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PROGRESS REPORT: TRANSPOSON TAGGING OF DISEASE RESISTANCE GENES.
Richard Michelmore. 5/1/1988 to present. (1992)**Introduction**

Our goal is to clone genes in lettuce determining resistance to downy mildew. One approach involves the mobilization of transposons into resistance genes to mutate and tag the target gene. Because transposons have yet to be isolated and characterized from lettuce, we have been testing heterologous transposons from corn and snapdragon for transposition in lettuce. The majority of our experiments have involved *Ac* from corn as this is increasingly the best characterized transposon.

The efficiency of a transposon mutagenesis approach will depend on the degree to which transposition can be controlled and the ease with which insertion events can be selected and characterized. Over the past several years, various labs have contributed to a detailed understanding of the biology of *Ac* in corn and heterologous plant species. We have collaborated closely with several of these labs, exchanged materials and incorporated their advances into our analysis of transposition in lettuce. The development of transposon mutagenesis in lettuce now does not lag behind that in other species due the generous contributions of several of these labs as detailed below.

The original proposal described the development of a transposon mutagenesis system for lettuce and its subsequent use to tag disease resistance genes. The development phase involved characterization and manipulation of *Ac* transposition, identification of suitable whole plant selectable markers for the construction of chimeric non-autonomous elements, and investigation of the stability of resistance genes. Due to funding constraints, the last two years of this project have been predominantly the efforts of a graduate student, Chang-Hsien Yang, with additional technical support as needed for labor intensive procedures.

Investigation of *Ac* transposition in lettuce has received the majority of our attention. Initially, we made a simple construct with wildtype *Ac* and introduced it into lettuce. No transposition was observed; although other labs demonstrated that the same construct was functional in tomato. We then focused on assaying for *Ac* transposition with constructs of increasing sophistication that had been demonstrated by others to be functional in other species. In the initial absence of transposition in lettuce, we made constructs to dissect the transposition process and to test the various components of *Ac* independently. The function of these constructs was tested in transient assays of tobacco and in stable assays in tobacco and lettuce. The latest constructs for transposon mutagenesis (made by others and demonstrated first to function in other species) clearly demonstrated transposition in lettuce. This allowed us to generate seed stocks that we will start to screen for insertional inactivation of resistance genes this year.

Research progress**Ac in lettuce.**

Our first construct, pMAC, involved wildtype *Ac* from *waxy m-7* cloned into the *Agrobacterium* vector pMON200. Transgenic plants were analyzed for fragments characteristic of *Ac* excision and reintegration elsewhere in the genome using Southern analyses and later PCR. This approach was successful in tomato and tobacco as transposition of *Ac* was frequent. We initially regenerated 25 transgenic plants containing pMAC and analyzed them in Southern. No clear evidence of a characteristic excision band was observed. Banding patterns were detected that were not predicted from simple integration of the T-DNA sequences; however, using multiple digests and multiple probings

with *Ac* or flanking sequences, it was impossible to unambiguously distinguish between abnormal transposition events and rearrangements associated with T-DNA integration. This was in contrast to the results of John Yoder's lab, and later that of Brian Staskawicz, with the same construct introduced into tomato. The large majority of tomato plants containing pMAC clearly had an excision band of the expected size and new *Ac* integration sites (Yoder *et al.*, 1988). To check that our clone of pMAC was not rearranged or mutated, we conducted a transformation experiment of lettuce using the same bacterial suspension that was used simultaneously to transform tomato. Again, transposition occurred frequently in tomato but could not be unambiguously demonstrated in lettuce. We have now investigated over 75 R₁ lettuce plants containing pMAC either by Southern blots or by screening for an excision product using PCR primers of *waxy* sequences flanking the excision site. In no case did we obtain unambiguous evidence of transposition. A total of 80 R₂ progeny from six R₁ plants which contained *Ac* in the T-DNA were screened by PCR using primers from the flanking *waxy* sequences; again, the expected excision fragment was not detected, although the correct sized bands were readily amplified from the wildtype *waxy* sequence and from tomato DNA containing an excision event which were used as positive controls. The *Bam*HI site in the 5' region of *Ac* in plants containing pMAC was difficult to cut possibly indicating that this region was methylated.

As transposition of *Ac* appeared not occur frequently in lettuce, the next set of experiments involved an excision assay that had been used to assess excision frequency in tobacco (Jones *et al.*, 1989). This assay relied on excision of *Ac* from a chimeric *mas5'*::SPT (streptomycin phosphotransferase) gene resulting in resistance to streptomycin; resistance was scored as a lack of chlorosis in the presence of the drug. The same constructs as used in the tobacco experiments were kindly supplied by Jonathan Jones (Advanced Genetic Sciences, Oakland; now at The Sainsbury Lab, Norwich, UK). As positive controls, twenty-two transgenic lettuce plants were generated using pJJ2668 which contained the uninterrupted *mas5'*::SPT gene and selfed; all their R₂ progeny clearly segregated 3:1 for resistance to streptomycin. Sixty-five transgenic R₁ lettuce plants were generated using pJJ2853 which contained *Ac* interrupting the chimeric streptomycin resistance gene and selfed. Sixty-two of the resultant R₂ progeny clearly segregated 3:1 for streptomycin resistance with similar levels of resistance to the uninterrupted positive controls. Bleached and non-bleached plants were rescued; Southern analysis demonstrated that the resistant phenotype was correlated with the presence of the SPT gene in the T-DNA; however, no excision band was seen. These results suggest that *Ac* was efficiently processed from the mRNA in lettuce; such processing has now been indicated in tobacco (J. Jones pers. comm.) but the level of drug resistance in tobacco was low and only partial protection from chlorosis observed. *Ds* has been shown to be processed from *waxy* mRNA sequences in corn (Wessler *et al.*, 1987). Also, the *Ac* element in pJJ2853 contained an additional *Bgl*II site at the 3' end; this was subsequently shown to depress transposition in tobacco (J. Jones, pers. comm.). Therefore, studies with this construct in lettuce were discontinued.

To determine whether transposition in lettuce might be stimulated by expressing the putative transposase from a highly-expressed, heterologous promoter, the next experiments attempted to transactivate a *Ds* element that interrupted a chimeric GUS (β-glucuronidase) reporter gene. These constructs had been shown to promote frequent transposition of the *Ds* element in tomato and were kindly donated by Mike Lassner (now at Calgene Inc. Davis) in John Yoder's lab (UC Davis). One construct contained a *Ds* element between the 35S promoter and the GUS gene in pBI121. Other constructs contained the 35S or *Drosophila Hsp70* promoters replacing the 5' region of the *Ac* element from the *Bam*HI₁₈₂ or *Mlu*I₄₃₂ site (clipped-wing transposase constructs). Transactivation of the *Ds* element had been demonstrated in F₁ plants when tomato lines containing each of these constructs were crossed. Lettuce plants containing each of these constructs were generated and the integration events checked for rearrangements by Southern blots. Plants with complete

integrations of the transposase constructs were assayed by Northernblots. Fusions with the *Hsp70* promoter resulted in abnormally small message and showed no heat induction. Two plants with the 35S promoter at the *Mlu*I site expressed message of the predicted size; these plants were crossed to two plants with faithful integrations of the *Ds* construct. This combination had proved the most successful in tomato. Only two out of 48 F₁ plants contained both the transposase and *Ds* constructs, much less than the 25% expected (a similar paucity was seen in tomato; M. Lassner, pers. comm.). PCR and Southern analyses failed to identify any excision products in these two plants. One of these F₁ plants was selfed. Forty R₂ progeny were screened by histochemical staining for GUS activity; none was observed, although in retrospect this was not surprising as the positive controls, plants transformed with pBI121, also showed no GUS activity. A further 50 R₂ plants were screened by PCR for an excision product and again no evidence for transposition was detected.

The next experiments to detect transposition in lettuce involved the pKU series of constructs made by Barbara Baker (Baker *et al.*, 1987) and two derivatives made by Jeff Ellis (CSIRO, Canberra) (Fig. 1). These constructs provided a positive selection for excision events. pKU2 is the positive control containing a functional NPTII gene expressed from the 1' T-DNA promoter. pKU3 contains wildtype *Ac* between the 1' promoter and the NPTII gene; this construct does not confer drug resistance in plants unless *Ac* excises. pKU4 is the negative control, containing a *Ds* element between the 1' promoter and NPTII gene. pKU4-8 is derived from pKU4 by the addition of the putative transposase cDNA (kindly supplied by Nicole Houba-Herlin and Peter Starlinger, University of Koln, W. Germany) expressed from the divergent 2' promoter already present in pKU4 and with the PI-II terminator which may enhance message stability (courtesy Gyn An, Washington State University). pKU4-1 is a second negative control and identical to pKU4-8 except that the *Ac* cDNA is in the inverted orientation. In these experiments, no kanamycin resistant callus should occur in transformation experiments unless *Ac* or *Ds* excises from the chimeric NPTII gene. In experiments with flax, cocultivation with cointegrates of pKU3 resulted in infrequent sectors of kanamycin resistant callus; pKU4-8 resulted in frequent sectors of resistant callus similar to the positive control, pKU2; no callusing was seen with the negative controls (Jeff Ellis, pers. comm.). Southern analysis subsequently confirmed that excision had occurred in the resistant calli. In our experiments with lettuce, pKU3 resulted in infrequent drug resistant sectors (4/100 explants); pKU4-8 resulted in moderately frequent resistant sectors (130/750 explants); no callusing was observed in the negative controls. It therefore appeared that *Ac* is capable of low rates of excision in lettuce callus and that the cDNA expressed from the 2' promoter results in higher rates of excision of the *Ds* element. Transposition was confirmed by PCR amplification of an excision fragment of the expected size as well as by Southern analysis. The amplified excision fragment was sequenced from eight calli; sequences at the point of excision were characteristic of imprecise excision from the 8 bp duplicated *waxy* sequence observed in other species (Table 1). These experiments demonstrated that there was nothing inherently lacking in lettuce that was preventing transposition and that the low rates of transposition may have been due to either low promoter activity or inefficient processing.

Most recently we have focused our efforts on using a two element system based on the advanced pSLJ series of constructs (main proposal: Fig. 3); these were tested in tobacco and tomato (Schofield *et al.*, 1992 and B. Carroll *et al.*, unpublished) and kindly provided by Jonathan Jones (Sainsbury Lab., Norwich UK). In these constructs the genomic coding region for the *Ac* transposase is expressed from the 35S promoter which can result in elevated levels of transposition (Schofield *et al.*, 1992; Swinburne *et al.*, 1992). *Ds* elements are located in the 5' region of a spectinomycin gene that has a tobacco RuBP carboxylase small subunit transit peptide to provide cell autonomy for the resistance phenotype and is expressed from either the *Nos* or 35S promoters with the TMV omega leader sequence. We

initially tested their function in lettuce by cocultivating lettuce explants with both transposase and *Ds*-containing constructs simultaneously or sequentially (Fig. 2) and selecting on both kanamycin and spectinomycin. Only those cells containing both components and in which *Ds* had excised from the spectinomycin resistance gene should have grown. Some spectinomycin-resistant calli were obtained and a few green shoots developed. Calli and shoots were analyzed by PCR and Southern and bands of the size expected for excision fragments were obtained (Fig. 3). The plants were grown to maturity and selfed. Some of the progeny segregated 3:1 for resistance. Others segregated for the tendency to produce green sectors (Fig. 4); presumably these progeny were derived from R_1 plants in which *Ds* had excised somatically to provide resistance but had not excised in the germline. The number and size of the sectors varied from plant to plant, reflecting variation in the timing of the excision event. This is in contrast to the more constant timing of excision in tobacco (Scofield *et al.*, 1992) but similar to the variation reported for *Arabidopsis* (Swinburne *et al.*, 1992). The presence of green sectors in the R_2 plants was correlated with the presence of both transposase and *Ds*. These dual cocultivation experiments demonstrated that the pSLJ constructs were functional in lettuce and that transactivation could occur using a genomic coding region for transposase.

We then generated 110 R_1 plants of cv. Diana (*Dm1*, *Dm3*, *Dm7*, *Dm5/8*) containing a *Ds* construct and 24 R_1 plants of Diana containing a chimeric 35S::transposase construct (main proposal: Figs. 3 & 6). The transposase-containing R_1 plants were analyzed by Northern and four transposase expressing plants were crossed to the *Ds*-containing plants. F_1 seeds plated on spectinomycin showed green sectors; again the number and sizes of sectors varied from plant to plant. Selfed seed from R_1 plants containing only *Ds* showed no green sectors. DNA was extracted from nearly 1000 F_1 plants and screened by PCR for the presence of both transposase and *Ds* components. 150 F_1 progeny that contain both components were selected and the remainder rejected. The presence of fragments indicative of excision was confirmed by PCR and Southern analyses are currently in progress. These F_1 plants are now being grown to maturity. 3 to 6 x 10⁵ F_2 seed will soon be ready for screening for inactivation of each of the four *Dm* genes in Diana.

The *Ds* containing R_1 plants were also crossed to cv. Cobham Green that has no known *Dm* genes (main proposal: Fig. 6). F_1 plants were screened for the presence of *Ds* by PCR and are being selfed. The R_2 progeny will be screened for the cosegregation of each of the *Dm* gene and *Ds* using either PCR or a screen for basta resistance as appropriate. This will allow us to start screening those populations that contain a *Ds* element linked to a *Dm* gene first (see main proposal).

Studies on the Promoter and cDNA of *Ac*.

To determine the reasons for the apparent lack of transposition in lettuce and to manipulate *Ac* for efficient tagging experiments, various components of *Ac* were studied separately. The constructs were made while I was on sabbatical for six months at CSIRO ('89-'90). The constructs were initially tested in rapid transient assays of tobacco and then transformed in to tobacco and lettuce for comparison in stable transgenics.

To analyze the activity of the transposase promoter, putative promoter regions were fused to GUS. Fusions containing the whole 5' region, or starting at the *Cla*I₇₀ or *Bam*HI₁₈₂ sites to the *Hin*PI₃₈₀, *Mlu*I₄₃₂ or *Nhe*I₉₆₀ sites were constructed with and without four repeats of the octopine synthase enhancer (4OCS) element (Fig. 5). These fragments were cloned into pBI101.1 and then the GUS fusions were subcloned into pUC19 for transient assays. These constructs were electroporated into tobacco and corn protoplasts made from suspension cultures. After two days the protoplasts were assayed fluorimetrically for GUS activity using MUG. No activity was detected in the constructs without the 4OCS element. All constructs with the 4OCS element resulted in significant

activity in replicated experiments. In tobacco, the fusions were more active than the 35S::GUS fusion in pBI221 used as a positive control. In corn, lower levels were detected which were also lower than the 35S::GUS control. The presence of the putative leader sequence consistently resulted in slightly lower levels of expression; in these transient assays, however, the leader did not dramatically affect the levels of GUS activity.

A second series of constructs were made to investigate the long untranslated leader sequence of *Ac*. Three fragments of the leader sequence (*Dde*I280 to *Dde*I780, *Dde*I780 to *Dde*I980, and *Mlu*I432 to *Nhe*I960; Fig. 5) were cloned into a highly expressed promoter::GUS fusion that contained only a very short leader sequence (p4OCS35SGN; kindly supplied by David Last, CSIRO, Canberra). The promoter consisted of the 4OCS enhancer fused to a 35S promoter deleted to -90 and with 9 bases of untranslated leader. Transient GUS assays in tobacco and corn protoplasts showed that all of these fragments considerably increased GUS activity when compared to p4OCS35SGN (which in turn had much higher activity than the 35S::GUS in pBI221). This may be a non-specific effect and whether any sequence lacking an ATG would result in similar increases needs to be investigated. These results do confirm the first series of transient assays that the *Ac* leader sequence in transient assays seems not to decrease expression markedly.

A subset of the *Ac* promoter and leader reporter constructs were transformed into tobacco and lettuce to compare their activities when stably integrated into the chromosome. In both species, no activity was detected in R₁ plants with *Ac* promoter fragments lacking the 4OCS enhancer. Medium levels of expression (5 to 10 nmoles 4MU/min/mg protein) were detected with constructs containing the enhancer element. The presence of the leader fragment, *Dde*I780 to *Dde*I980, considerably enhanced GUS activity compared to the positive control (4OCS35SGN) as it had in the transient assays of tobacco. However, in both species the fragment, *Mlu*I432 to *Mlu*I960, considerably decreased GUS activity. This was in contrast to the transient assays in which it resulted in slight stimulation of GUS activity. This suggests that some of the effects of the long untranslated leader are at the transcriptional rather than the translational level. The results with lettuce paralleled those in tobacco except that GUS activity was lower on a per mg total protein basis. R₂ plants of both species are being selfed to provide R₃ individuals so that the stability of expression over several generations can be studied.

Tam3 in lettuce.

While *Ac* was proving refractory, we also tested *Tam3* from *Antirrhinum majus* for transposition in lettuce. A construct that had been used to demonstrate transposition of *Tam3* in tobacco (kindly supplied by Cathy Martin, John Innes Institute, Norwich, UK) was introduced into lettuce. Twenty-four transgenic R₁ plants were generated and analyzed by Southern using three endonuclease digests and multiple probings with *Tam3* or flanking *palida* sequences. In several plants, the hybridization patterns were consistent with at least partial excision; however, in no plant were the patterns in all digests and with all probes consistent with precise excision of *Tam3* from the *palida* sequences. Southern analysis of 10 R₂ progeny from each of 2 R₁ plants containing unrearranged integrations of *Tam3*, failed to reveal any evidence of transposition. Southern analysis of 20 R₂ progeny from each of 4 R₁ plants containing putative partial transposition events failed to demonstrate segregation of *Tam3* sequences away from *palida* sequences. As *Tam3* also appears frequently not to excise precisely in *Antirrhinum* and tobacco (Cathy Martin, pers. comm.), experiments with *Tam3* in lettuce have been discontinued.

Selectable markers for lettuce.

We have tested the efficacy in lettuce of several whole plant selectable markers for use either within a chimeric *Ds* element or as reporter genes for excision. Resistance to kanamycin, G418, or hygromycin is readily assayed in callus but is less easy to evaluate at the

whole plant level. The windows between levels at which all genotypes grow and those at which no genotypes grow is narrow and the optimal level varies from transformant to transformant. Experiments with several constructs of the *AroA* gene (Courtesy of Calgene Inc., Davis) failed to give significant resistance to the herbicide, glyphosate. The *Mas* 5':SPT gene in pJJ2668 (see above) conferred adequate levels of resistance to allow selection of lettuce seedlings for resistance to streptomycin. The spectinomycin resistance gene (SPC) in the pSLJ series of constructs (see main proposal) expressed from a 35S promoter and with chloroplast leader sequence provided clear resistance to spectinomycin (see above); it was better than SPT as resistance could be selected earlier and sensitive tissues bleached with spectinomycin rather than tending to remain green as with streptomycin with only the developing tissues becoming bleached. The acetolactate synthase gene from chlorsulfuron-resistant *Arabidopsis thaliana* (kindly supplied by Chris Sommerville, Michigan State) conferred high levels of resistance to the herbicide over a wide concentration range in R₂ progeny. The *bar* gene from *Streptomyces* (kindly supplied as part of pSLJ3621 by Jonathan Jones) providing resistance to the herbicide basta also functioned well in lettuce. Therefore, genes for resistance to spectinomycin, chlorsulfuron, and basta are all useful whole plant selectable markers in lettuce.

Stability of disease resistance genes.

To test for the stability of the targeted genes for resistance to downy mildew (*Dm*), a large F₁ population was generated and screened for expression of three *Dm* genes. Two inbred cultivars, Kordaat and Calmar, each expressing three different *Dm* genes, were crossed to generate 50,000 F₁ seed. So far, 5,000 F₁ seedlings have been simultaneously screened for resistance to three isolates of *Bremia lactucae*, each capable of detecting only one of the three resistance genes expressed in the F₁ population from the maternal parent (*Dm1*, *Dm3*, and *Dm4* from Kordaat). No susceptible seedlings have been detected. One hundred percent of the three positive control cultivars, each susceptible to only one of the isolates, were heavily infected. In control experiments, individual susceptible seedlings could be detected. Therefore, these three *Dm* genes appear not to be highly unstable. The remaining F₁ seed will be analyzed when we have sufficient time. As selfing could not be totally excluded, we found that we could only screen for expression of the three *Dm* genes from the maternal parent; selfs were not then detected as susceptible seedlings. We have also been screening for mutants in gamma irradiated M₂ progeny from cv. Saffier (*Dm1*, *Dm7*, *Dm16*) (see main proposal). Only infrequent mutants were detected, again indicating that these *Dm* genes are not highly unstable.

Conclusions

Ac transposes much less frequently in lettuce than in several other dicotyledonous species. However, *Ac* is capable of transposition in lettuce. *Ds* can be transactivated in both calli and whole plants by transposase expressed from a highly active promoter. Both cDNA and genomic transposase sequences can result in transactivation.

A selfing rather than test-cross strategy for transposon mutagenesis is preferable due to the inbreeding nature of lettuce and the opportunity to detect a greater range of genes. Large numbers of progeny will be available for screening for inactivation of *Dm* genes within the next few months.

Adequate numbers of progeny can be efficiently screened for tagging experiments. At least four *Dm* genes are not highly unstable.

Tam3 is not useful for transposon mutagenesis in lettuce.

Chlorsulfuron-resistant acetolactate synthase, spectinomycin resistance and basta resistance are useful whole plant selectable markers in lettuce. Streptomycin resistance is a

useful seedling selectable marker. These will be useful for monitoring excision and the position of *Ds* elements.

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Table 1

**Sequence comparison of excision sites from
different transgenic calli of *L. sativa*.**

<u>Wild type:</u>	5'--GGTCACGC GGTCACGC--3'	
<u>pKU4-8:</u>		
Callus#1:	5'--GGTCAC-- GGTCACGC--3'	missing GC
Callus#2:	5'--GGTCAC- C GGTCACGC--3'	missing G
Callus#3:	5'--GGTCACG- GGTCACGC--3'	missing C
Callus#4:	5'--GGTCACG- -GTCACGC--3'	missing CG
Callus#5:	5'--GGTCACGC <u>C</u> GTCACGC--3'	G to C
Callus#6:	5'--GGTCACG <u>G</u> <u>C</u> GTCACGC--3'	CG to GC
Callus#7:	5'--GGTCAG <u>T</u> C <u>CCGG</u> ACGC--3'	CGC GGTC to GTC CCGG
<u>pKU3:</u>		
Callus#1:	5'--GGTCACGC GG <u>C</u> CACGC--3'	T to C

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Figure 1. Schematic of components of the pKU series of constructs for detecting excision of *Ac*. pKU2 to 4 were made by B. Baker (Plant Gene Expression Lab., Albany); the derivatives pKU4-8 and pKU4-1 were made by J. Ellis (CSIRO, Canberra). 2'p 1'p = divergent 1' and 2' promoters from the T-DNA of *Agrobacterium tumefaciens*. Waxy fragment = original waxy sequences flanking *Ac* in maize.

Figure 2. Schematic showing the dual cocultivation experiment to rapidly demonstrate transactivation. The components of the constructs are shown in Figure 3 of the main proposal. + KAN = selection on media containing kanamycin. + SPC = selection on media containing spectinomycin.

Figure 3. PCR amplification of excision fragments resulting from excision of *Ds* after dual cocultivation with *Ds* and transposase containing plasmids. The sizes expected after excision of *Ds* from pSLJ2606 and pSLJ3621 are slightly larger than the fragments amplified from the positive control, pSLJ2561, due to the presence of *waxy* sequences flanking the excision site. In this experiment, the one plant obtained with pSLJ2581 had a smaller than expected excision fragment indicative of an abnormal excision event. (Excision fragments detected in F₁ plants with this construct were the expected 540 bp, not shown.)

SLJ Primers	3621	2604	2581	2557	2561	3232 + 2604	3232 + 3621	3232 + 2581
NOS + SPC (C15+C14)	—	—	—	—	520 bp	580 bp	580 bp	—
35S + SPC (C13+C14)	—	—	—	480 bp	—	—	—	470 bp

Figure 4. Range of phenotypes observed in some R₂ progeny generated by dual cocultivation as diagrammed in Figure 2 and plated on media containing spectinomycin (100 mg/l). Phenotype ranged from completely bleached (no excision or both *Ds*/SPT and transposase containing T-DNAs were not present) to completely green (very early excision). Some progeny (not shown) segregated 3:1 green:bleached indicating that excision had occurred in the germline of the previous generation (probably at the callus stage).

Figure 5. Regions of the 5' region of *Ac* used in GUS reporter constructs. wx = flanking waxy sequences from maize. A = regions cloned into pBI101.1 to generate promoter::GUS fusions. ■ = positions at which four OCS enhancers were added. B = regions of the untranslated leader sequence cloned into p4OCS35SGN (see text).

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