Journal of Biochemistry and Molecular Biology, Vol. 32, No. 1, January 1999, pp. 51–59

JOURNAL OF
Biochemistry and Molecular Biology

Regulation of the Korean Radish Cationic Peroxidase Promoter by Phytohormones and Other Reagents

Dong Ju Lee, Sung Soo Kim and Soung Soo Kim*

Department of Biochemistry and Bioproducts Research Center, College of Science, Yonsei University, Seoul 120-749, Korea

Received 18 September 1998, Accetped 28 October 1998

The Korean radish cationic peroxidase (KRCP) promoter, comprising nucleotides −471 to +704 relative to the transcriptional initiation site, was fused to the GUS gene and transformed to tobacco BY-2 cells. We examined how auxin (2,4-dichlorophenoxyacetic acid, 2,4-D), cytokinin (6-benzylaminopurine, BAP), gibberellic acid (GA_3), abscisic acid (ABA), methyl jasmonate (MeJA), and phosphatidic acid (PA) affect the GUS expression in the presence or absence of 2,4-D in a modified LS medium.

Exogenous 2,4-D or BAP greatly decreased the GUS expression regulated by the KRCP promoter in a modified LS medium containing 0.2 mg/l 2,4-D. GA_3 increased the GUS expression and ABA completely reduced the inductive effect of GA_3. The GUS expression was also increased dose-dependently by plant defense regulators, MeJA and PA.

In contrast to the above results, auxin deprivation from the modified LS medium increased the GUS expression after treatment with exogenous 2,4-D whereas BAP still greatly decreased the GUS expression dose-dependently. GA_3 or MeJA slightly decreased the GUS expression. The data suggest that auxin deprivation changes the sensitivity of the suspension cells to exogenous chemicals and that the regulation of the KRCP promoter by 2,4-D, GA_3, and MeJA is dependent on auxin, whereas the regulation by BAP is not. This study will be valuable for understanding the function and expression mode of the Korean radish cationic peroxidase in Korean radish.

Keywords: Korean radish, Methyl jasmonate, Peroxidase promoter, Phosphatidic acid, Phytohormones.

* To whom correspondence should be addressed.
Tel: 82-2-361-2698; Fax: 82-2-361-2698
E-mail: kimss518@bubble.yonsei.ac.kr

Introduction

The plant peroxidase isozymes (EC 1.11.1.7) are thought to be involved in various cellular functions, which include catabolism of auxin (Fox *et al.*, 1965), production of hydrogen peroxide (Mader *et al.*, 1980), the polymerization of phenolics into lignin (Lagrimini *et al.*, 1987), wound healing (Espelie *et al.*, 1986), and defense against pathogen attack (Lagrimini and Rothstein, 1987). The role of each plant peroxidase, however, has not been established clearly. Lagrimini reported that tobacco anionic peroxidase was the major lignin-forming peroxidase, and its promoter activity was tissue-specific and highly regulated in all organs throughout development (Klotz *et al.*, 1998). Also, the promoter of a basic isoperoxidase gene (*prxC2*) from Horseradish (*Armoracia rusticana*) had directed strong GUS activity in transgenic tobacco leaves, stems, and roots (Kawaoka *et al.*, 1992).

The induction of callus from dicotyledonous plants is most efficient by an approximately equal amount (typically around 10 μmol/l) of both auxin and cytokinin (Open Universiteit, 1993). It has been observed that the activity of tobacco anionic peroxidase promoter increases with distance from the shoot apex in transgenic tobacco plants (Klotz *et al.*, 1998) and this pattern may be related to auxin suppression of peroxidase expression (Klotz and Lagrimini, 1996). Generally, a high cytokinin to auxin ratio gives the best results of shoot induction (differentiation) (Open Universiteit, 1993) and it is well known that the calli on the low-cytokinin medium do not regenerate (Renaudin *et al.*, 1991). The correlation between organogenesis and expression of different isoperoxidases implies that each isozyme may have a specific role in plant growth and development (Kay and Basile, 1987).

GA has been reported to activate the peroxidase activity of the excised leaves of ragi plants (*Eleusine coracana*) (Kumar and Khan, 1983), and changes in the isoperoxidase pattern were reported in GA-treated tobacco tissue (Thorpe and Gaspar, 1978). Abscisic acid, on the other hand, has

multiple physiological effects such as stomatal regulation, bud or seed dormancy, and abscission that affect growth and development literally throughout the ontogeny of seed plants (Moore, 1979). Further, antagonistic effects of pairs of growth regulators have been reported in many plant experimental systems. For example, GA promotes the transcription of α-amylase, and ABA inhibits the production of its mRNA in aleurone layers of barley (Gubler and Jacobsen, 1992).

Lipid productions of different phospholipases have been demonstrated to play a role in defense responses to pathogens or parasites in animal systems (Samuelsson *et al.*, 1987). Linoleic acid and linolenic acids have been reported to play important roles in plant defense responses against wounding and pathogen attacks. Jasmonate (JA) and its methyl ester (methyl jasmonate, MeJA) are also potent inducers of an array of stress-related genes, such as L-phenylalanine ammonia lyase, chalcone synthase, protein inhibitors, and lipoxygenase (Constabel *et al.*, 1992). Hildmann *et al.* (1992) reported that jasmonate and abscisic acid induced several identical proteins in potato plants and supported that jasmonate played an important role of an intermediate in the signaling pathway that leads from ABA accumulation in response to wounding to the transcriptional activation of the genes. In addition to the stress-related genes, JAs affect a variety of processes such as root growth, fruit ripening, senescence, pollen development, and defense against insect predation and pathogens.

Recently, Curtis *et al.* (1997) reported that mRNA of two peroxidase, Shpx6a and Shpx6b, of the legume *Stylosanthes humilis* were rapidly induced after treatment with MeJA, and the Shpx6b promoter activity was also induced by MeJA in mature leaves of transgenic tobacco. However, the link between JA and the downstream responses is not well known, and the crosstalks among different transduction pathways triggered by various effectors are not well defined. Ryu and Wang (1996) reported that phosphatidic acid (PA) is accumulated rapidly at the wounded leaf and Lee *et al.* (1997) found that PA and lysophospholipids, like MJ, also accumulated rapidly in the neighboring non-wounded leaves of the wounded tomato seedlings.

Of those plant promoters which have been studied in detail, the general architecture of the promoter is such that sequence motifs important in qualitative expression are located close to the CAAT and TATA boxes and strong positive elements directing high levels of activity are located 5′ to these regions (Walden and Schell, 1990). However, the characterization of the cationic peroxidase promoter in plants has not been studied in detail.

In our laboratory, there are eight isoperoxidases distinguishable by starch gel electrophoresis; A1, A2, A3n, A3, C1, C2, C3n, and C3 (Lee and Kim, 1990; 1994), and two genomic genes (*prxK1* and *prxK2*) of putative cationic peroxidase from the Korean radish (*Raphanus sativus*) were isolated, restriction-mapped, and sequenced (Park and Kim, 1996a). Furthermore, the entire cDNA sequence of one cationic Korean radish isoperoxidase had been cloned and overexpressed in *E. coli* under the *lac* promoter (Park and Kim, 1996b).

In this study, we constructed a KRCP promoter/GUS gene and transformed tobacco BY-2 cells using an agrobacterium-mediated transformation method to study the effects of various phytohormones, MeJA, and PA, on the KRCP promoter activity.

Materials and Methods

Plasmid construction The Korean radish cationic peroxidase genomic clone, named *prxK1*, has been previously described (Park and Kim, 1996a). PCR amplifications of the KRCP promoter from positions –471 to +704 were performed with a DNA Thermal Cycler using *Pfu* DNA polymerase and sequence-specific primers in 50 μl reaction mixtures. Two primers, named primer 1 and primer 6, based on the nucleotide sequences of *prxK1*, were synthesized. The sequence of the upstream primer 1 (5′-GCGCTCTAGAAGCTTTCCTCTTCGTTTAC-3′) contains a *Xba*I restriction enzyme site. The sequence of the downstream primer 6 (5′-GCGCACCCGGGTCGAAGACTTCATATAGTT-3′) contains a *Xma*I restriction enzyme site to create the new restriction enzyme site. Purified PCR products were cloned into a pCR-Script Cam SK(+) cloning vector (Stratagene, La Jolla, USA), digested with a *Xba*I/*Xma*I, and subcloned into a pBI101 binary vector, a kind of Ti plasmid vector (Clontech, Palo Alto, USA). The new construct was verified by nucleotide sequencing. The pBK12 plasmid consisted of pBI101 plasmid containing the KRCP 5′ sequences between positions –471 and +704 fused 13 bp upstream of the ATG start codon of the GUS reporter gene.

Plant materials and transformation This plasmid construct (pBK12) was transferred into *A. tumefaciens* (LBA4404) by an electroporation method as previously shown in Melina *et al.* (1996). After electroporation, the transfected bacteria were transferred into 1 ml of fresh Luria-Bertani medium and incubated for 2 h in an orbital shaker at 150 rpm at 25°C. The transformed bacteria (named LBAK12) were selected on the basis of kanamycin and rifampicin resistance.

Tobacco (*N. tabacum* L. cv BY-2) cells were maintained in the modified LS medium containing MS salt, 0.37 g/l of KH_2PO_4, 1 mg/l of Thiamine-HCl, 0.2 mg/l of 2,4-D, 100 mg/l of myo-inositol, and 3% sucrose at 25°C with shaking at 100 rpm on a gyrotary shaker and subcultured every 2 wk with a 5% inoculum. BY-2 cells were transformed by co-culture with the LBAK12 cells. Transformed BY-2 cells were selected on the basis of kanamycin and carbenicillin resistance as described (An, 1985). The kanamycin-resistant transformant cells (named BYK12) could be detected for 1 month.

Analysis of transgenic tobacco cells by PCR Isolation of genomic DNA from BYK12 cells was carried out as previously described (Martin *et al.*, 1991). Cells (0.1 g) were ground to a fine powder with a small mortar and pestle after freezing in liquid N_2. The sample was mixed thoroughly in 800 μl of the extraction buffer and incubated for 30 min at 65°C. Then, the tube was filled

with chloroform:isoamylalcohol (24:1 vol/vol) and inverted repeatedly for 3 – 4 min. The sample was centrifuged at 17,400 × *g* for 10 min at 4°C. The aqueous phase was carefully transferred to a fresh tube, mixed with 2/3 vol of cold isopropanol and inverted until the DNA precipitates appeared. After centrifugation, the pellet was resuspended with 50 μl distilled water at 65°C for 10 min and stored at −20°C for later use. The amplification reaction was performed with two primers as previously described (Saiki, 1990). Two kinds of DNA primers based on the pBI101 sequence shown in Fig. 1B were prepared as follows: The primer For (5′-TACGCCAAGCTTGCATGCCTG-3′) is located at the portion upstream from the *Xba*I restriction enzyme site of the multicloning sites. The primer Back (5′-AGGACGTAACATAAG GGAATG-3′) is located at the portion downstream from the *Xma*I restriction enzyme site of the multicloning site. The reaction products were analyzed by 1% agarose gel electrophoresis.

GUS and protein assays Transgenic BYK12 cells (40 μl packed cell volume) were homogenized for 20 s at 4°C in 100 μl of GUS extraction buffer using pestles. The samples were then centrifuged at 17,400 × *g* for 30 min at 4°C to remove cell debris. The GUS extraction buffer consisted of 50 mM Na_2HPO_4 (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100. GUS assays were performed and evaluated by fluorometric methods using 4-methylumbelliferyl β-D-glucuronic acid (4-MUG) as previously described (Gallagher, 1992). After the cell extracts were incubated with 4-MUG at 37°C for 1 h, the reaction mixture was transferred to 0.2 M sodium carbonate stop buffer. Fluorescence was measured with 365 nm excitation and 460 nm emmision using a Kontron SFM 25 fluorospectrometer. Fluorescence calibration was done using 0.1 mM 4-methyl umbelliferone (4-MU) in 0.2 M sodium carbonate stop buffer. The GUS activity was detected by incubating transgenic BYK12 cells in X-gluc substrate solution containing 100 mM Na_2HPO_4 buffer (pH 7.0), 1 mM 5-bromo-4-chloro-indolyl glucuronide (X-gluc), 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100 for 24 h at 37°C. Yellow pigment was removed by incubation in 100% ethanol at room temperature. Total protein was determined by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Auxin starvation Transgenic suspension cell cultures were maintained as previously described (Lee and Kim, 1998). Seven-day old transgenic BYK12 cells were rinsed with 20-fold vol auxin-free medium (the modified LS medium without 2,4-D). The volume of suspension culture was adjusted to 50 ml with auxin-free medium and cells were grown at the same conditions for 3 d (Ishida *et al.*, 1993).

Effects of phytohormones, MeJA, and PA on the GUS activity of transgenic BYK12 cells Several concentrations of phytohormones (auxin, cytokinin, gibberellic acid, abscisic acid), MeJA, and PA (phosphatidic acid, didecanoyl acid) were added to 8 ml transgenic BYK12 cells 7 d after the subculture was performed. After 2,4-D starvation in the modified LS medium, GUS expression was also investigated by adding various concentrations of phytohormones, MeJA, and PA to the culture medium. Cells were then incubated for 4 h in a gyratory shaker at 100 rpm, 25°C for assays of GUS activity. All experiments were repeated four times.

Results

Characteristics of the Korean radish cationic peroxidase (KRCP) promoter Sequence analysis of about 1.2 kb of the KRCP promoter revealed elements common to most plant peroxidase promoters as well as several potential regulatory elements (Fig. 1A). The site of initiation of transcription, as determined by primer extension analysis, was the A at −717 bp from the ATG initiation codon (Park and Kim, 1996a). The untranslated leader sequence (717 bp) is very long compared to other known peroxidase promoters (Park and Kim, 1996a). Putative promoter sequences, the TATA and CAAT boxes, were found 64 and 117 bp upstream of the transcription start site. The putative gibberellin response element (GARE, TAACAAA) is located downstream of +151 relative to the transcriptional initiation site (Gubler and Jacobsen, 1992). The as-2 box, which is known to confer leaf and shoot expression, is conserved (Lam and Chua, 1989). The GT elements, which mediate transcriptional repression in roots, are also conserved (Villain *et al.*, 1996). The root motifs correlated with root specific expression in wheat (Hertig *et al.*, 1991) are also conserved in the KRCP promoter.

Transformation of tobacco BY-2 cells with a KRCP promoter/GUS construct The chimeric plasmid (pBK12) was constructed to express the GUS gene under the control of the KRCP promoter and to transform the tobacco BY-2 cells (Fig. 1B). The genomic DNAs isolated from transgenic BYK12 cells and plasmids from *Agrobacterium* transfected with pBK12 were subjected to PCR using two nucleotide primers (named For and Back or 1 and 6) in order to ascertain the presence of the KRCP promoter/GUS structure gene. The putative fragments (1.2 kb) were detected without any other amplified PCR products (Fig. 2A). However, in the case of the original BY-2 cells and BY-2 cells transformed with pBI101 plasmid, amplified PCR products were not detected (data not shown). The GUS activity in the transgenic BYK12 cells was also detected by histochemical staining with 1 mM X-gluc (Fig. 2B) (Gallagher, 1992). The endogenous GUS activity was not detectable in original BY-2 cells, and the expression of GUS was negligible in BY-2 cells transformed with promoterless pBI101 plasmids (data not shown). When pBK12 plasmids were introduced into BY-2 cells, the GUS activity was greatly increased reaching ca. 2–3 nmole 4-MU/min/mg soluble protein, indicating that most of the detectable GUS activity was due to the GUS expression in the transgenic BYK12 cells. These results indicate that the KRCP promoter/GUS construct is inserted on the tobacco BY-2 genome and the GUS expression is regulated by KRCP promoter, as we expected.

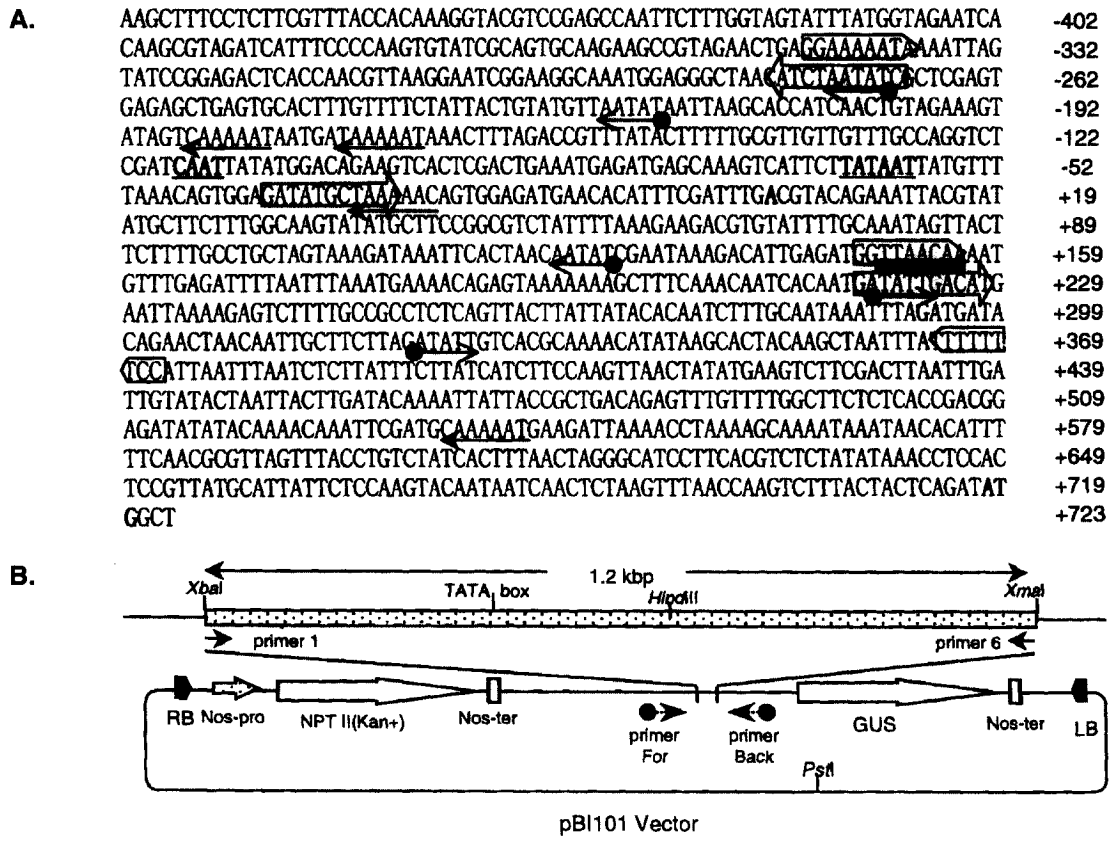

Fig. 1. A. Schematic representation of potential regulatory regions in the KRCP promoter. The numbering scheme for the nucleotide sequence specifies the A (bold) of the transcription start site as +1. The putative promoter sequences, the TATA and CAAT boxes, are bold and underlined. Bold letters indicates the start codon (ATG). The as-2 box, conferring leaf and shoot expression, are shown (⇨) (Lam and Chua, 1989). The GT elements, mediating the transcriptional repression in roots, are indicated (⇨) (Villain *et al.*, 1996). The root motifs, correlating with root specific expression of peroxidase in wheat, are shown (•→) (Hertig *et al.*, 1991). The KRCP promoter also contains a putative gibberellin response element marked with a bold line (Lee and Kim, 1998). B. Construction of the chimeric plasmid (pBK12) in pBI101 plasmid. Primers (For and Back, or 1 and 6) are indicated by arrows, used for PCR cloning and detection.

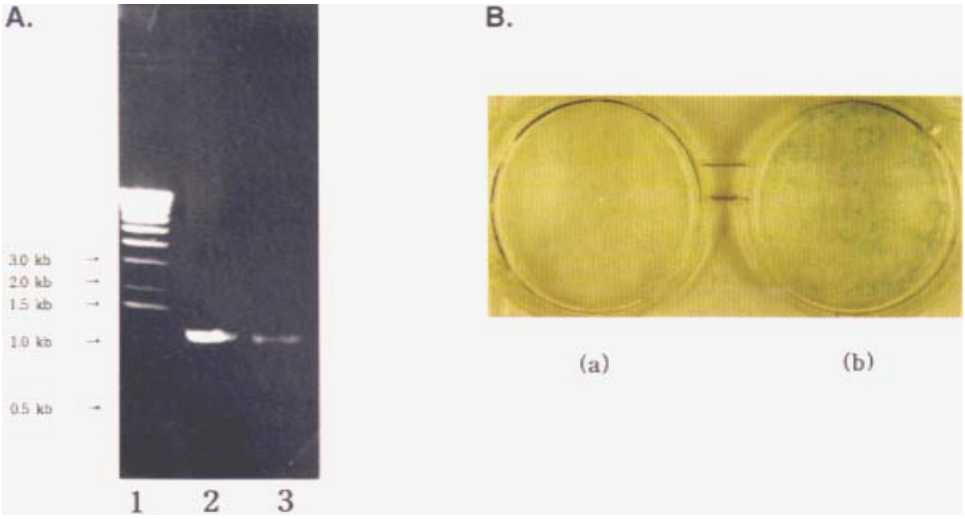

Fig. 2. A. PCR detection of KRCP promoter in the genomic DNA of transgenic BYK12 cells. Lane 1, 1 kb ladder marker; Lane 2, PCR products using two primers (named For and Back); Lane 3, PCR products using two primers (named 1 and 6). B. Cell stain of the GUS expression. (a). The original BY-2 cells. (b). The transgenic BYK12 cells. The GUS expression (blue color) was detected by incubating cells in 1 mM X-gluc substrate solution for 24 h at 37°C. Yellow pigment was removed by incubation in 100% ethanol at room temperature.

Effects of phytohormones, MeJA, and PA on the GUS activity in the transgenic BYK12 cell In order to better understand the function of the KRCP gene expression, we have characterized the response of KRCP promoter to phytohormones, MeJA, and PA. Dose dependence was studied 4 h after treatment with 2,4-D. Figure 3A shows that the GUS activity decreased when compared to the control (with no added auxin) after treatment with 2,4-D from 0.4 to 8 mg/l. Treatment with 2 mg/l of 2,4-D inhibited the GUS activity by approximately 35% when compared with the control. This suppression may be related with the report that IAA and NAA suppressed the GUS expression from the tobacco anionic peroxidase promoter (Klotz and Lagrimini, 1996).

It is well known that cytokinin can regulate the expression of the plant peroxidase gene. The putative anionic peroxidase (P17) accumulates in *Petunia hybrida* calli under non-regeneration conditions of an extremely low concentration of cytokinin in the medium (Renaudin *et al.*, 1991). Dose dependence of the GUS gene expression was studied 4 h after treatment with BAP. Incubation of BYK12 cells with BAP indicated that the GUS activity was rapidly inhibited, reaching 50% inhibition at 8 mg/l of BAP (Fig. 3B).

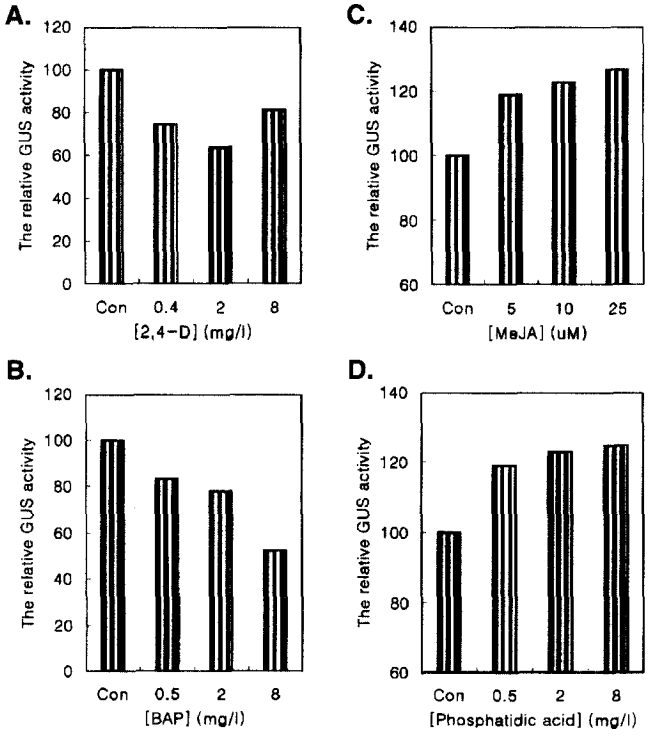

Fig. 3. Effect of 2,4-D, BAP, MeJA, and PA on the GUS expression of transgenic BYK12 cells. A. Treated with 2,4-D from 0.4 to 8 mg/l. B. Treated with BAP from 0.5 to 8 mg/l. C. Treated with MeJA from 5 to 25 μM. D. Treated with PA from 0.5 to 8 mg/l. All values have been shown relative to the GUS activity of non-treated BYK12 cells, which was given a value of 100.

Treatment with GA_3 was effective in activating the GUS expression of the KRCP promoter. Dose dependence of the GUS gene expression was studied 4 h after treatment with GA_3 (Lee and Kim, 1998). Concentrations of GA_3 between 288 and 1150 nM were effective and increased the GUS activity by approximately 40% at 577 nM when compared with the control (with no added GA_3) (Fig. 4). The fact that the GUS activity increased within narrow limits of the concentration of GA_3 suggests that the responsiveness of the KRCP promoter is very specific to the concentrations of GA_3. Dose dependence of the GUS expression was also studied 4 h after treatment with ABA. The GUS activity slowly decreased when compared with the control (with no added ABA) after treatment with ABA from 10 to 100 μM (Fig. 4). It is well known that ABA can counteract the effect of added GA (Moore, 1979; Gubler and Jacobsen, 1992). GA increases the GUS activity and ABA inhibits this effect of GA when barley aleurone protoplasts prepared after germination of grains were transfected with the α-amylase/GUS constructs (Gubler and Jacobsen, 1992). When 100 μM ABA and 577 nM GA_3 were applied simultaneously to the transgenic BYK12 cells, the GUS activity decreased to the control level (Fig. 4).

MeJA from 5 to 25 μM increased the GUS expression dose-dependently (Fig. 3C). Incubation of BYK12 cells with MeJA indicated that the GUS expression was activated to a 30% increase at 25 μM MeJA. Curtis *et al.* (1997) recently reported that two transcripts of isoperoxidase (Shpx6a and Shpx6b) were rapidly induced after treatment with the defense regulator, MeJA. The GUS expression was also increased dose-dependently by exogenous application of 0.5 to 8 mg/l of phosphatidic acid. Treatment with 8 mg/l of PA increased the GUS

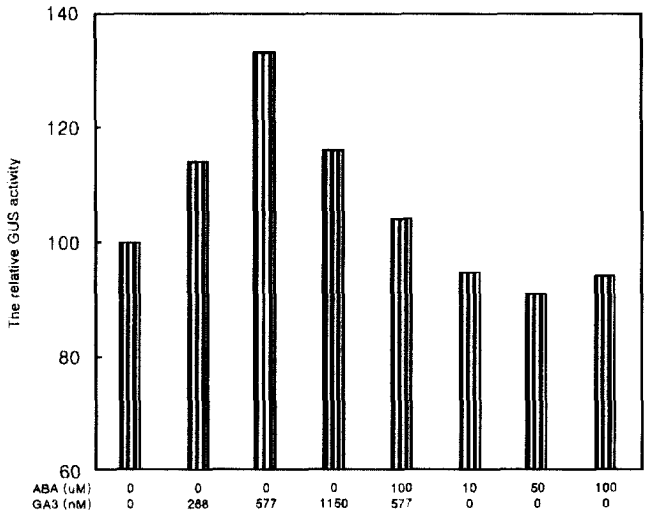

Fig. 4. Effect of GA_3, ABA, and GA_3/ABA on the GUS expression of the transgenic BYK12 cells. All values have been shown relative to the GUS activity of non-treated BYK12 cells, which was given a value of 100.

activity by approximately 25% when compared with the control (Fig. 3D).

Effects of phytohormones and MeJA on the GUS activity in the transgenic BYK12 cell after auxin starvation The effects of phytohormones and MeJA were also examined after depletion of auxin from cultured BYK12 cells. As shown in Fig. 5A, exogenous 2,4-D was effective in activating GUS expression in BYK12 cells after auxin starvation for 3 d. Concentrations of 2,4-D between 0.2 and 4 mg/l were effective and the GUS expression increased approximately 40% as the auxin concentration increased above 1 mg/l.

On the other hand, BAP is effective in inhibiting the GUS expression after auxin starvation in BYK12 cells. BAP suppresses the GUS expression at a concentration of 0.5 to 8 mg/l, and approximately 40% inhibition was observed from 2 to 8 mg/l when compared to the control (with no added BAP) (Fig. 5B). GA_3 between 288 and 577 nM slightly decrease the GUS expression after auxin starvation in BYK12 cells (Fig. 5C). Exogenous MeJA, from 5 to 10 μM, also decreases the GUS expression by approximately 20% after auxin starvation in BYK12 cells (Fig. 5D).

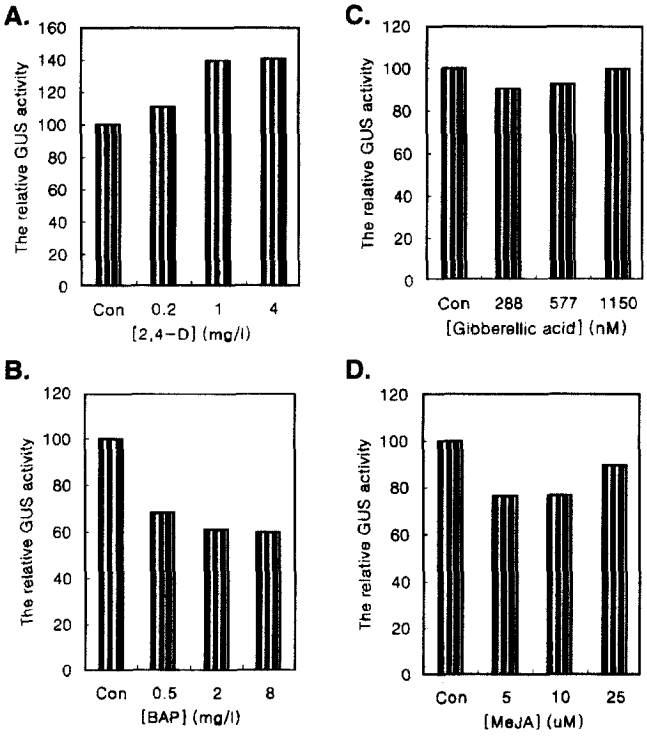

Fig. 5. Effect of 2,4-D, BAP, GA_3, and MeJA on the GUS expression of transgenic BYK12 cells after auxin starvation. A. Treated with 2,4-D from 0.2 to 4 mg/l. B. Treated with BAP from 0.5 to 8 mg/l. C. Treated with GA_3 from 288 to 1150 nM. D. Treated with MeJA from 5 to 25 μM. All values have been shown relative to the GUS activity of non-treated BYK12 cells, which was given a value of 100.

Discussion

A large number of experimental analyses have revealed the relationship between the regulation of the *cis*-acting elements of various stress-inducible plant genes and their physiological roles. In the present investigation, we analyzed the hormone-responsive expression of a KRCP promoter/GUS gene. Dose-response analyses indicate that the KRCP promoter may be able to activate or inhibit the GUS gene expression when exogenous phytohormones, MeJA, and PA were applied to the transgenic BYK12 cells.

We have examined the auxin and cytokinin sensitivity of the KRCP promoter in transgenic tobacco BYK12 cells. Addition of 2,4-D to the transgenic BYK12 cells slightly decreased the GUS activity when compared to the control (no added 2,4-D) (Fig. 3A). This expression mode may be similar to the auxin suppression mode of peroxidase expression. It has been observed that anionic peroxidase activity in tobacco increases with age, maturity, and distance from the shoot apex (Theologis, 1986; Klotz *et al.*, 1998). It has also been reported that auxin dramatically suppresses the expression of tobacco anionic peroxidase promoter and the extent of suppression was similar for either IAA or NAA (Klotz and Lagrimini, 1996). Further reports claim that one cationic peroxidase from young stem sections of dwarf peas was repressed by application of exogenous IAA (Ockerse *et al.*, 1966). Upon treatment of BYK12 cells with BAP, the GUS activity was rapidly inhibited in a dose-dependent manner (Fig. 3B). This suppression of the GUS expression may be considered to be associated with a phenomenon triggered by the high level of cytokinin, such as the onset of plant regeneration or senescence-retarding activity when applied exogenously (Smart, 1994), and with the expression of tobacco cationic peroxidase C1 and C2 suppressed by the high level of cytokinin (Kay and Basile, 1987). The interactions between auxin and cytokinin throughout plant growth, development, differentiation, and senescence are complex, and genetic investigations upon auxin and cytokinin signaling have been limited. Although the molecular mechanisms of auxin-cytokinin interactions are not known, these are thought to include mutual control of auxin and cytokinin metabolism, interactions in the control of gene expression, and post-transcriptional interactions (Coenen and Lomax, 1997). Several genes have already been shown to be down-regulated by cytokinins (Crowell and Amasino, 1991). For example, lowering the total cytokinin content of calli, which ultimately results in growth arrest and senescence, induces the expression of PeTh3 and Peperl at an early stage of this process (Tournaire *et al.*, 1996). However, BAP did not affect the activity of tobacco anionic peroxidase promoter (3 kb) in transiently transformed tobacco mesophyll protoplasts (Klotz and Lagrimini, 1996). So the function of the KRCP gene may be different from that of the tobacco anionic peroxidase gene or horseradish cationic peroxidase gene (*prxC2*).

GA has been reported to activate the peroxidase activity of the excised leaves of ragi plants (Kumar and Khan, 1983), and characteristic changes in isoperoxidase patterns in GA-treated tobacco tissue were reported. In particular, the intensities of the cationic isoperoxidases C1a and C4 increased more in starch gel electrophoresis upon GA treatment when compared to that of non-treated tobacco tissue with gibberellin (Thorpe and Gaspar, 1978). These data are similar to the promoter studies of a barley high-pI α-amylase gene, which can respond highly to the added GA or ABA. The present study provides evidence of peroxidase promoter regulation by GA_3 and ABA (Fig. 4). The fact that the GUS activity increased within narrow limits of the concentration of GA_3 suggests that the responsiveness of the KRCP promoter is very specific to the concentrations of GA_3. On the basis of collective data, it may be concluded that exogenous GA_3 and/or ABA antagonistically control 5′ upstream regions of the KRCP gene. The putative gibberellin response element (GARE, TAACAAA) was identified in α-amylase gene promoters of barley, wheat, and rice, and this element appears to be the site of action of ABA (Gubler and Jacobsen, 1992). As this conserved GARE sequence is located in the 5′ untranslational region in the case of the KRCP gene, there may be possible interaction between the promoter and the gibberellin response element. However, the presence of other gibberellin response elements in the promoter region cannot be excluded. It has been reported that the tobacco anionic peroxidase gene expression was not affected by gibberellic acid (Klotz and Lagrimini, 1996). Therefore, the physiological role of the KRCP gene in the present study may be different from that of the tobacco anionic peroxidase, which has been thought to be involved in the lignification of secondary cell walls (Lagrimini *et al.*, 1987). Thus, the increase of the GUS expression in the transgenic BYK12 cells by GA_3 may correlate with the function of gibberellins, which promotes cell elongation and gradually increases the intensity of peroxidase patterns (Waltraud and Rudolf, 1992).

Addition of MeJA slightly increased the GUS activity when compared to the control (no added MeJA) (Fig. 3C). The motif TGACG from lipoxygenase 1, which previously identified as being bZIP transactivating factors, is very much responsible for MeJA-responsive expression (Rouster *et al.*, 1997). This motif, which is conserved in the KRCP promoter, may control the KRCP gene expression in case of pathogen attacks. JA and MeJA are well known to be potent inducers of stress-related genes (Constabel *et al.*, 1992) and it was reported that exogenous MeJA induced the GUS activity of mature transgenic tobacco leaves transformed with Shpx6b isoperoxidase promoter (Curtis *et al.*, 1997). Upon treatment of BYK12 cells with PA, the GUS activity of transgenic BYK12 cells was increased dose-dependently (Fig. 3D). It was recently reported that PA might have some important roles in plant defense response elicited by wounding in various plants (Lee *et al.*, 1997). Hence, these results suggest that the KRCP promoter may contain *cis*-elements associated with plant defense. These inductions of the GUS expression by MeJA and PA support that the KRCP gene may also be associated with plant defense responses (Constabel *et al.*, 1992; Lee *et al.*, 1997) as it has been reported that the plant peroxidase is thought to be involved in wound healing (Espelie *et al.*, 1986) and defense against pathogen attack (Lagrimini and Rothstein, 1987). Hildmann *et al.* (1992) reported that jasmonate and abscisic acid induced several identical proteins in potato plants. This fact supports that jasmonate signaling can be coupled from ABA signaling in response to wounding. MeJA or PA increased the activity of KRCP promoter but not by ABA. Therefore, this finding supports that jasmonate signaling on the KRCP promoter is independent of ABA and can be uncoupled from abscisic acid signaling in Korean radish, in contrast to the wound-induction signal cascade of proteinase-inhibitor II in potato.

After auxin starvation for 3 d, the induction of the KRCP promoter activity was observed in transgenic BYK12 cells when treated with 2,4-D (from 0.2 to 4 mg/l) (Fig. 5A). Since the removal of auxin from the culture medium stops the growth of cells, auxins should play a pivotal role in maintaining cell division activity (Ito *et al.*, 1994). It was reported that addition of 2,4-D (0.2 mg/l) to the original BY-2 suspension cells after auxin starvation induces BY-2 cell division (Ishida *et al.*, 1993). Although auxin response elements are not found in the KRCP promoter, we speculate that the induction of KRCP promoter activity may be related with cell division that auxin induces. After auxin starvation, the inhibition of the KRCP promoter activity by exogenous BAP (from 0.5 to 8 mg/l) was observed (Fig. 5B). The fact that exogenous BAP inhibited the KRCP promoter activity in the case of both before and after auxin starvation shows that the cytokinin signaling pathway may not be related with cell division that auxin induces, but with plant regeneration triggered by the high level of cytokinin.

GA_3 slightly decreases the GUS expression after auxin starvation (Fig. 5C), and exogenous MeJA from 5 to 10 μM also decreases the GUS expression after auxin starvation (Fig. 5D). These results probably reflect some links between GA_3 or MeJA and cell division because complete auxin starvation resulted in a decrease of the frequency of cell division in the original BY-2 cells.

In conclusion, treatment of transgenic BYK12 cells with GA_3, MeJA, and PA in the modified LS medium induced the GUS expression while treatment with auxins, cytokinins, and ABA had inhibitory effects. However, after auxin starvation, only 2,4-D addition induced the GUS expression while BAP greatly decreased the expression dose-dependently, and GA_3 or MeJA decreased it only slightly. These results suggest that the KRCP promoter (1.2 kbp) contains at least the putative gibberellin response

element and defense responsive element, and that GA_3-, MeJA-, and PA-inducible GUS expressions in the presence of auxin are related with active cell responses, such as cell division and cell elongation which auxins control, and that auxin deprivation changes the sensitivity of the suspension cells to exogenous chemicals. The data suggest that the regulation of the KRCP promoter by 2,4-D, GA_3, and MeJA is dependent on auxin, but that by BAP is not. This study will be valuable for understanding the function and expression mode of the Korean radish cationic peroxidase.

Acknowledgements This work was supported by the Korean Ministry of Education (Project No. BSRI-98-4420). We would like to thank Dr. Youngsook Lee, Pohang University of Science and Technology, Korea, for providing us with phosphatidic acid (didecanoyl acid), and Chang Myung Kim and Min Jung Sun for their technical assistance on the GUS assays and management of cells.

References

An, G. (1985) High efficiency transformation of cultured tobacco cells. *Plant Physiol.* **79**, 568–570.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Coenen, C. and Lomax, T. L. (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. *Trends Plant Sci.* **2**, 351–36.

Constabel, C. P., Bergey, D. R. and Ryan, C. A. (1992) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. USA* **89**, 4938–4941.

Crowell, D. N. and Amasino, R. M. (1991) Induction of specific mRNA in cultured soybean cells during cytokinin or auxin starvation. *Plant Physiol.* **95**, 711–715.

Curtis, M. D., Rae, A. L., Rusu, A. G., Harrison, S. J. and Manners, J. M. (1997) A peroxidase gene promoter induced by phytopathogens and methyl jasmonate in transgenic plants. *Mol. Plant Microbe Interact.* **10**, 326–338.

Espelie, K. E., Trancechi, V. R. and Kolattukudy, P. E. (1986) Immunocytochemical localization and time course of appearance of anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiol.* **81**, 487–492.

Fox, L. R., Purves, W. K. and Nakada, H. I. (1965) The role of horseradish peroxidase in indole-3-acetic acid oxidation. *Biochemistry* **4**, 2754–2763.

Gallagher, S. R. (Ed.) *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression,* Academic Press, San Diego, California, 1992.

Gubler, F. and Jacobsen, J. V. (1992) Gibberellin-responsive elements in the promoter of a barley high-pI-amylase gene. *The Plant Cell* **4**, 1435–1441.

Hertig, C., Rebmann, G., Bull, J., Mauch, F. and Dudler, R. (1991) Sequence and tissue-specific expression of a putative peroxidase gene from wheat (*Triticum aestivum* L.). *Plant Mol. Biol.* **16**, 171–174.

Hildmann, T., Ebneth, M., Pena-Cortes, H., Sanchez-Serrano, J. J. Willmitzer, L. and Prat, S. (1992) General roles of abscisic acid and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell* **4**, 1157–1170.

Huang, N., Sutliff, T. D., Litts, J. C. and Rodriguez, R. L. (1990) Classification and characterization of the rice alpa-amylase multigene family. *Plant Mol. Biol.* **14**, 655–668.

Ishida, S., Takahasi, Y. and Nagata, T. (1993) Isolation of cDNA of an auxin-regulated gene encoding a G protein beta subunit-like protein from tobacco BY-2 cells. *Proc. Natl. Acad. Sci. USA* **90**, 11152–11156.

Ito, M., Sato, T., Fukuda, H. and Komamine, A. (1994) Meristem-specific gene expression directed by the promoter of the S-phase-specific gene, cyc07, in transgenic Arabidopsis. *Plant Mol. Biol.* **24**, 863–878.

Kawaoka, A., Sato, S., Nakahara, K., Matsushima, N., Okada, N., Sekine, M., Shinmyo, A. and Takano, M. (1992) Expression and promoter activity of genes for isozymes of Horseradish peroxidase. *Plant Cell Physiol.* **33**, 1143–1150.

Kay, L. E. and Basile, D. V. (1987) Specific peroxidase isoenzymes are correlated with organogenesis. *Plant Physiol.* **84**, 99–105.

Klotz, K. L. and Lagrimini, L. M. (1996) Phytohormone control of the tobacco anionic peroxidase promoter. *Plant Mol. Biol.* **31**, 565–573.

Klotz, K. L., Liu, T. Y., Liu, L. and Lagrimini, L. M. (1998) Expression of the tobacco anionic peroxidase gene is tissue-specific and developmentally regulated. *Plant Mol. Biol.* **36**, 509–520.

Kumar, K. B. and Khan, P. A. (1983) Age-related changes in catalase and peroxidase activities in the excised leave of *Eleusine coracana* Gaetin. Cv. PR 202 during senescence. *Exp. Gerontol.* **18**, 409–417.

Lagrimini, L. M. and Rothstein, S. (1987) Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* **84**, 438–442.

Lagrimini, L. M., Burkhart, W., Moyer, M. and Rothstein, S. (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proc. Natl. Acad. Sci. USA* **84**, 7542–7546.

Lam, E. and Chua, N. H. (1989) ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. *Plant Cell* **1**, 1147–1156.

Lee, D. J. and Kim, S. S. (1998) The regulation of 5′ upstream regions of a Korean radish cationic peroxidase gene by gibberellic acid and abscisic acid. *Plant Science* (in press).

Lee, M. Y. and Kim, S. S. (1990) Purification and characterization of the far migrating anionic isoperoxidase A3 from Korean radish root. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **23**, 440–446.

Lee, M. Y. and Kim, S. S. (1994) Characteristics of six isoperoxidases from Korean radish root. *Phytochemistry* **35**, 287–290.

Lee, S., Suh, S., Kim, S., Crain, R. C., Kwak, J. M., Nam, H. G. and Lee, Y. (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. *Plant J.* **12**, 547–556.

Mader, M., Ungemach, J. and Schloss, P. (1980) The role of peroxidase isozyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. *Planta* **147**, 467–470.

Martin, G. B., Williams, J. G. and Tanksley, S. D. (1991) Rapid identification of markers linked to a pseudomonas resistance gene in tomato by using random primers and near-isogenic lines. *Proc. Natl. Acad. Sci. USA* **88**, 2336–2340.

Melina, L., Ignacio, E. M. and Crag, L. N. (1996) *Plant Tissue Culture Concepts and Laboratory Exercises*, Trigiano, R. N. (ed.), CRC Press, Ann Arbor, pp. 261–274.

Moore, T. C. (1979) *Biochemistry and Physiology of Plant Hormones*, Springer-Verlag, New York, pp. 181–207.

Ockerse, R., Siegel, B. Z. and Galston, A. W. (1966) Hormone-induced repression of a peroxidase isozyme in plant tissue. *Science* **151**, 452–453.

Open Universiteit and University of Greenwich. (1993) *In Vitro Cultivation of Plant Cells*, Butterworth-Heinemann Ltd, pp. 43–64.

Park, J. H. and Kim, S. S. (1996a) Isolation, restriction mapping, and promoter sequence analysis of an isoperoxidase gene from Korean radish, *Raphanus sativus* L. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29**, 52–57.

Park, J. H. and Kim, S. S. (1996b) cDNA cloning and overexpression of an isoperoxidase gene from Korean-radish, *Raphanus sativus* L. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29**, 137–141.

Renaudin, J. P., Tournaire, C. and Teyssendier de la Serve, B. (1991) Quantitative analysis of protein changes during meristem initiation and bud formation in protoplast-derived *Petunia hybrida* callus. *Physiol. Plant.* **82**, 48–56.

Rouster, J., Leah, R., Mundy, T. and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *Plant J.* **11**, 513–523.

Ryu, S. B. and Wang, X. (1996) Activation of phospholipase D and the possible mechanism of activation in wound-induced lipid hydrolysis in castor bean leaves. *Biochim. Biophys. Acta* **1303**, 243–250.

Saiki, R. K. (1990) in *PCR Protocols: A Guide to Methods and Applications*. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (Eds.), Academic Press, San Diego, California, pp. 13–20.

Samuelsson, B., Dahen, S.-E., Lindgren, J. A., Rouzer, C. A. and Sterhan, C. N. (1987) Leukotrienes and lipoxin: structure, biosynthesis and biological effects. *Science* **237**, 1171–1176.

Smart, C. M. (1994) Gene expression during senescence. *New Phytol.* **126**, 419–448.

Thorpe, T. A. and Gaspar, T. (1978) Changes in isoperoxidases during shoot formation in tobacco callus. *IN VITRO* **14**, 522–526.

Tournaire, C., Kushnir, S., Bauw, G., Inze, D., Teyssendier de la Serve, B. and Renaudin, J. P. (1996) A thiol protease and an anionic peroxidase are induced by lowering cytokinins during callus growth in Petunia. *Plant Physiol.* **111**, 159–168.

Villain, P., Mache, R. and Zhou, D. X. (1996) The mechanism of GT element-mediated cell type-specific transcriptional control. *J. Biol. Chem.* **271**, 32593–32598.

Walden, R. and Schell, J. (1990) Techniques in plant molecular biology — progress and problems. *Eur. J. Biochem.* **192**, 563–576.

Waltraud, R. and Rudolf, M. (1992) Stress effects on hormonal growth factors in tobacco tissues indicated by special changes in the isoelectric peroxidase patterns. *Electrophoresis* **13**, 798–799.