Tr. J. of Agriculture and Forestry
22 (1998) 313-321

Development of the Downy Mildew Pathogen *Bremia Lactucae* on Transgenic Lettuce Expressing a Bacterial ß-1,3-Glucanase

Yeter DEDE
Çukurova Üniversitesi Ziraat Fakültesi, Bitki Koruma Bölümü, Adana-TÜRKİYE

Received: 01.05.1995

Abstract: A β-1,3-glucanase gene from *Arthrobacter* sp. driven by the 35S promoter was singly transformed into two lettuce cultivars, Cobham Gree and Diana, using the binary vector system of *Agrobacterium tumefaciens.* Tansformation was confirmed by using Southern and Northern analysis, Npt II enzyme assays and segregation of resistance to kanamycin.

Transgenic plants were infected with the letuce downy mildew fungus, *Bremia lactucae* which contains β-1,3-gluncan in its cell wall and alternations in the development the fungus could easily be monitored. Transgenic plants inoculated with *B. lactucae* showed different levels of resistant responses compared to the control and they were examined microscopically.

Bakteriyel bir ß-1,3-Glukanaz Geni Transfer Edilen Marul Bitkisinde Mildiyö Patojeni *Bremia Lactucae*' nın Gelişimi

Özet: Marul çeşitleri Diana ve Cobham Creen'e *Agrobacterium tumefaciens*'in bitkilere gen transfer etme özelliğinden faydalanarak *Archrobacter* sp. orjinli bir β-1,3-glukanaz geni 35S promoter kontrolünde transfer edilmiştir. Gen transferi Southern ve Northern hibridizasyon teknikleriyle, NPT II enzim aktivite testi ve kanamisine dayanıklıktaki genetik açılma ile kanıtlanmıştır.

Gen transferi yapılan bitkiler hücre duvarında β-1,3-glukan içeren ve gelişimi kolayca izlenebilen mildyö etmeni *Bremia lactucae* ile infekte edilmiştir. Kontrole oranla bu bitkilerde değişik seviyelerde dayanıklık reaksiyonları gözlenmiş ve bunlar mikroskobik olarak incelenmiştir.

Introduction

Induced resistance in many plants is accompanied by the induced synthesis of PR (Pathagenesis-Related) proteins including chitinase and β-1,3-glucanase. β-1,3-glucanases are involved in the natural defence of plants against fungal infection (1). Plant β-1,3-glucanases degrade β-1,3-glucan in fungal cell walls and can inhibit fungal growth (2). In higher plants, β-1,3-glucanase activity increases in response to pathogen infection or hormonal treatments. Several studies suggest that plant β-1,3-glucanase may be components of a general plant defence mechanism against pathogen invasion in a number of different plant species (3, 4, 5). Plant chitinases and β-1,3-glucanases have been studied in detail.

Since chitinases and β-1,3-glucanases are induced by pathogen attack, strees and ethylene treatment and their substrates, chitin and β-1,3-glucan are important structural elements of the cell walls of many fungi and bacteria, these enzymes are thought to play multiple roles in resistance. First, they may release signalling molecules (β-glucan and chitosan oligomers) that act as elicitors of active host defence responses and, secondly, they may inhibit pathogen growth by degrading the pathogen cell wall or disrupting its deposition. Purified plant hydrolases have been shown to inhibit the growth of several fungi *in vitro.* The genral conclusion from in vitro tests in that plant chitinases are effective against chintin containing fungi whereas β-1,3-glucanase are effective against glucan containing fungi, and they are more effective in combination (5, 6, 7).

Although β-1,3-glucanases are a limiting factor in the defence reaction, some pathogens can still overcome the effect of this hydrolase probably due to in sufficient amount of the enzyme and relatively late upon pathogen attack or the pathogen may develop a resistance mechanism to this enzyme. Therefore it is important to use a β-1,3-glucanase different from plant one.

Chitinases and β-1,3-glucanases have also found in several fungi and bacteria during their developmental stages or to supply nutrient from their environment. Some of these hydrolases genes have been cloned and analysed (8, 9, 10). The chitinase gene from *Seratia mercencens*, a gram negative soil bacterium, was cloned and expressed in *Pseudomonas* ssp. The *Pseudomonas* ssp. expressing chitinase activity inhibited *Fusarium oxysporum* f. sp. *redolens* germ-tubes and reduced disease of radish caused by the same fungus (11). This chitinase was also expressed in *Escherichia coli* and when tested on *Sclerotium rolfsii* and *Rhizoctonia solani* in cotton under greenhouse conditions, it was found to be effective as a control agent (12). Logemann *et al.* (13) expressed this gene constutitvely in tobacco, resulting in plants that when infected with *R. solani* showed markedly less infection symptoms than control and also grew as fast as uninfected plants. A chitinase gene has also been cloned from *Bacillus circulans* that showed possible homology to other prokaryotic chitinases at the amino acid level (14). Chitinolytic activity has been shown other in bacteria including *Aeromonas cavia* (15) and *Gliocladium virens* (16).

Since bacterial hydrolases showed encouraging anti fungal activity *in vitro* and some *in vivo,* we were interested in the use of microbial hydrolases in plant defence mechanisms. A β-1,3-glucanase gene from *Arthrobacter* which was cloned by Doi and Doi (17) was chosen. The β-1,3-glucanase has been shown to digest long glucan molecules to a minimum of a pentaglucan chain and it is molecules of this length or longer that have been shown to be most effective elicitors of plant defence responses (18).

Doi *et al.* (19) first analysed the two glucanases (I and II) in the liquid culture of a *Arthrobacter* sp. strain YCWD3. They found that the enzyme was stable at 30℃ for 100 min but lost activity when incubated above 60℃ and at pH values from 3 to 8, the optimum pH was found to be 5.5-6.5. Both enzymes were found to be endo-glucanases. Glucanase I produced laminaripentaose from a linear chain of β-1,3-linked glucose units but it was incapable of attacking short chain laminaridextrans, while th second glucanse II was capable of hydrolysing short chain laminaridextrans to laminaribiose and glucose. Both were secreted extracellularly and acted synergistically in the degradation of yeast glucan (20). Doi and Doi (17) cloned the glucanase I from the *Arthrobacter* sp. strain YCWD3. The gene was recloned and expressed in a *Streptomyces lividans* secretion system by Beynon (unpublished data).

This paper reports studies on the interactions between *Bremia lactucae* and lettuce plants that had been transformed with the β-1,3-glucanase gene using *Agrobacterium*- mediated transformation system. *B. lactucae*- lettuce interaction was chosen as a model system because *B. lactucae* is an obligate parasite of lettuce and resistant and susceptible reactions can easily distinguish. The other important point is that *B. lactucae* contains β-1,3-glucan, which is the substrate of β-1,3-glucanase, in its cell wall and also the most important pathogen of lettuce.

Materials and Methods

Plant and fungal materials

Lettuce plants (*Lactucae sativa*) cultivar (cv) Diana (Tozer Ltd.) with *Dml, Dm3, Dm5/8* and *Dm7* and Cobham Green with no *Dm* gene determined for resistance to downy mildew disease (21, 22, 23) were used for the transformation and pathogneicity tests. Seedlings were grown in a peat based compost in a growth room at 21℃ under 16 hours photoperiod. Larger plants with 6-8 leaves were transferred to the glasshouse. Seedlings for pathogenity tests were grown on 3MM paper moistened with fungicide, Rovral (Rhone Poulenc) solution (20 mg/L) in clear perspex boxes in a growth room under the same conditions. Transformed lettuce plants were grown in approved containment conditions, in a designated cubicle in the greenhouse.

Bremia lactucae isolates, CL9W and TV, used for the experiments were maintained by mass transfer of spore suspension to lettuce seedlings grown on 3MM paper in a perspex box. The fungal isolates were kindly provided by Mark Bennett and Matthew Gallagher, Wye College, Department of Biological Sciences.

Preparation of spore suspension was based on the method of Maclean and Tommerup (24). Cotyledons showing profuse sporulation but no sign of bacterial infection were harvested and transferred to 30 ml

plastic Sterilin centrifuge tubes containing 20 ml sterile double distilled water (SDDW) at 15°C. The tubs were shaken a few min to dislodge spores and the suspension was filtered through a 50 µm sieve (Endecotts Ltd.). The spores were then washed at least twice with SDDW at 15°C by centrifugation at 200 g for 1 min. Spore concentration was adjusted to 10^5 spores/ml by counting in a haemocytometer.

To determine infection profiles in long term experiments in a large population, 7-10 days seedlings were sprayed with a spore suspension and incubated in a illuminated incubator at 15°C with a 12 hour photoperiod. As soon as sporulation had been seen, about 5-6 days, after inoculation, infection was scored as percentage of sporulating cotyledons. Scoring was carried out until sporulation reached 100% on the controls. The cotyledons with no sporulation were clared and prepared for microscopy.

Leaf discs from transformants and non transformants were laid with the abaxial surface uppermost, on a plastic mesh in a perspex box baselined with wet tissue paper. 20 µl of the spore suspension was placed on the abaxial surface and then the boxes were incubated at 16°C under a 12 hour photoperiod. The discs were blotted on dry tissue paper after 24 hours and incubated under the same conditions. As soon as first sporulation was seen, infection was scored on a scale of 0-5: 0, 0 sporulation visible; 1, a few sites of sporulation; 2, about 25%; 3, 26-50%; 4, 51%; 5, >75% of disc covered by sporangia (25). About 11 days after inoculation, leaf discs with no sporulation were examined microscopically. To examine the progress of fungal development in cotyledons an leaf dtiscs, they were decolorized overnight in 100% methanol and cleared in chloral hydrate solution (0.6 g/ml water). After fungal infection structures were clearly seen, tissues were mounted in 50% glycerol to have semipermanet slides for light microscopy.

Non transformed parental plants were used as controls in the pathogenity tests.

Plasmid construct and DNA transformation

The plasmid carrying the β-1,3-glucanase gene contains a napoline synthase (NOS) promoter expressing the neomycin phosphotransferase (NPT II) gene for kanamycin resistance and cauliflower mosaic virus 35S promoter (26) driven the β-1,3-glucanase gene cloned from *Arthrobacter* sp. (17). The soybean ribulase biphosphate carboxylase small subunit (SRS1) gene was used to increase the level of β-1,3-glucanase gene expression. Downstream from the β-1,3-glucanase and NPT II gene is a fragment carrying the polyadenilation signals from the *A. tumefaciens* (Nos polyA). pLMH24 was introduced into *A. tumefaciens* strain LBA 44004 by conjugation using tri-parental mating as described earlier (27). Disarmed *A. tumefaciens* starin LBA 4404, competent cells of *Escherichia coli* starin JM 109 containing pLMH24 and *E. coli* starin MM294 harbouring pRK 2013 helper plasmid were used for triparental matings.

Lettuce cultivars Cobham Green with no *Dm* genes and Diana with 4 *Dm* genes were used for the transformation. Lettuce cotyledons were infected with *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid, pLMH24. Transformation procedure was carried out as described earlier (28) and kanamycin-resistant plants were regenerated.

Blot analysis of nucleic acids

Plant DNA was isolated from transformed and untrasformed plants according to Dellaporta *et al.* (29). 10 µg of total DNA were digested with *Hind* III in the appropriate restriction enzyme buffer for the β-1,3-glucanase gene. The digested DNA were separated on 0.8% agarose gels in TAE buffer (40 nM Tris-Acetate and 1 nM EDTA, pH 7.8). After electrophoresis DNA fragments were transferred to the charged membrane (Hybond N+ from Amersham RPN 203B) according to Maiatis *et al.* (30). The filters were separately probed with β-1,3-glucanase gene fragments labelled with 32P using a commercial random primer kit (Oligolabelling kit, Promega). RNA was isolated and purified as previously described (27) and separated on a denaturing 1.2% agarose/formaldehyde gel and transferred to nylon membranes. The filters were hybridised with the probes used in Southern analysis.

Results

Expression of bacterial ß-1,3-glucanase in transgenic lettuce

The chimeric β-1,3-glucanase gene was introduced into lettuce cultivars Cobham Green and Diana by leaf disk transformation with the Ti-plasmid binary vector pLMH24. This vector contanis the β-1,3-glucanase gene from *Arthrobacter* sp. under the control of the expression signals of the 35S promoter of cauliflower mosaic virus. The vector also contains a NPTII gene with nopaline-syntase expression signals to provide kanamycin resistance (KmR) as a selectable marker in

plant cells. Southern blot analysis showed that the β-1,3-glucanase transformants contained the 2.37 kb *Hind*III fragment of pLMH24 coding β-1,3-glucanase. The segragation of the KmR trait in the F1 generation (selfed primary transformants) and F2 generation (selfed F1 plants) revealed that the transformants contained only one T-DNA insert. Four transgenic Cobham Green and five Diana lines containing the β-1,3-glucanase gene and KmR were chosen for further Northern blot analysis and pathogenecity tests.

Transcription of the β-1,3-glucanase gene was confirmed by Nothern blot analysis (Fig. 1). Total cellular RNA was extracted from non-transformed control plants and KmR plants of each trasgenic line. The same but freshly prepared probe used in Southern blots was used as a probe to the Nothern blots. As it is seen from Fig. 1, some lines showed higher transcription level than others while controls showed no expression.

Pathogenicity of *Bremia lactucae* on lettuce plants expressing a bacterial b-1,3-glucanase gene

Development of isolate CL9W isolate of *B. lactucae* was examined on leaf discs of β-1,3-glucanase transformed plants and controls. The results of these inoculations are summarised in Figure 2. Sporulation was recorded daily from the first appearance of sporulation until 11 days after inoculation (data presented are from duplicated experiments). As can be seen from figure 2, on the first day of sporulation (day 6) about 60% of leaf discs from the control plants showed sporulation with levels varying up to category 4. By contrast, in the transgenic lines, a maximum of 35% of leaf discs showed sporulation and mostly at level 1. Transgenic line CG1-20 showed more susceptibility compare to other transgenic lins, but sporulation was still less than control. On the lines, CG1-21 and CG1-23, only a few disc showed (5%) sporulation on the first day of sporulation and after 11 days only 50% sporulation was recorded indicating that these lines showed intermediate resistance as described by Maclean and Tommerup (25). The intermediate phenotype has been categorised as being compatible during the initial phase of infection but becoming incompatible in older regions of the infection site and producing low sporulation overall. On the other hand, line CG1-29 showed only 10% sporulation 11 days after inoculation. This line showed almost compete resitance in leaf disc experiments. It was noted that on leaf discs from transgenic plants without sporulation, necrotic flecks, similar to those that Crute and Norwood (25) reported as incomplete resistance, were visible. These flecks did not appear on the control. In addition, no spores were produced on some discs, these discs were microscopically examined to ensure that germinated spores were present at the microscopic level. The fungus developed primary and secondary vesicles, short intercellular hyphae and a few

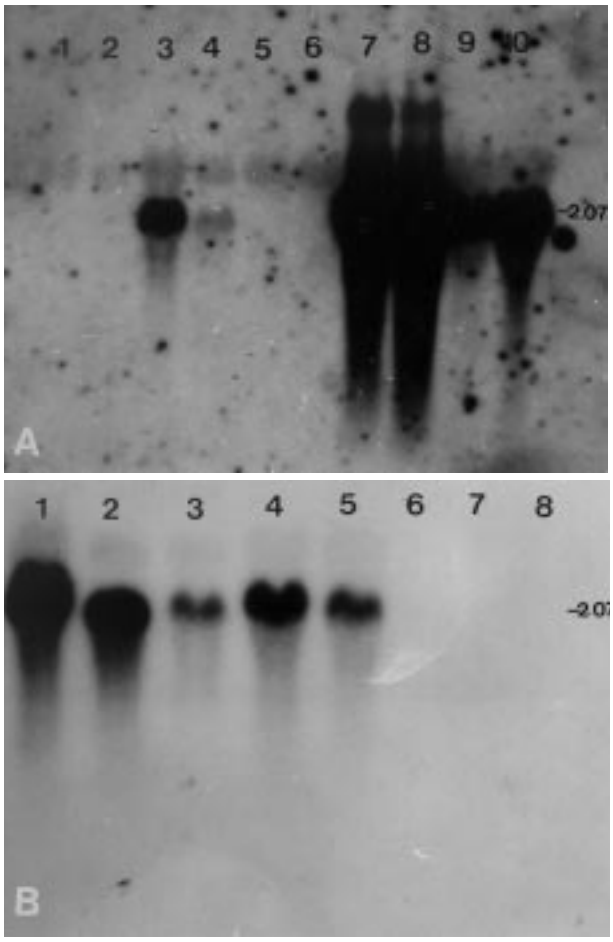

Figure 1. Northerm blot analysis of the plants transformed with the β-1,3-glucanase carried on pLMH24. (A) RNA was isolated from individual kanamycin resistant F_2 plants of each line, lanes are; 1 and 2, untransformed controls, 3 and 4 transgenic line CG1-20_2 and-20_4; 5 and 6, CG1-21_1 and 21_4; 7 and 8, CG1-23_4 and-23_5; 9 and 10, CG1-29_2 and -29_4. (B) Northem blot analysis of cultivar Diana transformants tarnsformed with pLMH24 carrying the β-1,3-glucanase gene, Lanes are; CG1-23, (a-Cobham Green transformant included as a positive control); 2 to 6, Diana transformants, D1-7, D1-11, D1-5, D1-3, D1-2; 7 and 8, untransformed controls. Northem blots were prepared and hybridised with β-1,3-glucanase fragment extracted from pLMH23. Values at the right are the size in kilobases of the hybridizing RNA estimated from RNA markers.

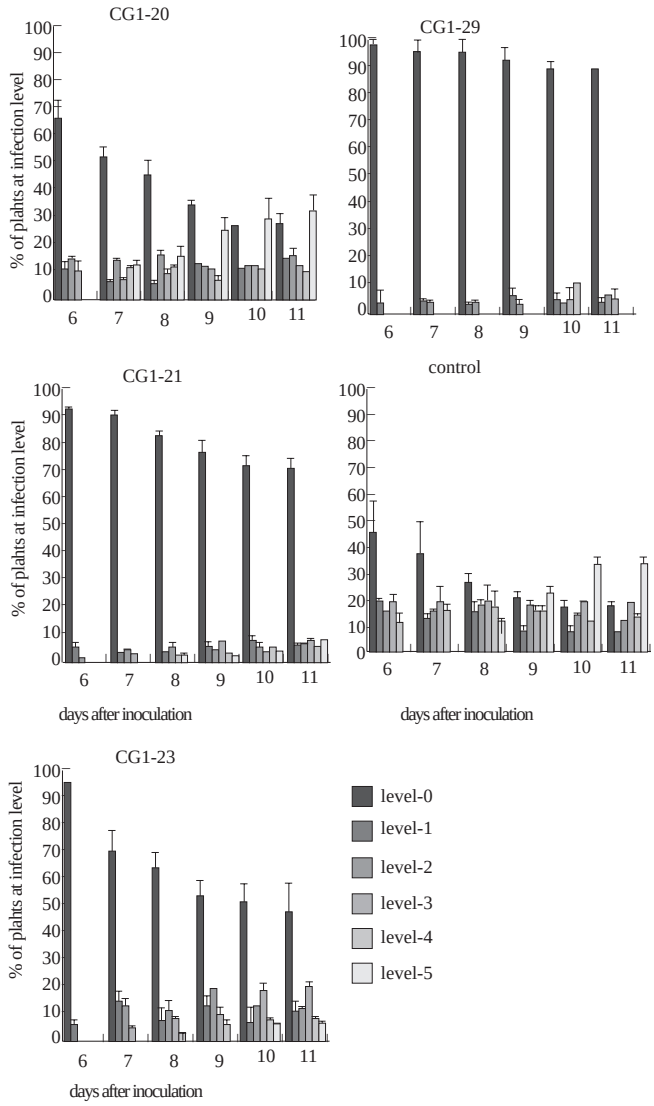

Figure 2. Sporulation of *B. lactucae* isolate, CL9W, on leaf discs of letuce plants transformed with β-1,3-glucanase gene and untransformed controls. Sporulation on leaf discs was recorded using a five point assessment scale; 0, no sporulation; 1, a few sites of sporulation; 2, about 25%; 3, 26-50%; 4,51-75%; 5, >75% of disc covered by sporophores. The data shown represent the mean of two separate experiments. CG1-20, CG1-21, CG1-23 and CG1-29 are different transgenic lines.

haustoria. Penetrated cells appeared to undergo the fleck to become necrotic and autofluorescent. It was clear that macroscopically visible necrotic flecks were due to necrosis of host cells.

Development of *B. lactucae* on transgenic cotyledons

To extend the number of plants of each transgenic line analysed for differences in resistance compared with controls, at least 50 seedlings were tested for reaction to *B. lactucae.* Seedlings were grown for 10 days and then inoculated by spraying them with a 10^5/ml spore suspesion and incubated as described in materials and methods. The numbers of cotyledons with and without sporophores were recorded from the fewer sight of sporulation until sporulation reached 100% in the controls. In this experiment two *B. lactucae* isolates, CL9W and TV, were used. Isolate CL9W has avirulence gene (A 7) matching Dm 7 genes but TV has no matching avirulence genes in cultivar Diana (22, 31). So the interaction between isolate CL9W and Cobham Green is compatible whereas that between CL9W and Diana is incompatible. Therefore, CL9W was used to analyse the Cobham Green transformants and cultivar Diana was included as a resistant control. In contrast, isolate TV was used on cultivar Diana transformants since it is compatible with this cultivar. Results are shown in Figure 3 and 4. The controls, untransformed and trasformed with vector, showed almost the same sporulation intensity (100%), 7 days

after inoculation (Figure 3). As expected no sporulation was seen on the resitant control, Diana. Trasgenic line CGI-20 showed lower sporulation than susceptible controls for first two days but reached almost control levels after the third day of sporulation. On the first day of sporulation, CG1-23 showed very low sporulation, high sporulation started one day later than others and after 7 days it reached the same severity as line CG1-21. However, no sporulation was seen on line CG1-29 which behaved in exactly the same manner as the resistant control, Diana. When high concentrations of inoculum were applied to this line, sparse sporulation was seen two weeks later, similar sporulation was also noted on Diana.

When Diana transformants were tested with isolate TV, sporulation began 5 days after inoculation and reached 100% on the controls within 10 days (Figure 4). Sporulation on both control cultivars Cobham Green and Diana was very similar. Sporulation intensity on tranformants was greatly delayed compared with controls. Lines D1-3 and D1-11 showed lower sporulation intensity than other transgenic lines and controls. Line D1-5 showed more sporulation than other lines until day 8 but D1-2 was the highest on day 9 and 10. Seedlings were cleared daily for microscopic examination until sporulation started. In the case of transformants in which sporulation was not observed 11 days after inoculation, samples were also prepared for microscopy at this time to check for the presence and activity of the pathogen. Inoculation of Diana and transgenic line CGI-29 with isolate CL9W showed necrosis after producing primary and secondary vesicle and sometimes short hyphae whereas on the other transgenic lines the pathogen produced long intercellular hyphae and then became necrotic.

Discussion

There is considerable direct and indirect evidence for the role of chitinases and β-1,3-glucanases in defending plants against bacterial and fungal infections (32, 33). However some fungal pathognes still cause disease on plant. The fungus has probably adapted to the defence mechanisms of plants, therefore it might be a good idea to express hydrolases from unrelated species in plants. The new activities represented by these hydrolases may be effective against the pathogenic fungi so that the new hydrolases cannot be overcome by the invading fungus.

Therefore, we used the β-1,3-glucanase from *Arthrobacter* sp. in order to test its activity in the transgenic lettuce against β-1,3-glucan containing pathogens.

In order that the enzyme has an increased change of coming into contact with the pathogen, the signal sequence from a hydroxyproline rich glycoprotein was used to secrete the β-1,3-glucanase from the plant cells. Sela-Buurlage et *al.* (6) modified the *chi*I and *glu*d, gene products that are normally located in vac-

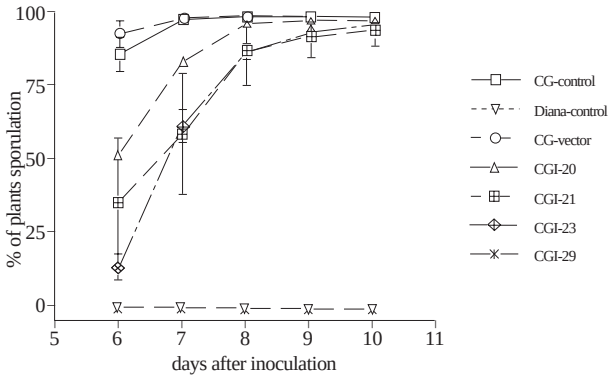

Figure 3. Pathogenity results on Cobham Green transformed with β-1,3-glucanase gene and controls inoculated with isolate CL9/W. CG1-20, -21, -23, -29; transformants, CG-vector; Cobham Green transformed with vector, CG-control and Diana control; untransformed susceptible and resistant controls, respectively. The data shown represents the mean of three separate experiments. Note; curves for Diana and CG1-29 are superimposed.

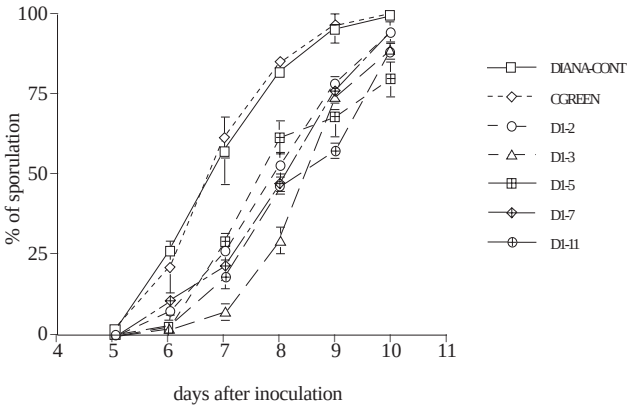

Figure 4. Pathogenicity result on Diana transformed with β-1,3-glucanase inoculated with *B. lactucae* isolate TV. D1-2, -3, -5, -7, -11; different transformants. Diana and Cobham Green; untrasformed susceptible controls. The data shown here represents the mean dof two separate experiments.

uoles, so that they were secreted extracellularly. Some transgenic plants expressing the β-1,3-glucanase of *Arthrobacter* extracellularly clearly showed resistant reactions, such as delayed and low sporulation.

In the first experiments, the development of *B. lactucae,* isolate CL9W, was examined on leaf discs of transgenic lines, CG1-20, CG1-21, CG1-23 and CG1-29, and the control. It has been shown that spore productivity can be used to assess host resistance to fungal pathogens (34). Sporulation was initially recorded on the 6th day after inoculation, in the contro about 60% of leaf discs showed varying levels of sporulation (level 1 to level 4), whereas in transgenic lines a maximum of 35% of laf discs showed sporulation (level 1 only). Transgenic line CG1-20 gave more sporulation compared to other lines, but still less than control. The β-1,3-glucanase expression was quite low in this line as well (Figure 1), possibly explaining why it was more susceptible than others. On the other hand, no gene expression was obtained from line CG1-21, and this line showed a more pronounced reaction than line CG1-20. These phenotypes were possibly due to somaclonal variatons during tissue culture processing, as earlier reported by Brown *et al.* (35). They found that plants regenerated from callus derived from cotyledons showed enchanced and reduced susceptibility to lettuce mosaic virus (LMV) and dowyn mildew as well as other morphological changes such as albinism, and changes in chlorophyll content. In Brown's studies two lines did segregate in a ratio of 3: 1 non-sporulation: sporulation, suggesting non-sporulation was dominant. However when higher inoculum concentrations (>10^5 spores/ml) were used, this result was not confirmed. They concluded that reduced or delayed sporulation may be components of partial or field resistance and may not be fully expressed in seedling tissues.

Transgenic line CGl-23 showed a low sporulation level. On the first day that sporulation was observed on the control, only 5% of leaf discs were covered with sporophores at level 1 (a few sites of sporulation), while in the control 55% of discs showed sporulation up to level 4. Another important point with this line is that the β-1,3-glucanase gene expression is much higher than others. This may explain why CG1-23 showed delayed and less sporulation. Interestingly, line CG1-29 showed the most resistant reaction among the lines tested, although the gene expression was lower than CG1-23.

Eleven days after inoculation, the leaf discs that did not show any sporulation were microscopically examined to check whether non-sporulation was due to lack of inoculum. All of the non-sporulated leaf discs of transgenic lines had inoculum on them. The fungus produced intercellular hyphae and haustoria but hyphae became necrotic and stopped growing further. This reaction type, reduced sporulation and necrotic flecks, were similar to those of Crute and Narwood (25) results, who called it intermediate resistance.

In order to determine the differences in terms of pathogen development between transgenic lines and controls, large numbers of cotyledons from each transgenic line were used for pathogenicity test. The *B. lactucae* isolate CL9W was used for a transgenic cultivar Cobham Green, because this isolate has a compatible interaction with cultivar Diana. In this experiment cultivar Diana was included as a resistant control, and untransformed and vector transformed Cobham Green were also in included as susceptible controls. As can seen from Figure 3, susceptible controls showed 100% sprolation 7 days after inoculation while transgenic lines except CG1-29 reached 95% sporulation 10 days after inoculation. This indicates clear differences between fully susceptible controls and the β-1,3-glucanase transformed line. Line CG1-20 showed less sporulation at day 6 and 7 but after day 7 sporulation intensity in the population was very close to the susceptible controls. This line showed more susceptibility than other transgenic lines confirming the leaf discs experiment. As mentioned earlier, the level of glucanase gene expression is less than CG1-23 and CG1-29, suggesting a correlation between resistance and level of β-1,3-glucanase expression. However, line CG1-21 was more resistant than CG1-20 but did not show expression of the β-1,3-glucanase gene. This suggests that the resistance is not due to the expression of the β-1,3-glucanase gene, but that it might from somaclonal variations during regeneration in tissue culture. However, line CG1-23 was more resistant than CG1-20 and CG1-21 and this line also showed the highest expression of the gene, suggesting a correlation with resistance. It was obvious that the pathogen development particularly during the first two days of sporulation was delayed on this line. So this line showed the partial resistance as initially expected. It has been also reported from other studies in which PR proteins had been overexpressed that some transgenic plants showed enhanced but not complete resistance against the fungal pathogen tested (13, 36).

The most interesting result was obtained from line CG1-29 which demostrated resistance similar to that

of the resistant control, cultivar Diana. Only transgenic line CG1-29 was checked with isolate TV because this line gave a completely resistant reaction with isolate CL9W. From these data, line CG1-29 showed sporulation with TV, but sporulation intensity was lower than with the untrasformed controls Diana and Cobham Green. This suggests that this line is not fully resistant to isolate TV, the strong resistance might be specifically to isolate CL9W. However, resistance to TV is also increased in this line which suggests that the transgenic line may have been made more generally resistant.

Combination of these hydrolases in the same plant could have improved the recorded impact on pathogenicity. The would have been expressed from an appropriate promoter in combination or individually. If the promoter was specifically activated when the pathogenic fungus attacks, the plant would produce large amounts of hyrolase only when it is challenged by the pathogen. In this way the hydrolases can be used more effectively by the plant against fungal attack.

In conclusion, since the resistance phenotype was obtained and was shown to be heritable, it is a worthwhile approach to analyse these transgenic plants to find out the reason for the resistance. The answer might be interesting such as the formation of new resistance gene that may be used in the future.

References

1. Meins, F.J., Alh, P., Induction of chitinase in tobacco plants infected with Psedomonas tabaci and Phytophthora parasitica var. nicotianae, Plant Science, 61, 155-1671, (1992).
2. Fleet, G.H., Phoff, H.J., Fungal Glucans- Structure and Metabölism, Encycyopedia of Plant Physiology Plant Carbohydrates II, Tanner, W. and Loewus, F.A. (Eds.). New York, Springer-Verlog Pub., (1981).
3. Behamou, N., Grenier, J., Asselin, A., Legrand, M. Immunogold localization of β-1,3-glucanases in two plants infected by vascular with fungi, The Plant Cell, 1, 1209-1221 (1989).
4. Joosten, M.H.J., De Wit, J.G.M., Identificarion of several pathogenesis-related proteins in tomato leaves inoculated with Cladosporium fulvum (syn. Fulvia Fulva) as β-1,3-glucanases and chitinases, Plant Phsiol., 89, 945-951, (1989).
5. Mauch, F., Mauch-Mani, B., Boller, T., Antifungal hydrolases in pea tissue. II. inhibition of fungal growth by combination of chitinase. Plant Physiol., 88, 936-942, (1988).
6. Sela-Buurlage, M., Ponstein, A.S., Bres-Volemans, S.A., Melchers, L.S., Elzen, P.J.M., Comelissen, B.J.C., Only specific tobacco (Nicotiana tabacum) chitinases and β-1,3-glucanases exhibit antifungal activity., Plant Physiol., 101, 587-863, (1993).
7. Brogli, K., Chet, I., Holloday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvalis, C.J., Broglie, R., Transgnic plants with enhanced resitance to the fungal pathogen Rhizoctania solani., Science. 254, 1194-1197, (1991)
8. Flach, J., Pilet, P.E., Jolles, P., What's new in chitinase research? Expreientia, 48, 701-716, (1992).
9. Vasseur, V., Arigoni, F., Andersen, H., Defago, G., Bompeix, G., Seng, J., Isolation and characterization of Aphanocladium album chitinase-overproducing mutants, J. Gen. Microbiol., 136, 2561-2567, (1990).
10. Ulhoa, C.J., Peberdy, J.F., Regulation of chitinase synthesis in Trichoderma harzianum., J. Gen. Microbiol., 137 (9), 2163-2169, (1991).
11. Sundheim, L., Poplawsky, A.R., Ellingboe, A.H., Molecular cloning of two chitinase genes from Serratia marcecens and their expression in Pseudomonas specis. Phsiological and Molecular Plant Pathology, 33, 483-491, (1988).
12. Shapira, R., Ordentich, A., Chet, I., Oppenheim, A.B., Control of plant diseases by chitinase expressed from cloned DNA in Escherichia coli., Phytopathol., 79, 1246-1249, (1989).
13. Logemann, J., Jach, G., Logemann, S., Leah, R., Wolf, G., Mundy, J., Oppenheim, A., Schell, J., Expression of a ribosome inhibiting protein (RIP) or a bacterial chitinase leads to fungal resistance in transgenic plants, In: Mechanisims of Plant Defence Responses. Friting, B., and Legrand, M., (Eds). Netherlands, Kluwer Academic Publishers. (1993).
14. Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K., Tanaka, H., Structure of the gene endocing chitinase D of Bacillus circulans WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases, J. Bacteriol., 174, (1992).
15. Inbar, J., Chet, I., Evidence that chitinase produced by Aeromonas caviae is involved in the biological control of soil-bome plant patpogens by this bacterium, Soil Biol. Biochem., 23, 973-978, (1991).
16. Pietro, A., Lorito, M., Hayes, C.K., Broadway, R.M., Harman, G.E., Endochitinase from Gliocladium virens: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin, Phytopathol, 83, 308-313, (1993).
17. Doi, K., Doi, A., Cloning and expression in Escherichia coli of the gene for an Arthrobacter β-(1,3)-glucanase. J. Bacteriol. 168, 1272-1276, (1986).
18. Waldmüller, T., Cosio, E.G., Grisebach, H., Ebel, J., Release of highly elicitor-active glucans by germinating zoosprose of Phytophthora megasperma f. sp. glycinea, Planta, 188, 498-505, (1992).
19. Doi, K., Doi, A., Fukui, T., Prufication and Properties of lytic β-glucanse from an Arthrobacter bacterium, Agr, Biol. Chem., 37 (7), 1619-1627, (1973).

20. Doi, K., Doi, A., Ozaki, T., Fukui, T., Further studies on the heterogeneity of the lytic activity for isolated yeast cell wall of the components of an Ardthrobacter glucanase system: properties of the two components of a β-1,3-glucanase, Agr, Biol. Chem., 40, 1355-1362, (1976).

21. Crute, I.R., Johnson, A.G., The genetic relationship between races of Bremiae lactucae and cultivars of Lactucae sativa (1976).

22. Norwood, J.M. Crute, I.R., The Genetic control and expression of specificity in Bremia lactucae (lettuce downy mildew), Plant Pathology, 33, 385-400, (1984).

23. Paran, I., Keseli, R., Michelmore, R., Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markesrs linked to downy mildew resistance genes in lettuce, using near-isogenic lines, Genome, 34, 1021-1027, (1991).

24. Maclean, D.J., Tommerup, I.C., Histology and physiology of compatibility and incompatibility between lettuce and the downy mildew fungus. Bremia lactucae Regel, Physiological Plant Pathology, 14, 291-312, (1979).

25. Crute, I.R., Norwood, J.M., Incomplete specific resistance to Bremia lactucae in lettuce, Ann. Appl. Biol., 89, 467-474, (1978).

26. Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G., Fraley, R.T., Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants, Nucleic Acids Res., 15, 1543-1559, (1889).

27. Ainsworth C., Beynon, J., Buchanan-Wollaston, V., Techniques in plant molecular biology. Wye College Press, 150, (1991).

28. Dede, Y., The use of bacterial chitinase and β-1,3-glucanase genes to engineer disease resistance in lettuce. PhD Thesis, University of London, 223, (1994).

29. Dellaporta, S.L., Wood, J., Hicks, J.B., A plant DNA minipreparation: Version II. Plant Mol. Biol. Reporter, 1, 19-21, (1983).

30. Maniatis, T., Frisch, E.F., Sambrook, J., Molecular Cloning, a Labortory Manual. Cold spirng harbor labarotory press, N.Y., (1982).

31. Farrara, B.F., Ilott, T.W., Michelmore, R.W., Genetic Analysis of factors for resistance to downy mildew (Bremia lactucae) in species of lettuce (Lactua sativa and L. serriola), Plant Pathology, 36, 499-514, (1987).

32. Linthorst, H.J.M., Pathognesis-Related Proteins of Plants, Critical Reviews in Plant Science, 10, 123-150, (1991).

33. Broglie, R., Broglie, K., Chitinases and plant protection. Mechanisms in plant defence respond. Fritig, B. and Legnard, M. (Eds.) Netherland, Kluwer Academic Publishers, (1993).

34. Johnson, R., Taylor, A.J., Spore yield of pathogenesis in investigations of the racespecifity of host resistance, Annual Review of Phytopathology, 14, 97-119, (1976).

35. Brown, C., Lucas, J.A., Crute, I.R., Walkey, D.G.A., Power, J.B., An assessment of genetic variability in somacloned lettuce plants (Lactuca sativa) and their offspring. Ann. App. Biol., 109, 391-407, (1986).

36. Vierheilig, H., Alt, M., Neuhaus, J.M., Boller, T., Wiemken, A., Colonization of transgenic Nicotiana sylvestris plants, expressing dtifferent forms of Nicotiana tabacum chitinase, by the root pathogen Rhizoctonia solani andt by the mycorrhizal symbiont Gomus mosseae, Molecular Plant-Microbe Interaction, 6, 261-264, (1993).