Grapevine Protoplasts as a Transient Expression System for Comparison of Stilbene Synthase Genes Containing cGMP-Responsive Promoter Elements

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A method for preparing elicitor-responsive protoplasts from grapevine cells kept in suspension culture was established. The protoplasts were employed in order to perform transient gene expression experiments produced by externally added plasmids. Using the gene coding for bacterial β-glucuronidase as the reporter gene, the transient expression under the control of various promoters of stilbene synthase genes were analyzed. The elicitor-responsiveness of promoters from grapevine genes and heterologous promoters were assayed: the grapevine stilbene synthase gene VST-1 and pine stilbene synthase genes PST-1, PST-2 and PST-3. Compared to the expression effected by the cauliflower mosaic virus 35S RNA-promoter, the stilbene synthase promoters caused a 2–5-fold increase in GUS-activity. Incubation of transformed protoplasts with fungal cell wall further stimulated the stilbene synthase promoters but not the 35S RNA-promoter. An even more pronounced differentiation between the promoters was observed when cGMP was included in the transient expression assays. Instead of treating transformed protoplasts with fungal cell wall we administered simultaneously cGMP and the plasmid to be tested. The cGMP-responsive increase was (a) specific concerning the nucleotide applied, (b) characteristic of grapevine protoplasts, and (c) not seen with shortened promoter-GUS constructs or GUS under the control of the 35S RNA-promoter. The highest cGMP-dependent reponse to stress was shown by the promoter of the grapevine stilbene synthase gene VST-1.

Introduction

A broad range of functional proteins arises in plants from signalling processes elicited by fungal compounds. Among the proteins *de novo* synthesized are diverse molecules such as enzymes acting in phytoalexin production (Hahlbrock and Scheel, 1989), pathogenesis-related proteins (Kauffmann *et al.*, 1990; Renault *et al.*, 1996; Hammond-Kosack and Jones, 1996), and devices to enhance the strength of the cell wall structure by oxidative cross-linking of proline-rich cell wall proteins (Bradley *et al.*, 1992). The inducible mechanisms depend on the presence of an efficient signal transduction chain and a transcriptional activation of the specific defence-related genes.

For studying the molecular mechanisms underlying the transcriptional activation, both transgenic organisms carrying the respective promoter elements and protoplasts have been used as tools for the transient expression of promoter constructs. Several plants, besides the widely used tobacco, have been employed as sources of protoplasts suited for transient expression (Hain *et al.*, 1985; Dangl *et al.*, 1987; Liu *et al.*, 1994; Rohrig *et al.*, 1995). The accessibility of the cytosol during transient expression provides the opportunity of analyzing not only the promoter constructs but also the effect of putative members of a signal transduction chain which eventually affects transcriptional control.

We were interested to test plasmid-located promoter constructs in a plant which possesses an established signal transduction chain acting on closely related genomic elements. In grapevine, a multigene family coding for stilbene synthases differently susceptible to elicitation by UV-light or fungal elicitors has been described (Melchior and Kindl, 1991; Hain *et al.*, 1993; Wiese *et al.*, 1994; Sparvoli *et al.*, 1994; Preisig-Müller *et al.*, 1997). Here we report the properties of grapevine protoplasts and their characterization as an assay system

for stress-responsive gene activation. In the transient expression assay, the cell contains both the endogenous stilbene synthase promoters and the identical or closely related promoter located on a plasmid to be introduced into the cell.

Our interest concentrated on the comparison of elicitor-regulated genes; the pinosylvin synthase multigene family present in pine (Fliegmann *et al.*, 1992; Schwekendiek *et al.*, 1992) and the grapevine stilbene synthase multigene family. Pinosylvin synthase (Schöppner and Kindl, 1979; Gehlert *et al.*, 1990) belonging to the family of stilbene synthases is restricted to the genus *Pinus* and very few other plants (Kindl, 1985). It was interesting to see how promoters of the pine gene family acted in transient expression assays performed in grapevine cells known to house the multigene family coding for resveratrol-forming stilbene synthases (Schröder *et al.*, 1988; Melchior and Kindl, 1991; Preisig-Müller *et al.*, 1997). By expressing the inducible synthase genes we emphasized the stimulating and differentiating effect of cGMP, a putative link in the signal transduction chain.

Materials and Methods

Stilbene synthase genes and preparation of promoter-GUS constructs

The promoter region of the grapevine gene VST-1 (Wiese *et al.*, 1994) was subcloned in *Pst*I/*Bam*HI-cleaved pBI221 (CLONTECH, Heidelberg). By *Pst*I/*Bam*HI cleavage, an 800 bp fragment of the caulimo virus 35S RNA promoter was previously removed from pBI221. In addition, promoter fragments (Pr-7, Pr-6, Pr-2, Pr-1) were created by PCR using specific oligonucleotide primers which introduced *Bam*HI and *Pst*I restriction sites. The PCR products were digested with *Bam*HI and *Pst*I and ligated into the *Pst*I/*Bam*HI-cleaved vector upstream of the chimeric GUS gene.

For experiments with the pine genes PST-1, PST-2 and PST-3 obtained by screening a genomic DNA library prepared from pine seedlings, pBI221 was linearized with *Bam*HI and ligated with the *Sau*3A-cleaved promoter fragments of PST-1, PST-2 and PST-3 (Preisig-Müller *et al.*, 1998). As probe, we used pSP-54 cDNA (Schwekendiek *et al.*, 1992) obtained from a cDNA library prepared from poly-(A)$^+$-RNA of fungus-infected pine seedlings. The correctness of all constructs was verified by sequencing using an ABI PRISM 377 automatic sequencer (Perkin Elmer). All plasmids were amplified in *Escherichia coli* NM522 and purified by chromatography on anion exchange resin columns (QIAGEN, Hilden).

Preparation of protoplasts and transient expression

Cell suspension cultures of grapevine were grown as described (Melchior and Kindl, 1991). Protoplasts were isolated from midlog phase suspension-cultured cells using the procedure of Schwer and Kindl (1993). After 7 days of subculture, 50 ml of grapevine cells were sedimented and resuspended in 50 ml of digestion mixture (buffer A containing 0.6% cellulase Onozuka R10 and 0.4% maceroenzyme R10) (both Boehringer Ingelheim, Heidelberg). Buffer A consisted of 400 mM mannitol, 50 mM $CaCl_2$, and 25 mM N-morpholino-ethanesulfonic acid/KOH, pH 5.5. The cells were transferred to an Erlenmeyer flask and shaken at 116 rpm for 1 h at 26 °C. Light microscopy was used to monitor the time course of protoplast preparation. The protoplasts were pelleted at 650 rpm and then gently suspended in 20 ml (per 50-ml culture) of buffer A. This procedure was repeated twice. The number of protoplasts was counted using a Fuchs-Rosenthal chamber. From a 50-ml culture, the yield was 9×10^7 protoplasts.

For the polyethylene glycol-mediated transformation of protoplasts according to Hain *et al.* (1985), 20 µl plasmid preparation (1 µg/µl) were added to 200 µl protoplast suspension (1×10^6). To this mixture, 200 µl 25% (w/w) PEG (polyethylene glycol type 6000, Acros), or the concentrations indicated, dissolved in 100 mM calcium nitrate and 450 mM mannitol (adjusted to pH 9.0) was added. In cases indicated, the incubation with the plasmid preparation (20 min) was in the presence of cGMP, usually at a final concentration of 50 µM. Controls were run with H_2O (in the absence of cGMP) or with a vector lacking the promoter region of the 35S RNA promoter. Subsequently, the protoplasts were incubated with 5 ml of 275 mM calcium nitrate (adjusted to pH 6.0), washed with 5 ml of W5 medium (Hain *et al.*, 1985) consisting of 1.25 mM $CaCl_2$, 1.55 mM NaCl, 5 mM KCl, and 5 mM glucose,

pH 5.5, and collected by centrifugation at 650 rpm for 5 min. The protoplasts were resuspended in 3 ml of buffer A. A recovery time of 24 h and 22 °C was used routinely after we had performed a series of experiments testing the optimal conditions for the grapevine protoplast system. At that stage and in cases indicated, 7 mg of cell wall of *Botrytis cinerea* (DSM 877) were added to the protoplast suspension (Liswidowati *et al.*, 1991). Following the recovery time, cell extracts were prepared in phosphate buffer, pH 7.0, containing 10 mM mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 50 mg Polyclar AT (Serva). The protein content was assayed according to Bradford (Bradford, 1976). Subsequently, the incubation time with methylumbelliferyl-β-D-glucuronide was either 15 h or 22 h.

To analyze the quality and quantity of protein synthesis *in vivo*, either intact cells or protoplasts were incubated with 5.5 MBq [^{35}S]L-methionine (specific activity 1.0 TBq/mmol). The incorporation of the radioactive amino acid into stilbene synthase was determined by immunoprecipitation, PAGE and fluorography.

Enzyme assays and analytical procedures

GUS activity was determined according to Jefferson (1987) using 4-methylumbelliferyl-β-D-glucuronide as the substrate and 50 μg total protein of the protoplast extract. For detection of fluorescence, a Baird-Atomic fluorescence spectrophotometer (model SF-100) was used. Controls were run to exclude the possibility of resveratrol fluorescence interfering with the fluorescence of methylumbelliferone. Stilbene synthase activity was assayed according to Liswidowati *et al.* (1991). The concentration of cGMP was determined using the detection kit of Amersham based on an immunological procedure. A linear calibration curve was used in the cGMP range of 0.5–8 pmol. The extraction and calibration procedure was checked by running tests using cGMP as internal standard externally added to the homogenate.

Results

Elicitor-responsive grapevine protoplasts: preparation and plasmid application

To establish a reliable expression system for plasmids comprising various promoters controlling the bacterial GUS gene, we looked for protoplasts characterized by responsiveness towards fungal elicitors. For preliminary experiments with promoter-reporter gene constructs, tobacco protoplasts prepared from leaves (Wullems *et al.*, 1981) were used. By this means, we standardized our plasmid transfer protocol, recovery time and GUS assay. Based on this experience, we started our investigations on grapevine cell suspension cultures using them at midlog stage as the source for the preparation of protoplasts. Thus far, differentiated, cultured grapevine cells have not been utilized for preparing and assaying protoplasts. However, protoplasts from leaf mesophyll have been found valuable for studies on isoenzyme patterns (Siminis *et al.*, 1994), and protoplasts from embryogenic cells may become a very valuable tool for stable transformation (Mauro *et al.*, 1995; Reustle *et al.*, 1995). In order to test the viability and biochemical competence of the protoplasts, the capacity for *de novo* protein biosynthesis and response to fungal elicitor were assayed by administering [^{35}S]L-methionine as precursor (data not shown). By testing a great number of independent preparations, we eventually found that it was crucial for the subsequent expression studies, and for the responsiveness of the protoplasts, to (a) select suspension cultures which appeared almost white/creme and were not induced at all, and (b) to treat the cells with the digestion mixture not longer than 1.5 hours.

To establish optimal conditions for the transient expression experiments, we tested the application of plasmids in the presence of PEG at various concentrations. Adjusting the pH value to 9.0 and ending up with a final PEG concentration between 10.6 and 12.0% during the plasmid administration were found to be essential. For the initiation of protoplast permeation by slowly mixing a drop of protoplast/plasmid suspension with a drop of PEG-solution, the concentration of PEG had to be in the range of 22–30%. Even using 27% PEG, instead of 25% PEG, in most cases caused a marked difference in transformation values. This held true for most promoter constructs tested (Table I). On top of the stress caused by the protoplast formation which led to a slight formation of GUS we also demonstrated an elicitor-dependent effect which became most evident by using a cell wall preparation from *Botrytis cinerea*. The time

allowed for the recovery of protoplasts was varied between 4 and 24 hours, and the incubation time with the GUS substrate was for 15 and 22 h.

The promoter regions tested in detail below were taken from various members of the multigene families of stilbene synthase genes in *Vitis vinifera* and *Pinus sylvestris*. The nucleotide sequences summarized in Fig. 1 have been deposited in the EMBL data base.

Table I. Polyethylene glycol concentration exerting influence on the efficiency of plasmid transfer and expression of the plasmid-localized and promoter-controlled information.

Promoter	Polyethylene glycol concentration % (w/w)	GUS activity pmol/min x mg
35S-RNA	25	20
	27	90
A-5	25	620
	27	1400
PST-1	25	780
	27	630
PST-3	25	230
	27	300

cGMP regulates the promoter activity of grapevine stilbene synthase gene VST-1 when assayed in grapevine protoplasts

The protoplasts are subjected to considerable stress during the induction of the plasmids. This was assessed by analysing the PEG-treated transformed protoplasts: a protein labelling pattern was observed which was distinct from the pattern of unstressed cells in suspension (data not shown). If the elicitor mediates gene activation via a signal transduction chain it is feasible that the introduction of a putative member of the signal chain together with the plasmid would modulate the effect on gene expression. Testing a variety of potential intracellular signalling compounds leading to effects, we eventually found a very pronounced effect brought about by cGMP. This pronounced and specific stimulating effect, caused by transferring cGMP simultaneously with the plasmid, was seen using grapevine protoplast but did not occur with the same intensity with tobacco protoplasts. Using VST-1 promoter constructs we compared the effect of cGMP applied simultaneously with the plasmid, to grapevine protoplasts which first received the plasmid, then recovered and subsequently were exposed to the influence of the cell wall elicitor. Both conditions led to substantial increases in gene activation (Fig. 2). Controls were run using H_2O plus vector instead of cGMP.

The results (Fig. 2) show that using the VST-1 promoter instead of the caulimo virus 35S RNA promoter led to increased expression of GUS activity even in the absence of elicitor. Although the protoplasts represent a system already stressed by both the preparation and the PEG treatment we observed with stilbene synthase promoters a 2.5–5-fold increase compared to the 35S RNA promoter construct. Furthermore, addition of fungal cell wall intensified the transient expression markedly, i. e. 25-fold in the case of the VST-1 promoter. Under the conditions described, the expression controlled by the pinosylvin synthase promoters yielded results indicating that the promoter strength varies within a multigene family (data not shown).

By including cGMP in the transient expression assays we found that, when applied in combination with the plasmid, the same increase as a subsequent treatment with fungal cell wall was produced. Furthermore, we demonstrated that the cGMP-enhanced expression of the VST-1 promoter-GUS construct (Fig. 3A) was dependent on the dosage of the nucleotides. The cGMP concentrations designated in the figure indicate the concentrations in the total expression assay containing the protoplast suspension and do not characterize the actual concentration within the resealed protoplasts. The comparison of the effects of various nucleotides in Fig. 3B clearly shows that cGMP is a rather specific means for the activation of the VST-1 promoter. cCMP, which causes small increases in the expression of GUS activity, was the only other positive effector.

Comparing cGMP-responsiveness of various stilbene synthase gene promoters

On top of the stress caused during the preparation of the protoplasts and subsequent introduction of the plasmids cGMP contributes to the increased gene expression. This marked effect of cGMP on the transient gene expression is not confined to the promoter of gene VST-1 (Fig. 4). Comparable data were obtained when we used the

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               10        20        30        40        50        60        70
VST-1   AAGTATTAAGTTTAAAGTCAATAATTAAAAATTATTTAATTTAAAA--TCAATTTAAATCATTAATTAAT
SV-25   TTATATTAAACTGCTTTAATATATTTAAGAATGTTATAAAGTATTG--TTAAAATGCTCTTATAGTCAAC
PST1    TAGATGAGGAGATCTTAAGCGATCTTGGAAATTTGACAAGAGAGAG--GAGGGAGGACGTCACAGTGGAA
PST2    ------------------GATCTTGGAAATTTGACAAGAGAGAG--GAGGGAGGACGTCATAGTGGAA
PST3    -------CTGCAGGTCAACGGATCTTGGAAATTTGACGAGAGAGAGAGGGAGGGAGGACGTCATAGTGGAA


VST-1   AAATACTC------AGTTTTAAGAACTACACACTTATTCCAT-TAAAAATATAATTATATATAAATACTC
SV-25   ATCCACTGTCGTG-GGTCCTATGATATGCACATATGATAAATATACACGTATAATGCTATTATATGATGC
PST1    AACCGCCTCA--GCAGCCACATGGCTAGCAAAGCCGCATTGAAAACCCAAGTATGAATAATGTGGCAAAC
PST2    AACCGCACCGCCGCAGCCACATGGCTAGCAAACCCGCATTCAAAACCCAAGTATGAATAATTTGTTTCAA
PST3    AACCGCCTCA-----GCCACAGGGCTAGCAAACCCGCAT--GTCTCAAAGACTAAGATGGTTTGGTATGT


VST-1   AGTTTTTGCTAGAAGTAAATTATTATTTAAAGCTGTTGAATCTTGGAATTAGACT---------CATTTC
SV-25   ACACGTTATTACACGATATTTAATTTGTGACTTTCTTATGGGTACAAATTAAAATT---TGTACCAATTT
PST1    CCAAGTGTGAATATA-TAATTTGGCA-AACCCGCATTGAAAACCCAAGTGTGAATA-ATTTGG-CAAACC
PST2    ATAAATAGTAAGATG-GTTGGGGAAATAATTTGTTTCCAAGACTAAGACCTAAGTATATTGGG-TGGATT
PST3    GACTGTGTTCACACT-CACTATATTTTCTTCCTTTTCCTCATTGCCGGCTTCACAG--TTGCG-TGAGTC


                                                                      ◊ Pr7
VST-1   GTAA---GGCAAGTGAAGTCAATTATTAACGCTCACTTATTATTAAAACCTTT----ACCCGATGAATCG
SV-25   TTGACTTGGCAACCAAAGTCATTGATATTTTCCAAGTTAAAATTTGTACCAATTAAAATTTGTACAATTT
PST1    CGCATTGAAAACCCAAGTATGAATAATTTGGCAAACCCGCATTGAAAACCACAGT--ATGAATAATTTGT
PST2    TGAGGTAATTAGCTCGCAGTGCG------GTTGGATTAATGTAAGGAATCACTGT--AATTATATATTAT
PST3    ACTCATATATATTGGCGGTTTCACAGTTGCGTCACTCGATTTCG--ACCGACTTG--ACGGATAATCTGA


VST-1   CCCCCAATACCTCCTAACTTGTGAT--CAGAAAGACACCTGCG--CCTACTTTGAACGACCTATGAGCCT
SV-25   TCCAATGAGTTTGCAAAATTCTAATGCTATGAAGATATTTTCAAATCAAATTTATGTGAACAAAAATTAA
PST1    GTGAAAGACTAAGATGGTTTGGGTTGGGAAATAATCTGTTTCAAAGACCAAGACTAAGACTGGGTGGATT
PST2    AT---ATTCCACGGTTAATTGGTTCCCTAA---ACTCGATATACATGGCTTTGCTGCATCTGCTTCTTCT
PST3    AT---ATTCATCGTCCACACGTGTTTCTGA-----TT--TACAGTCTCCTCTCCTCTCCTCTCCTCTCCT


VST-1   CCCAAAG--------TCAAGTTGTTTCGTGGATC----------ACTTAAGTATTTACTTTTCCTTCTT-
SV-25   AGTAGTAGTTATTTTCCAAGTTATTTCGTGGATATTTGATGGAGACTTGTATTTTTGATCTACTTATTTA
PST1    TGTAATTA-------CCTCGCAGTGCACCAGAAGGGAAG-GTTGGTTGGTGAGGTTAGATTAATGTGATA
PST2    AGGATTGG-------CTTTG--GTTC--TGGCAATCCAA-GTGGCTCCC---GGTAAAGCTACTACGGGT
PST3    CTCGTGGA-------GTATGT-ATGTA-TGAATATTCTG-ATTTACAGTCTCGTCTCCTCTCCTCTCGTG


VST-1   -TCAATTATTTTTGGGAATCAAACGAGTG-GCCCAA----CTTGGATGACGAAACAGGTCAAATAAATTA
SV-25   TTTATTTATTTTTTAAGAATCAAACGAATGCGCCCCAGCGTCTTGTATCGCGGAATAGGTCAGATACAAAT
PST1    CTGGG-TGGATTTGTAATTACCT-CGCAGTGCACCAGAAGGAAGGAAGGTTGGTGAGGTTAGATTCATGT
PST2    ACGGG-TGCTACTGCTACTGTGAGTATAGTATATGCTCTGTGACTGTGTTCGATACGTCACGTCACTCAT
PST3    GAGTA-TGTATGTATGAATATTC---TGATTTACAGTCTCGTCTCCTCTCCTCTCGTGGAGTATGTATGT


                                    ◊ Pr6
VST-1   AAATGAGATATTT--TTGTTAAATGA-CATGTTACGT---ATTGGCTATTGGGTTGTTGAGGTTTGAAAT
SV-25   GAGATATTTGTTCAATTACTATGTGAATAGGTTAAATGACATGTGACGTTATCCTGGAGAAGAAAGATAG
PST1    GATATGTCACGTCACTCACTATATTA-TA-TTTTCTTCCTTTTCCTCATT-GGCGGGCGGCTTCACAGTT
PST2    TATATTTTCTCCCTTTTCCTCATTGG-CG-GCTTCTCAC---TCGTTATA-TATTGGCGGCTTCACAGTT
PST3    ATGAATATTCAAAACCCAAGATATAG-CA-GTATGGTTG--GTTGGTGTT-GACTCAAGATATTCCAACA


VST-1   TCGGACATTGAGTGACTTTGATTTCATTAACATTACGAAGGTTGGTAGCAGCTTCCCTCCCGTGGGATTG
SV-25   TGGGTTGTTGAGATTATTACTTCTAATTGAGAAATCCATCTTTGAATGACCTTGTCATGAGGAATGATGG
PST1    GTGTCACTCAAATACAAATACATTGATTGGCGGGCGGCTTCACAGTTGTGTCACTCATATACATTGGCGG
PST2    GAGTCACTCGATTCCGACCGACTTGACTATTCATCGTCCACACGTGTTTCTGATTTACAGTCTCCTCTCC
PST3    AAGCAAGCATAAGATTATAATATTAATAAATCGGCGGCTTCGC---TGTTGCGGCTGGCGGCTTCACAGT
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VST-1    CCTCCTTTTC---------AAAAGTCAAGTGGAAAATGTATTAATTTCCAGTTCAAGGGGCTCGAAAAG
SV-25    TAGCAGCTTCCCAGCCGTGGAAAAGTCAAATGAACAAAGTATTAATTTCCAGT--AAGAGGTTGGAGAAG
PST1     CTTCACAGTC---GATTTCGACCGAGTTGATGGATAA--TCTGAATATTCAAAACGTGT------ACAAT
PST2     TCTCCTCTCC---TCTCCTCTCCTGGAGTATGTATG---TATGAATATTCAAAACCCATTCATCCACAAT
PST3     TGCGTCACTC---GATTTCGACCGACTTGATGGATAA--TCTGAAAATTCAAAACCCATTCA---TCATC

                                                                       ◊  Pr2
VST-1    TCTTTATCTCTCTATATAAGAAATGTTATAATGA-GAAACCGTAAGCAGATTGATTGCTCTCTCCTTCCT
SV-25    TCTTTGTCTCTCTATATAAAAAATGTTTCTCTT---TAGCCGTGACCGGATTGAATGCTCGCTCCTTTCT
PST1     CACGTTTCTGATGATTTACAGTCTCGTCTCCTCTCCTCTCCTCTCCTGGAGTATGTACGTATGAATATTC
PST2     CACGTGTTTC-TGATTTACAGTCTCCTCTCCTCTCCTCTCCTCTGCTGGAGTAT----GTATGAAAATTC
PST3     CACACGTTTC-TGATTTACAGTCTCCTCTCGTCTCGTCTCCTCTCCTGGACTAT----GTATGAATATTC


VST-1    GTGGGTTGCATTCATCATATATGGGTAGGTGAAGAACAGAGAGCAAAA--------------CAAATAC
SV-25    GTGGAT-GCACGCATCGTATATGGGTAGGTGAAGAACAGATAAGAATGAAGAGACAACACGCGCGAATAC
PST1     AAAACCCATTCATA---CAGTATTGACCATGACATCGCTGTATGGTTTGGTTGGTGTTGACTCGAGATAT
PST2     AAAACCCATTCATCATCCACAATAAGATAAGATATAGCTGTATGGTT-GGTTGGTGTTGACTCCAGATAT
PST3     AAAACCCA-------------------AGATATAGCTGTATGGTT-GGTTGGCTTTGACTATAAAGC


VST-1    ATTAACAC--ATGCATACATGTCTGAAAATCCAAAAATAA-AATCACGCACACAAACTTTGAAGCCACCT
SV-25    ATAAACACCTACACATACATGTCTGAAAATCCAAAA-TAA-ACTCAAGCACACAAGCTTTGAAGCCAACT
PST1     TCAAACAA-AGCAAGCATAAGTCATCA----GTATGATATTAATAATAAATCGAACCCATGGTATTGGAC
PST2     TCAAACAA-AGCAAGCATAAGTCATCATATTAGATTATGGTATTAGAGTATAGAAGCCATGGTATTAGAC
PST3     CATGGTAT-TGGACTAATTGGTC-------------TATTGATGCATTATATAA-TAATCATATTGGAC


VST-1    GATCATT------------------------------GACTGCCGATGGATGAGAGTTGGTGAGA
SV-25    AATCATTCAAAACCCAAATTCAAATATCTAACATTAGTTATTGACCGCCAATAGATGAGAGTTGGTGAGA
PST1     TCGTGCACT-AATTCATCC-------ATTG-----ATGCAT-TATTATTATATAATCATATTTATCTCAT
PST2     TATAGAAGC-CATGGTATCGGACTGGACTA-----ATTGATGCATTATATAATAATCATATTTATTTCAT
PST3     T---------AATTGGCCT--------------ATTGATGCATTATATAATAATCATATTTATTTCAT


VST-1    CACAGCTAGCT **TATAAA**-TACCCAACACTCACACCCAGCTTTCTCAAGCCAGCTCCAAGCACTC----
SV-25    CA------GGC **TATAAA**-AGCCCGGCACCCACAACCAGCTTTCTCAAGCCAACTCCAAGCACTTGAG-
PST1     ATCAA--AGTG **TATAAA**ATAGCT---ATAGATTTAGAGATTCTTATAGCTATTCGACGAGAACAA---
PST2     ATCAA--AGTG **TATAAA**ATAGCTGCTATAGATTTAGAGATTCTTATAGCTATTCGACCAGACCAGACC
PST3     ATAAA--AGTG **TATAAA**ATAGCTGCTATAGATTTAGAGATTCTTATAGCTATTCGACCAGACCAA---


VST-1    --TTCTCTTTCCTT-CC-----TCAATCTTCAGCTTCAATTTGA------------------------
SV-25    --TTCTCTTTTCTTACCTCAACTTAATCTTAAGCTTCCATTTCA------------------------
PST1     -----------CAAAGTAAAGTAAATATTGAGATTTGTTTTCCAGGGTTTGGTACTAGGCTTAACCTCC
PST2     ATCAAAGTAAAGTAAAGTAAAGTAAATATTGAGATTTGTTGTCCAGGGTTTGGTACCACGCTTAACCGCC
PST3     --------------AGTAAAGTAAATATTGAGATTTGTTGTCCAGGGTTTGCTTAACCGCCTCATAGCA


VST-1    -GTTCGTAGCTAGGATCA<u>ATG</u>
SV-25    -TTACGTATCTAGCATCC<u>ATG</u>
PST1     TC--ATAGCATAGCAGAG<u>ATG</u>
PST2     TC--ATAGCATAGCAGAG<u>ATG</u>
PST3     TAGAATAGCAGAGCAGAG<u>ATG</u>
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Fig. 1. Comparison of promoter sequences applied in this study. The promoter regions of grapevine stilbene synthase gene VST-1 (acc. no. Y18532) as well as of the pine stilbene synthase genes PST-1 (acc. no. Y17594), PST-2 (acc. no. Y17595), and PST-3 (acc. no. Y17596) have been deposited in the EMBL gene bank. For the fractions of the VST-1 promoter, the 5'-ends of the deletion constructs are indicated by Pr-7, Pr-6 and Pr-2. The 5'-end of Pr-1 is just upstream of the TATAA box. The 3'-ends of all deletion constructs extend to nucleotide +79 of VST-1. The sequence of the construct A-5 of the VST-1 promoter includes, besides the 1 kb upstream region, the TATAA box (-48) and the transcription start (+1).
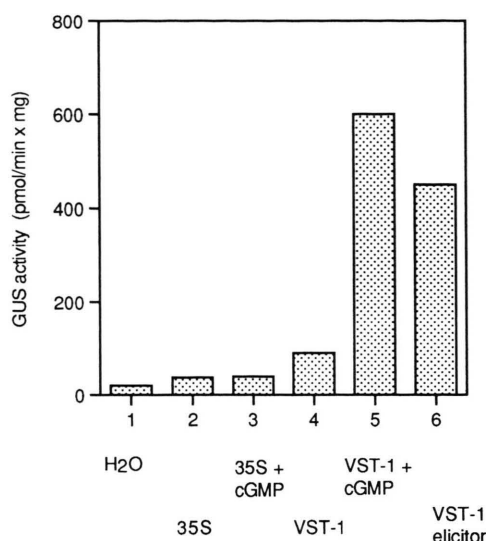
Fig. 2. Comparison of the 35S RNA promoter and the promoter A-5 of the stilbene synthase gene VST-1 (1 kb, full length) during exposure of protoplasts to stress. Transient transformation was performed either with a plasmid containing a 35S-GUS construct or with a plasmid encompassing the construct A-5 representing the VST-1 promoter and the intact coding sequence of GUS. Both constructs were also assayed by administering cGMP simultaneously with the plasmid (lanes 3 and 5). Column 6 shows the result of an experiment in which the plasmid containing A-5-GUS was used and the protoplasts were subsequently incubated in the presence of a *Botrytis cinerea* cell wall preparation. Column 1 ("H₂O") shows values either obtained with water alone or with plasmid containing GUS but lacking any promoter. The size of each column represents the average of the values obtained from five independent experiments.

promoters of pinosylvin-forming stilbene synthase genes (PST-1, PST-2, PST-3). Whereas PST-1 promoter showed highest response to cGMP, comparable to VST-1, the PST-2 promoter was characterized by a rather strong expression in the absence of a cGMP and a lack of response to cGMP.

The pronounced effect of cGMP provided a suitable means to further differentiate between the response elements present within the VST-1 promoter. Using four different constructs made by deleting various parts at the 5'-end of the 1 kb promoter (see Fig. 1), we found that increased elimination of DNA sequences in the upstream region of the promoter gradually reduced the responsiveness towards cGMP (Fig. 4). The highest cGMP effect was observed with Pr-7, a promoter
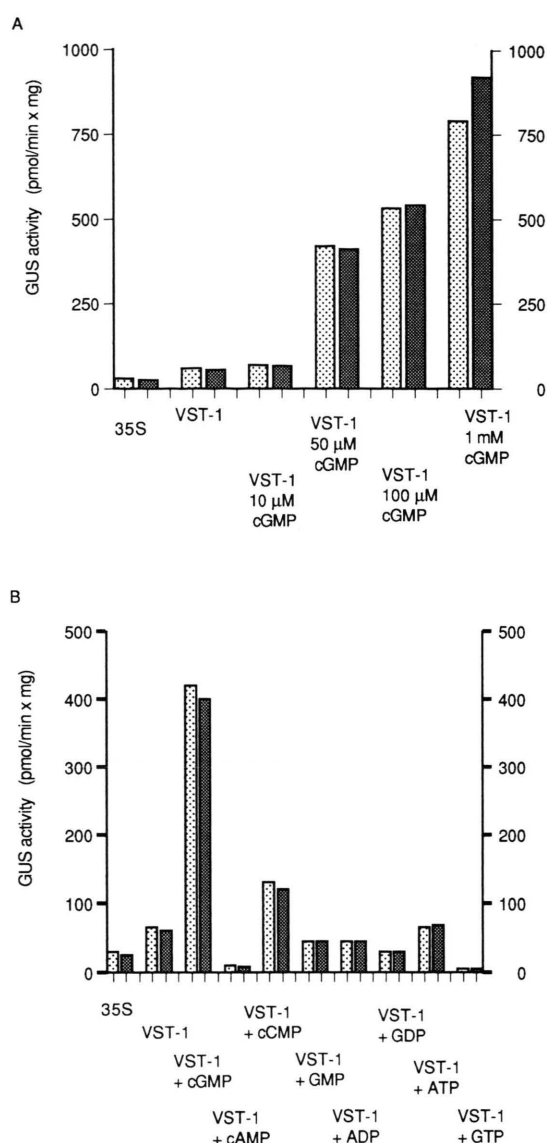

Fig. 3. Responsiveness of stilbene synthase VST-1 promoter towards cGMP and other nucleoside phosphates. With grapevine protoplasts, (a) various concentrations of cGMP were tested and (b) various cyclic nucleoside monophosphates and nucleotides (all at final concentrations of 50 μM) were compared. The combinations of promoters used in the reporter plasmids and the nucleotide effector applied are indicated below the diagrams. In all cases, the protoplast suspension was incubated with the GUS-expressing plasmid for 20 min. After incubation with calcium nitrate, washings and resuspension in buffer, the recovery time was for 24 h. In part a and b the enzyme extracts prepared were assayed twice either by incubation with methylumbelliferyl-β-D-glucuronide for 15 h (pointed column) or 22 h (shaded column). The results shown in these figures were reproducible in three independent experiments.
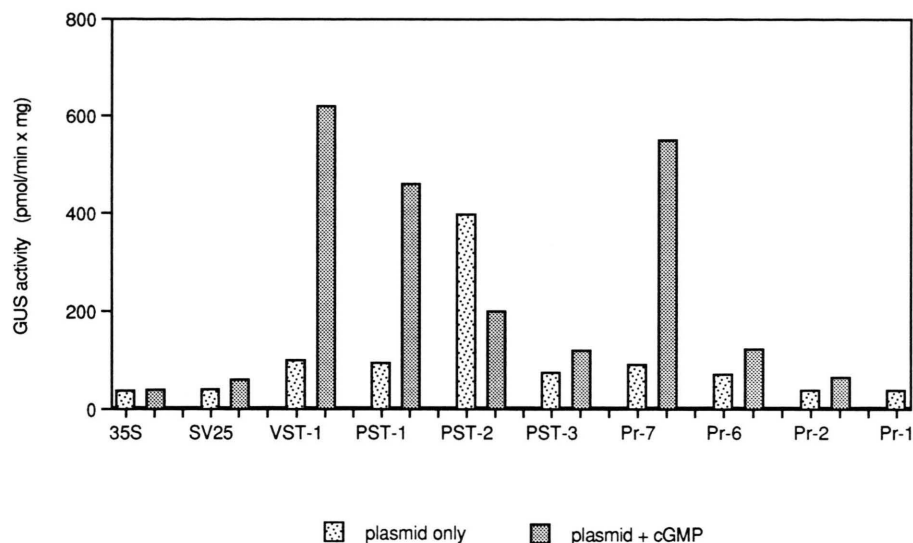
Fig. 4. Transient expression of GUS activity after transformation of grapevine protoplasts with plasmids containing promoter-GUS constructs in the presence and absence of 50 μM cGMP. The promoters originated from grapevine (VST-1, Pr-7, Pr-6, Pr-2, Pr-1) or pine (PST-1, PST-2, PST-3) stilbene synthase genes. For details of the promoter sequence see Figure 1. 35S: 35S RNA promoter (plasmid pBI221, CLONETECH, Heidelberg). These data represent the average of four independent experiments.

element encompassing the region up to nucleotide -956. Unlike VST-1 (construct with element A-5) which controlled the expression of wild type GUS, the constructs Pr-7 to Pr-1 coded for GUS fusion proteins including 15 amino acid residues originating from the stilbene synthase N-terminus.

Finally, the effects of cGMP led us to investigate the intracellular cGMP level and its alteration during elicitation by fungal cell wall or by other biological stress. We determined the concentration of cGMP in unstressed grapevine cell suspension cultures with 0.4 pmol per g fresh weight. Stress caused by fungal cell wall addition produced a transient increase up to 0.9 pmol cGMP per g fresh weight.

Discussion

This research was conducted in order to establish a transient expression system using as host a plant possessing inducible stilbene synthase genes. Earlier investigations had shown that in grapevine most of the stilbene synthase genes are responsive to stress exerted by fungal elicitors or other means (Melchior and Kindl, 1991; Wiese *et al.*, 1994). Unlike tobacco protoplasts which lack an elicitor-me-

diated pathway leading to phytoalexins, grapevine protoplasts possess their own set of stress-induced stilbene synthases. Although the grapevine protoplasts were slightly induced during their preparation by exposure of intact cells to cell wall-degrading enzymes, they still responded to the addition of fungal cell wall by synthesizing new proteins, with stilbene synthase being the most extensively expressed protein. In the future, grapevine protoplasts may be useful for comparing the expression of endogenous genes and exogenous DNA constructs, both responses being mediated by the same signal transduction chain.

cGMP as an external signal has been found to promote organogenesis in tobacco callus cultures (Mangat and Janua, 1987). The presence of cGMP in plant cells has been shown unequivocally, e. g. in seedlings of *Phaseolus vulgaris* (Newton *et al.*, 1984). Analysis of the cGMP level in stressed grapevine cell cultures showed an increase of up to 0.9 pmol/g fresh weight. In aleuron layers isolated from imbibed barley grains, cGMP levels of 0.07 pmol/g fresh weight (Penson *et al.*, 1996) were found. Gibberellic acid but not abscisic acid led to a transient increase of the cGMP level of up to 0.25 pmol/g fresh weight. Recently, a role of cGMP

in signal transduction of light-induced genes in plants has been demonstrated (Bowler *et al.*, 1994; Millar *et al.*, 1994). cGMP in the presence of Ca^{2+} triggers the production of anthocyanins and induces in phytochrome-deficient tomato mutants the formation of fully competent chloroplasts (Wu *et al.*, 1996). In those studies, a high level of GUS activity was observed by applying cGMP at a concentration of 70 μM. In grapevine protoplasts, 50 μM cGMP caused a 5 to 8-fold increase in the expression of the externally added VST-1 promoter-GUS construct (Fig. 2). Unlike the phytochrome signalling system investigated by microinjection into bean hypocotyls (Wu *et al.*, 1996), we do not observe a light-dependent activation of stilbene synthase genes in grapevine cells.

In suspension cultures of grapevine cells, it has been shown earlier by Northern blots that both VST-1 and SV-25 mRNAs are the stilbene synthase mRNAs exhibiting the highest increases upon elicitation of intact cells with fungal cell wall (Wiese *et al.*, 1994). To verify the exorbitant increase in gene activation and to localize roughly the stretches in the promoter region responsible for the cGMP-mediated gene activation, sections the gene VST-1 promoter were analyzed by the transient expression test. Figure 1 shows the nucleotide sequence of the 5'-end of gene VST-1 extending from nucleotide -956 to +58 (translation start) and provides an overview of the DNA fragments (Pr-7, Pr-6, Pr-2) used in this investigation. The 1 kb fragment of the promoter and the fragment Pr-7 conferred full cGMP-mediated response. They carry interesting regulatory elements. Pr-6 and Pr-2 are less responsive. When we applied as minimal promoter the fragment Pr-1 beginning shortly upstream of the TATAA box we measured a low expression not inducible at all, comparable to the effect observed with the 35S RNA-promoter. It is noteworthy that the decrease in cGMP-mediated effect seen by comparing the gene expression governed by Pr-7, Pr-6, and Pr-2 parallels the reduction of the number of TGAC elements within the promoters. Sequence-specific DNA-binding proteins have been shown to recognize elicitor-responsive W-elements (W1, W2, W3) in the promoter of pathogenesis-related proteins from parsley (Rushton *et al.*, 1996). Regions with similar TGAC consensus sequences are found in the grapevine VST-1 gene (Fig. 1) at nucleotides -508 to -495 and -463 to -450 (similar to W3), nucleotides -408 to -395 (W2), and nucleotides -93 to -80 (W1). The sizes of the promoter deletions were selected in such a way that the promoter constructs differed by their number of putative W boxes (Rushton *et al.*, 1996). It appears that constructs containing W-boxes conferred strong expression to the promoter.

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