Transgenic Resistance to Pathogens and Pests

Margareta Melander
Department of Crop Science
Alnarp

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Abstract

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Pathogens and pests constantly threaten plants and cause crop losses of significant economic importance for agricultural production worldwide. One way to reduce the damage caused by pathogens and pests is the development of new, resistant cultivars. However, conventional resistance breeding often suffers from limited access to suitable resistance sources. The development of gene technology has drastically increased the availability of genes conferring resistance, which can be derived from non-related plant species as well as non-plant sources. In this thesis three attempts to improve the resistance against a virus, some fungi and an insect by genetic engineering are described.

In the first example an approach to develop potato with improved resistance against *Potato mop-top virus* was evaluated. A modified version of a gene involved in viral movement from *Potato mop-top virus* was transformed into potato. Transgenic plants shown to transcribe the inserted sequence were evaluated in a field trial, where increased resistance to natural infection by *Potato mop-top virus* could be demonstrated.

The second example is the evaluation of doubled haploids of oilseed rape transformed with a chitinase and a β-1,3-glucanase gene from barley. Although the barley chitinase and β-1,3-glucanase did show some antifungal effects when evaluated *in vitro*, no significant effects could be demonstrated when the transgenic plants were challenged with four different oilseed rape fungi in greenhouse assays. The doubled haploids were also evaluated for stability of the transgenic inserts as well as their expression during five subsequent generations.

The third example evaluates pea lectin as a possible resistance factor against pollen beetles in oilseed rape. In feeding assays with pollen beetle larvae several plant proteins with potential insecticidal activity were tested. In these assays pea lectin was shown to have significant detrimental effects both on growth and survival of the larvae. Therefore, transgenic oilseed rape plants were produced that expressed pea lectin in pollen. When pollen beetle larvae were fed anthers from these plants significant reduction in larval weight was observed as well as some effect on survival.

Keywords: fungal resistance, insect resistance, molecular breeding, pathogen-derived resistance, PR proteins, transformation, virus resistance

Author's address: Margareta Melander, Plant Science Sweden AB, Herman-Ehles väg 3-4, SE 268 31 Svalöv, Sweden. E-mail: margareta.melander@plantscience.se

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List of papers

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The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Melander M, Lee M & Sandgren M. 2001. Reduction of potato mop-top virus accumulation and incidence in tubers of potato transformed with a modified triple gene block gene of PMTV. *Molecular Breeding* 8: 197-206.

II. Melander M, Kamnert I, Happstadius I, Liljeroth E & Bryngelsson T. Oilseed rape transformed with chitinase and β-1,3-glucanase genes from barley – stability of transgene integration, expression and effect on fungal infection. (Manuscript).

III. Åhman I & Melander M. 2003. Potato proteins, and other plant proteins, as potential transgenic resistance factors to pollen beetles in oilseed rape. *Annals of Applied Biology* 143: 253-260.

IV. Melander M, Åhman I, Kamnert I & Strömdahl A-C. 2003. Pea lectin expressed transgenically in oilseed rape reduces growth rate of pollen beetle larvae. *Transgenic Research* 12: 555-567.

Abbreviations

2-5 Aase, 2'-5' oligoadenylate synthetase; 2-5A, 2'-5' oligoadenylate; *Avr,* avirulence; Bt, *Bacillus thuringiensis*; CaMV, *Cauliflower mosaic virus*; CMV, *Cucumber mosaic virus*; con A, concanavalin A; CP, coat protein; CPMR, coat protein mediated resistance; CpTI, cowpea trypsin inhibitor; HR, hypersensitive reaction; ISR, induced systemic resistance; JA, jasmonic acid; MP, movement protein; PDR, pathogen-derived resistance; PI, proteinase inhibitor; PKR, RNA-dependent protein kinase; PLRV, *Potato leaf roll virus*; PMTV, *Potato mop-top virus*; PR protein, pathogenesis-related protein; PRSV, *Papaya ringspot virus*; PTGS, post-transcriptional gene silencing; PVX, *Potato virus X*; PVY, *Potato virus Y*; *R*-gene, resistance gene; RIP, ribosome-inactivating protein; RNAi, RNA interference; RNAseL, ribonuclease L; SA, salicylic acid; SAR, systemic acquired resistance; TGB, triple-gene-block; TMV, *Tobacco mosaic virus*; TRV, *Tobacco rattle virus*; TSWV, *Tomato spotted wilt virus*; WClMV, *White clover mosaic virus*; WGA, wheat germ agglutinin; WMV2, *Watermelon mosaic virus 2*; ZYMV, *Zucchini yellow mosaic virus*

Introduction

Attacks by plant pathogens and pests always have been and still is a threat to agricultural production. Plant diseases have had historical implications, where one of the most well known examples is the potato famine in Ireland of 1845-1847 caused by *Phytophthora infestans*, which spurred a huge wave of emigration to North America. In 1870 the coffee industry in Ceylon was completely destructed due to *Hemileia vastatrix* and a century later the same fungus hit many countries in South and Central America. More recent examples include gemini-virus infections wiping out vegetable crops in the Caribbean and Florida in the 1990s (James *et al.*, 1990; Moffat, 2001).

Agriculture worldwide suffers from production losses due to pathogens and pests. The crop losses in 1988-1990 of eight principal food and cash crops, which are grown on about 50% of the global cultivated land, was estimated to 29% of the attainable production, corresponding to 167 billion US$. It is estimated that without the application of various crop protection measures, crop losses due to pathogens and pests globally would have reached 40% of the attainable production (Oerke *et al.*, 1999).

Agrochemicals are commonly used to control many plant pests and diseases. In 1990 about 19 billion US$ were spent globally on insecticides and fungicides (Oerke *et al.*, 1999). Other alternatives, or complements, to protect plants include phytosanitary measures such as healthy seed, appropriate crop rotation and tillage methods.

Another very important factor in controlling plant pathogens and pests is the use of resistant cultivars. Classical plant breeding is continuously aiming at improving plant resistance to diseases and pest attacks by introgression of resistance from the same or closely related species. However, in many instances there are no sources of resistance available or the resistance is tightly genetically linked with undesirable traits.

The development of gene technology has drastically increased the availability of genes conferring resistance, since they can now be derived from non-related plant species as well as non-plant sources. Additionally, the problem with co-transfer of linked undesirable characters from the gene source as is often the case in traditional cross-breeding can be avoided. Gene technology in combination with the increasing knowledge of various plant defence mechanisms contributes with new possible strategies that might be used for improvement of resistance.

The aim of this thesis was to evaluate the possibilities to introduce transgenic resistance against some pathogens and pests that affect potatoes and oilseed rape in Northern Europe and elsewhere. The targeted parasites were *Potato mop-top virus* in potatoes and four fungal pathogens as well as pollen beetles in oilseed rape.

Plant defence against pathogens and pests

Plants are constantly exposed to potential pathogens and pests. However, most encounters result in resistance by the plant and only exceptionally the outcome is disease. Most pathogens and pests are very specialised and only affect a limited range of host plants, often only a single species. This specialisation is due to the need for certain host factors for host recognition and to the plant's defence system. The first line of defence includes constitutive barriers such as wax layers, cell walls and chemical defence compounds. These barriers are present in the plant prior to any attack by pathogens or pests and confer a broad resistance to a wide variety of parasites (Takken & Joosten 2000).

If the first line of defence is overcome a second line of induced responses may stop the invader. Further parasite attack on plants can cause either a compatible response with a successful infection of the plant or an incompatible response where the plant becomes resistant to the parasite. In compatible interactions, the parasite is often recognized too late and the outcome will be a diseased plant. In the case of an incompatible interaction, the plant rapidly recognizes the parasite and induces resistance mechanisms, which act very effectively against the invader (Métraux, 2001).

Induced responses to viruses, fungi, bacteria and certain insects generally require a specific interaction between plant and pathogen, which is described by the 'gene-for-gene' hypothesis (Flor, 1971). For a plant to be resistant to a specific pathogen race the plant must carry a resistance (*R*) gene that corresponds to an avirulence (*Avr*) gene of the pathogen. The *R*-genes encode receptors for *Avr*-specified ligands, and by a direct or indirect interaction between *R*-gene and *Avr*-gene products the induced defence response is triggered (Takken & Joosten, 2000; Jones, 2001).

The induced resistance response to pathogens can be divided into a local response at the site of infection and a systemic response in remotely located, yet unaffected plant parts. This induced systemic response is referred to as systemic acquired resistance (SAR) (Métraux *et al.*, 2002).

The local response includes modifications of the cell wall composition in surrounding cells as well as synthesis of antimicrobial compounds such as phytoalexins and pathogenesis-related (PR) proteins. Another feature of the primary, local response is the activation of a controlled process of cell death, which is called the hypersensitive reaction (HR). By HR the pathogen will be trapped in dead cells and is thus prevented from spreading from the original site of infection (Heil & Bostock, 2002).

The initial local defence response is followed by a signal spreading throughout the plant that induces changes in gene expression in uninfected distant parts of the plant. This systemic response involves the onset of PR protein production and sometimes also phytoalexin synthesis (Heil & Bostock, 2002). The systemic signal can also alert the remote tissue to a faster defence response, *e.g.* faster induction of cell wall lignification, after challenge infection (Métraux *et al.*, 2002).

Systemic acquired resistance is regarded to be active against the major pathogen groups, *i.e*. viruses, fungi and bacteria, and is conferring resistance not only to the pathogen initiating the response but also to other pathogens. This resistance is rather non-specific and long lasting (Heil & Bostock, 2002). SAR has been demonstrated in over 30 plant species belonging to both mono- and dicotyledonous plant families (Métraux *et al.*, 2002).

PR proteins induced by SAR can be divided into 17 different families (van Loon & van Strien, 1999; Christensen *et al*., 2002), where the function still is unknown for members of many families. Some families contain proteins with chitinase or β-1,3-glucanase activity. These proteins hydrolyse chitin and β-1,3-glucan, respectively, which are important components of many fungal cell walls. Chitinases and β-1,3-glucanases have also been shown to possess antifungal activity *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993).

Salicylic acid (SA) is a key component of the signal transduction pathway leading to SAR. The levels of SA increase both locally and systemically after an infection event and the SAR induction is preceded by an increase of SA in the phloem. Transgenic plants that overexpress a bacterial salicylate hydroxylase and thus cannot accumulate SA are blocked in SAR response. However, it is unlikely that SA is the primary signalling molecule. In addition to SA other molecules, such as the volatile methyl salicylate acting as a signal also between plants (Shulaev *et al*., 1997), seem to be involved in systemic signalling for SAR (Heil & Bostock, 2002).

The SAR signalling pathway is not a linear chain of events but rather a complex network, where several pathways interact causing defence responses targeted at various pathogens (Métraux *et al.*, 2002). One distinct branching point occurs downstream of SA with one branch with PR protein production involved in resistance to fungal and bacterial pathogens and another branch conferring resistance to viruses. The virus-specific branch includes, depending on virus, different mechanisms for virus resistance such as inhibition of replication, inhibition of cell-to-cell movement and inhibition of long-distance movement (Murphy *et al.*, 2001).

An additional defence process involved in virus resistance is an RNA degradation mechanism called post-transcriptional gene silencing (PTGS). This is a way for plants, as well as other organisms, to degrade aberrant, unwanted excess or foreign RNA, such as viruses, in a homology-dependent manner. The elicitor for this defence reaction is double-stranded RNA that is produced during virus replication. The double-stranded RNA is degraded into small interfering RNAs of ~21 nucleotides by the enzyme Dicer-1. The small interfering RNAs then serve as a guide that in a complex with Dicer-1 and other cellular factors degrade RNA molecules with homology to the elicitor RNA. This degradation process spreads within the plant in a systemic fashion from the originally invaded cell (Waterhouse *et al.*, 2001).

While SA-dependent SAR is generally induced by and active against various pathogens, a wound response is active mainly against herbivores. This response is induced by insect feeding and wounding and results in a local defence reaction at

the wound site as well as systemic responses that are transmitted throughout the plant. The defence response can be divided into direct and indirect responses. The direct responses include the production of antinutritive and toxic compounds such as proteinase inhibitors, polyphenol oxidases, alkaloids, terpenoids and phenolics as well as the formation of physical barriers. The indirect responses are constituted by the production of volatile compounds that attract predators and parasitoids of the attacking insects and repel herbivorous insects (Kessler & Baldwin, 2002).

Jasmonic acid (JA) is a central signalling molecule of induced plant responses to herbivores. Insect feeding or mechanical wounding is triggering the induced response where both oligosaccharides and oligogalacturonides released from damaged cell walls as well as systemin have been proposed as the primary elicitor. The second step of the cascade is the release of linolenic acid from membrane lipids, which is then enzymatically converted to JA. Genes encoding proteinase inhibitors and enzymes involved in production of volatile compounds or secondary compounds, such as nicotine and numerous phenolics, as well as other defence-related compounds are transcriptionally activated by JA (Heil & Bostock, 2002).

Although the SA pathway is generally associated with pathogen defence and the JA pathway with insect defence there are many exceptions to this rule. Defence mechanisms to certain pathogens, especially to necrotrophic and saprophytic fungi and bacteria are independent of SA. Instead, systemic defence responses to necrotrophs, termed induced systemic resistance (ISR), are induced by the JA pathway together with an ethylene-induced pathway. Defence responses to phloem feeding insects are induced by the JA/ethylene pathway as well as the SA pathway. Thus, the SA, JA and ethylene pathways may control different sets of plant parasites but may also overlap (Walling, 2000; Hammond-Kosack & Parker, 2003; Rojo *et al.*, 2003).

The different signalling pathways are also influencing each other and there is evidence for both synergistic and antagonistic effects. Cross-resistance occurs when resistance that is induced by one set of enemies, *e.g.* pathogens, is active also against other groups, *e.g.* herbivores. Antagonistic effects have been demonstrated where plants expressing resistance against microbial pathogens become more susceptible to insect attacks and *vice versa* (Pieterse *et al.*, 2001; Heil & Bostock, 2002).

Plant reproductive organs, such as seeds and tubers, are rich sources of energy and amino acids that are needed for subsequent growth. These vital organs are very important to protect against parasites if the plant is to survive and reproduce. Plants have thus developed efficient constitutive defence mechanisms, such as morphological (*e.g.* thorns), physical (*e.g.* thick seed coats) and chemical protections. The chemical defence includes both toxic low molecular weight compounds (*e.g.* saponins and alkaloids) and toxic or antinutritive proteins. One group of toxic/antinutritive proteins is the lectins, which are often found in substantial amounts in storage organs as they simultaneously serve as storage proteins. Also proteinase inhibitors are included in this constitutive defence and is an example that the same kind of compounds may be involved in induced as well as constitutive defence (Peumans & Van Damme, 1996; Murdock & Shade, 2002).

Despite the plant defence array against pathogens and pests, including both constitutive and induced defences, plants are still not always able to combat the invader. Even though new plant defence responses have evolved, *e.g*. new *R*-genes, there is also a constant evolution of the parasites so that defence mechanisms may be overcome. One advantage of gene technology is that new defence factors that the parasites have not encountered before and have thus not yet started to adapt to can be introduced into plants.

Resistance by genetic engineering often takes advantage of components that are included in normal defence responses against pathogens and pests but also other approaches utilising non-plant sources have been applied. The first examples of transgenic resistance against pathogens and pests generally included the expression of single proteins by constitutive promoters. Since then more sophisticated resistance mechanisms have been utilized sometimes involving expression of more than one gene. Additionally, the discovery of new promoters, *e.g*. tissue specific or parasite induced, has contributed so that the transgenic resistance can be more specifically controlled.

Transgenic resistance to viruses

The strategies for conferring virus resistance in plants by genetic engineering can be divided into two groups. The first group includes various strategies in which a plant is transformed with a gene, or part of a gene, from the virus against which resistance is desired. This strategy is named pathogen-derived resistance (PDR) and can normally be explained by the virus-derived transgene interfering with essential steps in the life cycle of the infecting virus. The second group includes various strategies that do not include viral genes, such as host resistance genes and other genes involved in defence responses.

PDR can be subdivided into different groups depending on which viral gene is involved. The most commonly used genes are coat protein genes, replicase genes and movement protein genes.

Coat protein

The first example of virus resistant transgenic plants was published by Powell-Abel *et al.* (1986) describing tobacco plants transformed with the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) displaying improved resistance against TMV. Seedlings of transgenic plants that expressed the coat protein demonstrated significant delay in symptom development when inoculated with TMV. The delay in symptom development was correlated with the expression level of the CP.

Since this first demonstration that introduction of the coat protein gene of a virus can confer resistance to the corresponding virus, this approach has been extensively used for resistance against numerous viruses belonging to different groups in many crop plants as well as ornamentals (*e.g.* Pang *et al.*, 2000; Lehman

et al., 2003; Liao *et al.*, 2004; Yang *et al.*, 2004). The approach of CP-mediated resistance (CPMR) has also been shown to be commercially applicable. In 1992 *Papaya ringspot virus* (PRSV) was discovered in Puna, Hawaii and the Hawaiian papaya industry was soon severely damaged. Fortunately, experiments with CPMR against the virus had already been initiated, and in 1998 two varieties were available for commercial use and the papaya industry started to recover (Gonsalves, 2002). Other commercial examples include squash resistant to *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus 2* (WMV2) as well as potato resistant to *Potato virus Y* (PVY) (http://www.aphis.usda.gov/brs/not_reg.html; 21-Aug-2004).

The resistance mechanism involved in CPMR seems to vary between different viruses. For some viruses the resistance is protein-mediated requiring the expression of the coat protein (Bendahmane *et al.*, 1997; Lehman *et al.*, 2003). For TMV it has been shown that the transgenically produced CP has a tendency to form aggregates. It is proposed that this CP prevents virus disassembly and thus viral infection by driving the virus assembly-disassembly reaction towards assembly (Bendahmane & Beachy, 1999). For other viruses resistance is mediated also by untranslatable forms of the CP-gene implicating an RNA-based mechanism. It has been shown that this mechanism is due to post-transcriptional gene silencing (Masmoudi *et al.*, 2002; Liao *et al.*, 2004).

Replicase protein

Another type of genes used for conferring PDR are genes encoding viral replicase proteins. By introducing either full-length, read-through portions, truncated or mutated versions of replicase genes resistance to at least 14 different viruses representing 10 different taxonomic groups have been accomplished (Palukaitis & Zaitlin, 1997). Replicase genes are structured differently in different virus genera, and this has an influence on choosing a suitable sequence for replicase-mediated PDR.

Introduction of full-length replicase genes has been shown to be effective for conferring resistance to *Potex-*, *Poty-, Luteo- Sobemo-* and *Poleroviruses* (Braun & Hemenway, 1992; Audy *et al.*, 1994; Koev *et al.*, 1998; Pinto *et al.*, 1999; Thomas *et al.*, 2000). The efficiency of this strategy has also been confirmed in field trials and a commercial product combining resistance to the *Polerovirus Potato leaf roll virus* (PLRV) with resistance to Colorado potato beetle in transgenic Russet Burbank potatoes has been developed (Lawson *et al.*, 2001).

Viruses of the genera *Tobamo-* and *Tobravirus* encode a read-through portion of about 54 kDa in the C-terminal part of the replicase gene. Resistance by introduction of this read-through portion was first demonstrated for TMV in *Nicotiana tabacum* (Golemboski *et al.*, 1990) and subsequently also for other *Tobamo-* and *Tobraviruses* (MacFarlane & Davies 1992; Tenllado *et al.*, 1995; Melander, in preparation).

Replicase genes with mutations in a conserved GDD motif, which is characteristic of replicase proteins, as well as other mutated or truncated forms of

replicase genes have also been shown to be able to confer virus resistance (Anderson *et al.*, 1992; Brederode *et al.*, 1995; Longstaff *et al.*, 1993; Tsukasa *et al.*, 2002).

Also for DNA plant geminiviruses, PDR by transformation of plants with replication-related genes has been obtained (Hong & Stanley, 1996; Noris *et al.*, 1996).

Resistance mediated by replicase genes often confers a high level of protection but is generally only effective against viruses closely related to the source of the transgene. This, combined with other observations such as a correlation between high levels of resistance with low levels of transgene mRNA implies that this kind of resistance is mediated by RNA rather than protein. Some studies have shown that gene silencing mechanisms related to post-transcriptional gene silencing are involved in the resistance mechanism (Marano & Baulcombe 1998; Jones *et al.*, 1998; van den Boogaart *et al.*, 2001). However, in other cases, *e.g.* the use of modified replicase genes, protein-mediated mechanisms are also involved possibly combined with RNA-mediated mechanisms (Wintermantel & Zaitlin, 2000; Goregaoker *et al.,* 2000).

Movement protein

Plant viruses encode movement proteins (MPs) that are required for viral cell-to-cell transport. The MPs contain nucleic acid binding domains and localize to plasmodesmata where they facilitate the viral transfer to adjacent cells. By transgenic expression of dysfunctional MPs resistance to plant viruses can be achieved. This has been shown by expression of mutated versions of MP genes from the *Tobamovirus* TMV and the *Luteovirus* PLRV, while no effect was seen when expressing wild-type MPs. The resistance was shown to be efficient not only to the homologous virus and related viruses but also to viruses belonging to other virus groups (Lapidot *et al.*, 1993; Cooper *et al.*, 1995; Tacke *et al.*, 1996).

For viruses of the genera *Pomo-*, *Potex-, Carla-, Hordei-, Beny-* and *Pecluvirus* a movement protein complex is expressed by a set of three overlapping genes called the triple-gene-block (TGB). Expression of mutated versions of TGB genes can confer virus resistance as has been shown for *White clover mosaic virus* (WClMV) (Beck *et al.*, 1994) and *Potato virus X* (PVX) (Seppänen *et al.*, 1997) that both belong to the genus *Potexvirus*. In both cases the introduced resistance was very broad, affecting other potexviruses as well as other TGB-containing viruses such as *Potato virus S* and *M* that belong to the genus *Carlavirus*.

Virus protection mediated by modified versions of proteins involved in viral movement normally results in a relatively broad resistance. This kind of resistance is generally protein-mediated requiring the expression of the dysfunctional protein. It is thought to be the result of competition between wild-type virus encoded MPs or TGB proteins and preformed, dysfunctional transgene encoded proteins that disrupt the viral transfer system through plasmodesmata (Lapidot *et al.*, 1993; Tacke *et al.*, 1996; Seppänen *et al.*, 1997).

Paper I

Paper I of this thesis describes an attempt to introduce resistance against the *Pomovirus Potato mop-top virus* (PMTV), which causes severe quality problems in potato due to the formation of brown arcs or rings in the tuber flesh known as spraing. As modified TGB proteins have been shown to confer a very broad spectrum resistance this strategy was chosen and the study describes the production and analysis of transgenic potato plants containing a mutated version of the second TGB gene. The TGB2 gene was isolated from a Swedish isolate of PMTV by RT-PCR, and thereafter mutated by PCR-mutagenesis in a region highly conserved between TGB2 genes from different viruses. The mutated TGB2 gene was cloned into the binary vector pBI121 after the *Cauliflower mosaic virus* (CaMV) 35S-promoter. The derived construct was used for transformation of potato (*Solanum tuberosum*, cv. Hulda) by *Agrobacterium*-mediated transformation using kanamycin-selection.

Potato plants confirmed to be transgenic by PCR-analysis for presence of the *nptII* gene were further analysed for transcription of the introduced mutated TGB2 gene by a ribonuclease protection assay. Ten lines, all confirmed to transcribe the mutated TGB2 gene, were selected for resistance evaluation. These lines were assayed in a field trial in Halland, Sweden, by natural infection with PMTV transmitted by the fungal vector *Spongospora subterranea*. The different lines were grown in a randomised pattern with five potato plants per line in each of four

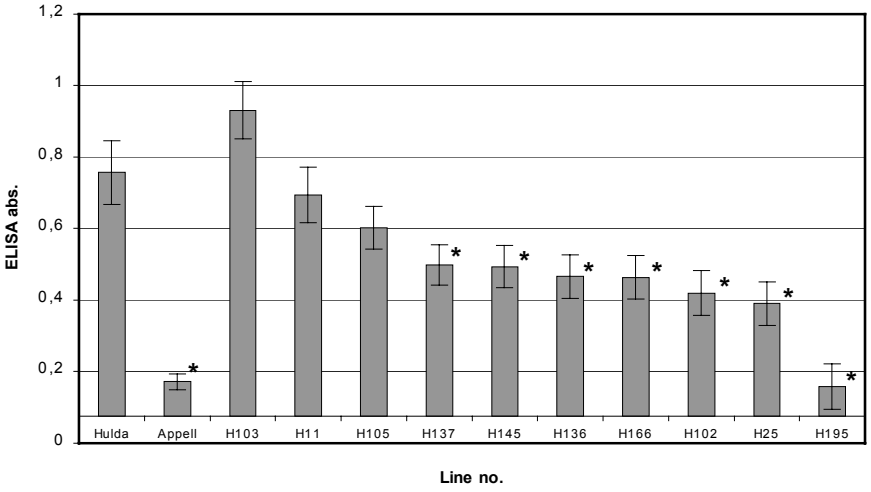

Figure 1. Mean ELISA absorbance of lines from field trial. Hulda is the non-transgenic parental variety, Appell is a variety known to have a high level of resistance to PMTV and H11 – H195 are transgenic Hulda lines. Bars indicate standard error. Negative tuber controls for the ELISA have an absorbance of 0.06-0.09. Lines having a significant difference of ELISA absorbance compared to the Hulda control line as determined with Dunnett's test are marked with *.

blocks. Non-transgenic cv. Hulda and cv. Appell were used as a susceptible and a resistant control, respectively. After harvest, tubers from the field were analysed for virus content by ELISA.

In seven of the analysed transgenic lines there was a significant reduction in ELISA values when compared to the non-transgenic Hulda control line according to Dunnett´s test (Figure 1). In the three lines that had the lowest ELISA readings this reduction was manifested both as a reduction in the proportion of infected tubers and as a reduction in virus levels in those tubers that were infected. In the line showing the highest level of resistance, the total ELISA value was reduced by 79%. This is the first study showing TGB2 mediated resistance to be effective against infection transferred by a natural vector in the field. These results demonstrate that the introduction of a mutated TGB2 gene into potato can confer increased resistance to PMTV.

Satellites and defective interfering nucleic acids

Some strains of certain RNA viruses contain satellite RNAs, which are small RNA sequences that are fully dependent on the host virus for replication and transmission. The presence of satellite RNAs can either suppress or enhance symptoms induced by the host virus. Transgenic expression in plants of suppressing satellite RNA sequences may attenuate symptoms when plants are infected with the host virus as has been shown *e.g.* for CMV in tobacco, tomato and pepper (McGarvey *et al.*, 1994; Baulcombe *et al.*, 1986; Kim *et al.*, 1997).

For several viruses the occurrence of truncated genomic sequences of the virus have been detected in infected tissue. These, so called defective interfering nucleic acids, may interfere with replication of the full-length virus genome and attenuate symptoms. Plants engineered with defective interfering nucleic acids display reduced or delayed symptoms and reduced replication (Kollar *et al.*, 1993; Frischmuth *et al.*, 1997).

Antisense RNA and RNA interference

The expression in transgenic plants of antisense RNA complementary to viral sequences has been used as a way to suppress expression of certain genes from the invading virus and thus improving resistance. This RNA-silencing strategy has been tested with antisense expression of *e.g.* coat protein and replicase genes (Cuozzo *et al.*, 1988; Powell *et al.*, 1989; Kawchuk *et al.*, 1991; Bendahmane & Gronenborn, 1997), but in some instances the resistance was only efficient at low inoculum concentrations.

RNA interference (RNAi) has later been shown to be a more efficient way to suppress the expression of homologous genes than antisense RNA. Various constructs containing the *Pro*-gene of PVY in sense + antisense direction were transformed to tobacco resulting in a large proportion of plants immune to PVY (Waterhouse *et al.,* 1998; Smith *et al.*, 2000).

Resistance genes

An alternative to use pathogen derived genes for introduction of resistance is to transform *R*-genes conferring virus resistance into heterologous plant species or varieties. The *N* gene of tobacco confers resistance to TMV by localizing the viral infection by an HR-response. When the *N* gene was transferred to tomato the resulting transgenic tomato plants showed typical HR-responses as well as blocked systemic movement of the virus when the plants were challenged with TMV (Whitham *et al.*, 1996). In a similar way also other *R*-genes, such as the potato *Rx* gene conferring resistance to PVX and the tomato *Sw5* gene conferring resistance to *Tomato spotted wilt virus* (TSWV), have been shown to be functional when transferred to tobacco (Bendahmane *et al.*, 1999; Spassova *et al.*, 2001).

Ribosome-inactivating proteins and protease inhibitors

Many plant species contain ribosome-inactivating proteins (RIPs), which have antiviral properties. A RIP from pokeweed (*Phytolacca americana*) was expressed in tobacco and potato, and was shown to confer broad-spectrum virus resistance both when inoculated mechanically and by aphids (Lodge *et al.*, 1993). RIPs, however, may also be toxic to the transgenic host plant. Therefore, less toxic forms of RIPs have been used that still confers virus resistance but without affecting the host ribosomes (Wang *et al.*, 1998; Zoubenko *et al.*, 2000). Another approach is to fuse the *RIP* gene to a promoter that is active only upon infection by a specific virus (Hong *et al.*, 1996).

Viruses from some genera are dependent on cysteine proteinase activity for processing of virus-encoded polyproteins. By expression of cysteine proteinase inhibitors in transgenic plants virus replication and propagation can be blocked. In transgenic tobacco plants expressing oryzacystatin, a cysteine proteinase inhibitor from rice, there was a clear positive correlation between resistance to potyviruses and expression of oryzacystatin (Gutierrez-Campos *et al.*, 1999).

Plantibodies

The expression in transgenic plants of antibodies, so called plantibodies, recognizing plant viruses is another approach for conferring virus resistance. Single chain variable regions of monoclonals raised against a virus have been expressed in transgenic plants, which upon challenge with the corresponding virus demonstrated delayed symptom development and reduced incidence of infection (Tavladoraki *et al.*, 1993; Fecker *et al.*, 1997). In another example transgenic plants encoding the single chain variable region of a broad-spectrum monoclonal antibody showed reduced number of local lesions when challenged with the corresponding virus but also with other viruses of the same genera (Xiao *et al.*, 2000). Simultaneous resistance to viruses of different genera was obtained when single chain antibodies against a conserved region of a plant viral RNA-dependent RNA-polymerase was transformed to plants (Boonrod *et al.*, 2004). Also genes encoding full size monoclonal antibodies have been transformed into plants resulting in the expression of heavy and light chains, which were assembled into

functional antibodies conferring reduced symptom development upon challenge infection (Voss *et al.*, 1995).

Interferon system enzymes

Interferons are mammalian proteins inducing various defence mechanisms that ultimately inhibit viral replication. One of these defence reactions is the 2-5A system, where 2'-5' oligoadenylate synthetase (2-5 Aase) makes 2'-5' oligoadenylates (2-5A) in response to double stranded viral RNA. The formed 2-5A then activates ribonuclease L (RNAseL), which degrade RNAs. Transgenic potato plants constitutively expressing 2-5 Aase have been shown to be protected against PVX infection, also under field conditions (Truve *et al.*, 1993). Expression of 2-5 Aase has also been combined with expression of RNAseL resulting in plants with improved resistance to several different viruses (Mitra *et al.*, 1996; Ogawa *et al.*, 1996).

Another interferon-induced enzyme is the double stranded RNA-dependent protein kinase (PKR). The human PKR gene was fused to a wound-inducible promoter and introduced into tobacco. The transgenic tobacco showed significantly reduced viral symptoms or no viral symptoms at all when challenged with various RNA viruses (Pyung *et al.*, 2002).

Transgenic resistance to fungi and bacteria

While there are many successful examples of transgenic resistance to viruses, transgenic resistance to fungi and bacteria has generally been more difficult to accomplish and so far there are no commercial applications. The first efforts included the expression of single proteins, such as PR proteins and other proteins and peptides with antimicrobial effects. More recently, thanks to the increasing knowledge about genes involved in plant defence pathways, the utilisation of more complex defence responses has become possible.

PR proteins

The hydrolytic PR proteins chitinase and β-1,3-glucanase have been extensively studied in transgenic plants. In 1991 (Broglie *et al.*) it was demonstrated that heterologous expression of a bean chitinase in oilseed rape and tobacco could reduce the susceptibility to *Rhizoctonia solani*. Since then chitinases of various classes from different plant species, as well as other sources, have been expressed in a large number of crops with effects against various fungal pathogens (*e.g.* Yamamoto *et al.*, 2000; Oldach *et al.*, 2001; Mora & Earle, 2001; Pappinen *et al.*, 2002). The effects demonstrated have mainly been a reduction of fungal development and reduced number and size of lesions. Partial resistance to fungal infection has also been demonstrated in field trials (Howie *et al.*, 1994; Grison *et al.*, 1996).

In some examples it has also been shown that β-1,3-glucanases have the potential to improve resistance against fungal as well as *Oomycete* infections (Yoshikawa *et al.*, 1993; Lusso & Kuc, 1996). When chitinase and β-1,3-glucanase enzymes are combined they can act synergistically with improved suppressive effects on fungal infection. Expression of a class I chitinase and β-1,3-glucanase in tomato significantly enhanced resistance to *Fusarium oxysporum* f. sp. *lycopersici* while comparable expression levels of either gene alone did not (van den Elzen *et al.*, 1993; Jongedijk *et al.*, 1995). Similar results were obtained when a chitinase gene and a β-1,3-glucanase gene were combined in tobacco and challenged with *Cercospora nicotianae* (Zhu *et al.*, 1994) and when barley class II chitinase and β-1,3-glucanase were simultaneously expressed in tobacco (Jach *et al.*, 1995).

Also expression of PR proteins from other classes, such as osmotin and thaumatin-like proteins (PR-5), in transgenic plants have been shown to have negative effects on fungal and *Oomycete* infections such as delayed development of disease symptoms (Liu *et al.*, 1994; Datta *et al.*, 1999; Fagoaga *et al.*, 2001). PR proteins of class 1a are expressed at high levels in response to pathogen attack, but the biochemical function is still unknown. When PR-1a was constitutively expressed in tobacco the derived plants displayed enhanced resistance to oomycetes (Alexander *et al.*, 1993). The cysteine-rich peptides defensins (PR-12), thionins (PR-13) and lipid transfer proteins (PR-14) are thought to be involved in induced defence responses (van Loon & van Strien, 1999) and are also often found in seeds. Various such cysteine-rich peptides from different plant species have been expressed in transgenic plants where they have been shown to confer enhanced resistance to infections caused by fungi and bacteria (Epple *et al.*, 1997; Molina & Garcia-Olmedo, 1997; Gao *et al.*, 2000; Iwai *et al.*, 2002).

Paper II

As combinations of chitinases and β-1,3-glucanases have been shown to enhance resistance to various fungal diseases this approach was evaluated in paper II of this thesis. A class II chitinase and a β-1,3-glucanase from barley were studied with respect to the oilseed rape pathogens *Leptosphaeria maculans, Verticillium longisporum, Alternaria brassicae, A. brassicicola* and *Sclerotinia sclerotiorum.* These pathogens, together with *Trichoderma* sp. were evaluated in an *in vitro* assay where the β-1,3-glucanase caused growth retardment of *A. brassicicola* and *V. longisporum* and a combination of the chitinase and the β-1,3-glucanase severely affected the growth of *Trichoderma* sp. and slightly the growth of *A. brassicae*.

A construct containing both the chitinase gene and the β-1,3-glucanase gene after enhanced 35S promoters (Figure 2) was transformed into oilseed rape, cv Westar. Doubled haploid lines of primary transformants expressing both transgenes were produced and studied for five generations. The studies included copy number determinations, expression analysis and greenhouse assays with the oilseed rape pathogens. When analysing the transgenic lines for possible effects on fungal resistance an increased resistance to *L. maculans* was found in some of the

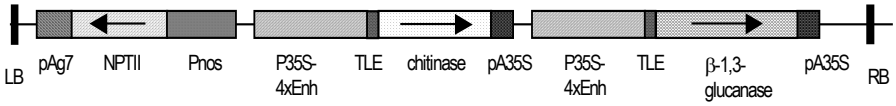

Figure 2. Organisation of T-DNA with chitinase and β-1,3-glucanase genes. LB = left border, pAg7 = polyA sequence from gene 7 on T-DNA of *A. tumefaciens*, *NPTII* = neomycinphophotransferase II gene, Pnos = nopaline synthase promoter from *A. tumefaciens*, P35S-4xEnh = 35S promoter from *Cauliflower mosaic virus* (CaMV) with 4 enhancer elements, TLE = translational enhancer sequence (Ω sequence) from TMV, chitinase = gene encoding chitinase, pA35S = polyA sequence from CaMV, β-1,3-glucanase = gene encoding β-1,3-glucanase, RB = right border.

transgenic lines, however this improvement was not correlated with the expression level of the PR proteins and is more likely due to a natural variation within the cultivar Westar. No significant effects on the other fungal oilseed rape pathogens were found.

The doubled haploid material was also used for studies on stability of the inserted sequences as well as stability of expression during the five generations evaluated. The number of inserted copies for both genes in the second doubled haploid generation was determined both with Southern blotting and real-time PCR, where there was full agreement between the two methods used. Seven out of ten doubled haploid lines contained two copies of each gene (which corresponds to one copy in the original transformant), while for the remaining three lines there was discrepancy between the number of chitinase and β-1,3-glucanase genes indicating the insertion of truncated T-DNAs. The copy numbers were also determined in generation five with real-time PCR. The results from this analysis were not always the same as the results obtained for the second generation. In one line only one copy each of the chitinase and the β-1,3-glucanase gene was found and in another line only one copy of the β-1,3-glucanase gene, whereas the analysis in generation two showed two copies in all cases. This indicates that at least part of the inserted sequences had been lost in one of the alleles.

The expression of the chitinase as well as the β-1,3-glucanase was analysed by western blotting in all five doubled haploid generations. The expression of the β-1,3-glucanase was normally higher than the chitinase expression except for one line that contained no β-1,3-glucanase gene. The β-1,3-glucanase expression was in general very stable over the five doubled haploid generations tested with the exception for one line where a rather strong β-1,3-glucanase expression in the three first generations was silenced in generation four and five (Figure 3a). For the chitinase the expression level was more variable between generations with bands disappearing and sometimes reappearing in later generations (Figure 3b).

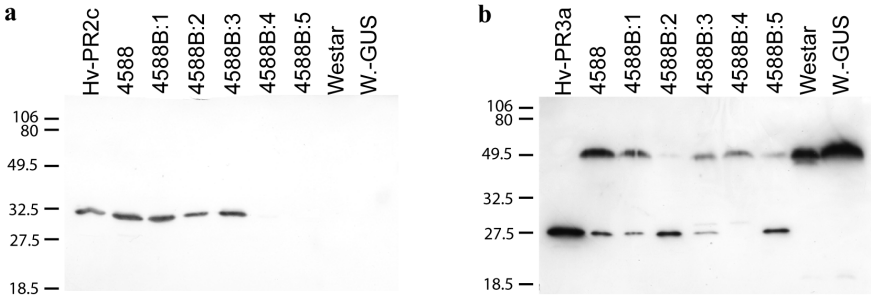

Figure 3. Immunoassay by western blotting of ***a)*** β-1,3-glucanase and ***b)*** chitinase in all five generations of doubled haploid line 4588B (4588B:1-5) and corresponding original transformant (4588). 1 µg of protein extract from leaves were assayed for each plant. The first lane contains a positive control (0.1 µg) of purified β-1,3-glucanase (Hv-PR2c) and chitinase (Hv-PR3a), respectively, and the last two lanes contain negative controls of untransformed cv. Westar and Westar transformed with a GUS-construct (W.-GUS). Band sizes (kDa) for the molecular weight marker are indicated.

Antimicrobial peptides and proteins

In addition to PR proteins also other peptides and proteins with antimicrobial activities have been evaluated as transgenic resistance factors. Some plant-derived antimicrobial peptides, *e.g*. puroindolines from wheat and a novel peptide from *Macadamia integrifolia* nuts, have demonstrated antifungal effects in transgenic plants (Krishnamurthy *et al.*, 2001; Kazan *et al.*, 2002). Antimicrobial peptides of animal origin have also been assayed in transgenic plants. When the sarcotoxin gene from flesh fly was expressed in transgenic tobacco (Mitsuhara *et al.*, 2000) enhanced resistance against both bacterial and fungal pathogens was obtained. Similarly, other antimicrobial peptides of animal origin, such as magainin and temporin from frog skin, have been shown to confer enhanced resistance against bacteria, fungi and oomycetes in plants (Li *et al.*, 2001; Chakrabarti *et al.*, 2003; Osusky *et al.*, 2004). In addition to naturally occurring antimicrobial peptides also synthetic or hybrid peptides have been designed and expressed transgenically in plants conferring improved resistance to bacterial, fungal and *Oomycete* pathogens (Osusky *et al.*, 2000; Cary *et al.*, 2000).

An example of proteins with antimicrobial activities are the ribosome inactivating proteins, that have been shown to confer resistance to fungal infection by inactivating foreign ribosomes (Logemann *et al.*, 1992). However, also a non-functional mutant of a RIP from pokeweed has been shown to confer improved fungal resistance by activating SA-independent constitutive overexpression of PR proteins (Zoubenko *et al.*, 1997). Other examples of antimicrobial proteins are the antifungal proteins derived from a virus infecting *Ustilago maydis* (Clausen *et al.*, 2000) and from *Aspergillus giganteus* (Oldach *et al.*, 2001; Coca *et al.*, 2004) that have shown potential as transgenic resistance factors against fungal infection. Also

antimicrobial proteins of animal origin, such as human lysozyme, have been demonstrated to improve bacterial and fungal resistance when expressed in transgenic plants (Nakajima *et al.*, 1997; Takaichi & Oeda, 2000).

Phytoalexins

Phytoalexins are normally synthesized via complex biochemical pathways and are thus more complicated to produce transgenically. However, expression of some enzymes involved in phytoalexin synthesis, such as resveratrol synthases and isoflavone O-methyltransferase, demonstrates the potential of phytoalexins in conferring enhanced resistance to fungal infections (Hain *et al.*, 1993; Hipskind & Paiva, 2000; He & Dixon, 2000).

Inactivation of pathogen produced compounds

During plant infection fungal pathogens often produce plant cell wall degrading enzymes, such as polygalacturonase, as well as various toxins, such as oxalic acid and mycotoxins. A possible target for transgenic resistance against fungal pathogens is the inactivation of these enzymes and toxins. The expression of a polygalacturonase inhibitor protein in tomato was able to reduce disease development by *Botrytis cinerea* (Powell *et al.*, 2000). Expression of oxalate oxidase as well as oxalate decarboxylase has been shown to confer enhanced resistance to fungal infections (Kesarwani *et al.*, 2000; Donaldson *et al.*, 2001). Resistance by transgenic detoxification has also been demonstrated against bacterial infection in sugarcane by introduction of an albicidin detoxifying gene (Zhang *et al.*, 1999).

Some fungal pathogens produce mannitol to suppress reactive oxygen-mediated plant defences. The expression in plants of heterologous mannitol dehydrogenase has been shown to enhance resistance to the mannitol-secreting fungus *Alternaria alternata* (Jennings *et al.*, 2002).

RNA interference

Gene silencing by RNA interference has been applied as a method to confer improved tolerance against the crown gall causing bacterium *Agrobacterium tumefaciens*. Transformation of *Arabidopsis* and tomato by inverted repeats of the bacterial oncogenes resulted in plants that could still be infected by *A. tumefaciens* but where the formation of crown galls was fully prevented (Escobar et al., 2001).

Resistance genes

The transfer of *R*-genes between related species has been shown to be a possible way to introduce disease resistance against both bacterial, *Oomycete* and fungal pathogens. The *Pto* gene of tomato and *Bs2* gene of pepper have been transferred to other *Solanaceous* species conferring resistance to the bacterial pathogens

Pseudomonas syringae and *Xanthomonas campestris,* respectively (Rommens *et al.*, 1995; Tai *et al.*, 1999).

Race non-specific resistance to the *Oomycete Phytophthora infestans* has been obtained in both potato and tomato by transfer of a resistance gene derived from the wild potato species *Solanum bulbocastanum* (Song *et al.*, 2003; van der Vossen *et al.*, 2003). Similarly, resistance to the fungus causing apple scab was introduced into a susceptible apple cultivar by transformation with the *HcrVf2* gene from the wild species *Malus floribunda* 821 (Belfanti *et al.*, 2004). Successful examples of transfer of *R*-genes have normally taken place between related species. However, for the *RPW.8* genes of *A. thaliana* it has been shown that these are functional against powdery mildew also when transferred to tobacco (Xiao *et al.*, 2003).

A possibly more broad-spectrum approach to make use of *R*-genes and the SAR-pathway has also been described. According to this approach transgenic plants are transformed with pathogen *avr*-genes under control of a heterologous infection-inducible promoter. If the generated transgenic plant carries the corresponding *R*-gene a defence response will be initiated upon infection. One example in this direction is the pathogen-inducible expression in tobacco of the elicitor cryptogein from *Phytophthora cryptogea,* a likely avirulence factor of *Phytophthora* spp. (Keller *et al.*, 1999). Challenge infection induced both HR and defence gene activation enhancing resistance to *Phytophthora parasitica* var. *nicotianae* as well as several fungal pathogens. In another example the *Cf9* gene from tomato conferring resistance to the fungus *Cladosporium fulvum* was expressed in oilseed rape plants also expressing the corresponding fungal *Avr9* gene (Hennin *et al.*, 2001). These oilseed rape plants were shown to have enhanced resistance to the fungus *Leptosphaeria maculans*.

Defence signalling components

Different steps in plant defence pathways have been evaluated to get a better understanding of defence responses and potentially produce transgenic plants with improved defence systems. One step in the defence-signalling cascade that has been evaluated is the *NPR1* gene from *Arabidopsis*, which regulates SA-signalling. When *NPR1* was overexpressed in *Arabidopsis* as well as rice, plants with stronger PR protein induction and enhanced bacterial and fungal resistance were generated (Cao *et al.*, 1998; Chern *et al.*, 2001). When bacterial SA-generating enzymes were expressed in transgenic tobacco, SA accumulation was substantially increased and PR proteins were constitutively expressed conferring enhanced resistance to fungal, as well as viral, infections (Verberne *et al.*, 2000). Also expression of a transcriptional regulatory protein gene, *Tsi1* from tobacco, induced constitutive expression of PR proteins and conferred broad-spectrum resistance to both bacterial and *Oomycete* pathogens as well as viruses (Shin *et al.*, 2002). When the *Prf* gene involved in resistance to *Pseudomonas syringae* in tomato was overexpressed in tomato the transgenic plants displayed constitutively activated SAR with enhanced SA accumulation, constitutive PR protein synthesis

and broad-spectrum resistance to bacterial and viral pathogens (Oldroyd & Staskawicz, 1998).

Another defence pathway step that has been modified is the expression of glucose oxidases that generates H_2O_2 production (Wu *et al.*, 1995; Murray *et al.*, 1999). Plants transformed with glucose oxidase genes displayed enhanced tolerance to a broad spectrum of bacteria, fungi and oomycetes but also distorted plant growth. Careful regulation of the glucose oxidase expression by pathogen-inducible promoters may overcome these negative effects (Kachroo *et al.*, 2003).

Plantibodies

As for virus resistance, expression in plants of antibodies binding to pathogen or pathogen products have been proposed as a strategy for conferring resistance to fungi and bacteria. However, so far the examples are very limited. Single-chain variable-fragments of an antibody specific to the wall-less bacteria stolbur phytoplasma expressed in tobacco resulted in symptomless shoots when inoculated by grafting onto stolbur phytoplasma-infected rootstocks (Le Gall *et al.*, 1998). Peschen *et al.* (2004) have expressed fusion proteins comprising chicken-derived single-chain antibody fragments against *Fusarium graminearum* linked to antifungal peptides in *Arabidopsis thaliana*. When the transgenic plants were challenged with *Fusarium oxysporum* a high level of protection was obtained, whereas expression of either antibody or antifungal peptides alone resulted in moderate levels of protection only.

Transgenic resistance to insects and nematodes

For transgenic resistance to insects the expression of *Bacillus thuringiensis* (Bt) toxins is the most well known approach, which is also used commercially on significant acreages. There are also many examples of the expression of other proteins with insecticidal activities, such as proteinase inhibitors and lectins. Some more complex approaches including expression of secondary metabolites and *R*-genes have also been initiated.

Bacillus thuringiensis toxins

Formulations based on the insect pathogenic bacterium *B. thuringiensis* have been used as a biopesticide for the last 50 years. The main insecticidal activity of *B. thuringiensis* is due to insecticidal crystalline inclusions formed during sporulation. These crystalline inclusions are composed of protoxin subunits, called δ-endotoxins or Cry proteins. The Cry proteins are classified into 24 major groups and are usually specific for a limited range of species within certain insect orders, mainly *Lepidoptera, Coleoptera* and *Diptera* (Hilder & Boulter, 1999). The protoxins are solubilized in the insect midgut, where they are cleaved by gut proteases to form the active toxin. The toxin binds to receptors of epithelial cells

in the midgut and then inserts into the cellular membrane. This leads to pore formation that lyses the cells and then causes death of the insect by starvation or septicaemia (Whalon & Wingerd, 2003).

Cloned genes encoding Cry proteins of *B. thuringiensis* were expressed in tobacco and tomato in the late 1980s (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987). In the first reports published the expression levels of the introduced *Cry* genes were very low, probably due to that the bacterial codon usage was suboptimal for plant expression and the occurrence of polyadenylation signals within the coding region. Later, attempts to express Cry proteins have made use of partially or totally synthetic genes optimised for plants resulting in considerably increased expression levels (Mazier *et al.*, 1997).

Cry genes have been transferred to a large number of different crop species and have in field trials been shown to confer resistance to various pests, mainly of the orders *Lepidoptera* and *Coleoptera* (*e.g.* Perlak, 1993; Tu *et al.*, 2000, Moellenbeck *et al.*, 2001; Kumar & Kumar, 2004). In 1995 the first insect resistant transgenic crops; corn, cotton and potato; expressing Cry proteins were approved for market release in the US (http://www.aphis.usda.gov/brs/not_reg.html; 21-Aug-2004) and in 2002 14 million ha were planted with Bt-crops globally (James, 2002). One concern with the use of Bt-crops has been the possibility of insects developing resistance to Cry proteins. Apart from resistance management strategies by mixing non-Bt- and Bt-cultivars prolonged durability may be achieved by pyramiding different *Cry* genes (Cao *et al.*, 2002; Zhao *et al.*, 2003; Estela *et al.*, 2004) or the development of hybrid Cry proteins (Naimov *et al.*, 2003).

Other toxic proteins produced by microrganisms have also been proposed as alternatives or complements to Bt-toxins. One example is the Vip1 and Vip2 proteins from *Bacillus cereus* as well as Vip3A from *B. thuringiensis* that have activities comparable to that of Bt-toxins (Estruch *et al.*, 1997). Other examples are the insecticidal toxin complexes produced by *Photorhabdus luminescens* and *Xenorhabdus nematophilus,* which are bacteria associated with entomopathogenic nematodes (ffrench-Constant & Bowen, 1999). A synthetic plant-codon-optimized gene encoding the toxin A protein from *P. luminescens* has been transferred to *A. thaliana* resulting in high levels of insect resistance (Liu *et al.*, 2003).

Proteinase and α-amylase inhibitors

Animals are dependent on proteinases for their amino acid metabolism. Thus production of proteinase inhibitors (PIs) may defend plants against herbivorous pests. Insects may contain proteinases of four different classes; serine, cysteine, aspartic and metallo proteinases. Different classes of proteinases predominate depending on insect species and gut pH. In lepidopterans, with alkaline gut pH, serine proteinases normally dominate while many coleopterans with neutral to mildly acidic pH use cysteine and aspartic proteinases. The antimetabolic effect of PIs is partially explained by inhibition of the corresponding proteinases and thereby a reduction in amino acid availability. Another important effect is an

induced hyperproduction of proteinases, which further reduces the availability of essential amino acids (Reeck *et al.*, 1997; Schuler *et al.*, 1998; Gatehouse & Gatehouse, 1999).

The first demonstration that transgenic expression of *PI* genes can confer insect resistance was when a gene from cowpea (*Vigna unguiculata*) encoding a serine PI with inhibitory activity against trypsin (CpTI) was expressed in tobacco. The transgenic tobacco plants displayed enhanced protection against a range of lepidopteran storage and field pests, typically shown as reduced larval growth and less plant damage (Hilder *et al.*, 1987; Hoffman *et al.*, 1992; Gatehouse *et al.*, 1992). CpTI as well as other serine PI genes have been transformed into many different plant species conferring resistance not only to lepidopterans but also to *e.g.* coleopteran insects as well as nematodes (Atkinson 1993; Duan *et al.*, 1996; Christeller *et al.*, 2002; Alfonso-Rubi *et al.*, 2003). Cysteine PIs, *e.g.* cystatins from rice and chicken egg-white, have also been demonstrated to confer enhanced pest resistance, mainly against coleopterans and nematodes (Leplé *et al.*, 1995; Urwin *et al.*, 1997; Urwin *et al.*, 2001) but also against aphids (Rahbe *et al.*, 2003). Some insects may overcome the effects of certain PIs by switching to production of alternative, insensitive proteinases (Zhu-Salzman *et al.*, 2003; Brunelle *et al.*, 2004). A strategy for more effective inhibition of insect proteolysis could be expression of linked or hybrid inhibitors active against different proteinase classes (Urwin *et al.*, 1998; Inanaga *et al.*, 2001).

In addition to proteinase inhibitors also α-amylase inhibitors may confer insect resistance. This has been shown by expression of an α-amylase inhibitor from common bean in peas resulting in resistance to various bruchid beetles (Shade *et al.*, 1994; Schroeder *et al.*, 1995).

Lectins

Lectins are sugar-binding proteins that can be found in various plant tissues but often in high amounts in seeds and other storage organs. Different lectins have specificities for different mono- or oligosaccharides. It has been shown that some lectins are toxic to certain insects and it is also well known that some lectins have toxic or antinutritive effects on mammals. The exact toxicity mechanism against insects is not fully understood but there is evidence for lectins binding specifically both to epithelial cells and to the peritrophic membrane in the midgut. In addition to this, a reduced intake of food due to feeding deterrence and a restriction in uptake of nutrients due to partial blockage of pores of the peritrophic membrane have been proposed (Gatehouse & Gatehouse, 1999; Murdock & Shade, 2002).

Enhanced insect resistance by transgenic expression of lectins was first shown when tobacco plants expressing pea (*Pisum sativum*) lectin displayed improved performance against tobacco budworm (*Heliothis virescens;* Boulter *et al.*, 1990). Lectins from other plant species, such as concanavalin A (con A; from jackbean, *Canavalia ensiformis*) and snowdrop (*Galanthus nivalis*) lectin, have also been demonstrated to confer improved resistance to lepidopteran insect pests when expressed transgenically (Fitches *et al.*, 1997; Gatehouse *et al.*, 1999). The

snowdrop lectin, as well as other monocot mannose-binding lectins, has also been shown to be effective against sap-sucking insects of the order *Hemiptera* as shown for various aphids (Hilder *et al.*, 1995; Yao *et al.*, 2003; Chang *et al*., 2003) and planthoppers (Rao *et al.*, 1998; Tinjuangjun *et al.*, 2000; Wu *et al.*, 2002).

Lectins, as well as proteinase inhibitors, are not as effective as Bt-toxins for insect control. However, combinations of different insecticidal factors with different modes of action could lead to appropriate levels of insect control and possibly also reduce the risk of insects developing resistance (Boulter *et al*, 1990; Maqbool *et al*., 2001).

Paper III

In paper III of this thesis the possibility of using plant derived resistance factors from edible plants for resistance to pollen beetles (*Meligethes* spp.) in oilseed rape was investigated. Feeding assays with pollen beetle larvae were set up where the larvae were fed anthers that had been soaked with solutions of various plant proteins that might have a negative effect on larval development and survival.

First, proteins with potential insecticidal activity was purified from potato tubers, which is a rich source of different groups of such proteins even though potato is a food crop. The proteins purified were a cysteine proteinase inhibitor, a pool of serine proteinase inhibitors, a pool of aspartic proteinase inhibitors, the esterase patatin and potato lectin. Additionally, a metallo proteinase inhibitor purified from potato was purchased from Sigma, thus all four groups of PIs were represented. When fed to pollen beetle larvae all tested proteins except the serine PIs had a significant growth-reducing effect when oilseed rape anthers were soaked in 100 g/l protein solutions (for cysteine PI only 50 g/l was used due to shortage of protein). With respect to influence on larval survival only potato lectin, at concentrations of 100 and 50 g/l but not at 10 g/l, displayed a significant effect.

As potato lectin was shown to be the most potent of the potato proteins tested, additional plant lectins with affinity for different sugars were purchased. These additional lectins were wheat germ agglutinin (WGA), concanavalin A and peanut (*Arachis hypogaea*) lectin. When fed to pollen beetle larvae all these lectins had a significant negative impact on both weight and survival at 100 g/l solutions, where con A was the most potent one followed by WGA (Figure 4). Con A and WGA were also tested at 10 g/l solutions where significantly reduced larval survival was still seen. Con A was further evaluated in an assay where the larvae could choose between con A treated and control anthers, but larvae did not discriminate between the treatments. When adult female pollen beetles were assayed for oviposition, feeding and survival on con A treated anthers and buds, significantly fewer eggs were laid in significantly fewer buds. The number of buds fed upon was also significantly lowered, but no effect on beetle weight or survival could be detected. The conclusion from paper III was that con A would be an interesting candidate to express in transgenic oilseed rape and study as a potential resistance factor against pollen beetles.

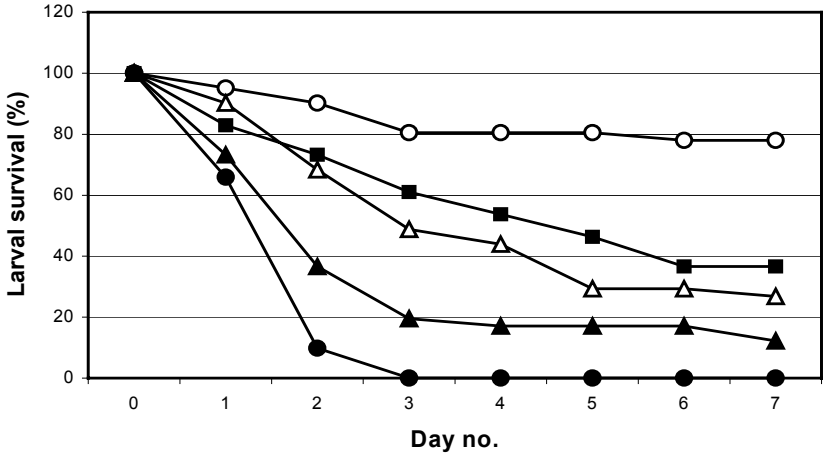

Figure 4. Survival rates of pollen beetle larvae during a period of 7 days when fed anthers soaked in 100 g/l solutions of the plant lectins -●- con A, -▲- WGA, -Δ- potato and -■- peanut or solvent treated controls -O-. The initial number of larvae per treatment was 41.

Paper IV

In paper IV transgenic oilseed rape plants expressing pea lectin were produced and evaluated. Pea lectin is structurally related to con A and bind mannose and glucose in essentially the same way. Due to a more complex posttranslational processing of con A in jackbean, which may not be effective in other plant species, and due to the fact that pea lectin is essentially non-toxic while con A is apparently toxic to mammals (Grant, 1989) pea lectin was regarded as a better alternative for use in genetically engineered plants. Feeding assays with anthers soaked in protein solutions showed that pea lectin had as strong influence on the survival rate of pollen beetle larvae as con A. When the larvae were fed oilseed rape anthers soaked in a 10 g/l solution of pea lectin there was a reduction in survival of 84% compared to larvae on control treatment and the weight of surviving larvae was reduced by 79%. When a 100 g/l solution of pea lectin was used all larvae were dead after four days of testing.

The pea lectin gene was isolated by PCR and fused to the pollen-specific promoter Sta44-4 from oilseed rape (Hong *et al.*, 1997) in a derivative of the binary vector pGPTV-Kan (Becker *et al.*, 1992), which also contains the *nptII* gene as a plant selection marker. Spring oilseed rape, cultivar Westar, was transformed with this construct by use of *Agrobacterium*-mediated transformation.

The generated transgenic material was assayed for effects on pollen beetle larvae both in the T_0- and T_2-generations. In 11 out of 20 tested plants of the T_0-generation there was a significant reduction in larval weight, which ranged up to 46% compared to the control (Figure 5). A small but significant reduction in larval survival rate was also observed. In the T_2-generation significant weight reductions,

with a maximum of 32%, were obtained in 10 out of 33 comparisons between transgenic plants and their controls. Pea lectin concentrations in anthers of transgenic T_2-plants were estimated by western blotting. The pea lectin concentrations ranged up to 1.5% of total soluble protein. There was a negative correlation between lectin concentration and larval growth, which further supports the conclusion that pea lectin was indeed responsible for the negative effects seen on pollen beetle larvae. Plants from test groups with significant differences in larval weights had a significantly higher mean pea lectin concentration, 0.64% compared to 0.15% for plants from test groups without effect on larval weight. These results supports the conclusion that pea lectin is a promising resistance factor for use in *Brassica* oilseeds against pollen beetles. This is also the first time that transgenic plants expressing a plant lectin have been shown to confer improved resistance to a coleopteran insect.

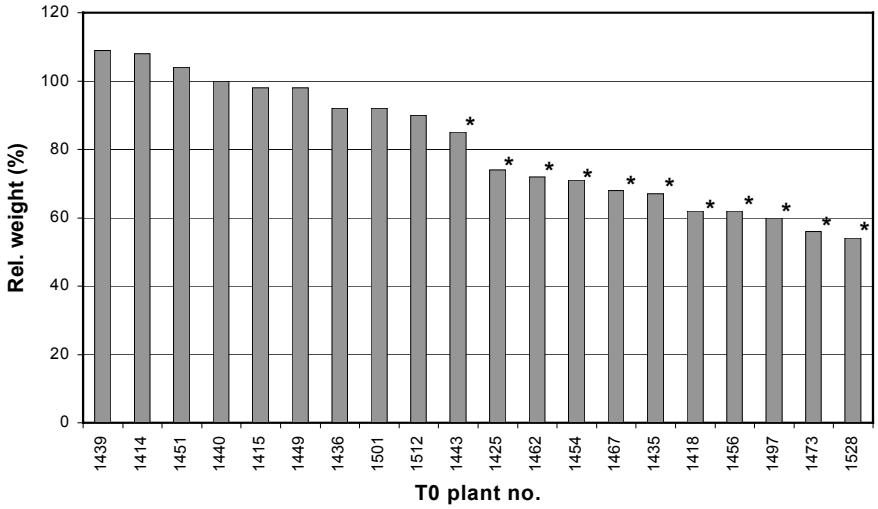

Figure 5. Weight of pollen beetle larvae fed anthers from plants transformed with pea lectin gene (generation T_0) relative to weight of larvae fed anthers from non-transformed plants. The initial number of larvae per test plant was 23-36. * = significantly different from control as determined by Scheffé's test at α=0.05.

Enzymes

Enzymes with different modes of action have also been proposed as alternative crop protection agents. Chitin is an important structural component of insects and transgenically expressed chitinase have shown some effect against insects (Ding *et al.*, 1998). Other examples of enzymes that potentially could be of interest for protection against insects include anionic peroxidase (Dowd *et al.*, 1998), cholesterol oxidase from *Streptomyces* (Corbin *et al.*, 2001) and the esterase

patatin from potato (Strickland *et al.*, 1995). Also the biotin-binding proteins avidin and streptavidin have been demonstrated to have deleterious effects on insects by causing biotin deficiency (Kramer *et al.*, 2000; Burgess *et al.*, 2002; Markwick *et al.*, 2003).

Secondary metabolites

Also non-protein, secondary metabolites, such as alkaloids, glucosinolates and terpenoids, that may take part in plant defence mechanisms against insects have been proposed as transgenic resistance factors. These compounds are normally produced via complex metabolic pathways and thus the transgenic production of such compounds is difficult. Several attempts in this direction, involving single enzymatic steps, have been made showing the potential of this strategy (Thomas *et al.*, 1995; Mikkelsen *et al.*, 2002; MacGregor *et al.*, 2003; Wang *et al.*, 2004). Additionally, an entire pathway involving three enzymatic steps for production of a cyanogenic glucoside has been transferred from *Sorghum bicolor* to *A. thaliana* conferring improved insect resistance (Tattersall *et al.*, 2001).

Resistance genes

Also for insects and nematodes, transgenic expression of *R*-genes is a possible way to improve resistance. *R*-genes targeted against nematodes from beet, tomato and potato have been shown to confer nematode resistance when expressed transgenically in susceptible varieties (Cai *et al.*, 1997; Milligan *et al.*, 1998; Paal *et al.*, 2004). The nematode resistance gene from tomato was demonstrated to have dual actions as the transgenic plants also displayed resistance to aphids (Rossi *et al.*, 1998; Vos *et al.*, 1998).

Conclusions and perspectives

This thesis describes examples of three different approaches to obtain transgenic resistance to pathogens and pests. Paper I evaluates an attempt to generate potatoes with resistance to *Potato mop-top virus* by introduction of a mutated triple gene block gene involved in viral movement. The field trial evaluation of the ten produced transgenic lines clearly demonstrated the potential of this approach to obtain PMTV resistant potatoes. To be practically applicable, however, a much larger number of transgenic lines ought to be produced and evaluated in repeated field trials to identify lines with a maximal reduction in virus incidence as well as symptoms when challenged with PMTV in the field. Furthermore, lines to be selected should not display any other alterations in agronomic performance or product quality and the transgenic inserts should be as few as possible and not contain any unintended transgenic sequences. In order to refine the expression of the introduced sequence alternative promoters, such as tuber specific promoters, could be considered. Resistance mediated by movement proteins is generally considered to be protein-mediated and normally efficient against a broader range of isolates than RNA-mediated resistance (Beck *et al.*, 1994; Seppänen *et al.*, 1997). Thus, the introduced sequence would be efficient enough on its own, but a combination with another approach, such as coat protein or replicase mediated resistance, could further strengthen broadness and durability of resistance. Another interesting feature would be to combine resistance to PMTV with resistance to *Tobacco rattle virus* (TRV), which also causes spraing symptoms in potatoes.

In paper II oilseed rape plants expressing PR proteins, a chitinase and a β-1,3-glucanase, were produced and evaluated for fungal resistance. Even though the chitinase and β-1,3-glucanase showed antifungal effects when assayed *in vitro*, no obvious antifungal effects were seen when the transgenic plants were analysed, despite good expression levels of the transgenes. The bioassays used for evaluating the fungal resistance and the transgenic material produced were not designed to detect very small antifungal effects, however minor antifungal effects would not be of interest for field application anyhow. Generally, transgenic resistance to fungal diseases has not been as successful as resistance to viruses and insects explaining the lack of commercial applications of transgenic plants with antifungal properties. Probably, more complex defence systems than expression of single proteins will have to be applied for successful fungal resistance. One such approach would be the use of transgenically expressed *R*-genes. This application has so far been limited by the availability, normally within related species, of suitable *R*-genes, and the fact that many *R*-genes have a narrow target spectrum. However, the increasing number of different *R*-genes being cloned in combination with genome information will facilitate the identification and exploitation of the natural diversity at *R* loci and the isolation of novel resistance gene analogs, including ones with a broader target spectrum. Additionally, with the increased knowledge about structure and function of *R*-genes it might become possible to design new variants with altered specificities. Other alternatives for durable resistance would be expression of various defence signalling components or the combined expression of *R*- and *avr*-genes. However, such approaches will have to

be very carefully controlled to restrict the effects to the site of infection by the use of pathogen-inducible promoters that are tightly regulated.

Another important aspect that is analysed in paper II is the stability of genetic integration of transgenes and the stability of expression of the transgenes in subsequent doubled haploid generations. The results demonstrate that such stability is not always obtained and that it is very important to carefully analyse over several generations the stability of an inserted trait in new transgenic lines if these are to be considered for commercial use.

Paper III and IV describe the screening for potential insecticidal proteins and subsequent evaluation of pea lectin in oilseed rape for conferring resistance to pollen beetles. When pollen beetle larvae were fed anthers from the transgenic oilseed rape plants there was a clear reduction in larval weight, but only a minor effect on larval survival. Further evaluation of the interaction between pollen beetles and the transgenic oilseed rape would be needed in order to properly examine this approach, including comparisons of damage rates on transgenic and non-transgenic lines. The subsequent effects on the pollen beetles after larval feeding also needs to be addressed, such as survival rates during following stages as pupae and adults and possible later effects on oviposition. If pea lectin would prove to be useful for obtaining pollen beetle resistant oilseed rape a careful analysis of effects on other insects would be needed. Although the expression pattern in this case was fine-tuned by a pollen-specific promoter for expression only in the target tissue for pollen beetles, pollinators, such as honeybees and bumblebees, are still exposed to the pea lectin. This aspect has to be carefully examined since detrimental effects on pollinators would certainly not be acceptable. In order to further improve resistance to pollen beetles and increase durability, a combination of pea lectin with another resistance factor would be favourable. If so, the lectin should be combined with a resistance factor with a different mode of action, such as proteinase inhibitors. Bt toxins with their high insecticidal activity would also be suitable to combine with, but so far no Bt toxin with effects on pollen beetles has been identified (Åhman, personal communication).

The general conclusion of the studies in this thesis is that depending on the target organism for the intended transgenic resistance the likelihood to be successful varies. This outcome reflects quite well the general situation for transgenic resistance where the number of proven successful strategies to choose from is highest for virus resistance and lowest for fungal resistance. In addition to requirements on protection level and durability of resistance also such aspects as possible environmental influences as well as toxic and allergenic effects have to be evaluated and excluded in order for a product with transgenic resistance to be commercially applicable.

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