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An analysis of the relative activities of a number of promoter constructs from genes which are expressed during late pollen development as determined by particle bombardment

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Summary. The promoters of a tobacco actin gene, a tobacco pectate lyase, a tobacco and maize polygalacturonase and a *Brassica S*-locus related gene have been fused to the β-glucuronidase reporter gene and their activities determined by biolistic transient assay in tobacco pollen. In stably transformed tobacco all the transgenes with the exception of Cauliflower Mosaic Virus-35S-β-glucuronidase appear to express efficiently in maturing pollen. Transient assay analysis showed that the tobacco pectate lyase and the polygalacturonase constructs were 8x more active than the tobacco actin construct, and that the tobacco polygalacturonase construct was some 33x more active than the maize polygalacturonase construct. Constructional manipulations that altered the lengths of the 5'-untranslated leaders including one which resulted in the removal of a 490 bp leader intron had little effect on the observed level of expression. However, the alteration of the context of the ATG from A/TnnATGG to CnnATGT resulting in a 70% reduction in the observed levels of activity, was obtained with the pectate lyase and polygalacturonase promoters. An identical reductional was also observed in transgenic plant populations transformed with the polygalacturonase transgenes.

Abbreviations: GUS = β-glucuronidase, LUC = luciferase, NosTer = nopaline synthase terminator, CaMV = Cauliflower Mosaic Virus, UTL = untranslated leader, PCR = polymerase chain reaction, PG = polygalacturonase, Npg = tobacco polygalacturonase, Pl = pectate lyase, Ac = actin.

Introduction

Microsporogenesis in higher plants is a complex process which in the initial stages is wholly dependent on the sporophyte. Following meiosis of the pollen mother cell the developing microspores are nurtured by the surrounding tapetal cells. The degeneration of the tapetal cells and mitotic division of the microspore is the point at which the development of the male gametophyte (immature pollen) becomes more or less independent of the sporophyte, though it is retained within the anther until it matures and is released.

A short time after the mitotic division of the microspore there is a rapid increase in both RNA and protein synthesis. It has been estimated that between 10-20% of the mRNA synthesised during this phase of development is not expressed elsewhere in the plant (Stinson *et al.* 1987). This observation and the subsequent use of differential hybridisation of pollen cDNA libraries has led to the identification of a number of genes (Rogers *et al.* 1991; Brown and Crouch 1990; Albani *et al.* 1990; Stinson *et al.* 1987; McCormick *et al.* 1987) whose expression is principally confined to post-microspore mitosis and to the larger vegetative cell of the maturing male gametophyte (Twell 1992).

A technique has been described (Twell *et al.* 1989) for the rapid analysis of promoter activities in pollen using a semi-*in vivo* transient assay system based on the microprojectile bombardment of tobacco pollen. This assay has been shown to be useful for the determining the cis-acting sequence elements within promoters which regulate gene expression (Twell *et al.* 1991). Here we have used this technique to compare the relative activities of a number of constructs which utilize promoters from the pollen-expressed genes we have previously isolated and characterised from maize and tobacco. A promoter of a *Brassica oleracea S*-locus related gene (Trick 1990, Hackett *et al.* 1992) and the CaMV 35S promoter (Odell *et al.* 1985), were also included in the comparison.

Materials and methods

DNA isolation and purification: The gene chimeras consisting of promoter, reporter gene (GUS or LUC) and the polyadenylation signal sequence (Noster) were constructed in pUC18 or pUC19. *E.coli* MC1022 were

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transformed with the plasmids and grown up as 0.4 l cultures overnight. Plasmid DNA was isolated according to the 'maxi-prep' procedure described by Lonsdale *et al.* (1986) and purified by banding twice in CsCl-ethidium bromide gradients. Ethidium bromide was removed by extracting 4 times with CsCl-saturated isopropanol. Alternatively DNA was isolated and purified by the Qiagen column procedure. DNA was precipitated and resuspended in water to a concentration of 1μg/μl. GUS and LUC plasmids were mixed in a 7:3 ratio.

Microprojectile Bombardment: The method of Twell *et al.* (1989) was employed for the bombardment of tobacco pollen grains. Ten μg of the GUS/LUC plasmid mixture was adsorbed to 12.5μg of Grade M17 tungsten powder (mean diameter 0.9μm) in 50μl 0.6M $CaCl_2$, 10mM spermine (Lonsdale *et al.* 1990). The tungsten-DNA precipitate was resuspended in 30μl of 0.6M $CaCl_2$, 10mM spermine and 2.5μl aliquots were used for each bombardment, with a minimum of 5 replicates for each construct.

Aliquots of the DNA mixture, prior to its adsorption onto tungsten, were digested with suitable restriction enzymes to generate fragments of both the GUS gene fusion and the LUC gene fusion. The restriction fragments were separated by agarose gel electrophoresis and the relative ratios of the two plasmids determined by densitometry using a Ultra Violet Products (Cambridge, UK) Gel Analysis System SW2000. Variations from the E^{260} determined 7:3 ratio were used to correct the calculated activities of the constructs. This correction overcomes the variation which occurs as a result of low molecular weight DNA contamination and/or residual ethidium bromide contamination of the plasmid preparations.

The particle accelerator used for these experiments was made by Shearline Precision Engineering Ltd, Milton, Cambridge, UK. Stopper plates and macroprojectiles were also obtained from Shearline Precision Engineering Ltd. 0.22 cartridges ('Gene Blanks') were manufactured to our own specification by Eley Ltd, Birmingham, UK.

Tobacco pollen: Nicotiana tabacum (var. Samsun) pollen was collected and stored at -70°C. Pollen was thawed and dispersed, to 20 mg/ml, in MS medium (Murashige and Skooge 1962), containing 30g.l^{-1} sucrose, 200m.l^{-1} carbenicillin and 0.5mg.l^{-1} Fungizone (GibcoBRL). A 3 cm^2 square of sterile Whatmans No.1 paper followed by a 2 cm^2 square of sterile nylon-membrane (Genescreen Plus™) were overlaid on MS medium, 0.8% agar, 30g.l^{-1} sucrose and 200mg.l^{-1} carbenicillin. Ten mg pollen (0.5ml) was pipetted onto the sterile nylon-membrane support so as to produce a target area of 1.0 to 1.5 cm^2. Following bombardment of the pollen samples, the plates were incubated for 16h at 25°C.

GUS and LUC assays : The bombarded pollen samples were harvested into an agate mortar and ground in 500 μl of 100 mM K-phosphate buffer pH 7.0, 1 mM DTT, 1 mg/ml BSA. Extracts were clarified by micro-centrifugation at 13,000 rpm for 5 min at 4°C and immediately assayed for LUC activity (Ow *et al.* 1986). Activity was measured in a Berthold luminometer (model: LUMAT LB9501); following injection of the luciferin, light emission was measured for 10s.

Glucuronidase activity was measured fluorometrically as detailed in Jefferson (1987). The kinetics of GUS activity were determined over 5 time points following measurements in a Fluoroscan II.

Results and discussion

Origin of the plasmids

We have isolated and characterised a number of genes whose expression is predominantly, if not totally, restricted to the maturing male gametophyte. These include a tobacco actin (Ac; Thangavelu *et al.* 1993), a tobacco pectate lyase (Pl; Rogers *et al.* 1992), a tobacco polygalacturonase (Npg1; Tebbutt *et al.* 1994), and a maize polygalacturonase (W2247; Allen and Lonsdale 1993). The promoter region of these genes including the transcription start domains and the UTL have been fused to the *E. coli* ß-glucuronidase gene (*uidA*, GUS) and their expression levels in mature pollen determined by 'biolistic' transient assay.

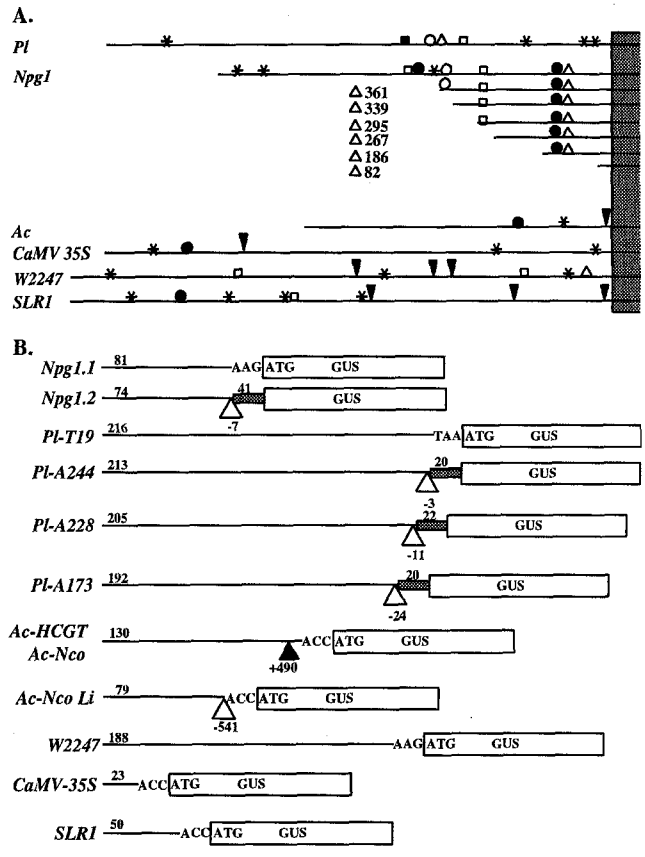

Fig. 1. Schematic representations of the promoter 5'-UTL fusions to GUS. A, promoter relative lengths of the promoters are shown as well as the positions of the LAT56/59 box (■) and core motifs (*); LAT52/56 box's (●) and core motifs (▼); Eh1 (○), Eh2 (⊖) and PG-box (Δ) motifs. The shaded area represents the -1 to -60 domain. B; Untranslated leader lengths are shown. Sequence deletions are indicated as open triangles. The position of the actin intron is shown as a filled triangle. Sequence derived from *uidA* (pRAJ255) is shown as a stippled rectangle. Abbreviations are described in the text.

The construction of the promoter-UTL::GUS fusions took advantage of pre-existing plasmids, pTAK and pKGT, comprising the GUS reporter gene fused to the nopaline synthase terminator fragment (NosTer) in pUC. The pTAK plasmid, derived from pRAJ255 (Jefferson 1987), retained the bacterial sequence domain surrounding the ATG (GGTCAGTCC**ATG**TT), while pKGT, derived from pRAJ275 (Jefferson 1987), was modified to the Kozak (1987) consensus (GTATCGACC**ATG**GG) and provided an *Nco*I cloning site. Directional deletion using ExoIII and S1 (Henikoff 1987) was performed to produce both transcription and translation fusions to the GUS reporter plasmids. Summary diagrams of the promoters and the UTL::GUS fusions are shown in Fig. 1A and 1B.

In addition a firefly luciferase (LUC) construct was made

from a CaMV 35S::LUC::NosTer plasmid (pJJ3792, a gift from J. Jones, Sainsbury Laboratory, Norwich, UK). Using the restriction endonucleases *Hind*III and *Nco*I, the CaMV 35S promoter and the untranslated leader in pJJ3792 were removed and replaced with a 1214 bp actin *Nco*I fragment which contains the promoter (594 bp) and untranslated leader (130 bp + 490 bp leader intron) of the tobacco actin gene Tac25. The resulting Ac-Nco::LUC construct was used in all the experiments to standardize results between bombardments. To standardize between different experiments utilizing different pollen samples the relative active activity of the Pl-A173::GUS plasmid to the Ac-Nco::Luc plasmid was set to 100 GUS/LUC units; the activity of all other constructs were expressed relative to this value.

Comparison of the tobacco pectate lyase and polygalacturonase promoter::GUS fusions.

The tobacco pectate lyase (Pl-T19) and polygalacturonase (Npg1.1) translational fusions were by far the most active constructs tested showing similar levels of activity (Fig. 2). The natural ATGs of both the pectate lyase and polygalacturonase genes were deleted using the exoIII/S1 procedure (Henikoff 1987) and the promoter-UTL deletions fused to pRAJ255. Seven bp from the 3' end of the polgalacturonase UTL was removed and 3, 11 and 24 bp in the three selected pectate lyase deletions (Fig. 1B).

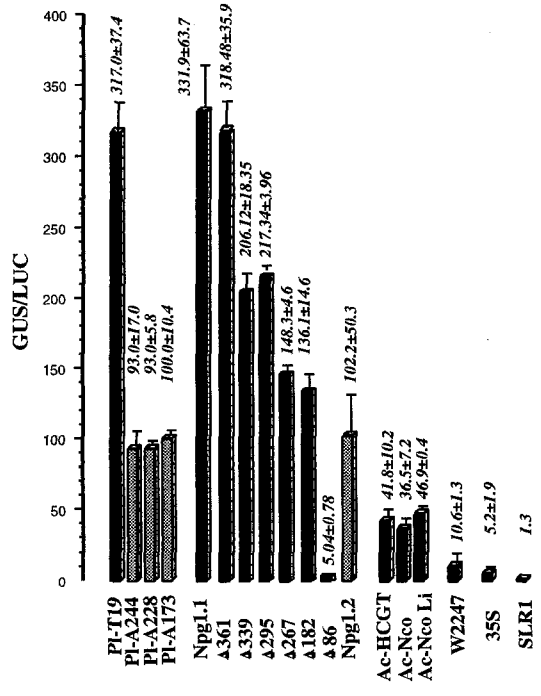

Fig. 2. Relative activities of the promoter::UTL::GUS fusions. Activities have been standardised against PL-A173 (100 GUS/LUC units). Experimental values and their standard deviations are given. Error bars represent represent half the standard deviation.

The activity of these transcriptional fusions, in contrast to the translational fusions, showed a 70% reduction in activity (Fig. 2).

As the reduction in activity was the same between the pectate lyase and polygalacturonase constructs it was independent of the promoter and independent of the length of the untranslated leader. Moreover the deletions in the 3' end of the pectate lyase-UTL had no effect on construct activity. The data strongly suggest that this consistent and reproducible reduction in the level of activity, at least in this comparison, is not due to either the length or composition of the UTLs. It appears to be solely a function of the ATG context which in the case of the transcriptional constructs was of *uidA* origin (GGTCAGTCCCTT**ATG**TT). In having a C at the -3 position it does not reflect the Kozak consensus - nAnn**ATGG** (Kozak 1987). Reductions in activity of 4 to 9 fold have been reported in rice and maize cells when the ATG sequence context of the expression vectors used had the *uidA* context (McElroy *et al.* 1991). A reduction of activity of 62% was observed by Lutcke *et al.* (1987) when mRNAs with a C at the -3 position relative to the ATG were tested in a reticulocyte lysate translation system though this was not observed in a wheat germ translation system.

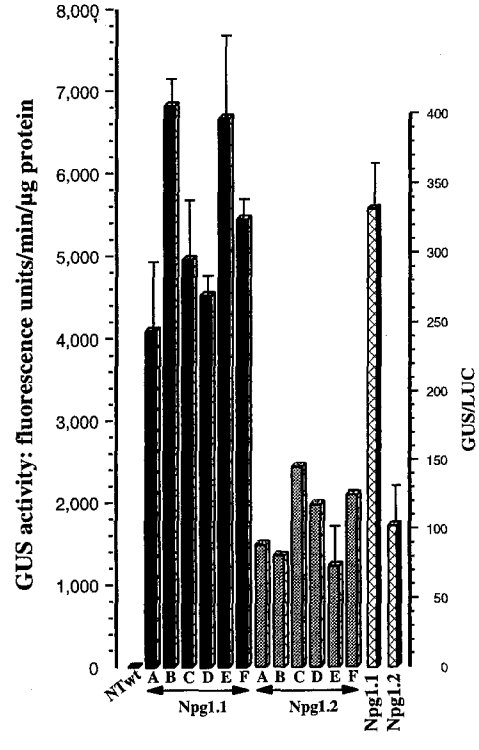

Fig. 3. Relative activities of Npg1.1 and Npg1.2, the translational and transcriptional fusions of the polygalacturonase promoter::UTL to GUS respectively. The GUS activity in mature pollen from 6 independentplants (A-F) has been compared to untransformed tobacco (NTwt) and to the activity of the same constructs following their biolistic introduction into mature pollen from untransformed plants (hatched bars). Error bars represent half the standard deviation.

The Npg1.1 and 1.2 transgenes, the translational and transcriptional fusions respectively, were transferred to

tobacco by agrotransformation and six randomly selected T1 progeny from each transformation analyzed for GUS expression. Gus expression was not observed in any tissues other than in maturing pollen (Tebbutt 1993; Tebbutt *et al.* 1994). Analysis of the levels of GUS activity in the pollen of the T1 transgenics showed that the Npg1.1 plants had high levels of GUS activity, averaging 5800 fluorescence units/min/μg protein while the Npg1.2 plants exhibited an average of 1900 fluorescence units/min/μg protein; a value which is approximately 68% lower than that in the pollen of Npg1.1 transgenics (Fig. 3). The reduction in the average activity is virtually identical to that observed in pollen samples which had been bombarded and identical to the reduction in activity of construct having a C at -3 (Lutcke *et al.* 1987).

Although the transgene copy number has not been determined by Southern blot analysis, the data do tend to suggest that transgene copy number is not playing a significant role in determining expression levels.

Based on our transient assays and in vivo expression experiments and the experiments described by McElroy *et al.* (1991) we can conclude that the ATG domain plays a crucial role in determining the levels of expression both *in vivo* and *in vitro*. This is certainly not the case in the wheat germ system which appears to be incapable of discriminating between constructs where the nucleotide at the -3 position had been altered (Lutcke *et al.* 1987).

The actin constructs

A tobacco actin gene, Tac25, has been isolated and characterised (Thangavelu *et al.* 1993; Lonsdale, EMBL Ac No.X63603). The isolated gene has a promoter of 2470 bp, a UTL of 130 bp interrupted by an intron of 490 bp, the 3' splice site being at -9 relative to the ATG. The ATG context was modified by PCR mutagenesis from AAAATGG to ACCATGG creating an *Nco*I restriction site. The entire promoter, UTL and intron was then fused directly to the *Nco*I site of pKGT (*Ac*-HCGT). In addition an *Nco*I subclone of the promoter, retaining only 594 bp was also created (*Ac*-Nco). The third construct, lacking the intron and 51 bp of the 3'-UTL sequence was created by PCR (*Ac*-Nco Li); it retains the *Nco*I site at the ATG.

The activity of these three constructs are statistically indistinguishable though all are significantly less active than either the polygalacturonase or the tobacco pectate lyase promoter constructs (Fig. 2). The removal of the 490 bp leader intron and 51 bp of the UTL had no significant effect on the level of expression. A similar lack of effect on CaMV::GUS expression was observed in tobacco cells when the castor bean catalase I intron was inserted into the N-terminal region of the GUS coding sequence (Tanake *et al.* 1990). These results are in sharp contrast to the general phenomenon of intron-mediated enhancement of gene expression observed in monocot cells and transgenic rice (Callis *et al.* 1987; Oard *et al.* 1989; Vasil *et al.* 1989; Tanaka *et al.* 1990; Luehrsen and Walbot 1991; McElroy *et al.* 1991).

The maize PG, Brassica SLR1 and CaMV promoters

Of the constructs tested these three were the least active. The maize polygalacturonase construct, W2247, has an expression level some 33 fold lower than the equivalent tobacco polygalacturonase gene construct. Nevertheless, despite its relatively low activity in transient assays, the tissue and developmental specificity of its expression is retained in stable transgenic tobacco lines (Allen and Lonsdale 1993). Retention of monocot promoter specificity has also been observed with other gametophytic genes both in transient assays and in transgenic plants (Guerrero *et al.* 1990; Hamilton *et al.* 1992; Allen and Lonsdale 1993) though the relative activity of these promoters to their tobacco homologues has not previously been reported.

The *Brassica oleracea SLR1* promoter from an *S*-locus related gene (Trick 1990) exhibits an extremely low GUS/LUC ratio in bombarded pollen. In transgenic tobacco a low level of GUS expression was detected in up to 50% of the pollen grains one day before anthesis. The level of expression observed was several orders of magnitude lower than that detected in stigma tissues of transgenic plants (Hackett *et al.* 1992).

Our results with the CaMV 35S promoter are consistent with the data of Twell *et al.* (1989) and van der Leede-Plegt *et al.* (1992) who observed a low level of GUS activity in bombarded pollen as determined fluorometrically. Unlike the maize W2247 promoter and the *Brassica SLR1* promoter which have detectable expression in the latter stages of pollen development in transgenic tobacco, the CaMV 35S::GUS transgene exhibits little or no activity (Plegt and Bino 1989; Guerrero *et al.* 1990; Mascarenhas and Hamilton 1992).

Analysis of the promoter sequences

Analysis of tomato pollen promoters has revealed a number of pollen-specific enhancer sequences and shared regulatory elements (Twell *et al.* 1991). Two of these elements, the LAT52/56 box (TGTGGTT) and the LAT56/59 box (GAAATTGTGA), have been shown by mutational analysis to be required for efficient promoter function in tobacco pollen. The critical core motifs within these elements are GGTT and GTGA.

The tobacco promoters were searched for sequences with homology to the LAT52/56 and LAT56/59 boxes. The pectate lyase promoter is devoid of GGTT motifs, though it does have 5 GTGA motifs, three of which are proximal to the transcription start. Only one of the five is embedded in a sequence which displays homology to the LAT56/59 box. In contrast the polygalacturonase promoter has a single GGTT motif embedded in a sequence with homology to the LAT52/56 box and 3 GTGA motifs. The actin promoter has

a single GGTT motif and two GTGA motifs the distal one of which from the transcription start lies within a LAT56/59 box.

A fine-deletion analysis of the Npg1 promoter has been carried out to identify sequence elements required for promoter activity (Tebbutt and Lonsdale 1995). The critical deletions from this analysis are shown in Figs. 1A & 2. The 745 bp promoter could be reduced to 361 (Δ361) without loss of activity. Removing the next 22 bp (Δ339) reduced promoter activity by 35%. No further reductions in promoter activity were observed until the sequence between -295 and -268 (Δ267) was removed. This further reduced promoter activity by 32%. This level of activity was retained by the -186 deletion - the 'minimal' promoter. Further reduction to -86 bp reduced the activity of the promoter to background levels, a level of activity defined by the CaMV 35S::GUS construct.

The two sequence elements between -361 and -339 and -295 and -267 and possibly including 9 bp (one helical turn) to either side play an important role in determining the activity of the Npg1 promoter. These unrelated sequence elements, which do not contain LAT52/56 or LAT 56/59 elements or their core motifs, have been termed Eh1 and Eh2 respectively (Tebbutt and Lonsdale 1995). Comparison of these sequence elements to the other promoter used in this study revealed related sequences in the pectate lyase and maize polygalacturonase promoters but nothing that could be considered significant. A sequence related to the 'PG' box, a 23 bp homology between the tobacco and maize polygalacturonase promoters (AACYYTYAATTAGTAAWACMAAG; Allen and Lonsdale 1993; Tebbutt and Lonsdale 1995), was identified in the pectate lyase promoter but the overall conservation was poor. An unrestricted sequence comparison, including a search against the transcription factor database and for reported plant transcription factor recognition sequences (Katagiri and Chua 1992, Sablowski et al. 1994) failed to identify any common sequence elements.

It is evident from this analysis of the promoters particularly the tobacco promoters, all of which are activated post first microspore mitosis, that identified sequence elements are dispensable either singly or in combination. It is also apparent that there is no association of sequence elements which contribute to the effectiveness of promoters or which can be implicated in determining late gametophytic expression. A study of the transcription factors which interact with the DNA sequences immediately adjacent to the RNA polymerase binding domain is required to address the questions of cell and tissue specificity and to understand the role of transcription factors in determining promoter activity. This work is currently in progress.

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References

Albani D, Robert LS, Donaldson PA, Altosaar I, Arnison PG, Fabijanski SF (1990) Plant Mol Biol 15:605-622.
Allen RL, Lonsdale DM (1993) Plant J 3:261-271.
Brown SM, Crouch ML (1990) Plant Cell (2):263-274.
Callis J, Fromm M, Walbot V (1987) Genes Dev 1:1183-1200.
Guerrero FD Crossland L, Smutzer GS, Hamilton DA, Mascarenhas JP (1990) Mol Gen Genet 224:161-168.
Hackett RM, Lawrence MJ, Franklin FCH (1992) The Plant J 2:613-617.
Hamilton DA, Roy M, Rueda J, Sindhu RK, Sanford J, Mascarenhas JP (1992) Plant Mol Biol 18:211-218.
Henikoff S (1987) Methods in Enzymology 155:156-165.
Jefferson RA (1987) Plant Mol Biol Rep 5:387-405.
Katagiri F, Chua N-H (1992) TIG 8:22-27.
Kozak M (1987) J Mol Biol 196:947-950.
Lonsdale D, Hodge TP, Stoehr PJ (1986) Methods in Enzymology 118:453-470.
Lonsdale DM, Onde S, Cuming A (1990) J Exp Bot 41:1161-1165.
Luehrsen KR, Walbot V (1991) Mol Gen Genet 225:81-93.
Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) EMBO J 6:43-48.
Mascarenhas JP, Hamilton DA (1992) Plant Journal 2:405-408.
McCormick S, Smith A, Gasser C, Sachs K, Hinchee M, Horsch R, Fraley R (1987) In: Tomato Biotechnology, Eds Nevins D, Jones R, New York, Alan Liss, pp 255-265.
McElroy D, Blowers AD, Jenes B, Wu R (1991) Mol Gen Genet 231:150-160.
Murashige T, Skooge F (1962) Physiol Plant 15:473-497.
Odell JT, Nagy F, Chua N-H (1985) Nature 313:810-812.
Oard JH, Paige D, Dvorak J (1989) Plant Cell Rep 8:156-160.
Ow DW, Wood KV, DeLuca M, DeWet JR, Helinski DR, Howell SH (1986) Science 234:856-859.
Plegt L, Bino RJ (1989) Mol Gen Genet 216:321-327.
Rogers HJ, Allen RL, Hamilton WDO, Lonsdale DM (1991) Biochim Biophys Acta 1089:411-413.
Rogers HJ, Harvey A, Lonsdale DM (1992) Plant Mol Biol 20:493 -502.
Sablowski RWM, Moyano E, Culianez-Macia F, Schuch W, Martin C, Bevan M (1994) EMBO J 13:128-137.
Stinson JR, Eisenberg AJ, Willing RP, Pe ME, Hanson DD, Mascarenhas JP (1987) Plant Physiol 83:442-447.
Tanaka A, Mita S, Ohta S, Kyozuka J, Shimamoto K, Nakamura K (1990) Nucleic Acids Res 18:6767-6770.
Tebbutt SJ (1993) PhD Thesis, University of East Anglia, UK.
Tebbutt SJ, Lonsdale DM (1995) Sex Plant Repro 8:in press.
Tebbutt SJ, Rogers HJ, Lonsdale DM (1994) Plant Mol Biol 25:283-297.
Thangavelu M, Belostotsky D, Bevan MW, Flavell RB, Rogers HJ, Lonsdale DM (1993) Mol Gen Genet 240:290-295.
Trick M (1990) Plant Mol Biol 15:203-205.
Twell D, Klein TM, Fromm ME, McCormick S (1989) J Plant Physiol 91:1270-1274.
Twell D, Yamaguchi J, Wing RA, Ushiba J, McCormick S (1991) Genes Dev 5:496-507.
Twell D (1992) Plant J 2:887-892.
van der Leede-Plegt LM, van der Ven BCE, Bino RJ, van der Salm TPM, van Tunen AJ (1992) Plant Cell Rep 11:20-24.
Vasil V, Clancy M, Ferl RJ, Vasil I, Hannah LC (1989) Plant Physiol 91:1575-1579.