge of phytoalexins has emerged from studies of isoflavonoid phytoalexins. The isoflavonoid phytoalexins are produced primarily in legume species (*Leguminosae*), and have been characterized especially from bean, soybean, pea, and alfalfa. Isoflavonoids have long been implied in the defence response to invading microorganisms in these species (Hahlbrock and Scheel 1989, Nicholson and Hammerschmidt 1992, van Etten and Pueppke 1976). Isoflavonoids accumulate rapidly in most organs of the plants in response to pathogen attack. For instance, during infection of alfalfa leaves with the fungal pathogen *Phoma medicaginis*, the level of the phytoalexins medicarpin and sativan increases from 4 hours post inoculation (Paiva et al. 1994).

A common substitution of flavonoids is the methylation of hydroxy groups on the flavonoid skeleton to yield methylated flavonoids. Methylated flavonoid phytoalexins are present in many plants. Flavonoids substituted with one or more methyl groups have, in several cases, been shown to be more potent inhibitors of fungal germination and/or growth compared to their non-methylated precursors (Kodama at al. 1992, Wiederbörner et al. 1989). A classical example is the methylated isoflavonoid phytoalexin pisatin, which is found in garden pea (*Pisum sativum*) and other legumes. Pisatin has a role as a determinant of disease resistance in pea, only isolates of the root rot fungus *Nectria haematococca* (anam. *Fusarium solani*) which are able to
demethylate and thereby detoxify pisatin show significant virulence on pea (Yoder and Turgeon 1985). Several pea pathogens other than *Nectria haematococca* can demethylate pisatin, and, as with *Nectria haematococca* their virulence is inversely correlated to the ability of the fungus to demethylate pisatin (van Etten et al. 1989). However, highly methylated flavonoids, lacking a unsubstituted phenol ring, were poor inhibitors of fungal growth (Wang et al. 1989) and that high polarity due to the presence of several hydroxyl groups seems to reduce anti-fungal activity (Weiderbörner et al. 1989).

Flavonoids are known to have not only the anti-fungal properties but also antibacterial (Haraguchi et al. 1998, Iniesta-Sanmartin et al. 1990, Linuma et al. 1994) as well as antiviral.

### 1.8 Protection against insect herbivory

Many cases of induction of flavonoid synthesis in cultivars resistant to herbivore attacks have been reported. One of the main pests of rice plants is the stem nematode (*Ditylenchus angustus*) (Harborne and Williams 2000). A flavonoid (sakuranetin) and a related phenylpropanoid (chlorogenic acid) have been recognised in rice leaves as providing resistance to nematode attack. They both increase their concentration in response to the infection (Plowright et al. 1996). The role of sakuranetin in response to fungal infection and UV-irradiation have also been reported (Dillon et al. 1997). Three other glycoflavones: shaftoside, isoshafortside and neoshafortside have been identified in rice phloem sap, where they act as sucking deterrents to the pest insects, *Niloparvata lugens* (brown plant hopper). They exhibit an ingestion inhibition activity and high levels of these glycoflavones are present in resistant cultivars (Grayer et al. 1994).

In certain cases, flavonol glycosides might act as phagostimulants to insects. Quercetin-3-glucoside present in the pollen of sunflower is phagoactive for the western corn rootworm, which feeds on this pollen (Lin and Mullin 1999). Several swallowtail butterflies, feeding on *Rutaceae* or *Umbelliferae* host plants, require flavonol, flavone or flavanone glycosides as oviposition stimulants (Harborne 1997).
1.9 Flavonoid content in wheat

In wheat extracts, flavonoids are often present as O- or C-glycosides. The O-glycosides have sugar substituents bonded to a hydroxyl group of the aglycone, whereas the C-glycosides have sugar substituents bonded to a carbon of the flavonoid aglycone, generally at position 6-C and 8-C (Waridel et al. 2001). Most of the flavonoids present in wheat occur as C-glycosyl flavones (Julian et al. 1971). So far several of wheat phenolic compounds have been identified: saponarin, isoorientin, 8-C-arabinosylhexoside of apigenin (Harborne and Hall 1964), lutonarin, lucenin-1, lucenin-3, isoorientin 7-O-rutinoside, wyomin, isoswertisin 4'-O-glucoside, vicenin-2, tricin, orientin, vitexin (Julian et al. 1971), schaftoside, isoschaftoside, vicenin-1 (Wagner et al. 1980).

1.10 Methods of detection and analysis of flavonoids

There are several simple as well as more complex methods of flavonoid analysis, based both on the chromatography and spectroscopy. Literature describing them is widely available (Lommen et al. 2000, Mabry et al. 1970, Markham 1982, Stobiecki 2000, Waridel et al. 2001). Here, only the methods used for the analysis of the wheat extracts during this work will be shortly presented.

**High pressure liquid chromatography - HPLC**

HPLC is a form of column chromatography which utilizes a column of packing material of small size and regular shape of particles. The high level of packing requires a high pressure of the solvent to obtain a reasonable flow rate through the column. The resolution and sensitivity of the method are very high and, moreover, it provides a quantitative analysis thanks to the UV detector and a chromatogram in the form of a diagram with peaks. The area below each peak on the chromatogram depends on the concentration of the respective compound in the mixture, therefore by use of the standard of known concentration, it is possible to quantify the analysed compounds with high accuracy (Markham 1982).
**UV/Vis absorption spectroscopy**

UV/visible light absorption spectroscopy is one of the most useful techniques for flavonoid structure analysis. The flavonoid spectrum measured in methanol, typically consists of 2 absorption maxima - in the range 240-285 nm (band II) and in the range 300 - 550 nm (band I). The position and relative intensities of those bands give the information about the nature of flavonoid and its oxygenation pattern (Mabry et al. 1970). However, this method requires the availability of good range of reference spectra for comparisons. The reference compounds may be used as well as the spectra published in the literature (Harborne 1967, Jay et al. 1975, Mabry et al. 1970).

**UV/Vis absorption spectroscopy with shift reagents**

The diagnostics of a flavonoid by UV/Vis spectroscopy can be greatly enhanced by using shift reagents, which influence the spectrum in a characteristic way, depending on the chemical structure of a compound (Markham 1982):

1. Sodium methoxide (NaOMe) - serves as a good “fingerprint” indicator of the hydroxylation pattern and useful for detection of the more acidic hydroxyl groups in unsubstituted form.
2. Sodium acetate (NaOAc) - causes significant ionization of only the most acidic of the flavonoid hydroxyl groups. It is used primarily to detect the presence of a free 7-hydroxyl group.
3. Sodium acetate in boric acid (NaOAc/H₃BO₃) - bridges the two hydroxyls in an ortho-dihydroxy group and is used to detect their presence.
4. Aluminium chloride (AlCl₃) and AlCl₃/HCl - by forming acid-stable complexes between hydroxyls and neighbouring ketones, and acid-labile complexes with ortho-dihydroxyl groups, these reagents can be used to detect both groupings. The AlCl₃ spectrum represent the sum effects of all complexes on the spectrum, while the AlCl₃/HCl spectrum represents the effect only of the hydroxy-keto complexes.

**Mass spectroscopy - MS**

Mass spectrometry is one of the physico-chemical methods applied to the structural determination of the organic compounds. The high sensitivity and possibility of coupling with chromatographical techniques makes it very useful for studying the natural
products from biological material, including flavonoids. The characteristic feature of MS is the use of different physical principles for sample ionization and separation of the ions. The ions are generated according to their mass to charge ratio (m/z).

Flavonoids are polar, non volatile and thermally labile compounds. For the long time, their analyses with the common MS techniques, like electron impact (EI) and chemical ionization (CI), were not possible. Both of those methods require the analyte to be in the gas phase for the ionization, and derivatization of the hydroxyl groups (for example methylation or acetylation) was necessary. The analysis of flavonoids became possible with the introduction of desorption ionization techniques, especially thermospray (TSP), electrospray (ESI) and atmospheric pressure chemical ionization (APCI) (Blakley and Vestal 1983, Horning et al. 1974, Stobiecki 2000, Whitehouse et al. 1985).

Coupling of a MS system with the liquid chromatography (LC) separation techniques enables identification of the compounds from various biological samples. Especially the introduction of the ESI and APCI into this field of research made such analysis possible. APCI systems may be coupled to different mass spectrometers such as quadrupole, ion trap, time of flight or Fourier transform ion cyclotron resonance (Niessen 1998, 1999a, 1999b). The temperature control of the APCI desolvation process is far less critical than in case of TSP and enables to maintain the same conditions of analysis for wide range of compounds. Splitting of the column eluate also provides an opportunity to use double detection systems - UV and MS (Wolfender et al. 1995, 1997, 1998).

Because of the structural diversity of flavonoid aglycones, as well as the diversity of the sugar components bonded to them, a single MS technique can not provide all necessary structural information allowing a proper assignment of the structure to an unknown compound. The structural information can be enhanced by using tandem MS with collision induced decomposition (CID MS/MS) and desorption ionization techniques (Stobiecki 2000, Waridel et al. 2001). Using different MS techniques following information can be obtained from the spectra:

- molecular mass,
- structure of aglycone (pattern of hydroxylation, point of attachment of the ring B on ring C),
- information about acylation of sugar hydroxyl groups,
- number of sugar rings and their configuration.

However, MS does not provide the information about stereochemistry of the glycosidic linkage or distinguish between diastereometric sugar units.

**Nuclear magnetic resonance - NMR**

NMR identifies the molecule by characteristic patterns of hydrogen and carbon relationships. The potential of NMR to quantify and identify a large number of compounds makes it a leading technique in the emerging area of metabolomic studies. Technically any metabolite with a hydrogen atom, providing that the quantity of the compound is above the NMR detection limit can be detected by the NMR. Recent studies have proven this technique to be useful for flavonoid identification when coupled with HLPC/UV and mass spectrometer (Lommen et al. 2000).

**1.11 Expression analysis using microarrays**

Changes observed in the metabolic pathways are consequences of changes in gene expression. Studying the molecular effects of particular conditions like environmental conditions or infections on the complex organism like plant require a method which allows to screen and compare expression of vast amounts of genes at the same time. The throughput of methods like RT-PCR or Northern blot is not high enough for such task. Genome-scale expression studies have become possible with the introduction of DNA microarrays. These arrays consist of a highly ordered matrix of thousands of different DNA sequences that can be used to measure RNA variation in applications that include gene expression profiling, comparative genomics and genotyping. Each array consists of a reproducible patterns of thousands of different DNAs (PCR products or oligonucleotides) attached to a solid support. Fluorescently labelled cRNA prepared from messenger RNA is hybridized to complementary DNA on the array and then detected by laser scanning. Hybridization intensities for each DNA sequence on the array are determined using an automated process and converted to a quantitative read-out of relative gene expression levels (Harrington et al. 2000).

There are two major types of DNA microarrays: spotted microarrays (c-DNA
Expression analysis using microarrays

microarrays) in which pre-synthesized single-strand or double-strand DNAs are bound onto glass slides (Schena et al. 1996) and high-density oligonucleotide arrays (produced by Affymetrix Inc. and recently also by Agilent Technologies) in which sets of oligomers are synthesized in situ on glass wafers using a photolithographic manufacturing process (Lockhart et al. 1996).

C'-DNA microarrays

On spotted arrays, templates for genes of interest are obtained and amplified by PCR. Following purification the aliquots are printed on coated glass microscope slides. Total RNA from both the test and the reference sample is fluorescently labelled with either Cye3- or Cye5-dUTP using a single round of reverse transcription. The fluorescent targets are pooled and allowed to hybridize under stringent conditions to the clones on the array. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo-coloured and merged. Information about the clones, including gene name, clone identifier, intensity values, intensity ratios, normalization constant and confidence intervals, is attached to each target. Data from a single hybridization experiment is viewed as a normalized ratio (that is, Cye3/Cye5) in which significant deviations from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression relative to the reference sample (Fig. 4) (Duggan et al. 1999).

Fig. 4 cDNA microarray scheme (Duggan et al. 1999).
Oligonucleotide GeneChips

On Affymetrix GeneChip® oligonucleotide arrays a given gene is represented by 15–20 different 25-mer oligonucleotides that serve as unique, sequence-specific detectors. Synthesis of oligos on the chip is based on photolithography and solid-phase DNA synthesis (Lipshutz et al. 1999). Synthetic linkers, modified with photochemically removable protecting groups are attached to a glass substrate and light is directed through a photolithographic mask to specific areas on the surface to produce localized photodeprotection. The first of a series of chemical building blocks, hydroxyl-protected deoxynucleosides, is incubated with the surface, and chemical coupling occurs at those sites that have been illuminated in the preceding step. Next, light is directed to different regions of the substrate by a new mask, and the chemical cycle is repeated until all oligos are synthesised (Fig. 5).

An additional control element on these arrays is the use of mismatch (MM) control oligonucleotides that are identical to their perfect match (PM) partners except for a single base difference in a central position. The presence of the mismatched oligonucleotide allows cross-hybridization and local background to be estimated and subtracted from the PM signal.

In the GeneChip expression assay eukaryotic mRNA is converted to biotinylated cRNA from oligo-dT-primed cDNA (Lockhart et al. 1996). Each sample is hybridized to a separate array. Transcript levels are calculated by reference to cRNA spikes of known concentration added to the hybridization mixture. Differences in mRNA levels between
samples are determined by comparison of any two hybridization patterns produced on separate arrays of the same array type.
In contrast to the cDNA arrays, the sequences of the ESTs (expressed sequence tags) synthesised on the chip must be known.

**Affymetrix Barley1 GeneChip**

Until recently, in the plant science, Affymetrix platform was available only for *Arabidopsis thaliana*. Its relatively small, fully sequenced genome as well as wide interest of the numerous research institutes allowed for a very fast development and improvement of the oligo-based arrays. At the moment already second generation of arrays is available from Affymetrix Inc. with 22 700 sequences and from Agilent Technologies with 21 500 sequences. Despite these advances, consistent data on parallel expression profiling from large-genome crop plants were infrequent (Close et al. 2004). Thanks to the combined efforts of the research communities, a 22K Barley1 GeneChip was made available for an Affymetrix platform in mid 2003, followed by the Wheat GeneChip in the early 2005. Barley1 chip was used for the analyses presented here.

The possibility of using the Barley1 GeneChip in profiling experiments of other crop plants was explored by Close et al. (2004). The studies revealed that hybridization of the cRNA from wheat leaves on the chip resulted in 5 392 present calls as compared to the 9 972 of present calls after hybridization of barley cRNA. A large number of probeset giving a good signal after hybridization had unknown functions, but there was also a large number having a putative functions in metabolism, signalling mechanisms, regulation of gene expression and oxidative stress. These results suggest that more than 5000 Barley1 probeset can produce good results when used in wheat experiments.

### 1.12 Field test

It has been shown that plants characterized by an anti-fungal or anti-viral properties when grown in the greenhouse conditions, did not show a significant increase of the resistance when challenged in the field (Annand et al. 2003, Sharp et al. 2002). This
clearly indicates that greenhouse conditions, being very often ideal for plant
development and not providing natural environment for the infection, do not represent
an ideal system for studying the complex metabolic mechanisms like disease and
pathogen control. Moreover, our research (Schlaich et al. 2005) showed a very high
impact of growing conditions on the overall gene expression as well as selected
metabolic pathway. These results together show the importance of small scale field
trials during assessment of new transgenic plant lines.

1.13 The aim of the study

The attempt to use genetically modified plants in agriculture provokes strong public
concerns. They range from ecological aspects (plants better adapted to the
environmental conditions may overcome the less adapted wild types) to health and
biosafety aspects (horizontal gene transfer, unexpected effects of transformation,
higher allergenicity or even toxicity of plant products) finally to economic aspects (big
companies distributing transgenic lines, high prices, inaccessibility for developing

We hypothesise that a single genetic transformation does not render major changes in
the overall metabolism of transgenic plants and we expect the pleiotropic effects to be
minimal. The aim of this study is to evaluate this hypothesis using plants transformed
with different anti-fungal transgenes. It is theoretically possible that pleiotropic effects
of genetic transformation might occur in not connected biochemical pathways.
Therefore we have focused our approach on global gene expression profile as well as
on specific metabolites accumulation pattern.

Flavonoids have been chosen because of their high importance in plant defence, not
only anti-pathogen but also stress-related. The new method of flavonoid extraction and
analysis for wheat has been developed and evaluated. Together with transcription
analysis tools, it provided valuable information about the influence of genetic
modification, infection and different growing conditions on plant metabolism.
Chapter 2 Materials and methods

2.1 Analysis of flavonoid metabolome

2.1.1 Plant material

Wheat plants (*Triticum aestivum* L., *Gramineae*) of Swiss spring varieties Frisai, Golin and Greina have been previously transformed with anti-fungal genes of broad spectrum effect like chitinase and glucanase (Bieri et al. 2003), ribosome-inactivating protein (RIP) (Bliffeld et al. 1999; Bieri et al. 2000) as well as transformed with a transgene of a specific effect - the KP4 against smuts and bunts (Clausen et al. 2000). Plants were transformed as described before (Bliffeld et al. 1999) using biolistic method (Finer et al. 1992). For all experiments, the homozygotic T4 generations were used. A compilation of the plant lines used is shown in table 3.

Plants were grown in the greenhouse under 15-h photoperiod conditions, at a temperature of 12-15°C, 60% relative humidity during the night and 16-19°C, 60% relative humidity during the day. Leaves of 10 plants were pooled into one sample. After collecting, leaves were frozen in liquid nitrogen (LN₂) and subsequently stored at -80°C before extraction.

Table 3: Plant lines used for the experiments.

<table>
<thead>
<tr>
<th>Variety</th>
<th>wt</th>
<th>KP4</th>
<th>Chitinase</th>
<th>RIP</th>
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<tr>
<td>Greina</td>
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</table>

2.1.2 Solvents and reagents

Acetonitrile (CH₃CN) for extraction use, was type Romil 230 gradient quality (Amman-Technik, Köliken, Switzerland). Methanol (MeOH) was HPLC-grade, type Romil 205 gradient quality (Amman-Technik, Köliken, Switzerland). HPLC-H₂O was prepared by ion-exchanger type Reinstwasser-System (Blanc-Labo, Tolochenaz, Switzerland).
Both HPLC solvents were passed through Millipore filters (H₂O: 0.45m HA; MeOH: 0.50m FH) (Bedford, MA, USA). Trifluoroacetic acid (TFA) Uvasol® for spectroscopy was purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fluka (Fluka Chemie, Switzerland).

2.1.3 Internal standard and reference standard

Apigenin-7-glucoside Rotichrom® used as internal standard was purchased from Roth (Carl Roth, Karlsruhe, Germany). Isoorientin used as reference compound has been previously isolated from Gentiana lutea L. (Gentianaceae) and was fully characterised in the laboratory of Pharmacognosy and Phytochemistry of University of Lausanne, Switzerland.

2.1.4 Extraction

Samples for analysis were extracted using a solid phase extraction (SPE) technique on C18 Chromabond® 6 ml cartridges (Macherey-Nagel, Düren, Germany). Before extraction, the leaf material was ground in LN₂. SPE cartridges were conditioned with 5 ml of 70% CH₃CN and 500 mg of material was placed in the cartridge together with 1 ml of apigenin 7-O-glucoside solution (1.17 mg/ml) used as an internal standard. During extraction, two fractions were collected: fraction A was extracted with 10 ml of 70% CH₃CN followed by 10 ml of 100% CH₃CN and fraction B was obtained after elution with 10 ml of 100% CH₃CN. Fraction B served as an extraction control, since all secondary metabolites to be detected are supposed to be in fraction A. After extraction, samples were evaporated to dryness using a Rotavapor® (Büchi, Flawil, Switzerland) and lyophylised with a Gamma 1-20 lyophilizer® (Blanc-Labo, Tolochenaz, Switzerland).

2.1.5 HPLC analyses

Reversed-phase HPLC was carried out using an HPLC system HP-1050 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a binary pump. A Hewlett-Packard 1050 series on-line photodiode array detector (DAD) was used for detection. This instrumentation was controlled by HP Chemstation software. The separation was
performed on a C-18 Symmetry column (250 x 4.6 mm i.d., 5 μm particle size, Bedford, MA, USA) with a MeOH (0.5% TFA):H₂O (0.05% TFA) stepped gradient 10:90 to 26:74 in 10 min, 26:74 to 30:70 in 10 min, 30:70 to 30:70 in 20 min, 30:70 to 100:0 in 25 min and 100:0 to 100:0 in 5 min. The column temperature was set at 25°C with a flow of 1 ml/min. The DAD-UV detector was set at 271 nm. HPLC/UV analyses were performed in duplicate by injection of a 20 ml volume of both fraction A and B samples, the latter being used as an extraction control.

2.1.6 LC-UV-MS analyses

LC/MS analyses were performed by atmospheric pressure chemical ionization (APCI) on a Finnigan LCQ ion trap mass spectrometer (FinniganMAT, San Jose, CA, USA). The APCI optimized parameters were: capillary temperature 150°C, vaporizer temperature 450°C, corona needle current 4.5 μA and sheath gas flow 65%. Spectra (160-900 amu) were recorded in the positive ion mode. MS detection was performed in the full scan mode set between 80 and 900 m/z. MS-MS experiments were performed after selection and consecutive fragmentation of the most intense ion of the MS. As solvents methanol + 0.5% formic acid and water + 0.5% formic acid were used. The gradient of elution was identical to the one of HPLC-UV analysis. TFA was substituted by formic acid because of its better compatibility for MS.

2.1.7 UV spectrography with diagnostic shift reagents

The Hewlett-Packard HP-1050 system with HP-1050 series on-line photodiode array detector (DAD) was used for spectral analysis. The conditions of measurements are presented in Table 4.
Table 4: Conditions of addition of diagnostic shift reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Pump 2</th>
<th>Flow (ml/min)</th>
<th>Pump 3</th>
<th>Flow (ml/min)</th>
<th>Temperature</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent</td>
<td>H₂O</td>
<td>0.2</td>
<td>H₂O</td>
<td>0.2</td>
<td>RT</td>
<td>2</td>
</tr>
<tr>
<td>NaOAc</td>
<td>NaOAc (0.5M)</td>
<td>0.2</td>
<td>NaOH (0.01M)</td>
<td>0.2</td>
<td>RT</td>
<td>5</td>
</tr>
<tr>
<td>KOH</td>
<td>KOH (0.3M)</td>
<td>0.3</td>
<td>H₂O</td>
<td>0.1</td>
<td>RT</td>
<td>14</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>AlCl₃ (0.3M)</td>
<td>0.2</td>
<td>NaOH (0.01M)</td>
<td>0.2</td>
<td>90°C</td>
<td>4</td>
</tr>
<tr>
<td>AlCl₃ acidic</td>
<td>AlCl₃ (0.3M)</td>
<td>0.3</td>
<td>H₂O</td>
<td>0.1</td>
<td>90°C</td>
<td>3</td>
</tr>
<tr>
<td>H₃BO₄</td>
<td>NaOAc (0.1M) H₃BO₄ (0.7M) 1:1</td>
<td>0.2</td>
<td>NaOH (0.01M)</td>
<td>0.2</td>
<td>RT</td>
<td>5</td>
</tr>
</tbody>
</table>

2.1.8 Data processing

After HPLC analysis, UV chromatograms were integrated and normalized on the internal standard present in each sample. To test the significance of changes, analysis of variance (ANOVA) and t-test were used. Additionally we applied Principal Components Analysis (PCA) (Raychaudhuri et al. 2000) to represent similarities between analysed samples. The statistical calculations were performed using SPSS software pack (http://www.spss.com) and R (http://www.r-project.org).

Finally, to show the distances between varieties, an appropriate method of clustering was implemented. The areas of each of the 13 analyzed peaks were correlated to the area of the standard peak of known concentration. Then relative areas of all 14 peaks were processed with "Genesis" software (Sturn et al., 2002). Hierarchical clustering analysis (Eisen et al. 1998; Guess and Wilson 2002) was performed with unweighted pair-group average linkage method. Distance between each sample was measured using Euclidian distance algorithm (Claverie 1999).
2.2 Analysis of transcription

2.2.1 Plant material

Plants were grown under the greenhouse conditions as described above (paragraph 2.1.1.).

For semi-open growth conditions, the vegetation hall of Swiss Federal Research Station for Agroecology and Agriculture (FAL, Zurich-Reckenholz, Switzerland) was used. The vegetation hall provides open conditions during dry weather with an automatic closure of roof and walls in case of rainfall, strong wind or darkness. No artificial lights or heating is used.

Wild type and transgenic plants of Swiss spring varieties Frisal, Golin and Greina were grown in randomised plots, in 3 repetitions. 50 seeds per repetition were sown and each pot was surrounded by wild type wheat plants for isolation.

During the same vegetation period (year 2003), wild type plants of Golin, Greina and Frisal varieties were grown on the experimental field of FAL (located in the vicinity of vegetation hall). Plants were grown in randomised plots, in 3 repetitions and were surrounded by wild type wheat plants for isolation.

The infection challenge of the KP4 plants was performed on the field and in the greenhouse as described by Schlaich et al. (2005). The field experiment was designed in randomised plots with four repetitions. In each plot, 100 seeds were sown in four rows. Five genotypes were tested wt Golin, KP4-Golin, wt Greina, KP4-Greina, and wt Frisal. From each genotype one batch was inoculated with stinking smut and another was not for control. The expression analysis was performed for wt and KP4 plants of variety Greina. For both genotypes, the infected and not infected plants were compared. We have analyzed the differences induced by the infection as well as those induced by the transgene.

Flag leaves of 10 plants were collected when plants were in the 3 leaf stage (developmental stage 13 according to Zadoks et al. 1974) and pooled. Since no biological replicates were possible on the basis of another time point and/or an
independent location, several independent samples were collected in the same time. Samples were stored in liquid nitrogen before RNA extraction.

2.2.2 Isolation of total RNA and labelling

RNA was extracted using TRIsol (Invitrogen) reagent according to manufacturer instructions. 15μg of total RNA was reverse transcribed by Superscript II reverse transcriptase (Invitrogen) with the T7-(T)24 oligo as recommended by the manufacturer. Immediately after the reaction cDNA was cleaned on Phase Lock Gel (Eppendorf) and precipitated.

The cDNA was labelled during in vitro transcription with labelled nucleotides using the ENZO BioArray kit (ENZO Biochem, Inc.) following the manufacturer's protocol. Immediately after labelling cRNA was purified on RNeasy mini columns (Quiagen) following the protocol supplied by the manufacturer.

2.2.3 Hybridization, staining and scanning of the GeneChips®

From the biotinylated cRNA, 15μg was fragmented at 95°C for 35 minutes in the presence of fragmentation buffer (200mM Tris acetate, pH 8.1, 500mM KOAc, 150mM MgOAc). The Affymetrix Barley1 GeneChips® were pre-conditioned with the hybridization buffer at 45°C for 10 minutes and afterwards each microarray was loaded with the hybridization cocktail containing 15μg of labelled cRNA mixed with BSA, herring sperm DNA, hybridization buffer, hybridization control mix and B2 hybridization control oligo. The microarrays were hybridized for 16 hours in 45°C with 60rpm rotation speed in the hybridization oven. All buffers used were prepared according to the standard Affymetrix protocols for GeneChip® hybridization.

The hybridized GeneChips® were placed in the Affymetrix FS450 Fluidics Station and washed and stained according to the Affymetrix protocols for eukaryotic samples. After staining the microarrays were kept in the dark before scanning and were scanned on GeneChip Scanner 3000 (Affymetrix).
2.2.4 Data analysis

Data obtained by scanning were exported to Silicon Genetics' Genespring 7.0 analysis software (www.silicongenetics.com). Data were normalized according to the following procedure: each measurement was divided by the 50th percentile of all measurements in that sample. Later-on all samples were normalized against the median of the control samples. Each measurement for each gene in those specific samples was divided by the median of that gene's measurements in the corresponding control samples. To allow the proper representation of the changes in gene expression among all samples analysed, each gene was divided by the median of its signal in all samples. If the median of the raw values was below 10, then each measurement for that gene was divided by 10.

In order to exclude false positives, a stringent gene selections based on Genespring's cross-gene error model and on identity of the hybridization of replicates were performed. Additionally as a negative control a comparison between not infected wt and transgenic plants was done. In this experiment only 0.98% of genes showed variation in mRNA level higher than 2-fold and these genes were excluded from further analysis. Genes that showed significant changes in mRNA levels (above 2-fold) between not-infected and infected transgenic plants were plotted on the graph showing expression trends among all compared samples. The proper annotations of probesets were confirmed with available cereal databases (www.barleybase.org; www.graingenes.org). Expression levels of those genes with known wheat sequence were confirmed by Real Time PCR.

2.2.5 Real Time PCR

To confirm the results from the GeneChip experiment, a group of PR-related genes was chosen for real-time PCR. About 100 mg of plant material was homogenized in liquid nitrogen and RNA was extracted using TRIzol (Invitrogen) according to the protocol of the manufacturer. DNA was digested by RNase-free DNAse (Quiagen). Of this RNA, 4 μg was reverse transcribed using Advantage-RT for PCR (Clontech). The cDNA was subsequently amplified using an ABI Prism 7700 Sequence Detector and the SYBR-Green I technique (Applied Biosystems). Actin was used as a standard. The primer sequences used were as follows:
Actin: 5'-TGG TAT ACA CGA AGC GAC ATA CAA T-3' and 5'-AAC CTC CAC TGA GAA CAA CAT TAC C-3'; Contig 2211 (Pathogenesis-related protein PRB 1-2): 5'-AAT GTC GTT GGA CAG AAA CCA TAC T-3' and 5'-AAG GAA CGA GGG ACT ACT GGA CTA T-3'; Contig 13350 (beta-1,3-glucanase): 5'-GGC CTA CAA CAT CCA GTT CAA GTA G-3' and 5'-GCA TTT TGC AGC TTA TTT AGG ATG A-3'; Contig 4056 (Pathogenesis-related protein 1A/1B): 5'-TGG GAT CAA TAA GGA ACA AGG ACT A-3' and 5'-GTC AAC ATA CAA GGC ACA GTC ATT C-3'; Contig 4324 (Chitinase II precursor): 5'-ACC TCA CTT GCT AGA TTC TTG ATC G-3' and 5'-ACA GGG AGG TAG AGC TGT AAG TAC G-3'.

The temperature program for the PCR reaction was: 50°C for 2 min, 95°C for 10 min, followed by 40 repetitions at 95°C for 15 s and 60°C for 1 min. RNA extraction and the PCR reaction were repeated with material from different developmental stages and gave comparable results.
Chapter 3 Development of the metabolite analysis method

A fast method of flavonoid extraction from wheat leaves was necessary in order to assess large amount of samples in relatively short time frame of this experiment. A number of different flavonoid extraction methods can be found in the literature (Molnar-Perl and Fuzfai 2005, Naczk and Shahidi 2004) however for our needs a fast and highly efficient method was necessary. Our aim was to design and optimise a method which would be easy to perform and which would yield enough extract for analysis from minimum starting material.

3.1 Extraction method

During the development of the extraction method several different approaches were tested. We have evaluated the use of extraction methods based on:

1. maceration of the sample with the solvent
2. Soxhlet extraction
3. medium pressure liquid chromatography
4. solid phase extraction cartridges

The evaluation was based on the repeatability of the method, its yield, ease of implementation, costs and availability of necessary equipment as well as the time needed for complete extraction. All four methods are briefly described below. In each of the approaches frozen, ground leaves were used as a starting material.
3.1.1 Maceration

Samples were macerated with solvent (methanol) on a shaking plate in 5 cycles, 2 hours each. After each cycle, the solvent was decantated (from above the sample), evaporated under low pressure and lyophylised. The chromatograms of extracts after each cycle are shown on Figure 6. Small peaks are visible on the chromatogram of the extract coming from 5th cycle of extraction. It shows that 5 cycles of maceration are not enough to extract the total flavonoid content of the plant. This method of extraction was very time consuming and did not give satisfactory results.

3.1.2 Soxhlet extraction

The frozen sample was placed in the thimble of the Soxhlet apparatus (Fig. 7) with methanol as a solvent. In the Soxhlet apparatus, the solvent is heated to the boiling temperature, evaporated and, after condensation in the cooler it is soaking the sample in the thimble, extracting the soluble compounds. After reaching a certain level the solvent is returned to the flask at the bottom of the apparatus through a syphon-shaped tube. We allowed 3 hours for one cycle of the extraction and we have used 2 cycles. After each cycle the solvent was evaporated under reduced pressure and lyophylised. The chromatograms of extracts coming from

Fig. 6 Chromatograms of the extracts obtained after each of 5 maceration cycles. Note, that even in 5th cycle flavonoids are detectable which indicate non-complete extraction.

Fig. 7 Soxhlet apparatus.
both cycles are shown on Figure 8. The method did not provide the complete extraction since the second fraction shows high abundance of flavonoids.

![Chromatograms of the two fractions after Sohxlet extraction.]

3.1.3 Medium pressure liquid chromatography (MPLC)

4g of frozen, ground sample was placed in the MPLC column filled with 12g of Silica 60 with grain size 70-200\(\mu\)m. The extract was eluted with a gradient of MeOH:
- 5% MeOH for 25 min (pressure ~6bar)
- 60% MeOH for 25 min (pressure ~8bar)
- 100% MeOH for 30 min (pressure ~4bar)

After the elution, extract was evaporated under reduced pressure and lyophylised. The initial trials have shown that the repeatability of the method was very low and, therefore, we have stopped the development of this method.
3.1.4 Solid Phase Extraction (SPE)

We have combined the acetonitrile gradient extraction with the Solid Phase Extraction (SPE) C18 Chromabond cartridges (Macherey-Nagel, D-52313 Düren) used for separation of the extracted compounds. These are commercially available cartridges filled with C18 absorbent (Fig. 9) which may be used for cleaning the extract before using it for another chromatography based analysis. The extract was eluted with a gradient of acetonitrile and evaporated under reduced pressure and lyophylised. After dissolving in methanol, it was injected into the HPLC chromatograph for analysis\(^1\).

Two fractions were eluted from the column and both were analysed with HPLC. The second fraction did not show any flavonoids which assured the complete extraction. Also the time needed for the complete extraction was the shortest of all methods tested.

The method proved to be very efficient (about 15mg of extract was obtained from 500mg of sample) and repeatable. The method was validated by performing 3 subsequent extractions of the same sample and injecting each extract twice into the HPLC chromatograph. The variation between the results was then calculated and compared with the variation between results of different samples (Fig. 10).

\(\text{Variance} \)

\begin{tabular}{|c|c|}
\hline
\textbf{Extracts of the same sample} & 0.006 \\
\hline
\textbf{Extracts of different samples} & 0.036 \\
\hline
\end{tabular}

Fig. 10 Comparison of the variance of the results of multiple extractions of the same sample with the variation observed between different samples.

\(^1\) See Chapter 2 “Material and methods” for a detailed description of the extraction procedure.
3.2 HPLC analysis

3.2.1 Columns

8 different columns, known to perform well for the separation of flavonoids, were tested for a quality of the separation:
- Nucleosil® 70mm
- Nucleosil® 150mm
- Nucleosil® 250mm
- Nova-Pak® 250mm
- Lichrocart® 250 mm
- Zorbax® 150mm
- Symetry® 150 mm
- Symetry® 250 mm

The Symetry® 250mm column yielded the best resolution of separation and therefore was used for further measurements (Fig. 11).

3.2.2 Separation conditions

The chromatographic separation was optimized using different elution systems (MeOH-H2O, CH3CN-H2O, and MeOH-CH3CN 1:1-H2O). The system MeOH-H2O gave the most satisfactory results. Temperature and elution gradient were modified to improve resolution. Temperature was set at 25°C and a gradient MeOH-H2O (10:90 to 26:74 in 10 min, 26:74 to 30:70 in 10 min, 30:70 to 30:70 in 20 min, 30:70 to 100:0 in
HPLC analysis

25 min and 100:0 to 100:0 in 5 min) was found to give an optimal chromatographic separation of the compounds of interest (Fig. 12).

![Diagram](image)

Fig. 12 Separation of the flavonoids on the Symetry® 250mm column with different solvents.

### 3.2.3 Standard

For proper interpretation of the HPLC results, the internal standard of known concentration is needed. It is added at the beginning of the extraction process, so that all sample-to-sample handling differences will be accounted for during final quantification of the separated compounds. To serve as a good standard, the compound must not appear in the analysed sample and its retention time must not overlap with any other compound present in the analysed mixture. Apigenin 7-O-glucoside has been chosen to serve as an internal standard as it combines all those properties. The retention time of apigenin 7-O-glucoside is different from retention times of all the analysed compounds and therefore on the chromatogram its peak does not overlap with other peaks. It was added at the beginning of each extraction to the ground plant material in the concentration of 1.17mg/ml. During the analysis we compared the area of the peak of apigenin 7-O-glucoside with all other compounds and estimated the concentration of each compound in the extract on this basis.
Chapter 4 Results

We focused on the flavonoids because they are known to interact in different ways with the environment. The plant can produce them in response to environmental stimuli and they can affect the environment e.g. as phytoalexins or inducers of symbiotic processes (Daniel et al. 1999; Harborne 1986). Changes in their synthesis would have important influence on plant characteristics.

Preliminary HPLC/DAD-UV experiments performed on wheat MeOH and CH$_3$CN extracts showed that the developed method yielded flavonoids as the major extracted compounds. Indeed, the UV spectra of the compounds detectable showed a characteristic pattern of flavones with the two maxima located around a wavelength of 270 and 350 nm respectively. This is in accord with the literature (Adom et al. 2003, Harborne et al. 1993, Julian et al. 1971).

4.1 Preparation of the sample

The differences between physico-chemical properties of different groups of flavonoids, for example their solubility, as well as differences between plant species required to optimize the extraction protocol for particular groups of compounds and particular plants, namely wheat.

The strategy for using SPE was to pre-purify the sample and to extract specific compounds of interest (flavonoids) in one single step from a small amount of available material. To avoid individual sample variation, leaves of 10 plants grown in the same time and conditions were pooled before extraction.

The extraction scheme provided 2 fractions: the first one after elution of 10 ml of CH$_3$CN 70% and 10 ml of CH$_3$CN 100% (fraction A), and the second one after a wash with 10 ml of CH$_3$CN 100% (fraction B). Fraction B was used to prove that all the compounds of interest were extracted. During the development of the method, MeOH was tested as an extraction solvent, but remaining compounds of interest were found
in B fractions. That indicated, that the extraction was incomplete and the results obtained could not be compared quantitatively. After using CH$_3$CN as a solvent, B fractions contained no compounds of interest indicating a complete extraction of the flavonoids. During this operation, SPE has proven to be a rapid, low-cost, non-sophisticated and highly reproducible method, consuming only low amounts of solvents.

4.2 Validation of the method

In order to validate and to test the repeatability of the method, every sample was extracted three times separately and each extract was injected twice into the HPLC system (see Chapter 3: "Development of the metabolite analysis method"). The variance between 6 measurements of the same sample was calculated and compared with the variance of measurements between the samples (Fig. 10, page 40). Very low variance of the measurements of the same sample as compared to the 10 fold higher variance of the measurements of different samples proved that the method is very sensitive and does not introduce significant errors to the measurements.

4.3 Identification of the compounds by mass spectrometry and UV spectrography

Most of the wheat flavonoids are present as glycosides. They are polar compounds and thus they are not amenable for analysis by GC/MS without an additional step of derivatization. Different LC/MS methods have been successfully applied to the analysis of flavonoid glycosides (Stobiecki et al. 2003). A rapid and convenient method based on reversed-phase HPLC combined with UV and mass spectrometric detection was developed using the ionization conditions optimized by Waridel et al. (2001).

This LC/MS method was applied to a sample of the Greina WT variety. The different detected peaks were numbered according to their elution time (Fig. 13). All of them exhibited a UV spectrum specific for flavonoid derivatives with two major absorption bands, band I (max 350 nm) and band II (max 270 nm). The major peak (7) was
identified as isoorientin after LC/UV and LC/MS comparison with the reference compound. Isoorientin was used as a standard compound in order to check the fidelity of the ionization method.

![Chromatogram of the wheat flavonoid extract](image)

**Fig. 13** Chromatogram of the wheat flavonoid extract. The standard used (apigenin-7-O-glucoside) is marked with an arrow. Additionally, the analysed peaks are marked with numbers (red) used in the text.

**Standard: Apigenin-7-O-glucoside**

For the proper quantification of the extracted flavonoids, the internal standard had to be added at the beginning of the extraction to account for all handling differences. We have chosen to use apigenin-7-O-glucoside as a compound which does not exist in wheat and, moreover, the retention time of which does not overlap with retention times of analyzed compounds. Figure 14 shows the sample chromatogram with the standard.
Identification of the compounds by mass spectrometry

chromatogram with marked standard as well as chromatogram of the standard alone.

Fig. 14 HPLC/UV spectrum of the sample chromatogram of wheat extract + standard (top) as compared to the standard alone (bottom).

Isoorientin (Compound 7)
The proper identification of isoorientin was performed by the comparison with the available pure compound. Thus, the MS/MS analysis of the compound could be used to check the accuracy of the mass analysis method.
The MS and MS² (second MS fragmentation) of the standard isoorientin shown on Fig. 16 were in agreement with those published previously by Waridel et al. (2001) and with those of compound 7 detected from sample of the Greina WT variety. The fragmentation of the parent ion at m/z 449 [M+H]+ led indeed to the observation of specific cleavages for C-glycosides in MS² with characteristic ions at m/z (molecular weight per unit charge) 431 [M+H-18]+, 413 [M+H-36]+, 395 [M+H-54]+, 383 [M+H-30-36]+, 353 [M+H-96]+ and 329 [M+H-120]+. The presence of the m/z 329 [M+H-120]+ fragment is of particular importance as it allows to identify unambiguously the 6-O-glycoside flavonoid isoorientin from its 8-O-glycoside analogue orientin (see Fig 14 and Waridel et al., 2001).
We have tested the compounds peak by peak (see Fig. 13) using MS/MS and UV with shift reagents. The resulting spectra with description can be seen in the Appendix 1: Identification of the compounds.

### 4.4 Identification of the compounds by NMR

Above methods provided good indication about the nature of compounds, however, in most cases they did not give a definite answers regarding their structure. Therefore additional analysis with nuclear magnetic resonance (NMR) were performed. The output of those analyses allowed for much more precise structure recognition.

Additional experiments involving 2D NMR are necessary to elude the interglycosidic bonds and identity of the glycoside substituent or/and position of the methoxyl group. This experiments are being performed in collaboration with the group of Prof. Hostettmann at the University of Geneva.

Here, the summary of the results obtained so far is provided.

**Compound 1**

Formula: $C_{27}H_{30}O_{16}$

Name: Isoorientin 6-O"-\(\beta\)-D-glucopyranoside (Fig. 17)

![Figure 17: The structure of Isoorientin 6-O"-\(\beta\)-D-glucopyranoside](image-url)
Compound 2 and 3
The compounds appear to be isomers and so far it is impossible to assign the names precisely.
Formula: C\textsubscript{26}H\textsubscript{28}O\textsubscript{15}
Name: Lucenin-1 or Lucenin-3 (Fig. 18)

![Fig. 18 Structures of Lucenin-1 (A) and Lucenin-3 (B)]

Compound 4 and 6
Also in case of those 2 peaks the final identification is not possible at the moment. Based on the formula - C\textsubscript{26}H\textsubscript{28}O\textsubscript{14} the possible compounds may be: Vicenin-1, Vicenin-3, Schaftoside or Isoschaftoside (Fig. 19).

![Fig. 19 Structures of Vicenin-1 (A), Vicenin-3 (B), Schaftoside (C) and Isoschaftoside (D)]
**Compound 5**
Formula: \( \text{C}_{27}\text{H}_{30}\text{O}_{16} \)
Name: Isoorientin 7-O-\( \beta \)-D-glucoside (Fig. 20).

![Fig. 20 The structure of Isoorientin 7-O-\( \beta \)-D-glucoside.](image)

**Compound 8**
Formula: \( \text{C}_{27}\text{H}_{30}\text{O}_{15} \)
Name: 4H-1-Benzopyran-4-one, 6-[6-O-(6-deoxy-\( \alpha \)-L-mannopyranosyl)-\( \beta \)-D-glucopyranosyl]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy- (9CI) (Fig. 21).

![Fig. 21 The structure of compound 8](image)
**Compound 9**
Formula: $\text{C}_{28}\text{H}_{32}\text{O}_{16}$
Name: Lutonarin (Fig. 22).

![Fig. 22 The structure of Lutonarin.](image)

**Compound 10**
3 different compounds are possible here. The formula of the compound has been identified as: $\text{C}_{22}\text{H}_{22}\text{O}_{11}$ meaning that possible compounds are: Isoscoparin, 6-D-Glucopyranosyldiosmetin or Swertiajaponin (Fig. 23).

![Fig. 23 Structures of Isoscoparin (A), 6-D-Glucopyranosyldiosmetin (B) and Swertiajaponin (C)](image)
4.5 Changes in flavonoid concentration under different growing conditions

The flavonoid concentration changed significantly when plants were grown under artificial (greenhouse) or natural (field) conditions (Fig. 25). These changes were of
Changes in flavonoid concentration under different magnitude and affected different compounds in 3 varieties tested (see Fig. 25 - 27).

The most visible changes were observed in the concentration of isoorientin and its derivatives. In most severe case (variety Golin) the concentration of isoorientin in the
Changes in flavonoid concentration under different field was 8 fold higher than in the greenhouse (Fig. 26 and 27). Note, that the height of the peak on the chromatogram must be correlated to the height if the peak of internal standard. Plants grown in the vegetation hall showed more moderate effects than those grown in the field. Such dramatic changes, however did not produce any visible phenotype. We assume that these effects were mostly caused by the variations in the light levels and light quality available for plants in specified locations.

Apparently for most of the compounds, plants grown in the vegetation hall show a concentration higher than in the greenhouse and lower than on the field, but this is a general conclusion (see for example components 2-4 on Figure 26). Multi-factor ANOVA (analysis of variance) showed that there are no variety-dependent differences between chromatographic profiles of plants grown in the greenhouse and vegetation hall. However when field-grown plants were analyzed, differences between cultivars can be seen (p-value = 0.02). Those differences are even more visible when concentration of selected compounds is compared. For example, note the 8 fold increase of concentration of isoorientin in plants grown on the field for Golin variety. Frisal under the same conditions shows only about 1.7 fold increase (Fig. 27).

![Greina](image_url)

**Fig. 26** Influence of the growing conditions on the flavonoid concentration in the variety Greina. Compound Nr 7 - isoorientin, Nr 12 - internal standard. Note: Points were connected for better illustration purposes.
Comparison of WT and GM plants for flavonoid composition

4.6 Comparison of WT and GM plants for flavonoid composition

In this experiment we tried to answer two questions. Are there any differences in flavonoid composition in extracts obtained from wt and transgenic plants of the same variety? And if such differences are discovered how do they compare to the naturally existing differences between plants of different varieties.

The flavonoid composition was compared between different transgenic lines and the respective wt varieties (Fig. 28). No new flavonoids have been found and the changes in their concentration were of much lower significance than those revealed when different wt varieties were compared against each other.

For all the varieties, the chromatograms of wt and transgenic samples look almost identical. The ratios between the peaks are kept and the only detectable difference is the area below certain peaks. We conclude, that even though some differences are detectable, they can be considered as minimal.

When different wt varieties are compared between each other, the changes are clearly visible on the chromatograms. Note for example the differences in the size ratio of 2nd and 3rd peaks on the chromatograms of Frisal wt, Golin wt and Greina wt or the striking differences of 7th peak between Greina wt and other 2 varieties.

The statistical significances of those differences were calculated by ANOVA and two-
Comparison of WT and GM plants for flavonoid com-
tail t-test. All calculations were performed with \( p = 0.01 \). The results are shown on the
histograms (Fig. 29). The differences introduced by the transgenes were in all cases
smaller than the differences occurring naturally between WT plants of different varieties.
Neither transgene-dependent, nor variety-dependent patterns of changes were
observed.
Comparison of WT and GM plants for flavonoid compo-

Fig. 28 Chromatograms of all compared transgenic lines with their respective wt varieties. Red arrows indicate internal standard (apigenin 7-O-glucoside). The account for the differences of the height of standard peak must be made (see Fig. 29).
Comparison of WT and GM plants for flavonoid comp-

A) Differences between Frisai wt, Frisai A5 and Frisai B12;
B) Differences between Golin wt and Golin KP4;
C) Differences between Greina wt and Greina KP4;
D) Comparison of three wt varieties.

Designation of significance (p=0.01):
- a - difference between Fr wt and Fr A5
- b - difference between Fr wt and Fr B12
- c - difference between Go wt and Go KP4
- d - difference between Gr wt and Gr KP4
- e - difference between Go wt and Gr wt
- f - difference between Go wt and Fr wt
- g - difference between Gr wt and Fr wt
In order to compare the different GM/nGM wheat varieties, a hierarchical clustering was performed using the data from HPLC/UV analysis. Distances between samples were calculated using Euclidean distance function (Claverie 1999), which is a square root of the sum of the squared distances of two vector values. To avoid the bias of the results by the largest peaks, data were log transformed before calculations. Clusters are formed between the most related sample pairs (Fig. 30). The bootstrap resampling was used to evaluate the accuracy of the clustering. Red numbers on the graph represent p-values calculated by the bootstrapping algorithm and are the measure of cluster relevance. They show the percentage in which the particular cluster appeared during multiple cluster analysis. They are not, however, a measure of similarities between the samples, which is represented by the length of the vertical lines: the longer the line, the bigger variations between the samples. The method has revealed similarities between wt and transgenic plants of the same variety. Different varieties have formed separated clusters.

![Hierarchical clustering of samples. Red values show the p-values (in %) computed by the bootstrap resampling. The Height axis is a measurement of the difference in the Euclidean scale. Note: the transgenic lines are clustering together with the respective wt.](image)

In order to get a synoptic view of the comparison, we reduced the number of factors responsible for variation between the samples to three main components. For this procedure, we used Principal Components Analysis (PCA) according to Raychaudhuri et al. (2000). The PCA begins by searching a linear combination of variables that
Comparison of WT and GM plants for flavonoid compo-
accounts for as much variation in the original variables as possible. It then searches for
another component which accounts for as much of the remaining variation as possible
and which is uncorrelated with the previous component. Usually, a few components will
account for most of the variation.
In this case, the initial variation factors were concentrations of all 13 compounds
detected by the HPLC. After calculations, three main components proved to carry
87.1% of sample-to-sample variability.
The main factors correlated with Component 1 are: compound 5, compound 7,
compound 9 and compound 10. Component 2 is correlated with compound 1, while
Component 3 with compound 3.
Component 1 and component 2, carrying 75% of sample-to-sample variability, after
plotting in the 2-dimensional space allowed for a separation of samples into clusters
(Fig. 31). In 2 out of 3 cases, transgenic plants (marked by the squares) are clustering
together with their wild type background (marked by triangles) and away from wild
types of other varieties (distinguished by colours). Data points of Golin Kp4 (red
squares) and Golin wt (orange triangles) are spread from the ones on the left part of
the graph up to the ones in the centre, mixed with the cluster of Frisal wt and transgenic
(green marks), but they still can be considered as a separate cluster when the range of
differences between varieties is taken into the account.

Fig. 31 PCA (principle component analysis) of samples.
Figure 31 shows that the Component 1 allows for a separation between varieties tested. Therefore it can be said, that the main differences between those varieties are carried by four detected compounds: 5, 7, 9 and 10.

4.7 Effect of growing conditions

The objective of this experiment was to asses the changes caused to the plant metabolism by the different environmental conditions and to compare the reaction of not infected wt and not infected transgenic plants. For this experiment, semi-open conditions of the vegetation hall were compared with the closed environment of the greenhouse. For a comparison, wt plants were grown also in the field.

Figure 32 shows the comparison of the flavonoid content of not infected wt and not infected KP4 Greina grown in the greenhouse and the vegetation hall as well as the field-grown wt. Wt plants show strong increase in the concentration of isoorientin (compound Nr 7) and moderate increase of several other compounds (Compounds 5, 6 and 8). In the vegetation hall, as opposed to the field, a decrease in concentration of compounds 2-4 can be seen. However, KP4 plants show a similar decrease in concentration of compounds 2-4, they do not show any significant changes of the concentration of compounds 5, 6, 7 and 8. The significance of observed changes, calculated by ANOVA and t-test, is presented in the Table 5. The concentration of those compounds is very much in accordance with the concentration revealed in the plants grown in the greenhouse (both wt and KP4).
Effect of growing conditions

Fig. 32 Comparison of the flavonoid concentration changes in WT and KP4 plants after the change of growing conditions from closed greenhouse to semi-open vegetation hall. Red arrows mark the internal standard.

Table 5: Significance of observed changes in the concentration of selected compounds between different growing conditions in Greina wt and KP4 plants.

<table>
<thead>
<tr>
<th>Greina wt Compound Nr</th>
<th>p-value</th>
<th>Greina KP4 Compound Nr</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.89E-07</td>
<td>2</td>
<td>1.86E-09</td>
</tr>
<tr>
<td>3</td>
<td>3.05E-08</td>
<td>3</td>
<td>4.05E-08</td>
</tr>
<tr>
<td>4</td>
<td>1.17E-05</td>
<td>4</td>
<td>0.002</td>
</tr>
<tr>
<td>5</td>
<td>3.46E-10</td>
<td>5</td>
<td>0.40</td>
</tr>
<tr>
<td>6</td>
<td>0.03</td>
<td>6</td>
<td>0.22</td>
</tr>
<tr>
<td>7</td>
<td>1.14E-12</td>
<td>7</td>
<td>0.24</td>
</tr>
<tr>
<td>8</td>
<td>9.20E-10</td>
<td>8</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Analysis of the flavonoid metabolome of KP4 plants grown in semi-open conditions of the vegetation hall revealed that the changes in the flavonoid content do not follow the pattern of the wild type plants under the same conditions, but are similar to the plants grown in the greenhouse.
4.8 Analysis of expression with Affymetrix Barley1 GeneChip

To address the question of changes in a broader way, we performed the transcription analysis with the Affymetrix GeneChip platform. By using a microarray we were able to track changes in the expression of vast amount of genes simultaneously. The expression pattern of wild type as well as transgenic plants of different varieties have been analyzed. The wt plants were grown in closed, semi-open and open conditions (described in chapter 2. Material and methods) while the transgenic (KP4) plants were grown only in the greenhouse and vegetation hall. The analysis was performed with the Affymetrix Barley1 GeneChip.

Hybridization efficiency
During the time of the experiment, Affymetrix provided only the Barley1 GeneChip for analysis of cereals. Though it is based on the barley ESTs, the high homology between wheat and barley enables to hybridize wheat samples with good efficiency. The total number of probesets on the chip is 22840. On average, after hybridization, about 30% of total probeset (6850) were assigned by Affymetrix MAS 5 as present (P) and about 2% of probesets (450) were assigned as marginal (M). In spite of the non homologous application of the chip, all the internal controls were in accordance with the Affymetrix guidelines.

Hierarchical clustering
The analysis of the expression results revealed strong influence of the growing conditions on the total expression pattern. Figure 33 shows the comparison of all samples tested. The red colour represents upregulated, green - downregulated and black - not changed genes. After hierarchical clustering of genes changed in this experiment, distinct blocks of up- and downregulated genes can be seen. The general pattern of expression in different varieties is similar when plants are grown under the same conditions. Note the big red block of overexpressed genes for Golin, Greina and Frisal varieties in the field (cluster A) as well as smaller red block for the same varieties in the vegetation hall (cluster B). The reaction of the plants in the greenhouse is very much different from this in the open and semi-open conditions. This can be seen as the inversion of the red-green pattern on the Figure 33 (cluster C). Interestingly when
transgenic plants are analyzed, their behaviour in the semi-open vegetation hall is different than of wild type. The expression pattern of those plants is very close to the one of plants (both wild type and transgenic) grown in the greenhouse. This expression pattern correlates with the results observed for the flavonoid concentrations (see paragraph 4.7). When comparing wt and transgenic plants grown in the greenhouse, the cluster have a similar appearance. The blocks of upregulated (red), downregulated (green) and not-changed genes (black) are similar. Certain differences are noticeable between them and they are described below.

Although the general expression pattern is highly altered by growing conditions, the differences between varieties can also be seen. For example the plants of Frisal variety grown in the field show considerably less downregulated genes than both Golin and Greina grown in the same conditions. However, the clustering of the samples shows that differences between varieties have are less pronounced, at least in the number of genes changed, than those introduced by the growing conditions.

However, the number of the genes changed does not always represent the magnitude of the physiological changes. Therefore, the nature of those genes have been studied in bigger detail.
Changes in expression induced by growing conditions

Changes in the gene expression influenced by the growing conditions, which are described above have been studied in greater detail. Figure 34 shows a typical pattern observed in all varieties tested. Two groups of genes can be distinguished when analyzing wild type plants. Genes of lower expression on the field and in the vegetation hall and higher expression in the greenhouse (red) and genes of lower expression in the greenhouse and higher in the vegetation hall and field (green). When this analysis is extended for the KP4 plants, the expression pattern in the vegetation hall resembles that of the greenhouse. This is in accord with the previous observation of gene expression clusters.
To study the changes in gene expression in different varieties all samples were plotted together on a single graph. Then all genes were clustered using the k-means method to reveal correlations between them. Figures 35-37 show 3 most interesting clusters. The first one (Fig. 35) consist of 252 genes which have higher expression in the field and vegetation hall than in the greenhouse for all 3 wild type varieties tested. The same genes do not show upregulation in the KP4 plants.
Fig. 35 Cluster 1. Genes upregulated in the vegetation hall and field when compared to the greenhouse. Each line represents a single gene.

Legend:
FR - Frisal wt; GoKP - Golin KP4; Go - Golin wt; GrKP - Greina KP4; Gr - Greina wt; _g - grown in the greenhouse; _v - grown in the vegetation hall; _f - grown in the field

This cluster contains genes of very different functions:
1. Chloroplastic proteins - 40 genes
2. Stress connected - 10 genes
3. Flavonoid biosynthesis - 7 genes
4. amino acid and protein metabolism - 22 genes
5. Cell growth - 7 genes
6. Unknown proteins - 73 genes

The complete list of genes in this cluster can be found in the Appendix 2: Genelist 1.

The second of chosen clusters is presented on Figure 36. It consists of 67 genes which expression is higher in the greenhouse then in the field and vegetation hall in wild type plants. As in the cluster presented above (Fig. 35) the expression pattern of KP4 plants,
both Golin and Greina, in the vegetation hall did not follow the behaviour of wt plants but were close to the one from the greenhouse.

The genes belonging to this cluster are listed in Genelist 2 (see Appendix 3). The functions of those genes can be assigned to:

1. Amino acid and protein metabolism - 6 genes
2. Stress related (heat and wounding) - 5 genes
3. Flavonoid metabolism - 2 genes
4. Transcription factors and regulators - 11 genes
5. Unknown proteins - 24 genes

Expression of genes in KP4 plants grown in the vegetation hall did not always follow the same pattern as for plants grown in the greenhouse. Therefore, the third selected
cluster (Fig. 37) revealed a group of genes with an expression similar to those seen in the wild type plants.

![Graph showing gene expression data](image)

Fig. 37 Cluster 3. Genes downregulated in the vegetation hall and field when compared to the greenhouse.

Legend:
FR - Frisal wt; GoKP - Golin KP4; Go - Golin wt; GrKP - Greina KP4; Gr - Greina wt; _g - grown in the greenhouse; _v - grown in the vegetation hall; _f - grown in the field

Cluster 3 consists of 35 genes listed in the Genelist 3 (see Appendix 4). Their function can be represented as follows:

1. Transcription factors and regulators - 4 genes
2. Stress related - 3 genes
3. Amino acid and protein metabolism - 6 genes
4. Unknown proteins - 10 genes

**Comparison: wt vs. transgenic**

Data from the above experiment were used also to assess the changes in gene expression between wt and transgenic plants. The analysis was performed for Golin and Greina plants carrying KP4 transgene, grown in the greenhouse. The lack of replicates and additionally cross-species hybridization made the analysis difficult from the validation point of view, therefore stringent selection criteria were used. In the end, only genes changed more than 3 fold were taken into consideration. In variety Greina
we have observed 53 genes changed: 24 upregulated and 29 downregulated (see Appendix 5), while in case of Golin variety 213 genes were changed more than 3 fold: 123 upregulated and 90 downregulated (see Appendix 6).

The analysis of the changes in the variety Greina is very difficult due to the high number of not-annotated or unknown genes present in both lists. However, the list of upregulated genes in variety Golin shows a pattern of 11 activated genes, directly involved in pathogen and disease resistance together with 4 putative protein kinases (Table 6).

In the Gene Ontology database (www.godatabase.org), in the Arabidopsis genome, the defence response genes represent about 1.7% of all genes described (378 out of 22810). In above experiment, the observed ratio of resistance genes was about 9% (11 genes out of 123). $\chi^2$ test was performed to calculate the significance of observed differences. Obtained $\chi^2$ value = 35.157, which with 1 degree of freedom resulted in p-value equal or less than 0.001. It proves that observed pattern of enrichment in defence related genes is significant.

Table 6: Pathogen and disease resistance genes and protein kinases upregulated in Golin KP4 plants.

<table>
<thead>
<tr>
<th>Probeset</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contig5974_s_at</td>
<td>pathogen-induced protein WIR1A</td>
</tr>
<tr>
<td>Contig25195_at</td>
<td>probable chitinase (EC 3.2.1.14)</td>
</tr>
<tr>
<td>Contig1637_at</td>
<td>(1-3)-beta-glucanase</td>
</tr>
<tr>
<td>Contig2211_at</td>
<td>pathogenesis-related protein precursor</td>
</tr>
<tr>
<td>Contig13350_at</td>
<td>beta-1,3-glucanase precursor</td>
</tr>
<tr>
<td>Contig4054_s_at</td>
<td>pathogenesis-related protein 1c precursor</td>
</tr>
<tr>
<td>Contig2214_s_at</td>
<td>PR-1a pathogenesis related protein (Hv-1a)</td>
</tr>
<tr>
<td>Contig4056_s_at</td>
<td>Pathogenesis-related protein 1A/1B precursor</td>
</tr>
<tr>
<td>Contig16386_at</td>
<td>putative disease resistance protein</td>
</tr>
<tr>
<td>HB30O24r_at</td>
<td>putative stripe rust resistance protein Yr10</td>
</tr>
<tr>
<td>Contig2992_s_at</td>
<td>chitinase (EC 3.2.1.14) cht2b precursor</td>
</tr>
<tr>
<td>Contig6958_s_at</td>
<td>serine/threonine kinase-like protein</td>
</tr>
<tr>
<td>Contig15820_at</td>
<td>putative serine/threonine protein kinase</td>
</tr>
<tr>
<td>Contig8678_at</td>
<td>putative aspartate kinase (EC 2.7.2.4)</td>
</tr>
<tr>
<td>Contig8678_s_at</td>
<td>putative aspartate kinase (EC 2.7.2.4)</td>
</tr>
</tbody>
</table>
4.9 Gene expression in KP4 plants after fungal infection during field trial

In the previous experiments performed in our group, KP4 plants under greenhouse conditions showed significant increase of resistance against *Tilletia caries*. In the following field trial plants were challenged with the same pathogen. The analysis of gene expression in both null segregants and transgenic plants was part of the experiment evaluation and was meant to address the issues of pleiotropic gene expression and biosafety of transgenic crops.

Transgenic and null segregant plants were infected with *Tilletia caries* and grown on the small scale field plots together with not infected controls. Null segregants are plants which have lost their transgene due to the segregation. They, however were subjected to the transformation and tissue culture procedures and, therefore they provide almost ideal background for comparisons with the transgenic plants. By using null segregants, only the influence of the transgene itself can be studied.

The high costs of the genechip experiments allowed us to focus only on one variety. Since null segregants have been available only for variety Greina and not for Golin, we have hybridized the microarrays only with the samples coming from variety Greina.

After hybridization of the samples on Affymetrix Barley1 GeneChip, 44 genes have been identified as changing more than 2-fold in wild type plants upon infection with *T. caries*. 22 of those genes were upregulated, suggesting a function in plant defence or change due to the activity of the pathogen itself (Fig. 38).
Gene expression in KP4 plants after fungal infection dur-

Fig. 38 Expression pattern of PR genes after infection with Tilletia caries.
Legend:
null seg. - null segregant not infected
null seg. inf - null segregant infected with T. caries
KP4 - KP4 Greina not infected (2 replicates)
KP4 inf - KP4 Greina infected with T. caries

Out of those genes, 18 were also identified as upregulated in KP4 plants (Table 7). Ten of those 18 genes were directly related to resistance and pathogen response (Table 7, genes No. 2, 4, 7-10, 12, 15, 16, 18) and the possibility that the remaining 8 have a role in plant defence as well cannot be excluded. All of those genes showed lower signals, however not always significantly lower, in KP4 infected plants when compared to the wt infected, which suggests lower expression level.
Gene expression in KP4 plants after fungal infection dur-

Table 7: Endogenous wheat genes induced upon infection with *T. caries* in KP4 wheat, variety Greina.

<table>
<thead>
<tr>
<th>No</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33 kDa secretory protein [Oryza sativa]</td>
</tr>
<tr>
<td>2</td>
<td>Putative calcium-dependent protein kinase [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>3</td>
<td>Putative lipid transfer protein [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>4</td>
<td>Pathogenesis-related protein 1a - barley emb</td>
</tr>
<tr>
<td>5</td>
<td>Serine carboxypeptidase II-like protein; protein id: At5g23210.1 [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>6</td>
<td>PHD-finger family homeodomain protein [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>7</td>
<td>Glutathione S-transferase GST 22 [Zea mays]</td>
</tr>
<tr>
<td>8</td>
<td>Putative ethylene-forming enzyme [Oryza sativa]</td>
</tr>
<tr>
<td>9</td>
<td>Glycosyl hydrolase family 38 (alpha-mannosidase); protein id: At3g26720.1, supported by cDNA: gi_14517402</td>
</tr>
<tr>
<td>10</td>
<td>Chitinase II precursor [Triticum aestivum]</td>
</tr>
<tr>
<td>11</td>
<td>DEAD/DEAH box RNA helicase protein, putative; protein id: At3g18600.1 [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>12</td>
<td>Glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39) precursor - barley gb</td>
</tr>
<tr>
<td>13</td>
<td>Putative integral membrane protein [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>14</td>
<td>Unknown</td>
</tr>
<tr>
<td>15</td>
<td>Pathogenesis-related protein PRB1-2 precursor</td>
</tr>
<tr>
<td>16</td>
<td>Pathogenesis-related protein PRB1-3 precursor (PR-1B) (HV-8)</td>
</tr>
<tr>
<td>17</td>
<td>Similar to protein kinase [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>18</td>
<td>Beta-1,3-glucanase precursor [Triticum aestivum]</td>
</tr>
</tbody>
</table>

We analyzed several of the up-regulated PR genes in more detail by real-time PCR using primers derived from published sequences of *PR B1-2 precursor*, *beta-1,3-glucanase precursor*, *PR protein 1A/1B precursor*, and *chitinase II precursor* (Fig. 39); *actin* was used as reference. On the graph, different genes are distinguished by colour. The real time PCR confirmed that the four PR genes were significantly up-regulated on infection of wild-type plants by *Tilletia caries*. In KP4 plants the expression of these genes also apparently increased during infection, but unlike the presumably constant *actin* control, KP4 plants in general showed lower expression of these PR genes as compared with the null segregant, in both the non-inoculated and the infected states.
The real time PCR measurements were performed twice on the independently collected samples and showed the same results.

Fig. 39 Real time PCR results of selected PR genes confirm the GeneChip results.
Legend:
ns inf - Greina null segregant infected with T. caries
ns not inf - Greina null segregant not infected
KP4 inf - Greina KP4 infected with T. caries
KP4 not inf - Greina KP4 not infected
Error bars represent the standard deviation.
Different primers used are distinguished by colour.

In the list of downregulated genes observed in this experiment most of the genes were not annotated or unknown. Lack of information about the nature of the genes makes the analysis as well as the corresponding real-time PCR experiments very difficult.
Chapter 5 Discussion

Wheat plants, which were transformed in our laboratory with different anti-fungal transgenes, show 30% increase in resistance against pathogenic fungi in the greenhouse (Clausen et al. 2000) and did not show any apparent morphological alterations when compared with wild type plants. However we cannot exclude that components of the phenotype, which are not easily visible with the naked eye, might have been altered. An issue in the biosafety discussion of genetically modified crop plants are so called pleiotropic effects which might cause unexpected changes in expression pattern of endogenous genes or in the phenotype of metabolic pathways. We focused on the putative pleiotropic effects of anti-fungal transgenes on transcriptional and metabolic level. Transcription analysis was performed with the Affymetrix GeneChip technology and was based on the Barley1 microarray. For the metabolite analysis we focused on the flavonoids which have a wide spectrum of functions: photoprotection, anti-microbial, insects attractants or deterrents, toxins, colours, smells, taste, and others (Harborne and Williams 2000). Additionally they are one of the main groups of secondary metabolites in wheat (Adom et al. 2002). Within the work described here, a method of flavonoid extraction has been optimised for wheat leaves, which allows for a fast and effective processing of large amounts of samples. Obtained phenolic compounds have been analysed by HPLC-UV, MS and NMR techniques. These methods were used to compare transgenic with non-transgenic plants of the same variety as well as to assess the effect of different growing conditions on transgenic and wild type plants.

5.1 Comparison of transgenic and non-transgenic plants

Three different wheat spring varieties containing different anti-fungal transgenes were analysed and compared to the non-transgenic plants of the same variety. The transgenes used are active against a wide spectrum of fungal pathogens (chitinase and ribosome inactivating protein) as well as a very specific one - KP4 from Ustilago maydis. To assess the putative pleiotropic effects of the transgenic modification, the flavonoid content and gene expression patterns of transgenic plants were compared
with the wild type of the same variety.

In above comparison a reference to the naturally occurring variability was necessary. Therefore, the differences occurring between varieties were assessed by direct comparison of the wild type plants of all three varieties between each other. This helped us to obtain a necessary background for the further comparisons of the transgenic plants.

**Flavonoid content**

The flavonoid content of wheat leaves and kernels was studied before (Adom *et al.* 2003, Dietrych-Szostak and Oleszek 1999, Harborne and Hall 1964, Julian *et al.* 1971, Wagner *et al.* 1980).

The analysis of the flavonoid content, performed within this experiment, showed that the differences between wt and transgenic plants of the same variety were smaller than those occurring naturally between the three studied Swiss spring wheat varieties (see figures 28 and 29). Only the differences in compound concentrations were observed and no changes of the overall profile pattern.

Two additional analysis methods were used for interpretation of the data. First, a hierarchical clustering of the normalized compound concentrations was performed (Fig. 30). The method showed a distinct cluster of Greina plants (transgenic and wild type). The flavonoid patterns of varieties Golin and Frisai are more similar to each other, nevertheless they still form separate clusters.

Comparable observations have been made using principal component analysis (PCA). The concentration of all compounds in all samples, both wild type and transgenic, were analysed together to reveal the level of similarities between different plants. This allowed to interpret the results on a global scale and directly compare the level of differences seen between wt and transgenic plants with the overall relations between the samples.

When the total number of variable factors among the samples was reduced to two main components and the results are plotted in two-dimensional space, different clusters of samples could be distinguished (Fig. 31). The results are in accord with those seen after hierarchical clustering. Greina wt and KP4 together formed a cluster clearly separated from other samples. Frisai wt, A5 and B12 formed another cluster and the results obtained for the variety Golin show that the samples have the weakest similarity
Comparison of transgenic and non-transgenic plants
to each other, with two samples mixed with the cluster formed by the variety Frisal. This has probably its reason in the genetic background of the transgenic line which we compared with a deliberate sample from the original wild type variety.

**Analysis of gene expression**
The plants were grown at three different time points in the greenhouse and the extracted RNA was pooled before hybridization. This minimized the differences between the replicates. Technical replications of the hybridization itself can be omitted when using *in situ* synthesised arrays (Meyers et al. 2004, Zhu and Wang 2000). The expression patterns on KP4-transgenic plants in two varieties - Golin and Greina, were variety-dependent. In case of variety Greina, we observed changes in expression of 53 genes between wt and KP4 plants. However most of those genes were not annotated, which made the interpretation of results very difficult. Since the chip design was based on the expressed sequences (ESTs) and neither barley nor wheat genome is sequenced, all that is known about certain probesets is that they are expressed. Their function however is not known. Updates of the annotation databases as well as sequence BLAST databases are necessary before the analyses can be taken forward. What deserves attention at the moment is that observed changes in the expression of 53 genes represent only about 1% of all wheat genes hybridized on the chip. This percentage in reality might be different because most of the 22840 probesets on the Barley1 chip did not give good enough signal to be considered as "Present". This might be either because the corresponding transcripts were not present in the samples (genes not transcribed) or because the homology between transcript and the probeset was not high enough for a proper hybridization. If the former is true, the 53 genes changed observed in this experiment will represent much smaller percentage of the total. To avoid problems of homology between the probesets on the chip and the transcripts in the sample, wheat chip, which was not available at the time of the experiment, should be used. However, according to the performed analysis, the changes introduced by the transgene can be described as minimal. Easier to interpret are the results observed in the variety Golin. In this case the expression of 213 genes was changed, showing an upregulation of several genes directly and indirectly involved in plant defence and general resistance mechanisms. This might indicate that the presence of a KP4 transgene influenced other defence
mechanisms. Pathogenesis-related (PR) genes expression is precisely regulated by both, presence of the pathogen as well as by the endogenous mechanisms of the host (Jia and Martin 1999, Lin et al. 2004, Somssich et al. 1986, van Loon and van Strien 1999, Wang et al. 2005). Jia and Martin (1999) showed that tomato Pto kinase regulates the expression of certain defence genes, which have, so called "PR-box" (5'-AGCCGCC-3') sequence in the promoter. The effect of phytohormones on PR genes has been exploited as well. Abscisic acid (ABA) has been proven to downregulate the expression of beta-glucanase in tobacco (Rezzonico et al. 1998) while auxin and cytokinin seem to inhibit the accumulation of chitinase mRNA (Shinshi et al. 1987).

The differences in gene expression between varieties observed here, could be explained by several hypotheses. First of all, the different reaction of transgenic plants might be simply variety-dependent. Other experiments, described in this thesis, showing the gene expression of different varieties under the same conditions, demonstrated that such differences exist. Moreover, since the plants are coming from different transformation events, the insertion site is most likely in different locations of the genome and this might also influence the behaviour of transgenic plants. Since it is possible that there is a certain level of segregation within the established variety, the behaviour observed in this experiment might be accidental. To confirm the results, additional experiments with plants coming from different transformation events as well as using null segregants for control are necessary. Unfortunately, such experiments were beyond the time and financial frame of this thesis.

5.2 Comparison of different growing conditions

Small scale experiments with transgenic plants are easier to perform under the closed, greenhouse conditions. They do not require long and costly (as in many European countries) procedures to obtain permission for a field release, they attract less attention from biotech opponents and are, in general, easier for scientists. However, greenhouse conditions very often do not reflect reality, which plants would have to face when grown in the field. Plants grown in the field are constantly subjected to a variety of stress conditions, like drought, wind, temperature and light intensity changes, pathogen attacks (Pasquer et al. 2005). Therefore, the changes in gene expression patterns between greenhouse and field conditions are expected and, in fact, reported (Pasquer
et al. 2005, Rizhsky et al. 2002). Such changes may induce putative pleiotropic effects, which later-on might influence the results of research. Therefore, it is essential to perform a field trial in addition to the greenhouse tests not only when studying the effects of a particular transgene, but also when performing any biosafety research (Pasquer et al. 2005).

**Influence on the flavonoid metabolome**

Observed changes in the flavonoid content in wild type plants between environments were significant. In general, we noticed an increase in the concentration of most phenolic compounds when plants were grown under the open conditions in the field (Fig. 26 and 27). The levels of those changes are variety-dependent and vary from few percent up to the 800% difference in case of isoorientin concentration in the variety Golin. The results observed in the vegetation hall are intermediate between greenhouse and field. The concentration of some compounds increased while that of few others decreased as compared to the greenhouse. The increase of flavonoid concentration observed here is not surprising and is in accord with the one of the most important functions of the flavonoids, namely photoprotection and stress response (Berhow and Vaughn 1999, Harborne and Williams 2000, Stapleton and Walbot 1994, van de Staaij et al. 2002).

In the vegetation hall plants are protected only by the bird net during good weather conditions and in case of rain or wind the roof and walls are automatically closed. It creates a semi-open environmental system. The amount of sunlight is decreased when compared to the field but substantially higher than in the greenhouse (Schlaich et al. 2005). The same can be said about the fluctuation of temperature and speculated about the presence of pathogens. Therefore, observed changes of lower magnitude seem to be perfectly understandable. Although according to our knowledge similar experiments were not performed by other groups, the results are in accord with predictions based on the known functions of flavonoids.

The increase of direct sunlight, changes in light quality, especially according to the higher content of the UV light in the spectrum, together with higher temperature fluctuations and higher pathogen and insect pressure, is supposed to activate the defence mechanisms, of which flavonoids are an important part (Hahlbrock et al. 1982, Kootstra 1994, Tattini et al. 2000). Tattini et al. (2005) showed that the accumulation of
flavonoid glycosides is associated with high solar radiation-induced oxidative stress. It has been also shown that changes in flavonoid concentration are due to transient increases in the transcription rates of the respective genes, which indicates that rapid gene activation has an important role in UV and disease resistance (Chappel and Hahlbrock 1984).

No striking differences were observed in the flavonoid content of wt and KP4 transgenic plants in the greenhouse (see par. 5.1). However when plants were grown in the vegetation hall, the significant increase of the concentration of flavonoids, seen in the wt plants was not observed in the KP4 plants (Fig. 32 and Table 5). In fact, the concentration of most compounds was even reduced in the KP4 plants when compared to the same plants grown in the greenhouse, however this reduction did not prove to be statistically significant. This is in accord with the gene expression data and confirms that KP4 under non-greenhouse conditions reduces other defence related reactions. At the time of the experiment, we did not have the permission to conduct the tests on the field, therefore we could not extend this test to field conditions.

**Influence on gene expression**

Recent work of Pasquer *et al.* (2005) confirmed that different growing conditions may influence the changes in the gene expression patterns of wheat which is not surprising. The analysis of the natural changes of endogenous genes among the varieties provides an important background for the comparison of wt and KP4 plants and enables to judge about the pleiotropic effects of the transgene. We therefore discuss this in detail here.

In our experiments, the clustering of gene expression patterns shows close similarities between the varieties under the same growing conditions and big differences between each of the conditions. A big cluster of upregulated genes in the field is partially represented also in the vegetation hall. The downregulated genes show very similar clusters in both - vegetation hall and field. The expression of those genes is inverted in the greenhouse - those which were upregulated in the field are mostly repressed in the greenhouse and those downregulated in the field are induced in the greenhouse. Many of the upregulated genes in the open conditions are coding for chloroplastic
Comparison of different growing conditions

proteins and stress responses. A group of flavonoid biosynthesis enzymes is also strongly represented. This is in accordance with the results observed in the flavonoid metabolism. Change in light quantity and quality influences the chloroplast formation (Weston et al. 2000). This might explain the induction of genes coding for chloroplast proteins. Additionally, the open conditions, being more stress-inducing than greenhouse conditions are expected to trigger stress response mechanisms (Pasquer et al. 2005), therefore induction of stress related genes as well as flavonoid biosynthesis genes is conceivable.

Another group of genes strongly represented in this cluster consists of genes involved in the amino acid and protein metabolism, both synthesis and degradation, and in cell growth. This is in accord with Cushman and Bohnert (2000) who reported that increased cell growth together with stress adaptation mechanisms require higher protein turnover.

The field trials reported so far for transgenic wheat (Chen et al. 1999, Anand et al. 2003) showed no significant anti-fungal field performance of a transgene, although the same plants showed significant improvement when challenged in the greenhouse. In our experiments, KP4 transgenic plants of both Golin and Greina varieties grown in the greenhouse reacted very similar to their wt equivalents, but the same transgenic plants grown in the vegetation hall showed expression pattern which is closer to plants grown in the greenhouse than to the wt from vegetation hall or field. 14% of measurable genes showed more than 2 fold difference and 3% more than 3 fold in Golin variety and respectively 16% and 4% in Greina variety. Hierarchical clustering confirmed these results and is in accord with the results from flavonoid analysis. KP4 transgenic plants of both wheat varieties, show similar expression patterns. No clear morphological differences have been detected between wt and KP4 plants in any of the three tested environments, but the above analysis shows that important physiological processes, in fact, might have been altered.

Presented results, as well as previous research on transgenic plants with increased anti-fungal resistance (Chen et al. 1999, Anand et al. 2003), confirmed, that the artificial conditions of the greenhouse do not represent the high variability of field and might not reveal the true physiology of the studied organism. It supports the necessity of analysis of the new plant lines in the natural environment.
5.3 Gene expression after fungal infection during a field trial

Gene expression was analysed in the field-grown KP4 and null segregant plants challenged with *Tilletia caries* and compared with the not-infected controls. Transgenic plants showed a significant increase of the resistance (Schlaich et al. 2005). With the Affymetrix GeneChip technology, we identified an alteration of 44 genes in wt plants after infection with *T. caries*. This represents only about 0.6% of all genes hybridized on the chip (6850) and shows the small scale of the effects. 22 of those genes have been upregulated more than 2-fold and 18 of those genes have been identified as upregulated also in the transgenic plants. This suggests that they might have a function in plant defence or that their expression might have been altered by the pathogen attack itself. In fact, most of those genes were identified as having a direct role in pathogen defence and such role of the remaining genes can not be excluded. The experiment has been performed in duplicates from different replicates of the same field trial. Additionally, several PR genes were selected and, basing on the sequence data available in databases (www.barleybase.org), their expression was confirmed by the real-time PCR.

The kp4 transgene did not appear to inhibit the up-regulation of endogenous PR genes identified according to their cross-hybridization with the barley micro-array. However, quantitative expression analysis by real-time PCR of some PR genes indicated a lower expression in the KP4 lines than in the wild type. Both KP4 wheat lines, whether inoculated or not, exhibited lower PR mRNA content than the respective control. This observation does not apply to the actin control, which was similar in all four samples. It is possible that the plant regulates its anti-pathogenic protein titer at least in part by homeostasis. In the case of earlier reports (Chen et al. 1999, Anand et al. 2003), homeostasis might have impaired the testing of transgenic plants: glucanase or chitinase may be very well endogenously regulated and thus ectopic expression might not lead to accumulation of these proteins to a higher level than in the wild type.

The results presented in this work show that pleiotropic changes introduced by genetic engineering techniques, although detectable, proved to be minimal especially when compared to the natural variation between different varieties. Their magnitude differs according to the variety and it is very probable that those differences are caused by the
insertion point of the transgene. We have also observed, that the level of those changes may depend on the conditions under which plants are grown. This again points out the importance of field testing of new plant lines since the potentially unwanted effects of a modification might be revealed only under agricultural conditions. The clustering analyses performed for both transcriptomics and metabolomics, yielded very similar results. Therefore, we conclude that the inter- and intravariety similarities and differences in the flavonoid metabolome reflect a general change at the expression level.
References


References


References


Curriculum vitae

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Acknowledgements

I would like to thank Jean-Robert loset and Emerson Ferreira-Qeiroz from the University of Geneva for their help with establishing the flavonoid analysis method and with the NMR analyses.

Additionally I would like to thank all the members of the group for nice time and collaborative atmosphere and Katalin Konya and Sabine Klarer for the maintenance of the plants in the greenhouse. A very special thanks go to the present and former members of the lab, Marzanna Künzli, Nicole Malgras, Susanna Poletti, Thomas Schlaich and Luisa Mariconti for keeping up a very special atmosphere and their immediate help whenever needed.

Finally I would like to thank Prof. Wilhelm Gruissem for allowing me to work in his group and last but certainly not least, I thank my supervisor PD Dr. Christof Sautter for accepting me as his PhD student and for his continuous help.
Appendix 1: Identification of the compounds.

**Compound 1**
The mass of the ion is $m/z$ 611.8 (Fig. 17). MS$^2$ spectrum results in the fragments of $m/z$ 449.9 which corresponds to [M+H]$^+$ of isoorientin or one of its isomers, as well as $m/z$ 431.6 and $m/z$ 330 which are characteristic for fragmentation pattern of isoorientin. The difference between the mass of the analysed ion and mass of isoorientin is 162 m.u. ($611.8 - 449.9 = 161.9$), which is typical for a loss of an O-hexose. The flavonoid could be an isoorientin-O-glucoside.

Fig. 40 MS/MS spectrum of compound 1
Appendix 1: Identification of the compounds.

The changes of the spectra after addition of shift reagents are shown in the Figure 18 and summarized in table 5.

![Graph showing changes in UV spectrum](image)

Fig. 41 Changes in UV spectrum of compound 1 after addition of shift reagents.

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>347, 351</td>
<td>259, 269</td>
</tr>
<tr>
<td>NaOAc</td>
<td>359</td>
<td>235, 267</td>
</tr>
<tr>
<td>KOH</td>
<td>410</td>
<td>273</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>403</td>
<td>275</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>359, 389</td>
<td>275</td>
</tr>
<tr>
<td>NaOAc/H₃BO₃</td>
<td>357</td>
<td>257</td>
</tr>
</tbody>
</table>

Table 8: UV spectral data ($\lambda_{max};$ nm) of Peak 1

Decreasing intensity of the spectrum and the shift after addition of NaOAc indicates that the compound belongs to the group of flavanones. It can have the alkali-sensitive groups, like 6,7 or 7,8-diOH.

Strong bathochromic (to longer wavelengths) shift in AlCl₃ spectrum and a weaker one in AlCl₃/HCl indicates an A-ring O-diOH in position 6,7 or 7,8. The same conclusion can be drawn from the shift in the NaOAc/H₃BO₃ spectrum.
**Compound 2**
The mass of the analysed ion is m/z 582 (Fig. 19). The MS² spectrum is very complex, though represents the typical fragmentation of a C-flavonoid. The peak at 563.7 m.u. is characteristic for a loss of water (loss of 18 m.u.) and the peak at m/z 527.6 may indicate the presence of an isopentenyl (-CH₂-CH=C(CH₃)₂) substituent. The spectrum alone does not provide enough information for a proper identification of this compound, but based on the mass itself, it might be one of the lucenin isomers: lucenin-1 or -3.

![MS/MS spectrum of compound 2](image_url)
Appendix 1: Identification of the compounds.

The changes of the spectra after addition of shift reagents are shown in the Figure 20 and summarized in table 6.

![Figure 43](image)

Fig. 43 Changes in UV spectrum of compound 2 after addition of diagnostic shift reagents.

**Table 9: UV spectral data (λ max; nm) of Peak 2**

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>347</td>
<td>263, 269,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(271)</td>
</tr>
<tr>
<td>NaOAc</td>
<td>360</td>
<td>269</td>
</tr>
<tr>
<td>KOH</td>
<td>339, 415</td>
<td>277</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>407</td>
<td>277</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>355, 395</td>
<td>275, (297)</td>
</tr>
<tr>
<td>NaOAc/H₃BO₃</td>
<td>359</td>
<td>267</td>
</tr>
</tbody>
</table>

Decreasing intensity of the spectrum and the negative shift of a band II after addition of NaOAc suggests that the compound belongs to the group of flavones/flavonols/isoflavones and has a 7-OH group together with 6- or 8-OH as well as the alkali sensitive group like 6,7 or 7,8 or 3',4'-diOH. The spectra in AlCl₃ and AlCl₃/HCl show evidences for the compound being a flavone or flavonol (strong shift of band I) with a B-ring O-diOH group. This conclusion is also confirmed by a +12nm shift of band I after addition of NaOAc/H₃BO₃.
**Compound 3**
The mass of this ion is very similar to that of peak Nr2 - m/z 581.5 (Fig. 21). Given the fact that additionally the retention times of both peaks are very similar, they can be isomers. Also here the peak [M+H-18]^+ (loss of water) can be seen, but no [M+H-55]^+ (loss of isopentenyl) as in peak 2. If it is the isomer of compound 2, it might be lucenin-3 or -1.

![MS/MS spectrum of compound 3.](image)

Fig. 44 MS/MS spectrum of compound 3.
Appendix 1: Identification of the compounds.

The changes of the spectra after addition of shift reagents are shown in the Figure 22 and summarized in table 7.

![Graph showing changes in UV spectrum of compound 3 after addition of diagnostic shift reagents.](image)

**Fig. 45 Changes in UV spectrum of compound 3 after addition of diagnostic shift reagents.**

**Table 10: UV spectral data (λ max; nm) of Peak 3**

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>347</td>
<td>263, 269, (271)</td>
</tr>
<tr>
<td>NaOAc</td>
<td>363</td>
<td>271</td>
</tr>
<tr>
<td>KOH</td>
<td>419, (339, 417)</td>
<td>(271), 279</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>406</td>
<td>275</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>359, 395</td>
<td>275, (295)</td>
</tr>
<tr>
<td>NaOAc/ H₃BO₃</td>
<td>357</td>
<td>267</td>
</tr>
</tbody>
</table>

The shifts are almost identical with that of compound 2. This supports the hypothesis of both compounds being isomers.

**Compound 4**

The mass of ion is m/z 565.7 (Fig. 23). The loss of 18 m.u. and 36 m.u. (water) can be detected, as well as loss of 55 m.u., typical for isopentenyl. The mass is characteristic for schaftoside, isoschaftoside, neoschaftoside as well as several apigenin-glucoside
Appendix 1: Identification of the compounds.

derivatives.

Fig. 46 MS/MS spectrum of compound 4.
The changes of the spectrum after addition of shift reagents are shown in the Figure 24 and summarized in table 8.

![Fig. 47 Changes in UV spectrum of compound 4 after addition of shift reagents.](image)

**Table 11: UV spectral data ($\lambda_{\text{max}}$; nm) of Peak 4**

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O + TFA</td>
<td>336, (339)</td>
<td>271</td>
</tr>
<tr>
<td>NaOAc</td>
<td>(337), 341</td>
<td>271</td>
</tr>
<tr>
<td>KOH</td>
<td>331, 401</td>
<td>281</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>339, (373)</td>
<td>279, 299</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>339, (373)</td>
<td>277, 299</td>
</tr>
<tr>
<td>NaOAc/H₃BO₃</td>
<td>337</td>
<td>271</td>
</tr>
</tbody>
</table>

No shift, but lower intensity can be observed in the spectrum with NaOAc. This does not allow for the classification of the flavonoid type, but it suggests the alkali sensitive group in position 6,7 or 7,8 or 3',4'-diOH. This is also confirmed by the lack of spectrum shift after addition of a NaOAc/H₃BO₃. A very small (almost neglectable) shift of bands
I and II in AlCl₃ and AlCl₃/HCl spectra is probably due to the existence 5-OH group. The absorption spectra are typical for Apigenin 8-O-glucoside.

**Compound 5**

In this case the ion of the same molecular mass (m/z 611.8) as in Peak 1 can be seen together with fragments of m/z 449.4 (characteristic for isoorientin) and m/z 491.6, which represents loss of mass of 120 m.u. - typical for C-flavonoids (Fig. 25). Therefore it can be identified as a derivative of isoorientin with different substitution than Peak 1. The presence of fragment at m/z 329.6 (which can be represented as the lose of [M-(162+120)+H]⁺ indicates a lose of O-hexose. Therefore the compound could be an isoorientin -O-glucoside.

![MS/MS spectrum of compound 5.](image)

Fig. 48 MS/MS spectrum of compound 5.
Appendix 1: Identification of the compounds.

The changes of the spectrum after addition of shift reagents are shown in the Figure 26 and summarized in table 9.

![Graph showing changes in UV spectrum of compound 5 after addition of shift reagents.]

**Table 12:** UV spectral data (λ max; nm) of Peak 5

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>349</td>
<td>269</td>
</tr>
<tr>
<td>NaOAc</td>
<td>353, (363)</td>
<td>269</td>
</tr>
<tr>
<td>KOH</td>
<td>(335), 413</td>
<td>275</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>413</td>
<td>271, 311</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>353, 387</td>
<td>275</td>
</tr>
<tr>
<td>NaOAc/H₃BO₃</td>
<td>357</td>
<td>267</td>
</tr>
</tbody>
</table>

Decreasing intensity of the spectrum and the shift after addition of NaOAc indicates that the compound belongs to flavanones. It is probable, that it has alkali-sensitive groups, like 6,7 or 7,8-diOH.

Strong bathochromic shift of band I in AlCl₃ spectrum and a weaker one in AlCl₃/HCl.
indicates the A-ring O-diOH in position 6,7 or 7,8 and a 5-OH group. The shift in the NaOAc/H$_3$BO$_3$ spectrum also indicates the presence of the O-diOH groups attached to the A-ring.

**Compound 6**
The mass of the molecular ion is almost identical to the one of peak 4 - m/z 565.6 (Fig. 27). With the fragment of m/z 445.6 (loss of 120 m.u.) it can be identified as C-flavonoid. If compound 4 would be an isoschaftoside, the compound 6 might be a schaftoside.

![Fig. 50 MS/MS spectrum of compound 6.](image-url)
Appendix 1: Identification of the compounds.

The changes of the spectrum after addition of shift reagents are shown in the Figure 28 and summarized in table 10.

![Fig. 28 Changes in UV spectrum of compound 6 after addition of shift reagents.](image)

**Table 13: UV spectral data ($\lambda_{max}$; nm) of Peak 6**

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O + TFA</td>
<td>335, 339</td>
<td>271</td>
</tr>
<tr>
<td>NaOAc</td>
<td>339, (345)</td>
<td>271</td>
</tr>
<tr>
<td>KOH</td>
<td>331, 403</td>
<td>283</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>335, (373)</td>
<td>279, 299</td>
</tr>
<tr>
<td>AlCl₃/HCl</td>
<td>339, (377)</td>
<td>277, 301</td>
</tr>
<tr>
<td>NaOAc/H₃BO₃</td>
<td>337</td>
<td>271</td>
</tr>
</tbody>
</table>

The spectra are almost identical to those observed for compound 4. Together with MS data it supports the hypothesis that compound 6 might be an isomer of compound 4.
**Compound 8**
The mass of the analysed ion is $m/z$ 595.5 (Fig. 29). The MS$^2$ spectrum shows a typical fragmentation pattern for isoorientin and the mass of the analysed ion is 146 m.u. bigger than isoorientin, which corresponds to the mass of desoxyhexose like rhamnose.

Fig. 52 MS/MS spectrum of compound 8.
The changes of the spectrum after addition of shift reagents are shown in the Figure 30 and summarized in table 11.

![Fig. 30 Changes in UV spectrum of compound 8 after addition of shift reagents.](image)

| Table 14: UV spectral data ($\lambda_{\text{max}}$; nm) of Peak 8 |
|-----------------------------------|-------------------|-------------------|
| MeOH $+$ H$_2$O $+$ TFA          | 351               | (261), 269        |
| NaOAc                            | 359               | 269               |
| KOH                              | (339), 415        | 275               |
| AlCl$_3$                         | 401               | 275               |
| AlCl$_3$/$\text{HCl}$            | 361, 387          | 273               |
| NaOAc/$\text{H}_3\text{BO}_3$    | 359               | 265               |

In this case, the absorption maxima of the spectra have a strong similarity to those observed for isoorientin, however the intensities of the spectra are different. These data indicate a derivative of isoorientin and are in accord with the observation from mass spectrography.
**Compound 9**

The mass of the ion is $m/z$ 625.8 (Fig. 31). The MS$^2$ spectrum shows a fragment of $m/z$ 505.4 which means a loss of 120 m.u. (ion characteristic for C-flavonoids) as well as a fragment of $m/z$ 463.6 (loss of 162 m.u., characteristic for O-hexose). Another interesting fact is, that the mass of 463.6 is 14 higher that the mass of isoorientin (449), which indicates methoxylation.

![Fig. 54 MS/MS spectrum of compound 9.](image-url)
The changes of the spectrum after addition of shift reagents are shown in the Figure 32 and summarized in table 12.

![UV spectrum changes](image)

**Table 15: UV spectral data (λ\text{max}; nm) of Peak 9**

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>343</td>
<td>270</td>
</tr>
<tr>
<td>NaOAc</td>
<td>331, 341</td>
<td>273</td>
</tr>
<tr>
<td>KOH</td>
<td>(323, 331), 409</td>
<td>273</td>
</tr>
<tr>
<td>AlCl\textsubscript{3}</td>
<td>343, (375)</td>
<td>279, 297</td>
</tr>
<tr>
<td>AlCl\textsubscript{3}/HCl</td>
<td>341, 345</td>
<td>277, 294</td>
</tr>
<tr>
<td>NaOAc/H\textsubscript{3}BO\textsubscript{3}</td>
<td>331</td>
<td>269</td>
</tr>
</tbody>
</table>

The spectra are characterised by a very low intensity and clear distinction of absorption maxima is very difficult. In the NaOAc spectrum, the small shift of band II absorption towards longer wavelength and the decrease of intensity of the spectrum is characteristic for a 7-OH group as well as diOH groups in positions 6,7 or 7,8 or 3',4'.
The AlCl₃ together with AlCl₃/HCl spectra reveal a presence of a 5-OH group and a possibility of 6-oxygenation.

**Compound 10**
The mass of the ion (m/z 463.5) corresponds to methoxylated isoorientin (as in the Peak 9), but without substituted sugar. The MS² spectrum shows all fragments characteristic for isoorientin, in each case their mass is 14 m.u. higher which confirms the methoxylation (Fig. 33). It is highly probable that the compound is a methoxylated isoorientin.

Fig. 56 MS/MS spectrum of compound 10.
The changes of the spectrum after addition of shift reagents are shown in the Figure 34 and summarized in table 13.

Table 16: UV spectral data (λ max; nm) of Peak 10

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O + TFA</td>
<td>345</td>
<td>269</td>
</tr>
<tr>
<td>NaOAc</td>
<td>351, 355</td>
<td>269</td>
</tr>
<tr>
<td>KOH</td>
<td>(323, 331), 409</td>
<td>269</td>
</tr>
<tr>
<td>AICI3</td>
<td>349, 377</td>
<td>277</td>
</tr>
<tr>
<td>AICI3/HCl</td>
<td>347, 351, 379</td>
<td>277, (295)</td>
</tr>
<tr>
<td>NaOAc/H3BO3</td>
<td>349</td>
<td>269</td>
</tr>
</tbody>
</table>

The maxima of the methanol absorption spectrum have a very strong similarity to those of peak 9. The methanol spectrum is characteristic for flavonols or flavones. Shift in the NaOAc spectrum indicates the alkali-sensitive groups in A or B-ring. The changes of band I in AICI3 and AICI3/HCl spectra indicate the presence of the 5-OH group together...
Appendix 1: Identification of the compounds.

with a O-diOH group on the A-ring. The presence of the latter is also indicated by the small shift of band I in NaOAc/H$_3$BO$_3$ spectrum.

**Compound 11**

Also in this case the MS$^2$ spectrum (Fig. 35) reveals fragments characteristic for methoxylated isoorientin (compare with Peak 10 - Fig. 33) as well as a loss of 146 m.u. which is typical for rhamnose. This would support the compound being a methoxylated isoorientin-O-rhamnoside.

![MS/MS spectrum of compound 11](image)

Fig. 58 MS/MS spectrum of compound 11.
The changes of the spectrum after addition of shift reagents are shown in the Figure 36 and summarized in table 14.

![Fig. 36 Changes in UV spectrum of compound 11 after addition of shift reagents.]

<table>
<thead>
<tr>
<th></th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH + H2O+TFA</td>
<td>349</td>
<td>257, 269</td>
</tr>
<tr>
<td>NaOAc</td>
<td>351</td>
<td>271</td>
</tr>
<tr>
<td>KOH</td>
<td>337, 411</td>
<td>267, 277</td>
</tr>
<tr>
<td>AlCl3</td>
<td>351</td>
<td>277, (293)</td>
</tr>
<tr>
<td>AlCl3/HCl</td>
<td>351</td>
<td>(261), 273, (293)</td>
</tr>
<tr>
<td>NaOAc/H3BO₃</td>
<td>351</td>
<td>255, 269</td>
</tr>
</tbody>
</table>

Also in this case, the maxima of methanol absorption spectrum are similar to those of peak 9. This indicates that all 3 compounds belong to the similar group. The methanol spectrum is characteristic for flavonols. In NaOAc spectrum, the small shift of band II absorption towards longer wavelength and decrease of intensity of the spectrum is characteristic for a 7-OH group as well as diOH groups in positions 6,7 or 7,8 or 3',4'.
The very small shift in NaOAc/H$_3$BO$_3$ spectrum can be due to the presence of the O-diOH group on the A-ring.
Appendix 2: Genelist 1

1. Contig3057_s_atBEST BLASTX NR: 11/06/02 BAB08818.1 2e-44 contains similarity to ATFP3~gene_id:MLE2.16 [Arabidopsis thaliana]

2. HVSMEm0020L23r2_x_atBEST BLASTX NR: 11/04/02 RKWTS 1e-36 ribulose-bisphosphate carboxylase (EC 4.1.1.39) small chain precursor (clone pWS4.3) - wheat

3. Contig10375_atBEST BLASTX NR: 11/06/02 NP_566339.1 9e-77 (NM_111729) putative SCO1 protein; protein id: At3g08950.1, supported by cDNA: 142032., supported by cDNA:

4. EBma08_SQ002_M18_atBEST BLASTX NR: 01/27/03 BAC27870.1 .5 unnamed protein product [Mus musculus]

5. Contig7552_atBEST BLASTX NR: 10/25/02 NP_193006.1 3e-097 (NM_117339) putative protein; protein id: At4g12700.1 [Arabidopsis thaliana]

6. Contig13674_atBEST BLASTX NR: 11/04/02 AAK38482.1 1e-80 beta-D-xylosidase [Hordeum vulgare]

7. Contig10889_atBEST BLASTX NR: 10/26/02 BAA34675.1 4e-97 (AB011670) wpk4 protein kinase [Triticum aestivum]

8. Contig8346_atBEST BLASTX NR: 10/27/02 BAC10351.1 e-113 contains ESTs AU174016(S13261),D47643(S13261)~similarto Oryza sativa chromosome 4,OSJNBA0086B14.2~unknown protein

9. Contig7663_atEST04127 Fetal brain, Stratagene (cat#936206) Homo sapiens cDNA clone HFBDQ70 similar to EST containing Alu repeat, mRNA sequence.

10. HVSMEc0012J16f_atBEST BLASTX NR: 01/27/03 NP_114251.1 1e-052 RNA polymerase beta" chain [Triticum aestivum] sp[Q9XPS9]RPOD_WHEAT DNA-directed RNA polymerase beta chain

11. Contig18801_atBEST BLASTX NR: 11/07/02 BAB64184.1 e-104 (AP003266) hypothetical protein~similar to Arabidopsis thaliana chromosome 3, F22F7.15 [Oryza sativa (japonica]

12. Contig7141_atBEST BLASTX NR: 11/07/02 NP_564060.1 5e-66 (NM_101724) expressed protein; protein id: At1g18660.1, supported by cDNA: 205719. [Arabidopsis thaliana]

14. Contig6353_at: BLASTX NR: 11/04/02 BAB17203.1 5e-91 putative branched-chain amino acid aminotransferase protein [Oryza sativa (japonica cultivar-group)]

15. Contig12147_at: BLASTX NR: 11/04/02 NP_192634.1 5e-55 (NM_116964) putative phi-1-like phosphate-induced protein; protein id: At4g08950.1, supported by cDNA: 3552., supported by

16. HB19M13r_s_at: BLASTX NR: 10/31/02 NP_195080.2 5e-10 putative protein; protein id: At4g33540.1, supported by cDNA: gi_20260497 [Arabidopsis thaliana]

17. Contig7356_at: BLASTX NR: 11/08/02 P26018 1e-88 CHALCONE SYNTHASE 1 (NARINGENIN-CHALCONE SYNTHASE 1) pir||S16275 naringenin-chalcone synthase (EC 2.3.1.74) - barley

18. Contig610_at: BLASTX NR: 11/07/02 P40880 9e-98 CARBONIC ANHYDRASE, CHLOROPLAST PRECURSOR (CARBONATE DEHYDRATASE) pir||T04478 probable carbonate dehydratase (EC 4.2.1.1) - barley

19. Contig8830_at: BLASTX NR: 11/08/02 NP_173308.2 4e-32 unknown protein; protein id: At1g18730.1, supported by cDNA: gi_19310577 [Arabidopsis thaliana]

20. Contig13785_at: BLASTX NR: 11/07/02 NP_565511.1 8e-72 (NM_127710) Expressed protein; protein id: At2g21385.1, supported by cDNA: gi_15027870 [Arabidopsis thaliana]

21. Contig5333_s_at: BLASTX NR: 01/29/03 BAB68096.1 6e-18 B1088C09.1 [Oryza sativa (japonica cultivar-group)]

22. Contig11641_at: BLASTX NR: 11/06/02 CAB40792.1 1e-91 putative lectin [Hordeum vulgare subsp. vulgare]

23. Contig7287_at: BLASTX NR: 11/04/02 BAA87054.1 e-121 (AB024006) nicotianamine aminotransferase [Hordeum vulgare subsp. vulgare]

24. Contig5949_s_at: BLASTX NR: <none>

25. Contig6671_at: BLASTX NR: 11/06/02 CAD41248.1 e-121 (AL606627) OSJNBa0067K08.12 [Oryza sativa (japonica cultivar-group)]
26. Contig6315_atBEST BLASTX NR: 10/27/02 AAK09233.1 2e-99 putative fatty acid hydroxylase [Oryza sativa (japonica cultivar-group)]
27. Contig17756_atBEST BLASTX NR: 10/13/02 CAD40595.1 5e-52 (AL662945) oj000126_13.17 [Oryza sativa (japonica cultivar-group)]
28. HF18A22r_s_atBEST BLASTX NR: 11/04/02 Q9SXPP2 1e-49 Formate dehydrogenase, mitochondrial precursor (NAD-dependent formate dehydrogenase) (FDH)
29. Contig25699_atBEST BLASTX NR: 11/04/02 NP_178286.1 3e-06 putative membrane protein; protein id: At2g01770.1 [Arabidopsis thaliana]
30. Contig13382_atBEST BLASTX NR: 11/06/02 CAC16135.1 1e-34 endonuclease III homologue [Arabidopsis thaliana]
31. Contig19388_atBEST BLASTX NR: 10/02/02 Q42840 1e-80 Coproporphyrinogen III oxidase, chloroplast precursor (Coproporphyrinogenase) (Coprogen oxidase)
32. Contig5009_atBEST BLASTX NR: 11/08/02 Q9FVI2 8e-58 Actin-depolymerizing factor 1 (ADF 1) gb|AAG16973.1|AF183903_1 actin-depolymerizing factor 1 [Petunia x hybrida]
33. Contig6732_atBEST BLASTX NR: 11/06/02 NP_180350.1 2e-88 putative chorismate mutase/prephenate dehydratase; protein id: At2g27820.1, supported by cDNA: 37739. [Arabidopsis thaliana]
34. Contig19934_atBEST BLASTX NR: 11/07/02 NP_191224.1 9e-31 (NM_115524) thylakoid lumenal 20 kDa protein; protein id: At3g56650.1 [Arabidopsis thaliana]
35. Contig9582_atBEST BLASTX NR: 11/08/02 NP_568781.1 5e-72 thylakoid lumen 15.0-kDa protein; protein id: At5g52970.1, supported by cDNA: gi_15081647, supported by cDNA:
36. HK03H11r_x_atBEST BLASTX NR: <none>
37. Contig5599_atBEST BLASTX NR: 10/29/02 NP_188036.1 2e-36 hypothetical protein; protein id: At3g14200.1, supported by cDNA: gi_20268706 [Arabidopsis thaliana]
38. Contig16471_atfragment AL39; L. lactis DNA for export element AL39.
39. Contig6438_s_atBEST BLASTX NR: 01/29/03 BAC22243.1 8e-91 putative DNA-binding protein phosphatase 2C [Oryza sativa (japonica cultivar-group)]
Appendix 2: Genelist 1

40. Contig15369_at
   BEST BLASTX NR: 10/27/02 NP_568246.1 7e-41 bZIP protein HY5; protein id: At5g11260.1, supported by cDNA: gi_2244708, supported by cDNA: gi_2251084 [Arabidopsis]

41. Contig10232_at
   BEST BLASTX NR: 11/06/02 NP_201385.2 2e-63 unknown protein; protein id: At5g65840.1, supported by cDNA: gi_20466749 [Arabidopsis thaliana]

42. HV_CEa0012B15f_s_at
   BEST BLASTX NR: <none>

43. Contig7139_at
   BEST BLASTX NR: 10/13/02 AAK60502.1 7e-84 sucrose export defective 1 [Zea mays]

44. Contig26422_at
   BEST BLASTX NR: 11/04/02 AAM14591.1 2e-94 putative sulfate transporter [Oryza sativa]

45. Contig4685_at
   BEST BLASTX NR: 11/08/02 NP_568172.1 1e-40 thioredoxin-like; protein id: At5g06690.1, supported by cDNA: gi_4973263 [Arabidopsis thaliana]

46. Contig15329_at
   BEST BLASTX NR: 10/02/02 BAB86111.1 2e-43 putative auxin transport protein-like [Oryza sativa (japonica cultivar-group)]

47. Contig9531_at
   BEST BLASTX NR: 11/08/02 BAB07956.1 1e-90 putative extensin-like protein [Oryza sativa (japonica cultivar-group)]

48. Contig6964_s_at
   BEST BLASTX NR: 10/29/02 AAM01041.1 9e-62 Putative nodulin-like protein [Oryza sativa] [Oryza sativa (japonica cultivar-group)]

49. Contig13394_at
   BEST BLASTX NR: 11/08/02 NP_197168.1 1e-29 putative protein; protein id: At5g16650.1, supported by cDNA: 17002., supported by cDNA: gi_14326538 [Arabidopsis]

50. rbai38e14_x_at
   BEST BLASTX NR: 01/29/03 S61417 5e-04 ubiquitin-protein ligase (EC 6.3.2.19) - rice

51. Contig7987_at
   BEST BLASTX NR: 11/06/02 CAB50787.2 e-120 putative glyoxalase I [Triticum aestivum]

52. Contig7577_at
   BEST BLASTX NR: 10/27/02 AAF97273.1 4e-91 F2H15.16 [Arabidopsis thaliana]

53. Contig15205_at
   BEST BLASTX NR: 10/31/02 AAM34535.1 e-106 putative WD-40 repeat protein [Oryza sativa (japonica cultivar-group)]

54. Contig3981_at
   BEST BLASTX NR: 10/04/02 CAB85628.1 8e-38 putative ripening-related protein [Vitis vinifera]
55. Contig2672_at | xyloglucan endo-1,4-beta-D-glucanase (EC 3.2.1.-) - maize
    gb|AAC49012.1| xyloglucan endo-transglycosylase homolog; similar to Triticum
56. Contig12438_at | BEST BLASTX NR: 11/08/02 CAC35879.1 1e-59 (AL590346)
    NADH dehydrogenase-like protein [Arabidopsis thaliana]
57. HA11124u_s_at | ABC1 family protein kinase-like protein [Oryza sativa (japonica cultivar-group)]
58. Contig7027_at | BEST BLASTX NR: 10/13/02 BAB55792.1 e-114 putative neutral invertase [Oryza sativa (japonica cultivar-group)]
    dbj|BAB86156.1| putative neutral invertase [Oryza sativa (japonica cultivar-group)]
59. HVSME0015C12f_at | BEST BLASTX NR: <none>
60. Contig14285_at | BEST BLASTX NR: 11/04/02 NP_198552.1 2e-24 (NM_123095)
    putative protein; protein id: At5g37360.1, supported by cDNA: gi_16604481 [Arabidopsis thaliana]
61. Contig4437_at | BEST BLASTX NR: 01/29/03 Q07300 8e-40 ATP synthase delta chain, chloroplast precursor pir|S43728 H+-transporting two-sector ATPase (EC 3.6.3.14) delta chain
62. Contig10131_at | atvaline-tRNA ligase-like protein - Arabidopsis thaliana
    emb|CAC01835.1| valine-tRNA ligase-like protein [Arabidopsis thaliana]
63. Contig10131_s_at | atvaline-tRNA ligase-like protein - Arabidopsis thaliana
    emb|CAC01835.1| valine-tRNA ligase-like protein [Arabidopsis thaliana]
64. Contig5574_at | BEST BLASTX NR: 10/27/02 CAC83301.1 e-123 (AJ303373)
    cytochrome P450 reductase [Triticum aestivum]
65. HI12K05r_s_at | putative cation transporter [Beta procumbens]
66. Contig10417_at | contains similarity to Methanobacterium thermoautotrophicum transcriptional regulator (GB:AE000850) [Arabidopsis
67. Contig4774_s_at | BEST BLASTX NR: 11/07/02 AAM62950.1 6e-35 unknown [Arabidopsis thaliana]
68. Contig4905_at | BEST BLASTX NR: 11/06/02 AAL77110.1 e-111 unknown [Hordeum vulgare]
69. Contig5198_at | BEST BLASTX NR: 11/08/02 AAN06836.1 7e-76 Hypothetical protein [Oryza sativa (japonica cultivar-group)]
70. HE01K03u_at 
**BEST BLASTX NR: 11/06/02 NP_568749.1** 7e-28 (NM_124480) 
expressed protein; protein id: At5g51010.1, supported by cDNA: 27885, supported 
by cDNA: gi_15292668

71. Contig716_at 
**putative protein, with at least 2 transmembrane domains, a coiled 
coil-4 domain (1H231)** [Caenorhabditis elegans]

72. Contig2001_at 
**BEST BLASTX NR: 10/28/02 P35685 e-101** 60S RIBOSOMAL 
PROTEIN L7A 

73. Contig6341_s_at 
**BEST BLASTX NR: 11/04/02 AAL77589.1 4e-84** ribose-5-phosphate isomerase [Spinacia oleracea]

74. Contig4368_at 
**BEST BLASTX NR: 11/06/02 NP_192768.1 9e-41** putative protein; 
protein id: At4g10300.1, supported by cDNA: 1489. [Arabidopsis thaliana]

75. Contig4570_s_at 
**EST02416 Early embryo, Stratagene (cat. #937007)** 
Caenorhabditis elegans cDNA clone CEESU35, mRNA sequence.; ubiquitin thiolesterase, family 2 and ubiquitin interacting motif (144.2 kD) (1K87)

76. Contig7015_at 
**ABC1 family protein kinase-like protein** [Oryza sativa (japonica cultivar-group)]

77. HV_CEa0010M13r2_s at
**BEST BLASTX NR: <none>**

78. Contig5328_at 
**BEST BLASTX NR: 10/13/02 NP_196434.1 3e-69** putative protein; 
protein id: At5g08170.1 [Arabidopsis thaliana] pir||T50502 hypothetical protein 
T22D6.110 - Arabidopsis thaliana

79. S0001100150B08F1_s at
**46 Lambda-PRL1 Arabidopsis thaliana cDNA clone** 
SCF8T7P, mRNA sequence.; DnaJ protein family

80. Contig11259_at 
**BEST BLASTX NR: 10/13/02 AAK07735.1 e-108** (AY024351) 
phytoene synthase [Oryza sativa] [Oryza sativa (japonica cultivar-group)]

81. Contig8458_at 
**MATE efflux protein family** [Arabidopsis thaliana]

82. Contig15576_at 
**MATE efflux protein family** [Arabidopsis thaliana]

83. HVSMEF0002J15r2_s at 
**BEST BLASTX NR: 11/04/02 P33630 1e-21** TUBULIN 
BETA-1 CHAIN 
pir||S32668 tubulin beta-1 chain - fern (Anemia phyllitidis)

84. Contig497_s_at 
**BEST BLASTX NR: 11/04/02 BAB19812.1 6e-60** ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Triticum aestivum]
Appendix 2: Genelist 1

85. Contig 7800_atBEST BLASTX NR: 10/02/02 NP_567791.1 1e-75 (NM_118934) E2, ubiquitin-conjugating enzyme 9 (UBC9); protein id: At4g27960.1, supported by cDNA: gi_13358210 [Arabidopsis]

86. Contig 1797_atBEST BLASTX NR: 10/02/02 AAG02280.1 e-125 inducible phenylalanine ammonia-lyase [Triticum aestivum]

87. Contig 4222_s_atBEST BLASTX NR: 10/02/02 NP_196527.1 e-112 inorganic pyrophosphatase-like protein; protein id: At5g09650.1, supported by cDNA: 33506., supported by cDNA:

88. Contig 6052_s_atBEST BLASTX NR: 10/02/02 BAC10192.1 2e-63 (AP004380) putative protein kinase HvPKABA1 (Abscisic acid-inducible protein kinase) [Oryza sativa (japonica)]

89. HVSMEc0015H24f_atBEST BLASTX NR: 01/27/03 NP_114263.1 3e-092 NADH dehydrogenase subunit K [Triticum aestivum] sp|P26304|NUKC_WHEAT NADH-plastoquinone oxidoreductase subunit K

90. Contig 2000_atBEST BLASTX NR: 10/31/02 P35685 e-111 60S RIBOSOMAL PROTEIN L7A 31|S38360 ribosomal protein L7a, cytosolic - rice

91. Contig 1397_atactin [Oryza sativa (japonica cultivar-group)] dbj|BAC76319.1| actin [Oryza sativa (japonica cultivar-group)]

92. Contig 10929_atBEST BLASTX NR: 10/02/02 NP_567098.1 2e-78 FKBP-type peptidyl-prolyl cis-trans isomerase; protein id: At3g60370.1 [Arabidopsis thaliana]

93. Contig 6846_s_atBEST BLASTX NR: 09/30/02 NP_568922.1 1e-83 imidazolglycerol-phosphate synthase subunit H - like; protein id: At5g60540.1, supported by cDNA: 39485. [Arabidopsis]

94. Contig 934_x_atBEST BLASTX NR: 11/07/02 Q38793 2e-25 Ribulose bisphosphate carboxylase small chain, chloroplast precursor (RuBisCO small subunit)

95. Contig 15266_atBEST BLASTX NR: 11/04/02 NP_568750.1 4e-90 (NM_124486) ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor; protein id: At5g51070.1.


97. Contig 7522_atBEST BLASTX NR: 11/06/02 AAM93722.1 8e-61 putative oxygen evolving complex protein [Oryza sativa (japonica cultivar-group)]
98. Contig10328_atPutative ripening regulated protein [Oryza sativa (japonica cultivar-group)]

99. Contig10738_athydrolase, alpha/beta fold family [Arabidopsis thaliana]
   gb|AAK43858.1| Unknown protein [Arabidopsis thaliana]

100. Contig5680_atBEST BLASTX NR: 10/04/02 CAA77592.1 6e-53 plastid ribosomal protein CL15 [Arabidopsis thaliana]

101. Contig18640_atBEST BLASTX NR: 11/04/02 NP_566959.1 5e-48 Expressed protein; protein id: At3g52155.1, supported by cDNA: gi_14517465 [Arabidopsis thaliana]

102. Contig13030_atBEST BLASTX NR: 11/08/02 AAK63861.1 5e-79 AT5g06530/F15M7_6 [Arabidopsis thaliana] gb|AAN72282.1| At5g06530/F15M7_6 [Arabidopsis thaliana]

103. Contig7678_atBEST BLASTX NR: 10/13/02 NP_683480.1 8e-56 (NM_148639) auxin-regulated protein; protein id: At1g67700.2, supported by cDNA: 19973. [Arabidopsis thaliana]

104. Contig9948_atBEST BLASTX NR: 10/13/02 NP_565505.1 7e-60 expressed protein; protein id: At2g21280.1, supported by cDNA: gi_11908095, supported by cDNA: gi_12642905, supported

105. Contig10360_atBEST BLASTX NR: 10/27/02 BAA96146.1 1e-70 unnamed protein product [Oryza sativa (japonica cultivar-group)] dbj|BAA96188.1| unnamed protein product [Oryza sativa (japonica cultivar-gr

106. Contig4711_s_atBEST BLASTX NR: 10/13/02 AAF23902.1 2e-39 MAP kinase homolog [Oryza sativa]

107. Contig7342_atBEST BLASTX NR: 10/26/02 AAF26001.1 2e-53 (AC013354) F15H18.4 [Arabidopsis thaliana]

108. Contig3776_s_atBEST BLASTX NR: 10/29/02 AAM74427.1 2e-18 (AC123594) Putative lipid transfer protein [Oryza sativa (japonica cultivar-group)]

109. Contig3235_atBEST BLASTX NR: 10/02/02 AAD51733.1 e-125 methylenetetrahydrofolate reductase [Zea mays]

110. HVSMEb0014C02r2_s_atBEST BLASTX NR: 11/08/02 Q41739 .003 Thiazole biosynthetic enzyme 1-2, chloroplast precursor pir|S61420 thiamin biosynthesis protein thi1-2 - maize
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<th>Accession</th>
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<th>E-value</th>
<th>Protein Description and Gene Ids</th>
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<td>111.Contig12323_at</td>
<td>BAA65762.1</td>
<td>10/13/02</td>
<td>1e-78</td>
<td>cobalamin N-methyltransferase [Arabidopsis thaliana]</td>
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<tr>
<td>112.Contig2960_at</td>
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<td></td>
<td></td>
<td>putative cation transporter [Beta procvmbens]</td>
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<td>113.Contig12565_at</td>
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<td></td>
<td></td>
<td>uracil transporter-like protein - Arabidopsis thaliana</td>
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<tr>
<td>114.Contig11198_at</td>
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<td></td>
<td></td>
<td>atesterase/lipase/thioesterase family [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>115.Contig11912_at</td>
<td>BAA65762.1</td>
<td>10/13/02</td>
<td>2e-65</td>
<td>unknown protein; protein id: At1g15290.1 [Arabidopsis thaliana]</td>
</tr>
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<td>116.Contig15432_at</td>
<td></td>
<td>01/29/03</td>
<td>1e-89</td>
<td>expressed protein; protein id: At1g50450.1, supported by cDNA: gi_14517415, supported by cDNA: gi_20453276 [Arabidopsis]</td>
</tr>
<tr>
<td>117.Contig16504_at</td>
<td>BAA65762.1</td>
<td>11/08/02</td>
<td>5e-15</td>
<td>hypothetical protein [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>118.HV_CEa0002J17f2_s_at</td>
<td></td>
<td>11/04/02</td>
<td>7e-09</td>
<td>succinyl-CoA ligase beta subunit; protein id: At2g20420.1, supported by cDNA: 36959., supported by cDNA:</td>
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<td>119.Contig9220_at</td>
<td>BAA65762.1</td>
<td>11/08/02</td>
<td>2e-60</td>
<td>expressed protein; protein id: At2g37240.1, supported by cDNA: 98881., supported by cDNA: gi_15215633 [Arabidopsis]</td>
</tr>
<tr>
<td>120.Contig9199_at</td>
<td></td>
<td></td>
<td></td>
<td>hypothetical protein; protein id: At1g01320.1 [Arabidopsis thaliana]</td>
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<tr>
<td>121.Contig10130_at</td>
<td>BAA65762.1</td>
<td>11/08/02</td>
<td>3e-87</td>
<td>putative aldo/keto reductase [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>122.Contig24885_at</td>
<td></td>
<td></td>
<td></td>
<td>3e-46 3(2),5-BISPHOSPHATE NUCLEOTIDASE-like protein; protein id: At4g05090.1, supported by cDNA: 158702. [Arabidopsis]</td>
</tr>
<tr>
<td>123.Contig14168_s_at</td>
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<td></td>
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<td>4e-42 expressed protein; protein id: At1g74880.1, supported by cDNA: gi_12744982, supported by cDNA: gi_15294195 [Arabidopsis]</td>
</tr>
<tr>
<td>124.Contig24856_at</td>
<td>BAA65762.1</td>
<td>11/07/02</td>
<td>4e-76</td>
<td>hypothetical protein [Oryza sativa (japonica cultivar-group)]</td>
</tr>
</tbody>
</table>
125. Contig433_x_atBEST BLASTX NR: 11/08/02 1204205B e-110 protein 1B, chlorophyll binding
126. Contig1977_s_atBEST BLASTX NR: 10/13/02 AAC72193.1 6e-49 pyruvate dehydrogenase E1 beta subunit isoform 2 [Zea mays]
127. Contig2185_atBEST BLASTX NR: 11/08/02 CAA09935.1 e-115 chloroplast protease [Capsicum annuum]
128. Contig3231_atBEST BLASTX NR: 10/02/02 NP_192839.1 9e-81 nucleoside diphosphate kinase 3 (ndpk3); protein id: At4g11010.1, supported by cDNA: gi_14334559, supported by cDNA:
129. Contig7571_atBEST BLASTX NR: 11/06/02 AAM51838.1 4e-72 Unknown protein [Oryza sativa (japonica cultivar-group)]
130. Contig9795_atBEST BLASTX NR: 11/06/02 BAB60914.1 e-106 (AP003213) contains EST AU096093(S12136)—unknown protein [Oryza sativa (japonica cultivar-group)]
131. Contig6086_atBEST BLASTX NR: 01/29/03 CAD40675.1 e-112 OSJNBB0118P14.13 [Oryza sativa (japonica cultivar-group)]
132. Contig8236_atBEST BLASTX NR: 11/06/02 NP_564667.1 2e-77 thylakoid lumen 18.3 kDa protein; protein id: At1g54780.1, supported by cDNA: 3853., supported by cDNA:
133. Contig8699_atBEST BLASTX NR: 11/06/02 NP_568280.1 2e-93 putative protein; protein id: At5g12470.1, supported by cDNA: gi_20268751, supported by cDNA: gi_21281148 [Arabidopsis]
134. Contig14608_atBEST BLASTX NR: 10/02/02 NP_565077.1 4e-46 (NM_106058) SppA protease IV (SppA); protein id: At1g73990.1, supported by cDNA: gi_6690269 [Arabidopsis thaliana]
135. Contig5105_atBEST BLASTX NR: 01/29/03 BAA90524.1 2e-70 peroxiredoxin Q [Sedum lineare]
136. Contig16023_atBEST BLASTX NR: 10/13/02 AAF69175.1 5e-33 (AC007915) F27F5.3 [Arabidopsis thaliana]
137. Contig2165_atBEST BLASTX NR: 10/28/02 NP_193129.1 e-115 hydroxymethyltransferase; protein id: At4g13930.1, supported by cDNA: 29662., supported by cDNA: gi_13358183, supported
138. Contig14290_at
BEST BLASTX NR: 10/02/02 BAC05562.1 e-118 putative lycopene epsilon-cyclase [Oryza sativa (japonica cultivar-group)]

139. Contig2895_at
putative 37kDa chloroplast inner envelope membrane polypeptide precursor [Oryza sativa (japonica cultivar-group)]

140. Contig9489_at
BEST BLASTX NR: 11/07/02 BAB63797.1 1e-61 (AP003611) contains EST D46238(S10779)~unknown protein [Oryza sativa (japonica cultivar-group)]

141. Contig7648_at
BEST BLASTX NR: 11/08/02 NP_563902.1 5e-69 chloroplast lumen pentapeptide protein, putative; protein id: At1g12250.1, supported by cDNA: gi_14334897 [Arabidopsis]

142. Contig7882_at
BEST BLASTX NR: 11/07/02 NP_200240.1 5e-77 (NM_124809) cytochrome c biogenesis protein precursor (gb|AAF35369.1); protein id: At5g54290.1, supported by

143. HY05O16u_s_at
BEST BLASTX NR: 11/04/02 AAG46115.1 2e-07 (AC073166) putative sugar transporter [Oryza sativa]

144. Contig965_at
BEST BLASTX NR: 01/29/03 Q40004 3e-35 Ribulose bisphosphate carboxylase small chain, chloroplast precursor (RuBisCO small subunit)

145. Contig7908_at
BEST BLASTX NR: 11/08/02 NP_181637.1 8e-75 (NM_129669) hypothetical protein; protein id: At2g41040.1 [Arabidopsis thaliana]

146. Contig7244_at
SET-domain transcriptional regulator family [Arabidopsis thaliana] ref|NP_851038.1| SET-domain transcriptional regulator family [Arabidopsis]

147. HW01B13u_at
BEST BLASTX NR: <none>

148. Contig9613_at
BEST BLASTX NR: 11/06/02 P81760 2e-52 Thylakoid luminal 17.4 kDa protein, chloroplast precursor (P17.4)

149. Contig5513_s_at
BEST BLASTX NR: 11/08/02 ZP_00074952. 5e-08 (NZ_AAAU01000100) hypothetical protein [Trichodesmium erythraeum IMS101]

150. Contig6672_s_at
BEST BLASTX NR: 11/04/02 CAD41248.1 1e-05 (AL606627) OSJNBa0067K08.12 [Oryza sativa (japonica cultivar-group)]

151. Contig21949_s_at
BEST BLASTX NR: 11/07/02 AAK07734.1 7e-39 phytoene synthase [Oryza sativa]

152. HVSMEb0003G21r2_s_at
BEST BLASTX NR: <none>

153. Contig7995_at
plastid-lipid associated protein PAP/fibrillin family [Arabidopsis thaliana]
Appendix 2: Genelist 1

154. Contig5214_at BEST BLASTX NR: 10/02/02 BAB86142.1  e-115 putative zinc finger protein [Oryza sativa (japonica cultivar-group)]

155. Contig14999_at BEST BLASTX NR: 01/29/03 NP_191360.1  2e-52 putative protein; protein id: At3g58010.1, supported by cDNA: 1979. [Arabidopsis thaliana]

156. Contig5618_at BEST BLASTX NR: 10/13/02 BAC15472.1  e-122 (AP003833) putative thioredoxin reductase [Oryza sativa (japonica cultivar-group)]

157. Contig451_at BEST BLASTX NR: 09/30/02 BAB39219.1  e-124 putative dihydrolipoamide dehydrogenase precursor [Oryza sativa (japonica cultivar-group)]

158. Contig4243_at BEST BLASTX NR: 10/02/02 BAA82377.1  7e-74 ESTs AU070372(S13446), AU075541(S0353) correspond to a region of the predicted gene. ~ Similar to Arabidopsis thaliana BAC

159. Contig5775_at BEST BLASTX NR: 11/08/02 AAC64970.1  e-106 50S ribosomal protein L5 [Oryza sativa]

160. Contig2281_at putative metacaspase, having alternative splicing products [Oryza sativa (japonica cultivar-group)]

161. Contig3588_s_at BEST BLASTX NR: <none>

162. Contig13371_at BEST BLASTX NR: 11/07/02 BAB64790.1  2e-78 P0701D05.4 [Oryza sativa (japonica cultivar-group)] dbj|BAC00581.1| B1189A09.35 [Oryza sativa (japonica cultivar-group)]

163. Contig2249_at BEST BLASTX NR: 11/06/02 AAF23357.1  e-113 glutathione-S-transferase [Hordeum vulgare]

164. Contig3905_at BEST BLASTX NR: 11/07/02 NP_567210.1  3e-20 expressed protein; protein id: At4g01150.1, supported by cDNA: gi_14488087, supported by cDNA: gi_20147122, supported

165. Contig6517_at BEST BLASTX NR: 10/29/02 Q9LKJ3  e-113 Alpha-glucan phosphorylase, H isozyme (Starch phosphorylase H) gb|AAF82787.1|AF275551_1 alpha 1,4-glucan phosphorylase; cytosolic starch pho

166. Contig2716_s_at BEST BLASTX NR: 11/07/02 AAM74942.1  3e-79 ferritin [Oryza sativa (japonica cultivar-group)]

167. Contig1247_s_at Putative r40c1 protein - rice [Oryza sativa (japonica cultivar-group)]
168. Contig10570_at
BEST BLASTX NR: 10/26/02 NP_671953.1 8e-73 (NM_147404) similar to putative amino acid transport protein; protein id: At2g42005.1 [Arabidopsis thaliana]

169. Contig7913_at
BEST BLASTX NR: 01/29/03 AAK38177.1 e-117 violaxanthin de-epoxidase [Triticum aestivum]

170. Contig594_x_at
BEST BLASTX NR: 11/06/02 P26667 5e-97 Ribulose bisphosphate carboxylase small chain PW9, chloroplast precursor (RuBisCO small subunit PW9)

171. Contig909_s_at
BEST BLASTX NR: 10/13/02 NP_172650.1 6e-47 aminomethyltransferase-like precursor protein; protein id: At1g11860.1 [Arabidopsis thaliana]

172. Contig6150_at
BEST BLASTX NR: 11/08/02 BAB93184.1 5e-59 P0031D02.12 [Oryza sativa (japonica cultivar-group)]

173. Contig6531_at
BEST BLASTX NR: 11/06/02 AAM65419.1 9e-67 unknown [Arabidopsis thaliana]

174. Contig1988_at
BEST BLASTX NR: 11/08/02 CAD40830.1 e-104 OSJNBA0086B14.2 [Oryza sativa (japonica cultivar-group)]

175. Contig3237_s_at
BEST BLASTX NR: 01/29/03 AAD51733.1 3e-22 methylene-tetrahydrofolate reductase [Zea mays]

176. Contig12958_at
plastid-lipid associated protein PAP/fibrillin family [Arabidopsis thaliana]

177. Contig7753_at
BEST BLASTX NR: 11/06/02 AAL69988.1 e-101 (AF465840) cold acclimation WCOR413-like protein gamma form [Hordeum vulgare] [Hordeum vulgare subsp. vulgare]

178. Contig10268_at
BEST BLASTX NR: 10/27/02 AAK20054.1 e-115 putative cytochrome P450 monooxygenase [Oryza sativa (japonica cultivar-group)]

179. Contig12211_at
BEST BLASTX NR: 10/27/02 NP_565597.1 4e-60 F-box protein family, AtFBL6; protein id: At2g25490.1, supported by cDNA: gi_18176338, supported by cDNA: gi_20259112

180. Contig1244_s_at
AR40g2 protein [Oryza sativa (japonica cultivar-group)]

181. Contig6208_at
BEST BLASTX NR: 11/04/02 AAK20047.1 3e-84 putative C-4 sterol methyl oxidase [Oryza sativa (japonica cultivar-group)]

182. Contig4186_at
BEST BLASTX NR: 11/06/02 AAF79697.1 2e-11 (AC020889) T1N15.11 [Arabidopsis thaliana]
183. Contig5567_s_atBEST BLASTX NR: 10/02/02 NP_567405.1 e-115 putative protein; protein id: At4g13430.1, supported by cDNA: gi_15027970 [Arabidopsis thaliana]

184. Contig2277_s_atBEST BLASTX NR: 01/29/03 CAD30024.2 e-125 ferredoxin-NADP(H) oxidoreductase [Triticum aestivum]

185. Contig10628_atBEST BLASTX NR: 11/06/02 BAB86041.1 e-103 (AP003347) contains EST AU101879(S16511)~similar to Arabidopsis thaliana chromosome 1, F5I6.13~unknown protein [Oryza]

186. HV_CEa0010K11f_x_atBEST BLASTX NR: <none>

187. Contig3204_atBEST BLASTX NR: 11/07/02 BAB61039.1 e-117 iron-deficiency induced gene [Hordeum vulgare]

188. HZ54E21r_s_atBEST BLASTX NR: 11/04/02 Q9FVI1 4e-16 Actin-depolymerizing factor 2 (ADF 2) gb|AAG16974.1|AF183904_1 actin-depolymerizing factor 2 [Petunia x hybrida]

189. Contig1198_x_atBEST BLASTX NR: 10/04/02 Q40677 e-117 Fructose-bisphosphate aldolase, chloroplast precursor (ALDP) pir||T03679 probable fructose-bisphosphate aldolase (EC 4.1.2.13) precursor,

190. Contig6765_atBEST BLASTX NR: 10/04/02 Q41364 e-102 2-oxoglutarate/malate translocator, chloroplast precursor gb|AAA68148.1| 2-oxoglutarate/malate translocator

191. EBpi07_SQ002_O11_s_atputative glycine decarboxylase subunit [Triticum aestivum]

192. rbags16h12_s_atBEST BLASTX NR: 10/02/02 O48651 5e-33 Squalene monooxygenase (Squalene epoxidase) (SE) dbj|BAA24448.1| (AB003516) squalene epoxidase [Panax ginseng]

193. Contig2454_atBEST BLASTX NR: 11/06/02 CAB59893.1 e-113 GPX12Hv, glutathione peroxidase-like protein [Hordeum vulgare subsp. vulgare]

194. Contig9168_s_atBEST BLASTX NR: 10/13/02 BAA07785.3 e-111 plastid omega-3 fatty acid desaturase [Triticum aestivum]

195. Contig23045_atBEST BLASTX NR: 11/08/02 NP_564838.1 3e-27 expressed protein; protein id: At1g64680.1, supported by cDNA: 101924. [Arabidopsis thaliana]
196. Contig6603_atBEST BLASTX NR: 10/04/02 NP_175852.1 2e-49 (NM_104328)  
rubredoxin, putative; protein id: At1g54500.1, supported by cDNA: 19234., sup-
ported by cDNA:
197. Contig2985_s_atBEST BLASTX NR: 10/04/02 AAK72401.1  e-139 (AY039003)  
Mg-chelatase subunit XANTHA-F [Hordeum vulgare] [Hordeum vulgare subsp. vul-
gare]
198. Contig1879_atBEST BLASTX NR: 11/04/02 P00068 7e-61 Cytochrome c  
pir||CCWT cytochrome c [validated] - wheat
199. Contig5804_atBEST BLASTX NR: 10/13/02 NP_197703.1  e-106 photosystem II  
stability/assembly factor HCF136; protein id: At5g23120.1, supported by cDNA:  
gi_15010779 [Arabidopsis
200. Contig14005_atBEST BLASTX NR: 11/08/02 NP_566122.1 1e-49 expressed  
protein; protein id: At2g48070.1, supported by cDNA: gi_15027852, supported by  
cDNA: gi_19310716 [Arabidopsis
201. Contig8775_atBEST BLASTX NR: 11/08/02 BAC16410.1 3e-81 (AP003931)  
putative mRNA binding protein precursor [Oryza sativa (japonica cultivar-group)]
202. Contig3791_s_ataldose 1-epimerase family [Arabidopsis thaliana]  
dbj|BAB10929.1| apospory-associated protein C-like [Arabidopsis thaliana]
203. HVSMEm0001P16r2_s_atBEST BLASTX NR: 11/04/02 NP_200240.1 6e-32  
(NM_124809) cytochrome c biogenesis protein precursor (gb|AAF35369.1); protein  
id: At5g54290.1, supported by
204. HV12A05u_s_atBEST BLASTX NR: 11/04/02 AAM74943.1 4e-34 ferritin [Oryza  
sativa (japonica cultivar-group)]
205. Contig3247_s_atBEST BLASTX NR: 11/04/02 P35100 2e-69 ATP-dependent  
clp protease ATP-binding subunit clpA homolog, chloroplast precursor
206. Contig6111_atBEST BLASTX NR: 10/04/02 P46225 e-115 Triosephosphate  
isomerase, chloroplast precursor (TIM) pir||S53761 triose-phosphate isomerase  
(EC 5.3.1.1) precursor, chloroplast -
207. Contig1698_atBEST BLASTX NR: 10/13/02 AAF80560.1 e-101 omega-6 fatty  
acid desaturase [Sesamum indicum]
208. Contig1760_s_atprobable RNA binding protein [imported] - Arabidopsis thaliana  
emb|CAA75602.1| putative RNA binding protein [Arabidopsis thaliana]
Appendix 2: Genelist 1

209.Contig4025_s_atBEST BLASTX NR: 11/04/02 AAB57688.1 9e-31 (U96045) APS reductase [Arabidopsis thaliana] gb|AAM66987.1| (AY088665) 5'-adenyllyl-phosphosulfate reductase, putative

210.Contig15111_atBEST BLASTX NR: 11/04/02 NP_563937.1 2e-43 PsbQ domain protein family; protein id: At1g14150.1, supported by cDNA: 5665., supported by cDNA: gi_13926215 [Arabidopsis]

211.Contig12091_atBEST BLASTX NR: 10/27/02 P80471 5e-87 Light-induced protein, chloroplast precursor (Chloroplastic drought-induced stress protein CDSP-34)

212.Contig5175_atBEST BLASTX NR: 09/30/02 NP_568382.1 3e-44 Expressed protein; protein id: At5g19855.1, supported by cDNA: 42402. [Arabidopsis thaliana]

213.Contig7338_atBEST BLASTX NR: 11/06/02 AAL58119.1 1e-43 hypothetical protein [Oryza sativa (japonica cultivar-group)] gb|AAM76345.1|AC074196_3 hypothetical protein [Oryza sativa (japonica cultivar

214.Contig8038_s_atBEST BLASTX NR: 11/06/02 NP_566745.1 e-118 (NM_113324) expressed protein; protein id: At3g24190.1, supported by cDNA: gi_15294249 [Arabidopsis thaliana]

215.HU02C06u_atBEST BLASTX NR: 11/06/02 AAL24425.1 3e-77 GTP-binding protein typA (tyrosine phosphorylated protein A) [Arabidopsis thaliana]

216.Contig491_s_atglycine dehydrogenase (decarboxylating) (EC 1.4.4.2) [imported] - Hordeum sp. x Triticum sp

217.Contig6788_atchopper chaperone [Hordeum vulgare subsp. vulgare]

218.Contig2724_s_atBEST BLASTX NR: 09/30/02 AAK31789.1 e-119 sucrose-6F-phosphate phosphohydrolase SPP3 [Triticum aestivum]

219.Contig10062_atBEST BLASTX NR: 10/02/02 AAG13625.1 3e-67 putative cytochrome b-561 [Oryza sativa (japonica cultivar-group)] gb|AAN05553.1| putative cytochrome [Oryza sativa (japonica cultivar-group]

220.rbags18j12_s_atBEST BLASTX NR: 10/02/02 AAG40130.1 5e-29 peroxiredoxin [Oryza sativa] dbj|BAB93323.1| peroxiredoxin [Oryza sativa (japonica cultivar-group)]

221.Contig2189_s_atBEST BLASTX NR: 10/29/02 AAL40895.1 3e-26 phosphoethanolamine methyltransferase [Triticum aestivum]
223. Contig2993_atBEST BLASTX NR: 10/02/02 AAM92707.1 3e-82 putative glycine decarboxylase subunit [Triticum aestivum]
224. Contig12596_atBEST BLASTX NR: 11/08/02 AAM92707.1 3e-82 putative glycine decarboxylase subunit [Triticum aestivum]
225. Contig5351_atBEST BLASTX NR: 11/08/02 NP_566588.1 1e-80 expressed protein; protein id: At3g17800.1, supported by cDNA: gi_15577., supported by cDNA: gi_17064819, supported by
226. Contig1791_x_atBEST BLASTX NR: 10/04/02 P32112 e-132 Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase)
227. Contig2949_s_atBEST BLASTX NR: 10/25/02 P46285 e-136 Sedoheptulose-1,7-bisphosphatase, chloroplast precursor (Sedoheptulose-bisphosphatase) (SBPASE) (SED(1,7)P2ASE)
228. Contig8096_atBEST BLASTX NR: 10/13/02 NP_565018.1 7e-71 expressed protein; protein id: At1g71500.1, supported by cDNA: gi_20260263 [Arabidopsis]
229. HY02C22u_x_atBEST BLASTX NR: <none>
230. Contig592_atBEST BLASTX NR: 10/13/02 P49363 e-114 Aminomethyltransferase, mitochondrial precursor (Glycine cleavage system T protein) (GCVT)
231. Contig2191_atBEST BLASTX NR: 10/04/02 AAL40895.1 e-139 phosphoethanolamine methyltransferase [Triticum aestivum]
232. Contig48_atBEST BLASTX NR: 01/29/03 P12782 e-125 Phosphoglycerate kinase, chloroplast precursor pir||TVWTGC phosphoglycerate kinase (EC 2.7.2.3) precursor, chloroplast - wheat
233. Contig1239_s_at415 Lambda-PRL2 Arabidopsis thaliana cDNA clone 39C4T7, mRNA sequence.; Expressed protein
234. Contig3657_s_atBEST BLASTX NR: 11/07/02 CAA06667.1 9e-16 cytochrome b6f complex subunit [Arabidopsis thaliana]
235. HVSMEb0009E07r2_s_atBEST BLASTX NR: 11/08/02 NP_564563.1 3e-30 chloroplast FtsH protease; protein id: At1g50250.1, supported by cDNA: gi_20268683 [Arabidopsis thaliana]
236. Contig2262_at BEST BLASTX NR: 10/02/02 BAA87823.1  e-145 unnamed protein product [Oryza sativa (japonica cultivar-group)] dbj|BAA89564.1| unnamed protein product [Oryza sativa (japonica cultivar-gr]

237. Contig2019_s_at BEST BLASTX NR: 10/04/02 AAC78101.1  e-113 inorganic pyrophosphatase [Oryza sativa]

238. rbah53j14_s_at BEST BLASTX NR: 11/08/02 P09043 1e-13 Glyceraldehyde 3-phosphate dehydrogenase A, chloroplast precursor (NADP-dependent glyceraldehydephosphate dehydrogenase

239. Contig3908_at BEST BLASTX NR: 11/08/02 P80470 2e-20 Photosystem II core complex proteins psbY, chloroplast precursor (L-arginine metabolising enzyme) (L-AME) [Contains:

240. HVSMEc0011A03f_at BEST BLASTX NR: 01/27/03 NP_114269.1 2e-032 photosystem I assembly protein Ycf4 [Triticum aestivum] spj|P30982|YCF4_WHEAT Photosystem I assembly protein ycf4

241. Contig1727_s_at BEST BLASTX NR: 11/04/02 AAL08496.1  e-123 ascorbate peroxidase [Hordeum vulgare]

242. Contig2080_at BEST BLASTX NR: 09/30/02 P29790 2e-93 ATP synthase gamma chain, chloroplast precursor pir||PWNTG H+-transporting two-sector ATPase (EC 3.6.3.14) gamma chain

243. Contig4964_at BEST BLASTX NR: 10/27/02 Q9X6W8 6e-56 ATP-dependent Clp protease proteolytic subunit (Endopeptidase Clp) gb|AAD37435.1|AF150957_2 heat-shock protein ClpP [Azospirillum brasiliense

244. Contig2135_s_at BEST BLASTX NR: 11/08/02 AAD37017.1 2e-09 putative MtN3-like protein [Dianthus caryophyllus]

245. Contig5153_at BEST BLASTX NR: 09/30/02 AAG13554.1 4e-85 putative chloroplast inner envelope protein [Oryza sativa]

246. Contig3443_at BEST BLASTX NR: 11/08/02 NP_564801.1 e-117 elongation factor Tu family protein; protein id: At1g62750.1 [Arabidopsis thaliana]

247. Contig1916_s_at BEST BLASTX NR: 11/08/02 BAB64099.1 1e-82 putative photosystem II subunit (22KDa) precursor [Oryza sativa (japonica cultivar-group)]

248. baak1k18_s_at BEST BLASTX NR: 11/08/02 NP_174996.1 2e-05 glyceraldehyde-3-phosphate dehydrogenase; protein id: At1g42970.1, supported by cDNA: gi_14517407, supported by cDNA:
249. Contig1192_s_at BEST BLASTX NR: 10/13/02 Q40677 e-126 Fructose-bisphosphate aldolase, chloroplast precursor (ALDP) pir||T03679 probable fructose-bisphosphate aldolase (EC 4.1.2.13) precursor,

250. Contig446_at BEST BLASTX NR: 10/29/02 BAA85402.1 e-113 EST C74302(E30840) corresponds to a region of the predicted gene.~similar to glycer-aldehyde-3-phosphate

251. Contig888_at BEST BLASTX NR: 01/29/03 NP_172750.1 e-109 putative calcium-binding protein, calreticulin; protein id: At1g12900.1 [Arabidopsis thaliana]

252. rbasd17i02_s_at BEST BLASTX NR: 11/07/02 AAF74220.1 5e-40 fructose 1,6-bisphosphate aldolase precursor [Avena sativa]
Appendix 3: Genelist 2

1. rbaal9k07_atBEST BLASTX NR: 10/02/02 AAK72891.1  1e-58 (AC091123) putative myosin heavy chain [Oryza sativa]
2. rbaal35o19_s_atBEST BLASTX NR: <none>
3. rbaal23d16_s_atNADP-specific isocitrate dehydrogenase [Oryza sativa (japonica cultivar-group)]
4. rbaal1f12_s_atBEST BLASTX NR: <none>
5. S0000200079C09F1_s_atBEST BLASTX NR: <none>
6. M58754_atacyl carrier protein III precursor
7. HY09L01u_s_atCysteine proteinase 1 precursor pir||S59597 cysteine proteinase (EC 3.4.22.-) 1 precursor - maize
8. HV_CEa0018D10f_s_athypothetical protein wali7 - wheat (fragment) gb|AAC37416.1|wali7
9. HVSMEm0004L13r2_s_atBEST BLASTX NR: <none>
10. HVSMEc0009D09f_atBEST BLASTX NR: 01/27/03 NP_114257.1  2e-038 ribosomal protein S14 [Triticum aestivum] dbj|BAB47032.1| ribosomal protein S14 [Triticum aestivum]
11. HVSMEb0013P12r2_atBEST BLASTX NR: <none>
12. HV14L22u_s_atputative ethylene-responsive transcriptional coactivator [Oryza sativa (japonica cultivar-group)]
13. HO04H12S_s_atputative wound inductive gene [Oryza sativa (japonica cultivar-group)]
14. Contig9232_atBEST BLASTX NR: 11/06/02 NP_178122.1  5e-61 (NM_106654) adenine phosphoribosyltransferase; protein id: At1g80050.1 [Arabidopsis thaliana]
15. Contig8369_atBEST BLASTX NR: 11/08/02 AAD22495.3  4e-20 APETALA2 protein homolog HAP2 [Hyacinthus orientalis]
16. Contig7972_atBEST BLASTX NR: 10/29/02 BAA90807.1  2e-08 ESTs AU069906(E11917), D48439(S14636), D46007(S10373), AU030823(E60306) correspond to a region
17. Contig7958_atBEST BLASTX NR: 10/26/02 BAB62326.1  e-120 peptide transporter [Oryza sativa (japonica cultivar-group)] dbj|BAB62327.1| peptide transporter [Oryza sativa (japonica cultivar-group)]
18. Contig7792_at BEST BLASTX NR: 11/08/02 NP_199782.1 9e-23 putative protein; protein id: At5g49710.1 [Arabidopsis thaliana] dbj|BAA98159.1| gene_id:K215.7~pir||T05575~similar to unknown protein [Arabidopsis thaliana]

19. Contig7543_at BEST BLASTX NR: 11/06/02 AAK50136.1 e-107 (AC087797) unknown protein [Oryza sativa]


21. Contig7402_at BEST BLASTX NR: 11/07/02 BAB92459.1 4e-06 P0698A10.29 [Oryza sativa (japonica cultivar-group)] dbj|BAC07317.1| P0471B04.1 [Oryza sativa (japonica cultivar-group)]

22. Contig6873_at BEST BLASTX NR: 11/06/02 NPJ95563.1 e-126 unknown [Arabidopsis thaliana]

23. Contig6878_at BEST BLASTX NR: 11/07/02 AAM61357.1 6e-77 unknown [Arabidopsis thaliana]

24. Contig6358_at BEST BLASTX NR: 11/06/02 BAA33203.1 1e-69 (AB001885) zinc finger protein [Oryza sativa (japonica cultivar-group)]

25. Contig5508_at BEST BLASTX NR: 11/06/02 BAB21196.1 2e-38 P0480E02.2 [Oryza sativa (japonica cultivar-group)]

26. Contig5765_at BEST BLASTX NR: 11/06/02 AAL33648.1 e-116 MSI type nucleosome/chromatin assembly factor C [Zea mays]


28. Contig4772_s_at BEST BLASTX NR: 11/08/02 Q03200 3e-23 LIGHT REGULATED PROTEIN PRECURSOR pir||S33632 lir1 protein - rice

29. Contig4707_x_at BEST BLASTX NR: <none>

30. Contig4604_s_at BEST BLASTX NR: 11/06/02 NP_197314.1 9e-10 putative protein; protein id: At5g18130.1, supported by cDNA: 10299., supported by cDNA: gi_14532801, supported by
Appendix 3: Genelist 2

32. Contig4580_at BEST BLASTX NR: 10/04/02 CAC08239.1 6e-34 (AL392144) 
Pspzf zinc finger protein-like [Arabidopsis thaliana]

33. Contig4545_s_at BEST BLASTX NR: 11/04/02 BAB64285.1 2e-20 P0005H10.22 
[Oryza sativa (japonica cultivar-group)]

34. Contig4459_at BEST BLASTX NR: 11/06/02 S27762 e-103 Sip1 protein - barley gb|AAA32975.1| seed imbibition protein

35. Contig4350_at BEST BLASTX NR: 10/29/02 NP_181807.1 e-122 putative citrate synthase; protein id: At2g42790.1, supported by cDNA: 17416., supported by cDNA: gi_15081643

36. Contig4118_at BEST BLASTX NR: 10/02/02 AAL87174.1 e-116 putative apospory-associated protein C [Oryza sativa (japonica cultivar-group)]

37. Contig4095_s_at BEST BLASTX NR: 10/02/02 CAB61759.1 5e-47 putative amipropyltransferase [Oryza sativa (japonica cultivar-group)]

38. Contig3891_s_at BEST BLASTX NR: 11/08/02 AAF34802.1 1e-71 putative flavonol synthase-like protein [Euphorbia esula]

39. Contig3875_s_at BEST BLASTX NR: 10/02/02 NP_566088.2 1e-23 (NM_130250) MYB-related transcription factor (CCA1); protein id: At2g46830.1, supported by cDNA: gi_1777442 [Arabidopsis]

40. Contig3873_at BEST BLASTX NR: 11/08/02 CAD12767.2 5e-36 LHY protein [Phaseolus vulgaris]

41. Contig366_athypeothetical protein wali7 - wheat (fragment) gb|AAC37416.1| wali7

42. Contig3470_at BEST BLASTX NR: 11/08/02 CAC03605.1 2e-61 splicing factor RSZ33 [Arabidopsis thaliana]

43. Contig3281_s_at BEST BLASTX NR: 10/27/02 Q40082 e-124 Xylose isomerase emb|CAA64545.1| xylose isomerase [Hordeum vulgare subsp. vulgare]

44. Contig3002_s_at BEST BLASTX NR: 11/06/02 S53521 5e-40 histone H2A.4 - wheat dbj|BAA07278.1| protein H2A [Triticum aestivum]

45. Contig2909_s_at BEST BLASTX NR: 11/08/02 AAL67123.1 2e-16 AT3g06760/F3E22_10 [Arabidopsis thaliana] gb|AAM91471.1| AT3g06760/F3E22_10 [Arabidopsis thaliana]

46. Contig2640_s_at BEST BLASTX NR: 11/06/02 BAB32502.1 8e-75 (AB044747) 1-aminocyclopropane-1-carboxylate oxidase [Phyllostachys edulis]
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<th>Accession</th>
<th>Description</th>
<th>Completeness</th>
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<td>47</td>
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<td>BAA94511.1</td>
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Appendix 3: Genelist 2

63. Contig10289_at

BEST BLASTX NR: 11/06/02 AAD23910.1  9e-85 (AF073698)
cysteine synthase [Oryza sativa] [Oryza sativa (japonica cultivar-group)]

64. Contig10284_at

BEST BLASTX NR: 01/29/03 NP_191190.2  1e-95 glycosyl hydrolase family 27 (alpha-galactosidase/melibiose); protein id: At3g56310.1, supported by cDNA: gi_18377734,

65. Contig10189_at

BEST BLASTX NR: 11/06/02 AAG52423.1  8e-24 (AC011622)
unknown protein; 83181-85105 [Arabidopsis thaliana]

66. Contig10173_at

FB6E1 Fetal brain, Stratagene Homo sapiens cDNA clone FB6E1 3'end, mRNA sequence.; Transcribed sequences

67. Contig10037_at

BEST BLASTX NR: 11/06/02 BAB61155.1  4e-42 B1045D11.23 [Oryza sativa (japonica cultivar-group)] dbj|BAB61158.1| B1111C09.3 [Oryza sativa (japonica cultivar-group)]
Appendix 4: Genelist 3

1. Contig25646_atBEST BLASTX NR: 11/07/02 EAA00454.1  e-103 agCP9307  
   [Anopheles gambiae str. PEST]
2. Contig15752_s_atBEST BLASTX NR: 11/08/02 BAB93187.1  3e-31 (AP003230)  
   putative arm repeat containing protein [Oryza sativa (japonica cultivar-group)]
3. Contig23900_atBEST BLASTX NR: 11/08/02 P29522  6e-50 ELONGATION  
   FACTOR 1-BETA' pir||S35514 translation elongation factor eEF-1 beta chain - silk-worm  
4. Contig15974_atproline-rich protein-like [Oryza sativa (japonica cultivar-group)]
5. rbags16c12_s_atBEST BLASTX NR: 10/02/02 BAC06946.1  2e-31 putative GTP-binding protein [Oryza sativa (japonica cultivar-group)]
6. HV02I22r_atBEST BLASTX NR: 11/06/02 NP_174105.2  9e-33 (NM_102549) G-protein beta-subunit (transducin) family; protein id: At1g27840.1, supported by cDNA: gi_15982896, supported
7. Contig12040_atBEST BLASTX NR: 11/08/02 AAL77132.1  2e-64 Putative calcium-binding protein [Oryza sativa]
8. Contig11866_atBEST BLASTX NR: 11/08/02 BAC10827.1  2e-54 putative protein kinase Xa21, receptor type precursor [Oryza sativa (japonica cultivar-group)]
10. Contig7388_atBEST BLASTX NR: 11/06/02 NP_201074.1  5e-66 peptide transporter [Arabidopsis thaliana]
11. rbaal21j20_s_atRING/C3HC4/PHD zinc finger-like protein [Cucumis melo]
12. Contig5185_atBEST BLASTX NR: 11/06/02 AAF45043.1  e-118 RNase S-like protein precursor [Hordeum vulgare]
13. HM11K07r_atBEST BLASTX NR: <none>
14. Contig4942_atBEST BLASTX NR: 10/13/02 NP_568750.1  3e-80 (NM_124486)  
   ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor; protein id: At5g51070.1,
15. Contig10361_atBEST BLASTX NR: 11/08/02 BAA95829.1  2e-78 (AP002069)  
   Similar to Prunus armeniaca ethylene-forming-enzyme-like dioxygenase. (U97530) [Oryza sativa (japonica]
16. Contig2470_s_atBEST BLASTX NR: <none>
17. Contig7064_s_atBEST BLASTX NR: 11/04/02 CAD48130.1 8e-59 saccharopin dehydrogenase-like protein [Hordeum vulgare subsp. spontaneum]

18. Contig5353_s_atBEST BLASTX NR: 11/06/02 BAB91776.1 3e-19 (AP003287) contains EST D24276(R1622)~similar to Arabidopsis thaliana chromosome 5, K16F4.11~unknown protein [Oryza]

19. Contig7811_s_atfructan 1-exohydrolase precursor [Hordeum vulgare]

20. Contig346_atBEST BLASTX NR: 11/06/02 A05005 8e-10 ribulose-bisphosphate carboxylase (EC 4.1.1.39) small chain precursor (clone 234) - wheat (fragment)

21. Contig21245_atBEST BLASTX NR: 11/04/02 NP_200157.1 2e-30 (NM_124724) putative protein; protein id: At5g53450.1, supported by cDNA: 95459. [Arabidopsis thaliana]

22. Contig4317_atBEST BLASTX NR: 10/29/02 AAM80486.1 7e-33 DRE binding factor 1 [Zea mays]

23. Contig11103_atcopine-related [Arabidopsis thaliana]

24. Contig2448_s_atBEST BLASTX NR: 10/29/02 AAK73115.1 8e-55 ribosomal protein L35A [Zea mays]

25. HVSMEn0019F17r2_atBEST BLASTX NR: 11/04/02 BAC16492.1 5e-19 (AP005198) putative auxin-regulated dual specificity cytosolic kinase [Oryza sativa (japonica cultivar-group)]

26. Contig19861_atyb88a06.r1 Stratagene liver (#937224) Homo sapiens cDNA clone IMAGE:78226 5‘ similar to similar to gb:X64707 BREAST BASIC CONSERVED PROTEIN 1 (HUMAN), mRNA sequence.; ribosomal protein L13

27. Contig7416_atBEST BLASTX NR: 11/06/02 CAB97351.1 e-127 (AJ249143) MADS-box protein 3 [Hordeum vulgare subsp. vulgare]

28. Contig3246_s_atacetic ribosomal protein [Triticum aestivum]

29. Contig7038_atBEST BLASTX NR: 11/07/02 NP_172499.1 1e-83 unknown protein; protein id: At1g10280.1 [Arabidopsis thaliana] gb|AAD32878.1|AC005489_16 F14N23.16 [Arabidopsis thaliana]

30. Contig11229_atBEST BLASTX NR: 11/06/02 CAB97354.1 e-103 MADS-box protein 8 [Hordeum vulgare subsp. vulgare]
31. Contig4641_s_atBEST BLASTX NR: 11/07/02 AAF81309.1 9e-17 (AC061957)
   Contains a weak similarity to a farnesylated protein GMFP5 mRNA from Glycine
   max gb|U64916. ESTs
32. Contig5020_s_atBEST BLASTX NR: 11/08/02 BAC15484.1 1e-75 contains ESTs
   AU091627(C0596), D15410(C0596A)~similar to Arabidopsis thaliana chromosome
   2, At2g41250~unknown protein [Oryza
33. Contig3626_s_atBEST BLASTX NR: 11/06/02 AAN17464.1 e-126 hypersensi-
   tive-induced reaction protein 3 [Hordeum vulgare subsp. vulgare]
34. Contig5059_s_atBEST BLASTX NR: 01/29/03 AAM80567.1 e-105 RNase S-like
   protein [Hordeum vulgare]
35. Contig5075_s_atBEST BLASTX NR: 10/27/02 BAB90280.1 7e-13 P0470A12.5
   [Oryza sativa (japonica cultivar-group)] dbj|BAB92626.1| P0456E05.27 [Oryza
   sativa (japonica cultivar-group)]
Appendix 5: Genes changed more then 3 fold in Greina KP4 compared to wt

Upregulated genes:

1. HV12A05u_s_atBEST BLASTX NR: 11/04/02 AAM74943.1 4e-34 ferritin [Oryza sativa (japonica cultivar-group)]
2. HV_CEb0022G09f_atBEST BLASTX NR: <none>
3. HVSMEg0013l19r2_s_atBEST BLASTX NR: <none>
4. Contig2089_atBEST BLASTX NR: 10/13/02 AAD14440.1 4e-35 (AC005275) putative C-type U1 snRNP [Arabidopsis thaliana] emb|CAB77797.1| (AL161496) putative C-type U1 snRNP [Arabidopsis thaliana]
5. HY05O16u_s_atBEST BLASTX NR: 11/04/02 AAG46115.1 2e-07 (AC073166) putative sugar transporter [Oryza sativa]
6. rbaal10h14_atBEST BLASTX NR: 10/02/02 T03731 2e-53 abscisic acid-induced protein - rice emb|CAA61981.1| EFA27 for EF hand, abscisic acid, 27kD [Oryza sativa]
7. HVSMEi0017H19r2_s_atBEST BLASTX NR: <none>
8. Contig541_x_atBEST BLASTX NR: 10/26/02 S53050 1e-41 RNA binding protein - barley emb|CAA88558.1| glycine rich protein, RNA binding protein [Hordeum vulgare subsp.
9. Contig13457_s_atBEST BLASTX NR: 11/06/02 NP_567210.1 8e-38 expressed protein; protein id: At4g01150.1, supported by cDNA: gi_14488087, supported by cDNA: gi_20147122, supported
10. Contig4641_s_atBEST BLASTX NR: 11/07/02 AAF81309.1 9e-17 (AC061957) Contains a weak similarity to a farnesylated protein GMFP5 mRNA from Glycine max gb|U64916. ESTs
11. Contig2720_atBEST BLASTX NR: 10/13/02 T06489 e-110 probable peptidyl-prolyl isomerase (EC 5.2.1.8) FKBP77 - wheat emb|CAA68913.1| peptidylprolyl isomerase [Triticum aestivum]
12. Contig9351_s_atBEST BLASTX NR: 10/27/02 BAB17148.1 4e-60 (AP002868) hypothetical protein~similar to Oryza sativa chromosome 10, OSJNBA0051D19.6 [Oryza sativa (japonica}
13. Contig3588_s_atBEST BLASTX NR: <none>
14. Contig12784_atBEST BLASTX NR: 11/06/02 BAB89542.1 e-113 P0435B05.21 [Oryza sativa (japonica cultivar-group)]
15. EBpi01_SQ005_E16_atBEST BLASTX NR: 11/06/02 S10076 2e-14 histone H4.2 - slime mold (Physarum polycephalum) [Physarum polycephalum]
16. HV_CEa0012K03r2_x_atBEST BLASTX NR: 11/04/02 AAF02295.1 4e-24 (AF093006) PR-4 [Triticum aestivum]
17. HW03F16u_s_atBEST BLASTX NR: <none>
18. HVSEMeb0012C16r2_s_atBEST BLASTX NR: 11/08/02 AAL34937.1 1e-38 Putative purple acid phosphatase [Oryza sativa]
19. Contig4922_s_atBEST BLASTX NR: 10/26/02 T00607 2e-72 hypothetical protein At2g02560 [imported] - Arabidopsis thaliana gb|AAC18930.1| unknown protein [Arabidopsis thaliana]
20. Contig2333_s_atBEST BLASTX NR: 11/08/02 NP_190930.1 2e-94 inorganic pyrophosphatase-like protein; protein id: At3g53620.1, supported by cDNA: 40186. [Arabidopsis thaliana]
21. Contig7520_s_atBEST BLASTX NR: 11/08/02 AAK67149.1 8e-20 silencing group B protein [Zea mays]
22. Contig7618_atBEST BLASTX NR: 11/04/02 AAM63085.1 7e-31 putative ubiquinol-cytochrome-c reductase [Arabidopsis thaliana]
23. Contig8899_atBEST BLASTX NR: 10/13/02 NP_565645.1 1e-42 expressed protein; protein id: At2g27290.1, supported by cDNA: 32664. [Arabidopsis thaliana]
24. Contig3201_s_atBEST BLASTX NR: 10/27/02 NP_172334.1 1e-14 hypothetical protein; protein id: At1g08580.1 [Arabidopsis thaliana]

Downregulated genes:
1. Contig13036_atBEST BLASTX NR: 10/02/02 NP_191352.1 2e-12 (NM_115655) putative protein; protein id: At3g57930.1 [Arabidopsis thaliana]
2. HV_CEb0003B21r2_s_atBEST BLASTX NR: <none>
Appendix 5: Genes changed more than 3 fold in Greina

3. Contig4646_atBEST BLASTX NR: 10/02/02 NP_565878.1 6e-59 (NM_129356) expressed protein; protein id: At2g37990.1, supported by cDNA: 40538. [Arabidopsis thaliana]

4. baak4o13_s_atBEST BLASTX NR: <none>

5. EBro03_SQ007_P24_atBEST BLASTX NR: <none>

6. Contig17247_atBEST BLASTX NR: 11/07/02 AAD16139.1 9e-05 (AF096299) DNA-binding protein 2 [Nicotiana tabacum]

7. HS16L14r_x_atBEST BLASTX NR: <none>

8. HVSMEi0010i15r2_s_atBEST BLASTX NR: <none>

9. HM14E10r_atBEST BLASTX NR: <none>

10. Contig7096_atBEST BLASTX NR: 10/27/02 T02934 e-111 JUN-activation-domain-binding protein homolog - rice gb|AAC33765.1| jab1 protein [Oryza sativa subsp. indica]

11. HVSMEh0085C20r2_atBEST BLASTX NR: 10/02/02 BAB86225.1 4e-21 putative hydroxymethyltransferase [Oryza sativa (japonica cultivar-group)]

12. HB18P10r_atBEST BLASTX NR: <none>

13. HVSMEh0089C05r2_x_atBEST BLASTX NR: <none>

14. Contig17441_atBEST BLASTX NR: 10/02/02 AAL58959.1 2e-16 (AC091811) putative dehydrogenase [Oryza sativa]

15. HY04M24u_s_atBEST BLASTX NR: <none>

16. HO04D06S_atBEST BLASTX NR: 11/06/02 AAK71467.1 2e-42 (AY038983) nuclear transport factor 2 [Aspergillus nidulans]

17. HVSMEh0091C12r2_atBEST BLASTX NR: 11/04/02 AAL83672.1 5e-26 (AC092263) unknown protein [Oryza sativa (japonica cultivar-group)]

18. HVSMEg0007G16r2_atBEST BLASTX NR: <none>

19. Contig6376_atBEST BLASTX NR: 10/04/02 BAB78500.1 8e-62 (AB070259) 26S proteasome regulatory particle non-ATPase subunit5 [Oryza sativa (japonica cultivar-group)]

20. HD12C08r_atBEST BLASTX NR: <none>

21. S0000800041F09F1_atBEST BLASTX NR: <none>

22. EBro08_SQ011_K16_atBEST BLASTX NR: 11/06/02 AAK60140.1 2e-21 (AF365406) ribosomal protein L39 [Candida albicans]
23. Contig2517_s_atBEST BLASTX NR: 11/06/02 P23951 e-136 26 kDa endochitinase 2 precursor (CHI-26) pir||A38664 chitinase (EC 3.2.1.14) precursor - barley

24. Contig18946_atBEST BLASTX NR: <none>

25. HV_CEa0009C07r2_atBEST BLASTX NR: <none>

26. Contig15886_atBEST BLASTX NR: <none>

27. rbags21i05_s_atBEST BLASTX NR: 11/08/02 P35686 4e-36 40S RIBOSOMAL PROTEIN S20 pir||S38356 ribosomal protein S20, cytosolic - rice

28. HO05G15S_atBEST BLASTX NR: <none>

29. HVSMEn0019L21f_atBEST BLASTX NR: 11/08/02 AAL35760.1 5e-37 (AF370729) CBF-like protein [Secale cereale]
Appendix 6: Genes changed more then 3 fold in Golin

KP4 compared to wt

Upregulated genes:

1. Contig18527_atBEST BLASTX NR: 11/08/02 EAA04717.1 3e-68 (AAAB01008807) agCP3941 [Anopheles gambiae str. PEST]
2. Contig2433_s_atBEST BLASTX NR: 10/13/02 CAB71336.1 e-112 putative acid phosphatase [Hordeum vulgare subsp. vulgare]
3. Contig3946_atBEST BLASTX NR: 10/26/02 BAA89423.1 5e-67 allyl alcohol dehydrogenase [Nicotiana tabacum]
5. Contig4772_s_atBEST BLASTX NR: 11/08/02 Q03200 3e-23 LIGHT REGULATED PROTEIN PRECURSOR pir|S33632 lir1 protein - rice
6. Contig5974_s_atBEST BLASTX NR: 11/08/02 Q01482 1e-24 WIR1A PROTEIN pir|T06988 pathogen-induced protein WIR1A - wheat
7. Contig494_atBEST BLASTX NR: 10/02/02 S53518 1e-45 histone H2A.2 - wheat dbj|BAA07276.1| protein H2A [Triticum aestivum]
8. Contig25140_atBEST BLASTX NR: 11/07/02 AAK83858.1 2e-54 (AF395587) ribosomal protein L18 [Spodoptera frugiperda]
9. Contig628_x_atBEST BLASTX NR: 11/06/02 AAB18209.1 e-104 chlorophyll a/b-binding protein WCAB precursor [Triticum aestivum]
10.Contig22418_atBEST BLASTX NR: 11/08/02 EAA05461.1 e-104 (AAAB01008823) agCP10585 [Anopheles gambiae str. PEST]
11.Contig21607_atBEST BLASTX NR: 01/29/03 NP_502087.1 1e-27 Phosphate carrier, mitochondrial precursor (36.7 kD) [Caenorhabditis elegans]
12.Contig3667_s_atBEST BLASTX NR: 11/06/02 T06179 e-164 myb-related protein - barley emb|CAA61021.1| GAMyb protein [Hordeum vulgare subsp. vulgare]
13.Contig13265_atBEST BLASTX NR: 11/07/02 AAM92825.1 4e-97 unknown protein [Oryza sativa (japonica cultivar-group)]
Appendix 6: Genes changed more then 3 fold in Golin

14. Contig6170_s_atBEST BLASTX NR: 10/04/02 BAB78620.1 4e-30 (AP002845) contains ESTs D47958(S13774),(S13774)~unknown protein [Oryza sativa (japonica cultivar-group)]

15. Contig4470_s_atBEST BLASTX NR: 10/29/02 AAD38399.1 1e-94 (AF155121) apoplastic invertase [Oryza sativa subsp. indica] [Oryza sativa (indica cultivar-group)]

16. Contig25195_atBEST BLASTX NR: 11/07/02 T04484 3e-53 probable chitinase (EC 3.2.1.14) - barley gb|AAA56787.1| chitinase

17. Contig3197_atBEST BLASTX NR: 11/08/02 T06800 9e-83 superoxide dismutase (EC 1.15.1.1) (Cu-Zn) 2, chloroplast - wheat gb|AAB67991.1| Cu/Zn superoxide dismutase [Triticum aestivum]

18. Contig8678_s_atBEST BLASTX NR: 10/04/02 T03589 e-107 probable aspartate kinase (EC 2.7.2.4) / homoserine dehydrogenase (EC 1.1.1.3) precursor - rice

19. Contig1637_atBEST BLASTX NR: 11/06/02 D38664 e-162 glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39) precursor - barley gb|AAA32939.1| (M62907) (1-3)-beta-glucanase [Hordeum vulgare]

20. Contig4545_s_atBEST BLASTX NR: 11/04/02 BAB64285.1 2e-20 P0005H10.22 [Oryza sativa (japonica cultivar-group)]

21. Contig2211_atBEST BLASTX NR: 11/06/02 P35792 1e-80 Pathogenesis-related protein PRB1-2 precursor pir||S52627 pathogenesis-related protein precursor - barley

22. HVSMEm0003G16r2_atBEST BLASTX NR: 11/04/02 AAK38084.1 3e-57 putative cytochrome P450 [Lolium rigidum]

23. Contig8678_atBEST BLASTX NR: 10/04/02 T03589 e-107 probable aspartate kinase (EC 2.7.2.4) / homoserine dehydrogenase (EC 1.1.1.3) precursor - rice

24. Contig18088_atBEST BLASTX NR: 11/08/02 S39045 2e-35 probable finger protein WZF1 - wheat dbj|BAA03901.1| (D16415) WZF1 [Triticum aestivum]

25. Contig3199_atBEST BLASTX NR: <none>


27. HM11K07r_atBEST BLASTX NR: <none>

28. Contig12407_atBEST BLASTX NR: 11/07/02 AAM64770.1 2e-24 (AY087214) unknown [Arabidopsis thaliana]
Appendix 6: Genes changed more than 3 fold in Golin

29. Contig23697_at BEST BLASTX NR: 11/06/02 S61413 1e-14 DNA-binding protein ABF1 - wild oat (fragment) emb|CAA88326.1| DNA-binding protein [Avena fatua]

30. Contig5922_at BEST BLASTX NR: 10/27/02 AAK54299.1 2e-99 putative thiolase [Oryza sativa (japonica cultivar-group)]

31. Contig2089_at BEST BLASTX NR: 10/13/02 AAD14440.1 4e-35 (AC005275) putative C-type U1 snRNP [Arabidopsis thaliana] emb|CAB77797.1| (AL161496) putative C-type U1 snRNP [Arabidopsis thaliana]

32. Contig4250_s_at BEST BLASTX NR: 10/13/02 AAF25968.1 1e-71 F6N18.8 [Arabidopsis thaliana]

33. Contig15820_at BEST BLASTX NR: 11/07/02 AAD31900.1 7e-28 (AF145482) putative serine/threonine protein kinase [Mesembryanthemum crystallinum]

34. Contig10407_at BEST BLASTX NR: 11/08/02 CAD41532.1 5e-13 (AL606629) OSJNBb0091E11.1 [Oryza sativa (japonica cultivar-group)]

35. Contig2189_s_at BEST BLASTX NR: 10/29/02 AAL40895.1 3e-26 phosphoethanolamine methyltransferase [Triticum aestivum]

36. Contig10709_at BEST BLASTX NR: 11/06/02 BAA90806.1 5e-44 ESTs C26000(C11448),AU082130(C11448) correspond to a region of the predicted gene. ~ Similar to mRNA for zinc-finger protein

37. Contig13350_at BEST BLASTX NR: 11/08/02 AAD28734.1 7e-65 beta-1,3-glucanase precursor [Triticum aestivum]

38. Contig4873_s_at BEST BLASTX NR: 11/04/02 NP_568447.1 2e-23 (NM_122357) putative protein; protein id: At5g24490.1, supported by cDNA: gi_13877770 [Arabidopsis thaliana]

39. Contig8940_s_at BEST BLASTX NR: 11/06/02 BAB84490.1 3e-22 (AP004194) contains EST AU162644(S20225)~unknown protein [Oryza sativa (japonica cultivar-group)]

40. HA28E09r_at BEST BLASTX NR: 10/27/02 P25877 3e-14 Photosystem II reaction center protein K precursor (PSII-K) pir||S28768 photosystem II protein psbK-barley chloroplast

41. Contig21096_at BEST BLASTX NR: <none>
42. Contig4976_at: BEST BLASTX NR: 11/06/02 BAC05572.1 e-123 (AP003332) contains EST D24834(R2633)~similar to Arabidopsis thaliana chromosome 5, At5g12010~unknown protein [Oryza

43. Contig3006_at: BEST BLASTX NR: 11/08/02 BAA95821.1 7e-34 EST AU082567(S21715) corresponds to a region of the predicted gene.~Similar to S.tuberosum ubiquinol~cytochrome c

44. Contig3315_s_at: BEST BLASTX NR: 10/13/02 AAM46894.1 2e-17 early drought induced protein [Oryza sativa (indica cultivar-group)]

45. Contig8369_at: BEST BLASTX NR: 11/08/02 AAD22495.3 4e-20 APETALA2 protein homolog HAP2 [Hyacinthus orientalis]

46. Contig20398_at: BEST BLASTX NR: 11/06/02 NP_565979.1 3e-53 (NM_129823) F-box protein ORE9, AtFBL7; protein id: At2g42620.1, supported by cDNA: gi_15420161 [Arabidopsis thaliana]

47. ChlorContig17_s_at: BEST BLASTX NR: 01/27/03 P41096 e-129 CHLOROPLAST 50S RIBOSOMAL PROTEIN L2

48. Contig5135_s_at: BEST BLASTX NR: 10/29/02 NP_567775.1 5e-71 putative protein; protein id: At4g27450.1, supported by cDNA: 35207., supported by cDNA: gi_14030722, supported by

49. Contig4054_s_at: BEST BLASTX NR: 11/06/02 P32938 3e-84 Pathogenesis-related protein 1C precursor pir|S18035 pathogenesis-related protein 1c precursor - barley

50. Contig2214_s_at: BEST BLASTX NR: 01/29/03 S37166 5e-81 pathogenesis-related protein 1a - barley emb|CAA52893.1| PR-1a pathogenesis related protein (Hv-1a) [Hordeum vulgare subsp.

51. Contig5002_at: BEST BLASTX NR: 01/29/03 NP_568877.1 2e-13 Expressed protein; protein id: At5g58375.1, supported by cDNA: 17375. [Arabidopsis thaliana]

52. Contig5537_at: BEST BLASTX NR: 11/06/02 BAB19864.1 2e-95 monosaccharide transporter 3 [Oryza sativa] dbj|BAC10381.1| monosaccharide transporter 3 [Oryza sativa (japonica]

53. Contig6353_at: BEST BLASTX NR: 11/04/02 BAB17203.1 5e-91 putative branched-chain amino acid aminotransferase protein [Oryza sativa (japonica cultivar-group)]
54. Contig14732_at: BEST BLASTX NR: 10/27/02 AAG30977.1  4e-23 (AC012396)  
unknown protein [Arabidopsis thaliana]

55. Contig4056_s_at: BEST BLASTX NR: 11/04/02 P32937  7e-46 Pathogenesis-related protein 1A/1B precursor pir||S18033 pathogenesis-related protein 1a precursor - barley

56. HVSMEc0019G06f_at: BEST BLASTX NR: 01/27/03 T11812  .012 hypothetical protein ORF35 - Norway spruce chloroplast gb|AAC95502.1| orf35 [Picea abies]

57. Contig5687_s_at: BEST BLASTX NR: 10/13/02 BAB12694.1  3e-09 (AP002746)  
putative zinc finger transcription factor [Oryza sativa (japonica cultivar-group)]

58. Contig19684_at: BEST BLASTX NR: 11/06/02 AAG13627.1  5e-35 (AC078840)  
putative hypersensitivity-related (hsr) protein [Oryza sativa (japonica cultivar-group)]

59. Contig23900_at: BEST BLASTX NR: 11/08/02 P29522  6e-50 ELONGATION FACTOR 1-BETA' pir||S35514 translation elongation factor eEF-1 beta chain - silkworm

60. rbaa19h21_s_at: BEST BLASTX NR: 10/02/02 Q40070  2e-33 Photosystem I 10 kDa polypeptide, chloroplast precursor pir||T06173 photosystem II 10K protein precursor - barley

61. Contig15524_at: BEST BLASTX NR: <none>

62. Contig7031_at: BEST BLASTX NR: 10/27/02 NP_568850.1  2e-32 bHLH protein; protein id: At5g57150.1, supported by cDNA: 266884. [Arabidopsis thaliana]

63. Contig3776_s_at: BEST BLASTX NR: 10/29/02 AAM74427.1  2e-18 (AC123594)  
Putative lipid transfer protein [Oryza sativa (japonica cultivar-group)]

64. Contig4901_s_at: BEST BLASTX NR: <none>

65. Contig3096_s_at: BEST BLASTX NR: 11/04/02 CAB86384.1  e-121 (AJ251304)  
allene oxide synthase [Hordeum vulgare subsp. vulgare]

66. Contig3968_at: BEST BLASTX NR: 11/08/02 AAK62365.1  e-129 Lon protease [Dichanthelium lanuginosum]

67. Contig13460_at: BEST BLASTX NR: 11/07/02 NP_191408.1  6e-83 (NM_115711)  
putative protein; protein id: At3g58490.1 [Arabidopsis thaliana]

68. Contig6958_s_at: BEST BLASTX NR: 11/06/02 BAC20671.1  7e-83 serine/threonine kinase-like protein [Oryza sativa (japonica cultivar-group)]

69. Contig926_x_at: BEST BLASTX NR: 11/06/02 AAA80589.1  e-116 chlorophyll a/b binding protein
70. Contig8185_atBEST BLASTX NR: 11/07/02 JA0172  e-113 ferredoxin-nitrite reductase (EC 1.7.7.1) precursor - maize (fragment)
71. Contig8115_s_atBEST BLASTX NR: 01/29/03 BAB91924.1  1e-71 putative phytochrome-associated protein [Oryza sativa (japonica cultivar-group)]
72. Contig6622_atBEST BLASTX NR: 10/27/02 AAK39571.1  3e-82 putative gamma-lyase [Oryza sativa]
73. HVSMEm0004L13r2_s_atBEST BLASTX NR: <none>
74. Contig4641_s_atBEST BLASTX NR: 11/07/02 AAF81309.1  9e-17 (AC061957) Contains a weak similarity to a farnesylated protein GMFP5 mRNA from Glycine max gb|U64916. ESTs
75. Contig11160_atBEST BLASTX NR: 11/08/02 AAM78094.1  2e-57 (AY125502) AT5g47240/MQL5_10 [Arabidopsis thaliana] gb|AAN18132.1| (BT000563) At5g47240/MQL5_10 [Arabidopsis thaliana]
76. Contig6682_atBEST BLASTX NR: 10/28/02 NP_683523.1  5e-051 expressed protein; protein id: At3g03270.2 [Arabidopsis thaliana] gb|AAF26101.1|AC012328_4 unknown protein [Arabidopsis thaliana]
77. Contig9265_atBEST BLASTX NR: 10/13/02 CAB61839.1  e-111 (AJ242803) putative serine/threonine phosphatase type 2c [Sporobolus stapfianus]
78. Contig15777_atBEST BLASTX NR: 11/06/02 CAD39351.1  1e-15 (AL662935) OSJNBa0094O15.19 [Oryza sativa (japonica cultivar-group)]
79. Contig3235_atBEST BLASTX NR: 10/02/02 AAD51733.1  e-125 methylenetetrahydrofolate reductase [Zea mays]
80. Contig4250_atBEST BLASTX NR: 10/13/02 AAF25968.1  1e-71 F6N18.8 [Arabidopsis thaliana]
81. Contig16386_atBEST BLASTX NR: 10/13/02 AAG21897.1  7e-68 putative disease resistance protein (3' partial) [Oryza sativa]
82. Contig7711_s_atBEST BLASTX NR: 10/26/02 CAC00545.1  6e-85 (AJ277085) putative low-affinity nitrate transporter [Nicotiana plumbaginifolia]
83. Contig3237_s_atBEST BLASTX NR: 01/29/03 AAD51733.1  3e-22 methylenetetrahydrofolate reductase [Zea mays]
84. Contig3235_s_atBEST BLASTX NR: 10/02/02 AAD51733.1  e-125 methylenetetrahydrofolate reductase [Zea mays]
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85. Contig4899_s_at BEST BLASTX NR: 11/08/02 AAF86307.1 2e-46 EF-hand Ca2+-binding protein CCD1 [Triticum aestivum]
86. Contig5711_at BEST BLASTX NR: 10/27/02 NP_176075.1 1e-63 integral membrane protein, putative; protein id: At1g57620.1, supported by cDNA: 17602. [Arabidopsis thaliana]
87. Contig4307_s_at BEST BLASTX NR: <none>
88. Contig2305_at BEST BLASTX NR: 11/04/02 T06190 e-100 lipoxygenase (EC 1.13.11.12) 2 - barley gb|AAC12951.1| methyljasmonate-inducible lipoxygenase 2 [Hordeum vulgare]
89. Contig11524_j5_at BEST BLASTX NR: <none>
90. Contig7483_at BEST BLASTX NR: 11/04/02 BAB21218.1 3e-59 (AP002913) putative DNA binding protein RAV2 [Oryza sativa (japonica cultivar-group)]
91. Contig4707__at BEST BLASTX NR: <none>
92. Contig981_s_at BEST BLASTX NR: 10/02/02 AAA80589.1 3e-95 chlorophyll a/b binding protein
93. Contig23391_at BEST BLASTX NR: 11/04/02 CAD41257.1 5e-10 (AL606627) OSJNBA0067K08.21 [Oryza sativa (japonica cultivar-group)]
94. Contig3361_at BEST BLASTX NR: 11/06/02 BAA89798.1 5e-82 (AB028183) OsNAC4 protein [Oryza sativa] dbj|BAB64820.1| (AP003411) development regulation gene OsNAC4 [Oryza sativa]
95. Contig5324_s_at BEST BLASTX NR: 11/08/02 T06521 1e-97 pitrilysin (EC 3.4.24.55) - garden pea gb|AAA81472.1| metalloendopeptidase
96. HB30024r_at BEST BLASTX NR: 10/31/02 AAM94294.1 1e-05 putative stripe rust resistance protein Yr10 [Sorghum bicolor]
97. Contig2992_s_at BEST BLASTX NR: 09/30/02 S48848 e-133 chitinase (EC 3.2.1.14) cht2b precursor - barley emb|CAA55345.1| chitinase [Hordeum vulgare subsp. vulgare]
98. Contig6773_at BEST BLASTX NR: 11/08/02 BAC16043.1 4e-82 (AP004316) contains ESTs AU173886(S20483),AU173885(S20483)-phytochelatin
99. Contig21224_at BEST BLASTX NR: 11/06/02 AAM14877.1 3e-33 (AC003105) unknown protein [Arabidopsis thaliana]
<table>
<thead>
<tr>
<th>Entry</th>
<th>Gene Information</th>
<th>Description</th>
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<tr>
<td>100. Contig19861_at</td>
<td>BEST BLASTX</td>
<td>Hypothetical protein (imported) - sorghum (fragment) ( \text{gb</td>
</tr>
<tr>
<td>101. HVSMEa0019D22f_s</td>
<td>BEST BLASTX</td>
<td>Ribosomal protein L3 (S38359) - rice</td>
</tr>
<tr>
<td>102. Contig4271_at</td>
<td>BEST BLASTX</td>
<td>N-hydroxycinnamoyl/benzoyltransferase [Ipomoea batatas]</td>
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<td>103. Contig9981_at</td>
<td>BEST BLASTX</td>
<td>Acetyl-CoA carboxylase, putative; protein id: At1g36050.1 [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>104. Contig5826_at</td>
<td>BEST BLASTX</td>
<td>Tubulin beta-1 chain (S32668) - fern (Anemia phyllitidis)</td>
</tr>
<tr>
<td>105. Contig13562_at</td>
<td>BEST BLASTX</td>
<td>Hypothetical protein similar to Arabidopsis thaliana chromosome 4, At4g21700 [Oryza sativa (japonica)]</td>
</tr>
<tr>
<td>106. Contig3413_s</td>
<td>BEST BLASTX</td>
<td>Blue copper-binding protein homolog [Triticum aestivum]</td>
</tr>
<tr>
<td>107. Contig3209_s</td>
<td>BEST BLASTX</td>
<td>ESTs AU062670(C30068) similar to Arabidopsis thaliana chromosome 2, At2g30500 unknown protein [Oryza sativa]</td>
</tr>
<tr>
<td>108. Contig619_at</td>
<td>BEST BLASTX</td>
<td>ESTs AU058067(E20733), AU058070(E20873) correspond to a region of the predicted gene. Similar to Populus tremuloides</td>
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115. Contig8214_atBEST BLASTX NR: 11/08/02 BAB89591.1 6e-75 P0446G04.14 [Oryza sativa (japonica cultivar-group)]

116. Contig6502_atBEST BLASTX NR: 01/29/03 AAK92832.1 e-115 putative glycyl tRNA synthetase [Arabidopsis thaliana]

117. Contig3373_s_atBEST BLASTX NR: 11/06/02 NP_179460.1 4e-66 peptide transporter [Arabidopsis thaliana]

118. Contig26282_atBEST BLASTX NR: 11/08/02 AAF57081.1 2e-14 (AE003775) CG11314-PA [Drosophila melanogaster]

119. Contig13457_s_atBEST BLASTX NR: 11/06/02 NP_567210.1 8e-38 expressed protein; protein id: At4g01150.1, supported by cDNA: gi_14488087, supported by cDNA: gi_20147122, supported

120. Contig7388_atBEST BLASTX NR: 11/06/02 NP_201074.1 5e-66 peptide transporter [Arabidopsis thaliana]

121. Contig24309_atBEST BLASTX NR: 11/06/02 AAG00554.1 1e-06 (AF286475) retinitis pigmentosa GTPase regulator-like protein [Takifugu rubripes]

122. HVSEMe0013J23r2_s_atBEST BLASTX NR: 10/02/02 AAG46367.1 .021 (AY013869) antigen 38 [Trypanosoma cruzi]

123. Contig20580_atBEST BLASTX NR: 11/06/02 AAC36744.1 4e-40 33 kDa secretory protein [Oryza sativa] gb|AAC72363.1| 33 kDa secretory protein [Oryza sativa]

Downregulated genes:

1. Contig1927_atBEST BLASTX NR: 10/27/02 AAM08784.1 2e-58 Putative epimerase/dehydratase [Oryza sativa]

2. HV_CEbo0011G04r2_x_atBEST BLASTX NR: <none>

3. Contig25358_atBEST BLASTX NR: <none>
4. Contig5658_atBEST BLASTX NR: 10/13/02 Q9ZUC1 9e-90 Quinone oxidoreductase-like protein At1g23740 gb|AAC98029.1| (AC005990) Strong similarity to gb|U20808 auxin-induced protein
5. Contig19029_atBEST BLASTX NR: 11/08/02 AAD43561.1 7e-62 bacterial-induced peroxidase precursor [Gossypium hirsutum]
6. Contig23200_atBEST BLASTX NR: 11/07/02 AAM10317.1 8e-75 (AY091769) AT3g20810/MOE17_10 [Arabidopsis thaliana] gb|AAM78048.1| (AY125538) AT3g20810/MOE17_10 [Arabidopsis thaliana]
8. Contig5242_atBEST BLASTX NR: 11/04/02 BAB60931.1 4e-20 P0682B08.1 [Oryza sativa (japonica cultivar-group)] dbj|BAB67860.1| P0487H02.20 [Oryza sativa (japonica cultivar-group)]
9. HI04P16u_atBEST BLASTX NR: 11/06/02 NP_566652.1 1e-06 (NM_112887) expressed protein; protein id: At3g19970.1, supported by cDNA: gi_14532501 [Arabidopsis thaliana]
10. rbaal22i06_atBEST BLASTX NR: 10/13/02 BAA96219.1 3e-37 (AP002094) Similar to Medicago truncatula MtN3 (Y08726) [Oryza sativa (japonica cultivar-group)]
11. HZ54E21r_s_atBEST BLASTX NR: 11/04/02 Q9FVI1 4e-16 Actin-depolymerizing factor 2 (ADF 2) gb|AAG16974.1|AF183904_1 actin-depolymerizing factor 2 [Petunia x hybrida]
12. Contig5949_s_atBEST BLASTX NR: <none>
13. HV_CEb0008E13r2_atBEST BLASTX NR: 11/04/02 AAL67584.1 2e-26 (AC018929) putative inositol 1,3,4-trisphosphate 5/6-kinase [Oryza sativa]
14. Contig12480_atBEST BLASTX NR: 10/27/02 AAD25755.1 1e-90 (AC007060) T5I8.13 [Arabidopsis thaliana]
15. rbaal10h14_atBEST BLASTX NR: 10/02/02 T03731 2e-53 abscisic acid-induced protein - rice emb|CAA61981.1| EFA27 for EF hand, abscisic acid, 27kD [Oryza sativa]
17. Contig7534_atBEST BLASTX NR: 10/29/02 BAC10350.1 e-106 (AP003818) putative serine/threonine kinase [Oryza sativa (japonica cultivar-group)]

18. Contig22627_atBEST BLASTX NR: 11/06/02 AAL85324.1 e-103 (AF472629) putative iron-phytosiderophore transporter [Hordeum vulgare]

19. HD08L01r_x_atBEST BLASTX NR: 10/31/02 NP_175778.1 4e-20 26S proteasome AAA-ATPase subunit RPT1a; protein id: At1g53750.1, supported by cDNA: 36419., supported by cDNA:

20. Contig17320_atBEST BLASTX NR: 11/04/02 AAK92612.1 2e-53 (AC078944) Putative cytochrome P-450 like protein [Oryza sativa] [Oryza sativa (japonica cultivar-group)]

21. Contig7450_atBEST BLASTX NR: 11/08/02 NP_199517.1 6e-10 putative protein; protein id: At5g47060.1 [Arabidopsis thaliana]

22. HW01F10u_s_atBEST BLASTX NR: 11/08/02 BAB84606.1 7e-38 putative Ser/Thr protein phosphatase [Oryza sativa (japonica cultivar-group)]

23. HD04N15r_s_atBEST BLASTX NR: <none>

24. HU10l03u_s_atBEST BLASTX NR: 11/04/02 P27124 3e-08 FK506-binding protein 4 (Peptidyl-prolyl cis-trans isomerase) (PPIase) (Rotamase) (p59 protein) (HSP binding

25. Contig10719_atBEST BLASTX NR: 10/27/02 AAG50807.1 2e-30 (AC079281) hypothetical protein [Arabidopsis thaliana]

26. Contig15432_atBEST BLASTX NR: 01/29/03 NP_564570.1 1e-89 expressed protein; protein id: At1g50450.1, supported by cDNA: gi_14517415, supported by cDNA: gi_20453276 [Arabidopsis]

27. HY05O16u_s_atBEST BLASTX NR: 11/04/02 AAG46115.1 2e-07 (AC073166) putative sugar transporter [Oryza sativa]

28. HVSMEg0001H24r2_s_atBEST BLASTX NR: 11/08/02 BAB92188.1 6e-13 P0520B06.12 [Oryza sativa (japonica cultivar-group)]

29. EBem10_SQ001_N01_x_atBEST BLASTX NR: <none>

30. Contig6788_atBEST BLASTX NR: 11/08/02 T50779 6e-29 copper chaperone homolog CCH [imported] - rice gb|AAF15285.1|AF198626_1 copper chaperone homolog CCH [Oryza sativa]

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31. Contig3900_at BEST BLASTX NR: 11/06/02 T06207 e-137 cysteine proteinase (EC 3.4.22.-) - barley embryo [CAB09698.1] cysteine proteinase [Hordeum vulgare subsp. vulgare]

32. rbags21i05_s_at BEST BLASTX NR: 11/08/02 P35686 4e-36 40S ribosomal protein S20, cytosolic - rice

33. HVSMEa0004110f2_s_at BEST BLASTX NR: <none>

34. Contig8666_at BEST BLASTX NR: 10/02/02 NP_566373.1 e-102 expressed protein; protein id: At3g10420.1, supported by cDNA: gi_15810178 [Arabidopsis thaliana]

35. Contig10122_at BEST BLASTX NR: 10/13/02 AAL68843.1 e-106 (AF466199) aminoalcoholphosphotransferase [Sorghum bicolor]

36. rbaal14f06_s_at BEST BLASTX NR: 10/04/02 CAB97352.1 2e-04 (AJ249144) MADS-box protein 5 [Hordeum vulgare subsp. vulgare]

37. rbaal15n16_at BEST BLASTX NR: <none>

38. Contig14507_at BEST BLASTX NR: 11/08/02 BAB90336.1 2e-12 (AP003535) zinc-induced protein-like [Oryza sativa (japonica cultivar-group)]

39. Contig149_at BEST BLASTX NR: 10/13/02 P26517 e-119 Glyceraldehyde 3-phosphate dehydrogenase, cytosolic - DEBHG glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 4.1.-.-)

40. Contig3691_at BEST BLASTX NR: 11/06/02 T04419 e-135 aleurone ribonuclease (EC 3.1.-.-) - barley (fragment) gb|AAB58718.1| aleurone ribonuclease [Hordeum vulgare]

41. Contig7557_s_at BEST BLASTX NR: 11/08/02 BAC16110.1 2e-95 phosphoglucomutase-like protein [Oryza sativa (japonica cultivar-group)]

42. Contig11428_at BEST BLASTX NR: 10/13/02 AAD17855.1 6e-75 sigma factor SIG2B; ZmSIG2B [Zea mays]

43. HE01K03u_at BEST BLASTX NR: 11/06/02 NP_568749.1 7e-28 (NM_124480) expressed protein; protein id: At5g51010.1, supported by cDNA: 27885., supported by cDNA: gi_15292668,

44. Contig3299_s_at BEST BLASTX NR: 01/29/03 AAC72193.1 e-110 pyruvate dehydrogenase E1 beta subunit isoform 2 [Zea mays]
45. HU10J04u_atBEST BLASTX NR: 11/04/02 Q03033 1e-22 ELONGATION FACTOR 1-ALPHA (EF-1-ALPHA) pir||JC1454 translation elongation factor eEF-1 alpha chain - wheat

46. Contig13552_atBEST BLASTX NR: 01/29/03 CAD40663.1 8e-93 OSJNBB0118P14.1 [Oryza sativa (japonica cultivar-group)]

47. rbaal1m15_atBEST BLASTX NR: 11/08/02 T46636 2e-25 glycine dehydrogenase (decarboxylating) (EC 1.4.4.2) [imported] - Hordeum sp. x Triticum sp

48. Contig407_x_atBEST BLASTX NR: <none>

49. Contig20144_s_atBEST BLASTX NR: <none>

50. Contig16774_atBEST BLASTX NR: 11/08/02 NP_197776.1 8e-17 (NM_122293) cleavage and polyadenylation specificity factor; protein id: At5g23880.1, supported by cDNA: gi_9082325

51. Contig15205_atBEST BLASTX NR: 10/31/02 AAM34535.1 e-106 (AC113947) putative WD-40 repeat protein [Oryza sativa (japonica cultivar-group)]