Theor Appl Genet (1998) 96 : 1004–1012

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Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance

Received: 5 November 1997 / Accepted: 11 November 1997

Abstract Transformation of barley and wheat via particle bombardment with a gene derived from *Vitis vinifera* L. (*Vst1* gene) resulted in the expression of the foreign phytoalexin, resveratrol, in the transformed plants. Transgenic barley plants were regenerated from microspores and transgenic wheat plants from immature embryos were both selected on Basta. Stable integration of the gene in the genomes of transgenic barley and wheat plants, as well as their progeny, was analysed by Southern-blot analysis. The induction of the stilbene synthase promoter and the transient expression of stilbene synthase-specific mRNA after induction by wounding and infection were proofed in T_1 and T_2 progeny plants. An enhanced expression of the *Vst1* gene under control of the stilbene synthase promoter was observed with enhancer sequences from the cauliflower mosaic virus 35*s* (CaMV 35*s*) promoter. The enzyme activity of the stilbene synthase was analysed in T_1 progeny plants. The first pathological results indicated an increased resistance of transgenic barley plants to *Botrytis cinerea* used as a model experimental system.

Key words Cereals · Barley · Wheat · Biolistic transformation · Stilbene synthase · Resveratrol · Phytoalexin

Introduction

Diseases of crop plants, especially those of cereals, cause world-wide and dramatic yield losses. Fungal diseases are one of the principal causes of crop losses. Currently, the epidemic spread of fungal diseases is controlled by the application of agrochemicals, breeding for resistant varieties, and various crop-husbandry techniques, such as crop rotation. Classical plant breeding has created new cultivates with resistance to fungal diseases, but these plant-breeding programs are based on processes of crosses, back crosses and selection. These time-consuming techniques can hardly keep pace with the rapid evolution of pathogenic microorganisms and pests. Alternative strategies, like genetic engineering and molecular-marker-assisted selection techniques, are currently being tested for the efficient production of genetic variability in a plant population. These methods allow for the rapid identification and isolation of genes and gene segments, and for the transfer of traits from one species to another, in cases which are beyond the limits of sexual compatibility.

Plants respond to pathogen attack by inducing several defence reactions. Obviously, different protective mechanisms play an important role in the overall expression of disease resistance (Dixon and Harrison 1990). These include the synthesis of polymers, like cutin and lignin, forming physical barriers, and of pathogenesis-related proteins, like the hydrolytic enzymes chitinases and β-1,3-glucanases. Some of these hydrolytic enzymes have been shown to increase the resistance of plants against various pathogens in vitro (Mauch et al. 1988; Sela-Burrlage et al. 1993). This defence system has been successfully transferred to tobacco, where Transgenic plants showed an enhanced protection against fungal attack (Zhu et al. 1994; Jach et al. 1995). Another important part of the plant defence system is the induction of antimicrobial compounds called phytoalexins. These low-molecular-weight products of the secondary plant-metabolic pathway are often synthesised locally and accumulate after exposure to pathogens and/or stresses. Several unrelated plants like grapevine (*Vitis vinifera* L.), peanut (*Arachis hypogaea* L.) and pine (*Pinus sylvestris* L.) synthesise the

Communicated by G. Wenzel

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stilbene-type phytoalexin resveratrol when attacked by pathogens.

Stilbene synthase, also termed resveratrol synthase, is the enzyme which synthesises the phenolic phytoalexin trans-resveratrol. The precursor molecules for the formation of resveratrol are malonyl-CoA and p-coumaroyl-CoA. They are commonly present in all plants as substrates for chalcone synthase (Rupprich and Kindl 1978). Trans-resveratrol seems to have an antifungal activity (Hart 1981) and can also be induced by other stress factors like UV light (Schöppner and Kindl 1979), wounding (Langcake 1981) or elicitor treatment (Melchior and Kindl 1990).

To-date the genes coding for stilbene synthases (STS) have been transferred into tobacco (Hain et al. 1990, 1993), oilseed rape (Thomzik 1993) and rice (Stark-Lorenzen et al. 1997) and such transgenic plants showed an increased disease resistance. When only one STS gene was used, relatively low amounts of the foreign phytoalexin were detected in transgenic plants after induction (Hain et al. 1990). In peanut and grapevine, several STS genes were found and may be necessary to produce high levels of resveratrol. After the transfer of two STS genes isolated from grapevine (*Vst1* and *Vst2*), higher levels of the foreign phytoalexin were detected after elicitor or pathogen induction (Hain et al. 1993).

In the present paper we demonstrate the transformation of barley and wheat with the stilbene synthase gene from *V. vinifera* (*Vst1* gene) resulting in transgenic plants with an increased resistance to fungal attack. To increase the expression of the *Vst1* gene under the control of the homologous Vst1 promoter we modified the gene with the enhancer of the 35*s* promoter from CaMV (35*s*).

Materials and methods

Plant material and culture of the different target tissues

For stable transformation of barley the winter-type cv Igri was used. Growth conditions of the donor plants and the isolation, culture and selection of the microspores were performed according to the protocol of Jähne et al. (1994).

The wheat transformation was carried out using the spring-type cv Veery # 5 and the winter-type cv Florida. Growth conditions of the donor plants and the isolation, culture and selection of the immature embryos were as described by Becker et al. (1994).

Plasmid construction

For the transient transformation experiments of immature barley embryos the plasmids pVstPG and pVstEPG were employed. The construct pVstPG contains the *uidA* gene under the control of the stilbene synthase promoter. (Fig. 1 a). pVstEPG contains the *uidA* gene under the control of the stilbene synthase promoter with the 4-fold enhancer of the 35*s* promoter. (Fig. 1 b). These two plasmids were also used for the stable transformation of barley protoplasts isolated from the suspension culture DL17, via PEG, in co-transformation with the plasmid pCaIneo (S. Lütticke, unpublished). For the stable transformation of barley microspores and immature wheat embryos the plasmids pGBI (Fig. 1 c) and pGBII (Fig. 1 d) were employed. The plasmid pGBI contains the *Vst1* gene under the control of the Vst1 promoter and the 4-fold enhancer, together with the *pat* gene under control of the CaMV 35*s* promoter. The plasmid pGBII contains only the *Vst1* gene under the control of the Vst1 promoter. This plasmid was used in co-transformation with the plasmid p35SPAT.

Particle bombardment

Plasmid DNA was precipitated onto gold particles of an average size between 0.4 and 1.2 µm. The particles were re-suspended in either 240 µl of ethanol for immature wheat embryos or 120 µl of ethanol for barley microspores. For each bombardment, 3.5 µl of the suspended DNA-coated particles were spread onto the surface of the macrocarrier. The particle gun employed in these experiments was a PDS 1000/He gun (BioRad, München, Germany). Immature wheat embryos and barley microspores were bombarded with a helium gas pressure of 1550 psi or less. Immature barley embryos were bombarded with a helium gas pressure of 1550 psi and 116 µg of particles per bombardment and the transient *uidA* gene expression was analysed histochemically 2 days after bombardment.

Protoplast transformation

Cell suspensions were cultivated in L1D2-medium (Lazzeri et al. 1991) and L3D2-medium (Jähne et al. 1991). Protoplast isolation and transformation were performed as described by Lazzeri et al. (1991). Fourteen days after transformation the embedded protoplasts were transferred to liquid selection medium with 50 mg/l of G418. Selected microcalli were transferred to solidified L3D2-medium supplemented with 50 mg/l of G418 for further growth.

Histochemical and luminometrical GUS assays

GUS activity was determined either histochemically, as described by McCabe et al. (1988), or luminometrically, according to the protocol of Serva, version A (1993). Transient GUS activity in bombarded immature barley embryos was assayed 2 days after bombardment by incubating the embryos for 18–24 h at 37°C in staining buffer. GUS activity in stable transformed cell suspensions was measured by the luminometrical GUS assay.

Herbicide application

All selected regenerates, as well as all T_1 plants, were sprayed either completely or only over a few leaves with an aqueous solution of BASTA containing 125–250 mg/l of PPT and 0.1% Tween. The concentration of PPT was dependent on the age of the plants.

Stilbene synthase-activity test

Stilbene synthase and chalcone synthase activities were assayed according to the methods of Rolfs et al. (1981) and Schöppner and Kindl (1984) by the incubation of 4-coumaroyl CoA with [2-^{14}C]malonyl CoA for 90 min at 30°C followed by the separation of the radioactive products by thin-layer chromatography.

Extraction of DNA and Southern blotting

Total genomic DNA was isolated from leaf tissue of primary transformants and their progeny using the protocol of Dellaporta et al.

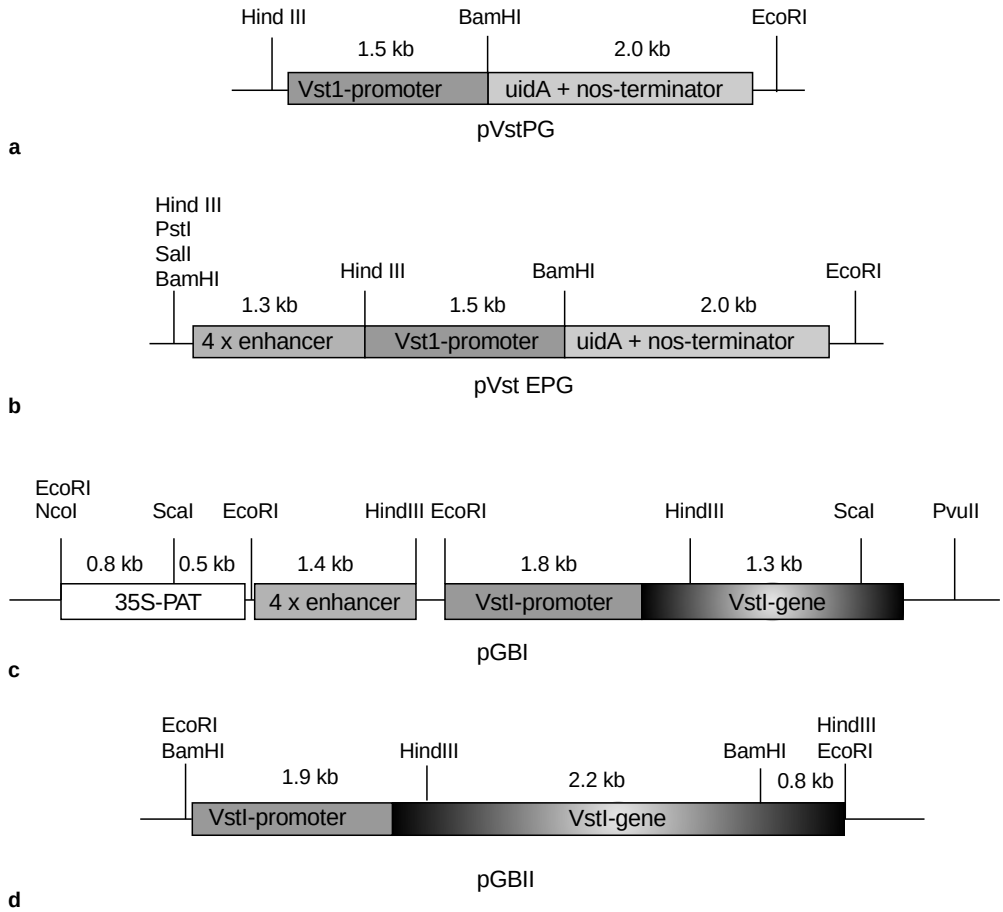

Fig. 1a–d Plasmid constructs used in transient transformation experiments (*a and b*) and in stable transformation experiments (*c and d*)

(1983). Twenty Micrograms of genomic DNA, uncut or digested with restriction enzymes, were separated by electrophoresis and transferred to a Hybond N membrane (Amersham/England). Introduced DNA was detected using a modified protocol of the non-radioactive digoxigenin chemiluminescent method (Neuhaus-Url and Neuhaus 1993). Filters were hybridised with a PCR-labelled *pat* or *Vst1* probe. Probe DNA was labelled as described by Becker et al. (1994). The primers used for the *Vst1* probe were 5′-GAGGAAAT-TAGAAACGCTCAACATGCC-3′ (position 1581 > 1607) and 5′-GACAGTTCCACCTGCATAG-3′ (position 2416 < 2434). The PCR was for 30 s at 94°C, 90 s at 58°C and 2 min 30 s at 72°C for a total of 30 cycles.

Extraction of RNA and Northern blotting

Total RNA was extracted from leaf tissue using the protocol of Gibco/BRL with TRIzol™ solution. For Northern analysis, 10–30 µg of total RNA were separated in 1.5% agarose gels and blotted on to Hybond N membrane. Filters were hybridised with the PCR-labelled DNA probes in a high-salt SDS-buffer at 50°C.

Inoculation with *Botrytis cinerea*

Transgenic progeny plants were grown under standardised conditions in growth chambers (Jähne et al. 1994) and after 14 to 21 days single leaves of the plants were harvested and placed on damp filter paper in a Petri dish. For inoculation either the whole leaf was sprayed with a spore suspension (2×10^5 spores/ml) or only 5–10 µl of the spore suspension was applied to the middle of the leaves.

Inoculation with *Erysiphe graminis*

Segments of transgenic progeny plants (2.5-cm long) were cut from the middle part of the primary leaf of 14–21 day-old seedlings, grown in a growth chamber under standardised conditions. These segments were deposited in Petri dishes on a water-agar medium (0.55% w/v) supplemented with 300 mg/l of benzimidazole. Powdery mildew spores (produced by the Resistenzlabor of the Saaten Union) were uniformly dispersed onto plant material using a settling tower with an inoculation of 300–500 spores/cm^2).

Results

Functional analysis of the Vst1 promoter in barley

The aim of the transformation experiments was to produce transgenic cereal plants which expressed the *Vst1* gene under the control of the homologous Vst1 promoter. Therefore, we first analysed the activity of the Vst1 promoter and the transcriptional activity of the 4-fold enhancer in barley by means of the *uidA*

reporter gene. For these experiments, the plasmids pVstPG and pVstEPG (Fig. 1 a, b) were used in transient and stable transformation experiments. To analyse transient *uidA* gene expression, immature barley embryos were subjected to biolistic transformation, stained histochemically 2 days after bombardment and then evaluated by counting the number of blue spots. The average number of spots per embryo was determined from four experiments with five independent bombardments per construct.

The promoter construct with the 4-fold enhancer gave between 19.4 and 34.3 transient transformation events per embryo. In comparison to the promoter construct without the 4-fold enhancer, with an average number of spots per embryo between 0.6 and 7.3, the transient transformation events increased 5-fold after transformation with the plasmid pVstEPG. In addition, some immature embryos were bombarded with a promoter fusion containing the enhancer fragment and the *uidA* gene and were analysed histochemically as well. With this construct no transient signals were detected (data not shown). These results indicate that the dicotyledonous Vst1 promoter of *V vinifera* L. is also active in monocotyledonous species like barley and that the CaMV (35*s*) virus enhancer sequences are transcriptionally active in cereals.

For analysing the induction of the Vst1 promoter in cereals, barley protoplasts from the suspension culture DL17 were transformed via PEG with the constructs pVstEPG and pVstPG in co-transformation with the plasmid pCaIneo, which contains the *nptII* gene under the control of the 35*s* promoter and the intron of the *AdhI* gene from maize. Stable transformation of the transgenes in the cell cultures was analysed using PCR. For each construct the induction of the Vst1 promoter was analysed in three independent transgenic suspensions lines. Suspension callus was taken off before and 16 h after subculture and GUS activity was analysed by chemiluminescent detection. Figure 2 represents the GUS activity measured in μU/10 μg of total protein from the different transgenic cell lines. In all cell lines analysed the enzyme activity increased 16 h after subculture to a maximal induction factor of 10.

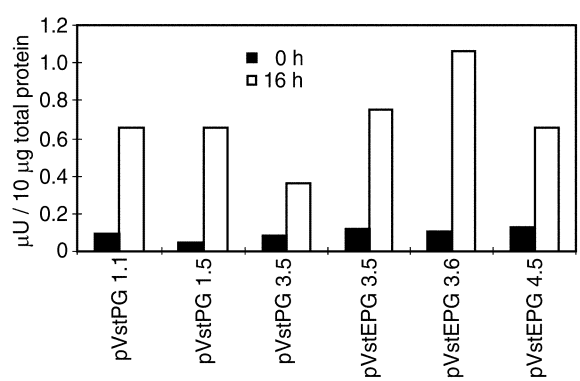

Fig. 2 Chemiluminescent GUS-assay. The enzyme activity was analysed in transgenic suspension lines transformed with the constructs pVstEPG and pVstPG without induction and 16 h after induction by changing the media

Stable transformation of barley and wheat with the Vst1 gene

The next step was the stable transformation of barley and wheat with the *Vst1* gene under the control of the Vst1 promoter both with the 4-fold enhancer and without the enhancer sequences. For these experiments we cloned the plasmids pGBI and pGBII (Fig. 1 c,d). pGBI contained the *bar* marker gene under the control of the 35*s* promoter and the *Vst1* gene under the control of its own promoter and the 4-fold enhancer. The plasmid pGBII contained only the *Vst1* gene under the control of its own promoter without the enhancer sequences and was co-transformed with the plasmid p35SPAT. Barley microspores and immature wheat embryos were used as target tissues for particle bombardment. The isolation, culture and transformation of microspores were carried out as described by Jähne et al. (1994). Immature wheat embryos were isolated, cultured and transformed following a protocol described by Becker et al. (1994). Putative transformants were screened for phosphinothricin acetyltransferase (PAT) activity by spraying the whole plants, 10–14 days after transfer to the greenhouse, with an aqueous solution of the herbicide Basta (200 mg/l) containing the active substance phosphinothricin (PPT).

The Basta-resistant T_0 plants were analysed by Southern-blot hybridisation for the presence of the transferred genes (Southern 1975)

In order to analyse T_1 plants as early as possible, immature embryos from two plants (MS53/1 and MS53/2) were removed from seeds and cultured on MS medium. The PAT activity in 24 T_1 plants of the two T_0- lines was analysed by spraying an aqueous solution of the herbicide BASTA. All progeny of the T_0 plant MS53/1 proved to be completely resistant with no visible necrotic lesions. The presence of the *Vst1* gene was first analysed by PCR. The DNA fragment of the expected site was amplified from the DNA of all progeny plants. Five of these plants were analysed by Southern-blot hybridisation. They showed an integration pattern identical to that of the parental line (Fig. 3 a). This indicates the homozygous nature of the T_0 plant MS53/1.

Only 4 out of 24 progeny plants of the T_0 plant MS53/2 showed no necrotic lesions after treatment with the herbicide BASTA. Amplification of a *Vst1* fragment was achieved from the DNA of 13 T_1-plants, and the *bar* fragment was be amplified from the DNA

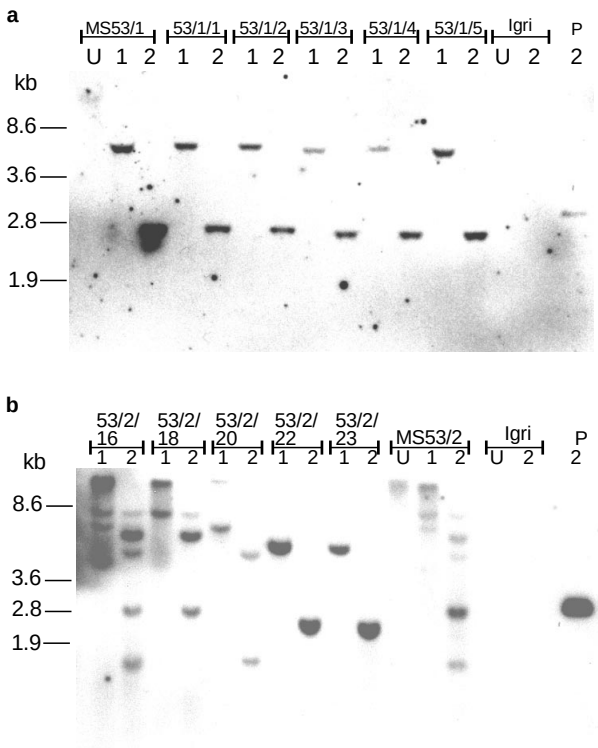

Fig. 3 a, b Southern-blot analysis of progeny plants of the T_0 barley plants MS53/1 and MS53/2. Fifteen Micrograms of genomic DNA from the untransformed control Igri, the T_0 plants MS53/1 (**a**) and MS53/2 (**b**) and five progeny plants of each T_0 plant were digested with *NcoI* (*1*) or with *Eco*RI/*Sca*I (*2*) and separated in a 0.8% agarose gel. Fifteen Micrograms of undigested genomic DNA (*U*) were separated in the agarose gel from the T_0 plants and Igri. The filter-bound DNA was hybridised to a DIG-labelled *VstI* fragment

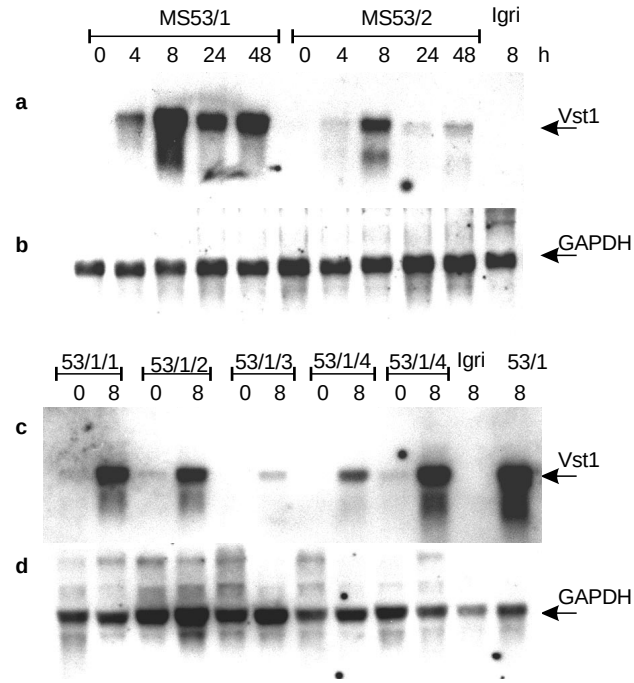

Fig. 4a–d Northern-blot analysis of T_0 and T_1 barley plants. Total RNA was isolated from leaf material of the T_0 barley plants MS53/1 and MS53/2 (*a*, *b*) at different time points after induction by rubbing with sea sand (0, 4, 8, 24 and 48 h after induction) and of five different T_1 plants and the parental line MS53/1 at the time point zero (0 h) and 8 h after induction by rubbing with sea sand. Hybridisation was carried out using a Dig-labelled *VstI* fragment (*a*, *c*) or a Dig-labelled *GAPDH* fragment (*b*, *d*)

of 11 T_1 plants. Southern-blot analysis indicated four different integration patterns for the *Vst1* gene in five *pat* and *Vst1* positive T_1 plants (Fig. 3 b). This indicates the hemizygous nature of the T_0 line MS53/2. Therefore, the integrations must have occurred after spontaneous chromosome doubling.

Expression of stilbene synthase-specific mRNA

The expression of the *Vst1* gene and the induction of the Vst1 promoter were analysed in transgenic barley and wheat plants. Transcription of stilbene synthase-specific mRNA was investigated in leaves of T_0 and T_1 plants. For induction, different leaves of the plants were wounded by rubbing with sea sand, and total RNA was isolated at different time points after induction.

Figure 4 a and b show the Northern–blot analysis of the T_0 plants MS53/1 and MS53/2 using a *Vst1* probe (Fig. 4 a) and, as loading control, a GAPDH probe (Fig. 4 b). At time-point zero, only weak hybridisation signals were visible. The accumulation of stilbene synthase mRNA reached a maximum after 8 h followed by a decrease after 24 h and then a second accumulation after 48 h.

Transcription of stilbene synthase-specific mRNA and the induction of the Vst1 promoter was also investigated in young leaves of progeny plants. Therefore leaves of these plants were wound-induced by rubbing with sea sand. Total RNA was isolated from leaf material which was harvested before induction and 8 h after induction. In Fig. 4 c the accumulation of stilbene synthase-specific mRNA is shown in five progeny plants of the line MS53/1 8 h after wound induction. A second hybridisation with the *GAPDH* probe is shown in Fig. 4 d as an internal loading control.

Induction analysis of the Vst1 promoter was also made with the biotrophic fungus *E. graminis*. For these experiments, leaf segments of progeny plants were inoculated with different isolates of the pathogen. When first symptoms were visible, total RNA was isolated. Control leaves were prepared in parallel with inoculated segments, expect for infection with the mildew. In Fig. 5 the results of the Northern-blot analysis after hybridisation with the *Vst1* probe (Fig. 5 a), and as a loading control with the *GAPDH* probe (Fig. 5 b), are shown. An accumulation of stilbene synthase-specific

mRNA was detected only in response to inoculation with the fungus (Fig. 5, lines A,B). No transcription of stilbene synthase mRNA was detected in non-inoculated leaves. Thus, the expression of the stilbene synthase was induced by the fungus and not by stress factors such as wounding at the cutting sites. Additionally, an infected and a non-infected leaf of the same T_1-plant of the line MS53/1 growing in the green house were harvested and analysed in the Northern-blot experiment. Only in the infected leaf was *Vst1*-specific mRNA detected (Fig. 5, line C).

Enzyme activity of stilbene synthase

The stilbene synthase activity was assayed in co-operation with Bayer AG, Germany. For protein extraction we used the protocol established for petunia and tobacco. As a positive control a petunia protein extract was employed. The enzyme activity was determined in crude protein extracts of leaves, 12 h after induction, through the incorporation of [2-^{14}C]malonyl CoA into the products resveratrol and naringenin, respectively. The incorporation of [2-^{14}C]malonyl CoA into the product resveratrol was definitely shown in the progeny MS53/2/16 and MS53/2/18, but only in very small amounts. In all analysed cereal crude-extracts the incorporation of [2-^{14}C]malonyl CoA into the product naringenin was detected, but again only in very low amounts. In comparison with the positive control, in which high incorporation rates were detected in both products, the total enzyme activity of the crude cereal-protein extract was very low.

In Table 1 a summary of all *Vst1*-positive barley and wheat plants is shown. All together nine *Vst1* positive plants were regenerated and the progeny plants were analysed. Three barley plants and three wheat plants were transformed with the plasmid pGBI, i.e. with the 4-fold enhancer in a 5′-position to the Vst1 promoter. Two barley plants and one wheat plant were transformed with the plasmid pGBII in co-transformation with the plasmid p35SSPAT.

Fungal-infection assay with *B. cinerea*

Progeny plants of the T_0 line MS53/1 were tested for enhanced disease resistance towards *B. cinerea*. Therefore, leaves of 3-week-old transgenic progeny, and of control plants, were placed between wet filter papers and inoculated with a spore suspension at a concentration of 2×10^5 spores/ml. For inoculation, 5–10 µl of the spore suspension were applied to the surface of the leaves. Already after 1 week, differences between the transgenic progeny plants and the control were detected (Fig. 6). After 14 days the leaves of the transgenic plants were still green and vital. At the infection site the formation of necroses could be detected which could probably be traced back to a hypersensitive reaction. The same necrotic lesions were observed in leaves of the control plant, but in this case the pathogen infected the entire leaf.

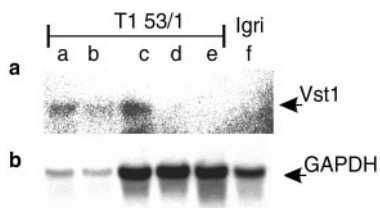

Fig. 5 a, b Northern-blot analysis after induction with the biotrophic fungus *E. graminis*. Leaf segments of transgenic progeny plants were inoculated with different isolates of the pathogen (111596 and 150496; isolated by the Saaten Union resistance Lab). Total RNA was isolated when first symptoms were visible. *a* and *b*: different isolates; *c*: infected leaf growing in the green house; *d*: non-inoculated leaves; *e*: non-infected leaf of a plant grown in the green house

Discussion

The aim of the present study was the stable transformation of a stilbene synthase gene under control of the stilbene synthase promoter in barley and wheat.

Table 1 Summary of stable transformation experiments. Immature wheat embryos and barley microspores were bombarded with the constructs pGBI and pGBII; +, positive signal in the assay; −, negative signal in the assay; n.a., not analysed

Transgenic plants	Cereal species	Construct	Southern positive	Northern positive	Transgenic progeny	STS activity in T_1 plants
MS53/1	Barley/Igri	pGBI	+	+	Homozygous	+
MS53/2	Barley/Igri	pGBI	+	+	Heterozygous	+
MS53/3	Barley/Igri	pGBI	+	−	−	−
MS60/1	Barley/Igri	pGBII	+	+	n.a.	n.a.
MS60/2	Barley/Igri	pGBII	+	+	n.a.	n.a.
W6/1	Wheat/Veery	pGBII	+	n.a.	Heterozygous	n.a.
W8/1	Wheat/Veery	pGBI	+	+	Heterozygous	n.a.
W8/4	Wheat/Veery	pGBI	+	+	Heterozygous	n.a.
W9/1	Wheat/Veery	pGBI	+	+	Heterozygous	n.a.

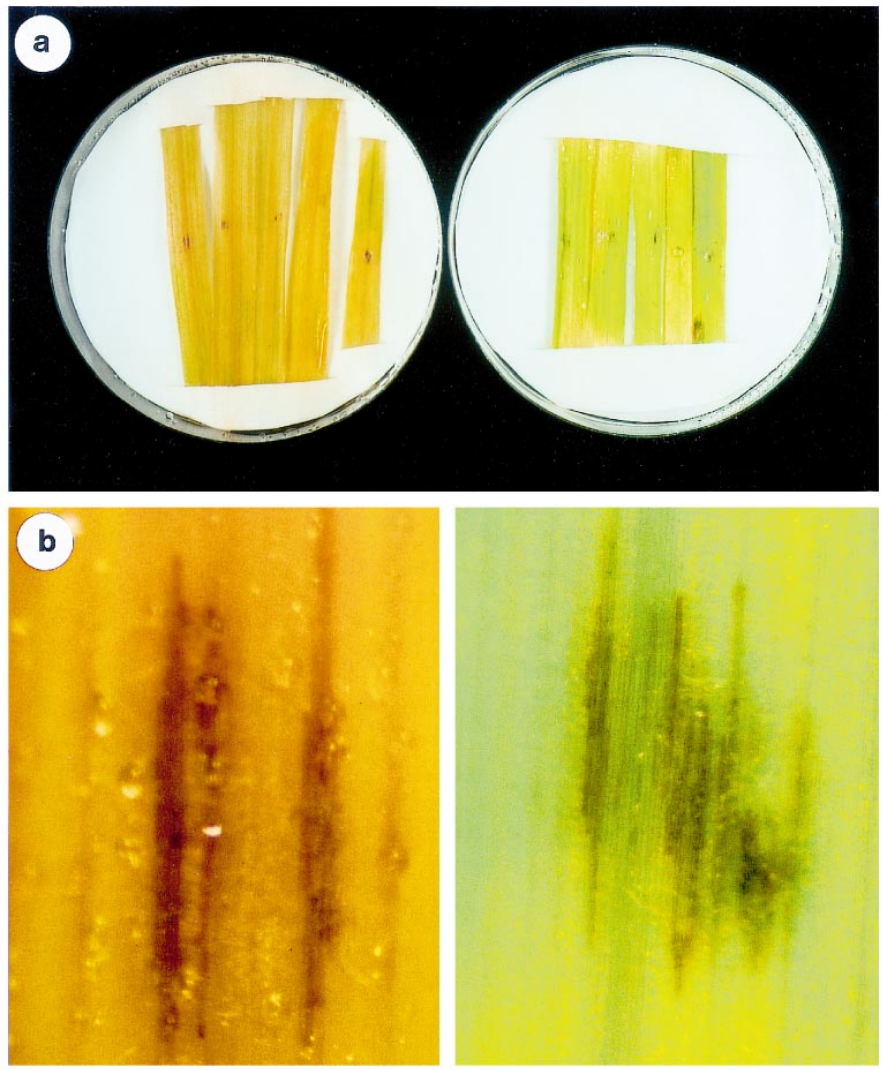

Fig. 6 a, b Enhanced resistance against *B. cinerea* infection in barley T_1 plants. Effect of *B. cinerea* inoculation on stilbene synthase expression in T_1 barley plants (right) and untransformed control plants (left) 14 days after infection. Leaves of 3 week-old seedlings of transgenic and control plants were inoculated with 10 µl of a 2×10^5 *B. cinerea* spore solution

Stilbene synthase genes have already been used to introduce novel phytoalexins into plants, resulting in enhanced disease resistance (Hain et al. 1990, 1993; Stark-Lorenzen et al. 1997). The effectiveness of grapevine STS gene expression in enhancing resistance was first reported by Hain et al. (1993). In these transformation experiments two STS genes, isolated from grapevine, were transferred to tobacco. In comparison to experiments in which only one STS gene was transferred, the transfer of two genes allowed enhanced STS mRNA accumulation and higher levels of the foreign phytoalexin. Another way to obtain high amounts of the foreign phytoalexin synthesised upon infection, is to modify the STS gene to allow higher levels of gene expression.

In this paper we used a modified *Vst1* gene with upstream fragments of the CaMV 35s as a 4-fold enhancer to increase the expression of the gene under control of its own promoter. Therefore, we first analysed the activity of the promoter and the enhancer in barley by means of the *uidA* gene. The two promoter constructs pVstPG and pVstEPG (Fig. 1 a,b) were used for initial transformation experiments of immature barley embryos via particle bombardment. With these experiments the activity of the dicotyledonous Vst1 promoter in barley, as an example of a monocotyledonous species, was proven and the enhancing effect of the 4-fold CaMV (35s) enhancer was detected. To analyse the induction of the Vst1 promoter in cereals we used transgenic suspension lines transformed with the constructs pVstPG and pVstEPG. Besides the induction by a fungus, or a fungal elicitor, the synthesis of stilbenes can also be induced by stress factors like UV light, wounding, or in cell cultures by changing the medium (Langcake 1981; Hain et al. 1990, 1993). Because of wounding through the particle bombardment the transient GUS expression in immature embryos was not suitable for analysing the induction of the Vst1 promoter. A constitutive expression of the *uidA* gene in the bombarded tissue was also possible. The Vst1

promoter was induced by changing the media as described by Rolfs et al. (1981). In all cell lines analysed, as well as in lines transformed with the enhancer sequences, an increased enzyme activity was observed after induction. This result indicated that the enhancer sequences have no influence on promoter induction and only work as transcription-activating elements. Omirulleh et al. (1993) have demonstrated an influence of the duplicated enhancer sequences (−208 to −46) on the tissue specificity of the wheat α-amylase promoter in transgenic maize. The tissue specificity of the α-amylase promoter was suppressed by the enhancer sequences.

All together nine *Vst1* positive transgenic barley and wheat plants were regenerated. Transcription activity was detected in T_0-and T_1-plants either with or without the 4-fold enhancer. Southern-blot analysis showed the integration of only one copy of the *Vst1* gene in the transgenic barley plant MS53/1, with multiple integration sites and rearrangements of one or both genes in other plants. Comparable results were also described for transformation experiments (Becker et al. 1994; Jähne et al. 1994). Normally, transgenic plants obtained from bombarded microspores are homozygous (Jähne et al. 1994). But in the T_0 barley plant MS53/2 an independent segregation of the transgenes was observed. In the Southern-blot analysis we analysed 24 progeny plants and detected five different integration patterns, as well as plants which had completely lost the integrated gene. Therefore, the integration must have occurred after spontaneous chromosome doubling followed by the regeneration of a hemizygous plant. Corresponding results were shown by Pedersen et al. (1997) using fluorescence in situ hybridisation (FISH).

Vst1 gene expression, and induction of the Vst1 promoter, were shown for transgenic barley and wheat plants. The kinetics of transcript accumulation in transgenic barley lines showed a corresponding result to grapevine (Wiese et al. 1994). A fast accumulation of stilbene synthase-specific mRNA followed by a decrease was also obtained in transgenic tobacco (Hain et al. 1993) and transgenic rice (Stark-Lorenzen et al. 1997). A second strong increase in barley occurred after 48 h, but was not observed in transgenic tobacco and rice. The repeated increase was only slight and the stilbene synthase mRNA synthesis disappeared completely 72 h after inoculation.

Corresponding experiments were made with the transgenic barley lines MS60/1 and MS60/2 which were transformed with the plasmid pGBII in co-transformation with p35SPAT. The accumulation of Vst1-specific mRNA after wound induction in these plants was very low. For the detection of Vst1-transcript accumulation in them, it was necessary to use 3-fold higher amounts of total RNA for Northern-blot analysis. The higher expression of the *Vst1* gene in the plants MS53/1 and MS53/2 in comparison to MS60/1 and MS60/2 is probably due to the 4-fold enhancer, although a position effect might also explain the increased gene expression. To prove the effect of the 4-fold enhancer more transgenic plants need to be analysed.

In view of the agronomic relevance of cereals, it is important to determine whether biosynthesis of the foreign phytoalexin is also induced by biotrophic fungal pathogens like *E. graminis*. Powdery mildew is an important disease of barley and wheat and is spread world-wide (Robe and Doussinault 1995). Genes for resistance to powdery mildew have been identified in wheat (Robe and Doussinault 1995) and barley (Freialdenhoven et al. 1996), and the genetic resistance has been used effectively to control powdery mildew (Hu et al. 1997). Several of these genes have only been effective a few years after having been widely applied. Therefore, the synthesis of the foreign antimicrobial phytoalexin is of interest for enhancing the vertical resistance. The induction analyses of the Vst1 promoter through infection with the fungus *E. graminis* indicated that the promoter is also inducible by a biotrophic fungus. The *Vst1*-specific mRNA was only detected in the surrounding area of the infection site. In non-infected leaves of the same plant no *Vst1* specific mRNA was detected.

The effectiveness of the expression of the *Vst1* gene in enhancing resistance in transgenic barley was first analysed with the necrotrophic fungus *B. cinerea*. This fungus was chosen as a pathogen because of the positive correlation between the resistance of certain varieties of grapevine against the fungus and the concentration of resveratrol formed when attacked by the pathogen. In contrast to *V. vinifera* L., only exceptionally were economic yield loses in cereals obtained after infection with *B. cinerea* (Jenkins 1974).

A significant increase in the resistance against the fungus in transgenic plants was first reported by Hain et al. (1993). Preliminary results in transgenic rice indicated that it may possess enhanced resistance to *Pyricularia oryzae* (Stark-Lorenzen et al. 1997).

After leaflet infection of the transgenic line and control plants of cv Igri with the fungus *B. cinerea* an increased resistance against the fungus was detected in the transgenic plants. These preliminary results indicated that the accumulation of the phytoalexin resveratrol in transgenic barley may enhance resistance against the fungus *B. cinerea*. Further experiments *in planta*, and especially with other fungi, are necessary in larger-scale experiments to prove the effectiveness of the foreign phytoalexin in cereals.

Acknowledgement This work was part of a Ph.D project at the Institut für Allgemeine Botanik der Universität Hamburg. The authors thank Bayer AG (Monheim, Germany) for their gift of the *Vst1*-gene and Ute Teuschel (Bayer AG, Monheim) for helpful assistance in the enzyme assays. We also thank Dr. Susanne Stirn for critical reading of the manuscript and Dr. Dirk Becker and Dr. Reinhold Brettschneider for helpful discussions. The work presented here was supported by the Körber Foundation and the Ministry of Education and Research BMBF (Bonn).

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