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TRANSPOSON TAGGING OF DISEASE RESISTANCE GENES
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Project Outline:

We aim to clone genes for resistance to lettuce downy mildew (*Bremia lactucae*). The approach involves the mobilization of transposons into resistance genes to generate mutants. The transposon sequences will then be used to identify the mutated flanking sequences. The functional wildtype gene will be selected using the flanking sequences as probes. The identity of the wildtype gene will be confirmed using other mutants and by introduction into susceptible genotypes. Because transposons have yet to be isolated and characterized from lettuce, we are manipulating heterologous transposons from maize and Antirrhinum. The majority of our experiments have been with *Ac* from maize as this is increasingly the best characterized plant transposon. The efficiency of the approach will depend on the degree to which transposition can be controlled and the ease with which insertion events can be selected and characterized. The majority of our current efforts are involved with developing an efficient mutagenesis system and to a lesser extent with characterizing the target genes.

Progress Over Past Year:

1) **Experiments to Detect Transposition of Wildtype *Ac*.** We have generated and analyzed several hundred transgenic lettuce plants over the past two years containing wildtype *Ac* elements in constructs which have been shown by others to allow transposition in other species. The simplest construct, which we made several years ago, contained *Ac* from *waxy-m7* cloned into the *Agrobacterium* vector pMON200. Transposition was monitored by extracting DNA from transgenic plants and either analyzing for changes in restriction fragments by Southern hybridization or, more recently, by changes in sizes of PCR (polymerase chain reaction) products. The majority of plants showed no clear evidence of transposition. This is in contrast to the results gained by Dr. John Yoder (UC Davis) with whom we collaborate, who observed high rates of transposition in tomato with the same construct. Southern hybridization of a few plants showed weak hybridization patterns consistent with infrequent transposition such that a minority of cells contained a transposed element and the diagnostic donor site after transposition. Bands the size of the donor site after transposition were also identified by PCR of DNA from several plants; however, while PCR is rapid and highly sensitive, it is very prone to false positives due to contamination. Therefore, we cloned and sequenced several PCR reaction products to confirm whether they were real excision products; all were identical to the wildtype *waxy* allele which was being used as the positive control. Therefore, either transposition in lettuce exactly and consistently restores the wildtype sequence rather than leaving a variable number of extra bases as in other species (unlikely) or the putative excision products were the result of contamination of the PCR reaction (more likely). We are refining experimental procedures to minimize the chances of contamination during PCR.

Another set of transgenic plants contained *Ac* interrupting the 5' region of a chimeric *Mas::streptomycin* resistance gene. This was constructed by Dr. Jonathan Jones (The Sainsbury Lab, UK) who observed high rates of transposition in tobacco with this construct; excision could be monitored by the appearance of streptomycin resistance in the progeny. Nearly all of 65 independent *T₂* lettuce families clearly segregated for

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2/10

resistance to streptomycin. This was surprising in view of the lack of frequent transposition with other constructs. Southern analysis of streptomycin resistant and susceptible plants, however, revealed no evidence of transposition. Therefore, the frequent and high levels of streptomycin resistance observed in lettuce must have been due to events during transcription or mRNA processing which remove the transposon sequences and restore a functional message. This is an interesting result but as it is not directly pertinent to the development of a transposon mutagenesis system we have not pursued it further. It does indicate the need to avoid making constructs with reporter genes for excision in which the transposon is in the correct orientation to allow the element to be processed out as an intron.

2) Experiments to Detect Transposition of Chimeric Transposons. Transgenic lettuce plants were generated containing *CaMV 35S* or *hsp70* promoters fused to the putative transposase gene from *Ac*. In parallel, plants were generated which contained a *Ds* element (a non-autonomous element derived from *Ac*) interrupting a chimeric *35S::GUS* (glucuronidase) gene. Both constructs were made by Dr. Mike Lassner in Dr. Yoder's lab who observed high levels of transactivation when tobacco or tomato plants containing each of these constructs were crossed. Excision of *Ds* could be detected by staining for GUS activity. T_1 plants containing each of these constructs were analyzed by Southern and those plants containing unrearranged T-DNA inserts were used in crosses. Of the 48 outcrossed T_2 progeny so far analyzed, only two have contained both the transposase and *Ds* constructs; Southern analysis and GUS assays of both have provided no evidence of transposition. The reasons for the large departure from the expected segregation ratio (one quarter should have contained both constructs) are currently being investigated. More progeny are being analyzed.

3) Experiments to Detect Transposition of *Tam3*. Over 20 transgenic T_1 lettuce plants containing the transposon *Tam3* from *Antirrhinum* (Courtesy of Dr. Cathy Martin, John Innes Institute) and several T_2 families were analyzed by Southern hybridization. Several had hybridization patterns in multiple endonuclease digests which were consistent with transposition; however, no plant had patterns that were consistent with simple excision in all endonuclease digests. It was therefore difficult to distinguish unambiguously between hybridization patterns resulting from rearrangements during T-DNA integration and those resulting from abnormal excision of *Tam3*. Large scale rearrangements have been observed by Dr. Cathy Martin in *Antirrhinum* and tobacco. Due these complications and the paucity of data on *Tam3* in other species, we have halted our analyses of *Tam3* in lettuce for the time being.

4) Whole plant selectable markers. Efficient transposon mutagenesis will involve whole plant selectable markers to indicate the occurrence of transposition and the genetic position of the transposon. We have so far analyzed the effectiveness of several herbicide resistance genes. Glyphosate resistance conferred by a modified bacterial *AroA* gene proved ineffective, although this may have been due to the lack of activity of the promoters (see below). A mutant *Arabidopsis* acetolactate synthase gene (courtesy of Dr. Chris Sommerville) provided high levels of resistance to chlorsulfuron in lettuce seedlings. Therefore this gene could be used as an effective whole plant selectable marker. A chimeric *Mas::streptomycin* resistance gene (Courtesy Dr. Jonathan Jones) also provided good resistance to streptomycin in lettuce under the correct assay conditions; this is therefore also a potential marker. Transgenic plants have been generated containing a bacterial nitrilase gene (fused to a *Mas* promoter, Courtesy of Dr. David Stalker, Calgene) which confers resistance to bromoxynil in other species; the T_1 plants are currently growing; the T_2 progeny will be tested for resistance.

5) **Promoters effective in lettuce.** These studies are part of a separately funded project but as they are pertinent to inducing and controlling transposition, they will be summarized here. The lack of transposition with constructs known to provide transposition in other species parallels our inability to express a variety of genes in lettuce using constructs known to be highly expressed in several species. As a consequence, we are currently analyzing a wide range of heterologous promoter::GUS fusions including fusions with 35S promoters from two sources, a RuBPC small subunit promoter, two ubiquitin promoters, the *Mas* promoter, and a Hsp70 promoter as well as several chimeric promoters. In several cases high levels of expression are observed in callus but restricted or no expression is observed in regenerated plants. High levels of expression in the leaves have not been observed with any promoter. These results may reflect the low activity of these promoters in lettuce, may be the consequence of non-specific modification of introduced or heterologous sequences or may be due to some other reason. We are currently trying to distinguish between these possibilities and to optimize gene expression in lettuce. We are also beginning to isolate endogenous promoters from lettuce.

6) **Stability of Resistance Genes.** Transposon mutagenesis requires that the likely rate of insertional inactivation by the characterized transposon is at least as frequent, and hopefully far more frequent than, the spontaneous rate of mutation of the target gene. The only data on the spontaneous mutation rates of disease resistance genes comes from maize and flax. In both species, different resistance genes exhibit different mutation rates. We have generated 50,000 F₁ plants heterozygous for several resistance genes. We have so far simultaneously screened 5,000 of these plants for the expression of each of three resistance genes. No susceptible plants were observed. Therefore, none of these genes in this genetic background are as unstable as the most unstable alleles in maize. We will continue to screen further heterozygous progeny.

7) **Dissection of *Ac*.** Except for the initial construct, much of the above data on *Ac* have been generated using constructs made by others for a variety of species. The advantage of this approach was that (apart from their availability) these constructs had been shown to provide transposition in other species and were not therefore inherently defective. This was important as *Ac* is sometimes unstable and can be difficult to manipulate. Now that several promoters have been shown to function poorly in lettuce and there is an increasing understanding of *Ac*, we are making constructs specifically to test whether the lack of transposition in lettuce is due to the lack of expression of the transposase gene or due to a post-transcriptional block such as mRNA processing or lack of transposase function. While on sabbatical for six months at CSIRO, Australia, I have constructed a series of *Ac* promoter::GUS fusions. These will be first tested in tobacco and then in lettuce (and flax in which again only low rates of *Ac* transposition have been observed; Dr. Jeff Ellis *et al.*, CSIRO). These promoter fusions will be modified to include enhancer elements likely to increase their expression. I am also making constructs containing genomic and cDNA transposase coding regions fused to chimeric promoters which should be highly expressed in lettuce. Again, these will be first tested for their ability to mobilize a *Ds* element from a reporter gene in tobacco and then in lettuce (and flax).

Efficient transposon mutagenesis requires the separation of the transposase functions from the element that is mobilized. The above experiments will define optimal promoter regions and transposase coding regions. To identify the minimum sequences necessary for transposition in *cis*, I am constructing *Ds* elements with an internal polylinker cloning site and varying amounts of terminal sequences either side. These will be tested in tobacco for their ability to transpose. The smallest functional *Ds* will then be modified to include selectable marker genes to allow genetic monitoring of the *Ds* in the

whole plant and to aid in cloning in bacterial and yeast artificial chromosome (YAC) vectors. After confirming the function of these elements in tobacco, they will be introduced into lettuce for resistance gene tagging experiments.

Research Plans:

In view of the data generated over the past two years on the recalcitrant behavior of *Ac* in lettuce, we are having to characterize and manipulate *Ac* and promoter sequences in greater detail than we originally anticipated. This has delayed the initiation of experiments to mutagenize resistance genes. However, even in those species where *Ac* transposes frequently, progress has been slow to clone genes by transposon mutagenesis using heterologous elements. This has at least in part been due to the lack of controlled transposition and the lack of *Ds* elements containing whole plant marker genes. The increased understanding of *Ac* generated by others, particularly Kunze *et al.* (1989) allows constructs to be made in my and other labs that will increase the efficiency of transposon mutagenesis as envisaged in the original proposal.

Control of transposition. As part of a companion project described in 5) above, we will identify promoters which provide high levels of expression in lettuce. If necessary we will clone and characterize endogenous sequences. As described in 7) above the *Ac* promoter will also be tested in lettuce. I am currently fusing chimeric promoters with multiple enhancer elements to cDNA and genomic coding regions of *Ac*; as described in 7) these will be first tested in tobacco to confirm their activity. The most highly expressed promoters will be fused to the effective transposase coding regions and tested in lettuce.

Ideally, transposition should be restricted to the germline and occur just prior to gamete formation so that the transposon, and therefore any mutations, are somatically stable, while each gamete is potentially a different mutation event. Most flower-specific genes so far isolated from a variety of species are either expressed in the vegetative pollen nucleus or in non-germline tissues. If we have sufficient resources, we will clone and characterize genes that are specifically expressed in the lettuce germline just prior to gamete formation. This will involve identification of differential using cascade hybridization of mRNA populations from target tissues against mRNAs from other tissues. Patterns of expression will be confirmed using *in situ* hybridizations. cDNAs showing expression in either male or female germline tissues in young flowers will be used to isolate the corresponding genomic sequences; their regulatory regions will be isolated and fused to GUS and to transposase coding regions. The GUS fusions will be used to analyze to confirm the specificity of the promoter regions. The transposase fusions will be used in experiments to mutagenize disease resistance genes. The isolation of such a promoter, however, will require a major commitment and will not be attempted until the other aspects of the system are functional. Such experiments would extend beyond the next year of funding.

Chimeric *Ds* elements. As described in 7) above I am constructing *Ds* elements with minimal terminal sequences flanking a polylinker cloning site. Selectable markers that function in whole plants, probably *acetolactate synthase* (chlorsulfuron resistance), yeast, probably *LYS* (lysine auxotrophy), and bacteria, *SupF*, will be cloned into the polylinker. The inclusion of the yeast *LYS* gene will allow us selectively to clone the *Ds* element and large amounts of flanking sequences into YAC vectors. YAC vectors are currently in use in my laboratory.

Transposon mutagenesis. It is probable that the above will not be completed by the end of the next year of funding. However, as soon as suitable patterns of transposition and functional *Ds* elements with marker genes are available, we will initiate mutagenesis experiments.

Whether *Ac* preferentially transposes to nearby locations is an important question as it determines the crossing strategy to be used to tag disease resistance genes. If insertion occurs at random relative to the point of excision, the transposon can be introduced to any genomic position (that allows expression). Large numbers of test cross progeny are then screened for inactivation of several genes; multiple unlinked would be targeted simultaneously. If *Ac* frequently reinserts close to the point of excision, it would be advantageous first to develop stocks in which the transposon is located close to a cluster of resistance genes. Fewer test cross progeny would have to be screened to isolate mutants. To determine the pattern of *Ac* insertion in lettuce, transposase constructs will be introduced into a line carrying no resistance genes. A *Ds* element containing selectable markers genes as described above, and interrupting the 5' region of another whole plant marker gene will be introduced into a cultivar (Diana) which contains four resistance genes and hundreds of transgenic plants will be generated. Plants containing the transposase construct will be crossed to plants containing the *Ds* construct (the latter as female). The F_1 plants containing both constructs will be selfed. F_2 progeny will be screened for the segregation of the marker genes representing the point of excision and the new position of *Ds*. Preferential transposition to nearby positions will be indicated by frequent cosegregation of the two markers. If this occurs, the F_2 will be screened for the segregation of the disease resistance genes relative to the marker within *Ds*. By screening several hundred F_2 families it should be possible to identify lines with *Ds* linked to each resistance gene. These lines will be used in subsequent mutagenesis experiments.

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Transposon Tagging of Disease Resistance Genes

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We are developing a transposon mutagenesis system for lettuce to clone genes for resistance to the fungal pathogen, *Bremia lactucae*. Activity of heterologous transposons is being studied in transgenic plants. Southern analysis of T₁ and T₂ plants containing *Tam3* from *Antirrhinum* provided ambiguous results. Multiple endonuclease digests indicated that transposition had occurred; however, in no plant were all endonuclease digests consistent with a simple excision event. Southern or PCR analysis of over 50 plants containing *Ac* from maize have also failed to reveal clear evidence of transposition; this is contrast to experiments by others with the same constructs who have observed high rates of *Ac* excision in other plant species. Nearly all of 65 T₂ families containing *Ac* interrupting a chimeric streptomycin resistance gene (Courtesy J. Jones, Sainsbury Lab., UK) clearly segregated for streptomycin resistance. Southern analyses, however, showed no evidence of transposition, indicating restoration of a functional message by other mechanisms, possibly mRNA processing. Transgenic plants have also been generated containing CaMV 35S or *hsp70* promoters fused to transposase coding sequences or a *Ds* element interrupting a chimeric GUS gene (Courtesy M. Lassner, UC Davis). F₁ plants containing both constructs were analyzed for transposition. Only two plants containing both constructs were obtained from 48 progeny, far fewer than expected, and neither showed evidence of transposition in Southern and GUS assays. We are currently constructing further chimeric transposase fusions. To test for the stability of the targeted disease resistance genes, 50,000 F₁ plants heterozygous for three resistance genes were generated; no mutants have been identified in the 5,000 so far screened.

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