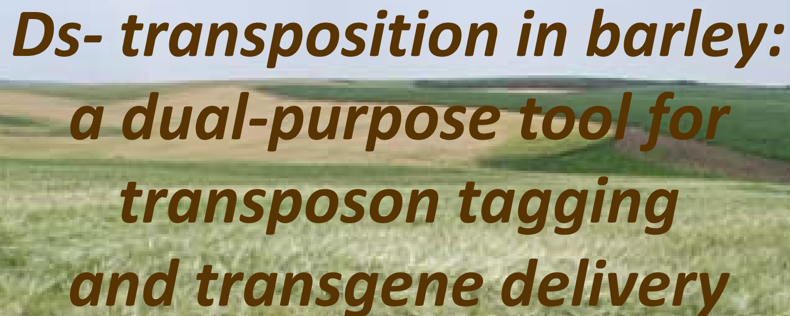


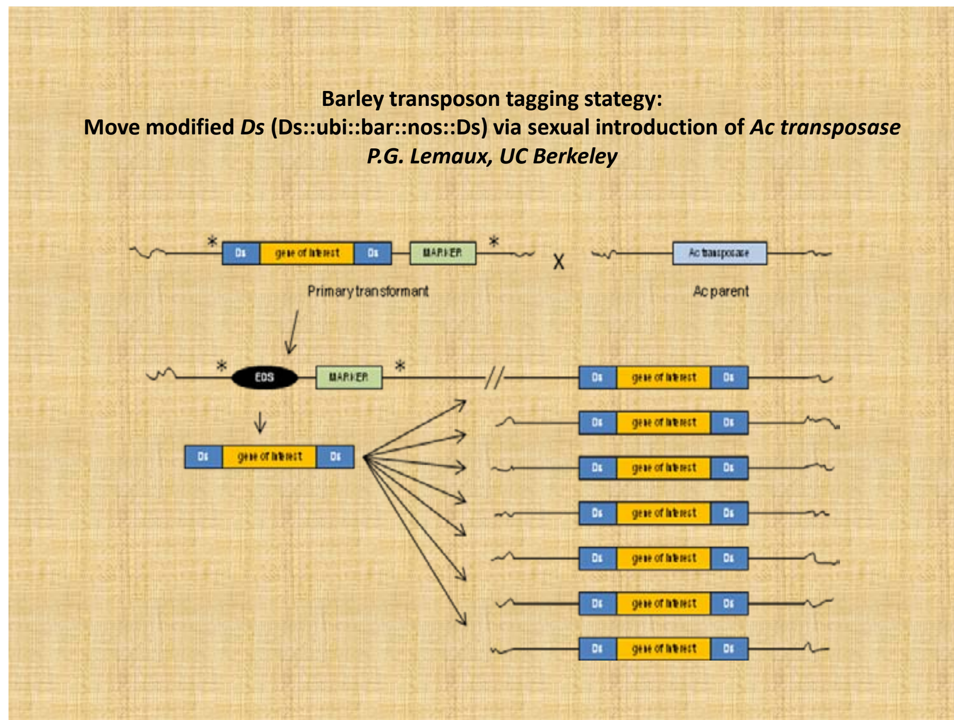
Phil Bregitzer
National Small Grains Research Facility
USDA-ARS, Aberdeen, ID

The cooperative development of barley transposon-tagging stocks has been brought to a finishing point. Initially conceived by Dr. Peggy Lemaux (UC-Berkeley), and followed by additional work by her, a succession of post-doctoral workers and students, Pat Hayes (OSU), and Phil Bregitzer (ARS-Aberdeen), we now have released 70 transposon-tagged lines, with approximately that many more lines being readied for release at a later date. Each has an single, independent *Ds* element at an independent position, in a homozygous state.

The key biological feature of this system is its basis on the maize (corn) *Ac* (*Activator*)-*Ds* (*Dissociation*) transposon system, and the ability to introduce functional features of this system into species other than maize. *Ac* elements encode an enzyme (*Ac transposase,* or *AcT*) that acts on terminal sequences of *Ds* elements, which can cause the element to excise and reinsert into a new location elsewhere in the genome. This is the process of tranpsosition.

Because the terminal sequences required for interaction with *AcT* are very short, modified *Ds* elements can be constructed where short (<300 base pairs) *Ds* termini flank genes of interest, and the process of transposition will move that gene around the genome. In our system, we are using a modified *Ds* element (*Ds-bar*) that includes the selectable marker gene *bar*, which encodes herbicide resistance. Slide 2 illustrates the system, where transposition is initiated by introducing

AcT into a plant carrying *Ds-bar* by hybridization. Note that the original transgene locus, which will include unwanted sequences (indicated by the asterisks) such as bacterial vector DNA, duplications, and rearrangements. Transposition of *Ds-bar* will result in a "clean" single copy of *Ds-bar* moving to a new location, leaving the unwanted sequences at the original location, where they can be removed by genetic segregation during generation advance. Any one of the seven barley chromosomes can be the new location of the transposed *Ds-bar* locus. Transposed loci can be induced to move again, simply by repeating the process of hybridization with *AcT*-expressing plants.



Slide 2--transposon tagging/gene delivery scheme

In some cases, transposition will insert *Ds-bar* into a gene or a sequence influencing the expression of a gene, and the so-called "tagging" of this sequence led to the name "transposon tagging". Because the transposon sequence is known, simple and accurate genetic assays can be conducted to discover the nature of the tagged sequence, and ultimately, its genetic function in the development of the plant.

The function of the *Ac-Ds* system is well-characterized in its native maize (corn), and studies in other species suggests that it behaves similarly regardless of the organism it is in. To verify this for barley, we examined various features of the transposon barley tagging population. For instance, we expected new transpositions to map to all chromosomes. However, there should be preference for locations linked to the site where any given *Ds-bar* insertion was prior to re-transposition as a result of hybridization of various lines to *AcT*-expressing plants. Slide 3 shows that *Ds-bar* insertions have been recovered on all chromosomes, although there appears to be some preference for certain chromosomes. Some of this pattern clearly results from the expected preferential transposition to locations near the excision site. For instance, transposition from

chromosome 3HL results in preferential transposition to other locations on chromosome 3HL. This pattern is more obvious in some lines produced at Aberdeen (Slide 4) than some lines produced at Berkeley (slide 5). It is not clear if differences in the methods used to identify new lines at the two labs influenced this result, but limited numbers of lines were examined, so firm conclusions should be avoided. Note also that in several cases multiple lines had *Ds-bar* mapped to the same position; however, none of the insertions at the same map location were in exactly the same spot, but just very near each other.

_Ds_-tagged Barley Populations, status—January, 2013

Independently tagged sequences, at least one flank determined: 162

Released lines: 70 **Lines to be released later: ~70**

Distribution by Chromosome Arm															
1H	1HS	2HS	2HL	3HS	3HL	4HS	4HL	5H	5HS	5HL	6HS	6HL	7HS	7HL	Total
5	4	9	25	5	16	6	3	1	9	20	1	10	22	12	148
Position (IPK FPC)															
11.8	7.0	2.3	59.5	3.2	50.8	26.4			31.7	85.6	56.0	108.3	0.4	74.3	
128.0		23.9	73.7	48.1	51.6	40.2			31.7	111.1		115.0	1.5	98.3	
137.8		27.5	86.0 (2)		83.7	75.6				111.3		117.5	2.5	116.1	
		27.7	86.8		95.5					139.6		119.1	6.8	118.3	
		30.4	99.2		141.9 (2)					139.9			12.7	122.3	
			107.2		155 (4)					151.9			12.7	124.2	
			110.9 (2)							159.5			17.6	129.4	
			113.9 (2)							160.5			21.4		
			128.8							168.5			30.6		
			132.6 (2)										37.6 (2)		
													53.2		
													68.4		

?? _Ds_ transpositon: Random or to Preferred Positions ??

Slide 3. Locations of Ds-bar insertions. Native genomic DNA sequences were determined and compared to barley genomic sequences determined by the International Barley Sequencing Consortium (http://www.nature.com/nature/journal/v491/n7426/full/nature11543.html) to identify chromosome arm position and map position.

Does launch site influence transposition site?
Expectation: High frequency of local transposition
DsT lines identified via PCR—ARS-Aberdeen

DsT 48 derivatives   Launch site 2HL ~161 cM OWB map

Distribution by Chromosome Arm														
1H	1HS	2HS	2HL	3HS	3HL	4HS	4HL	5HS	5HL	6HS	6HL	7HS	7HL	Total
1	1	3	15		3	1	1	2	3			3	3	36
Position (IPK FPC)														
138	11.8	27.5	59.5		51.6	40.2		44	85.6			53.2	129	
		30.4	99.2		155				140					
			107											
			114											
			133											

Ds T 31 derivatives   site 7HS 33 cM OWB map

Distribution by Chromosome Arm														
1H	1HS	2HS	2HL	3HS	3HL	4HS	4HL	5HS	5HL	6HS	6HL	7HS	7HL	Total
		1	1	1	1	1	1		1		1	10	1	19
Position (IPK FPC)														
		23.9	133	3.2	83.7				169		115	0.4	116	
												6.8		
												21.4		
												30.6		

Slide 4. Pattern of transposition from lines identified at Aberdeen, ID, using a PCR based technique to identify lines with new transpositions. This technique relies on the identification of plants with one wild-type allele (from the AcT parent) and one empty-donor-site band (which contains the "footprint" created by the excised Ds-bar that was originally there (see slide 6)), followed by the identification of plants with Ds-bar located somewhere else in the genome.

Does launch site influence transposition site?
Expectation: High frequency of local transposition
DsT lines identified via transposition to
novel RFLP fragments (Southern)—UC Berkeley

TNP 24 derivatives site 3HL 150 cM OWB map

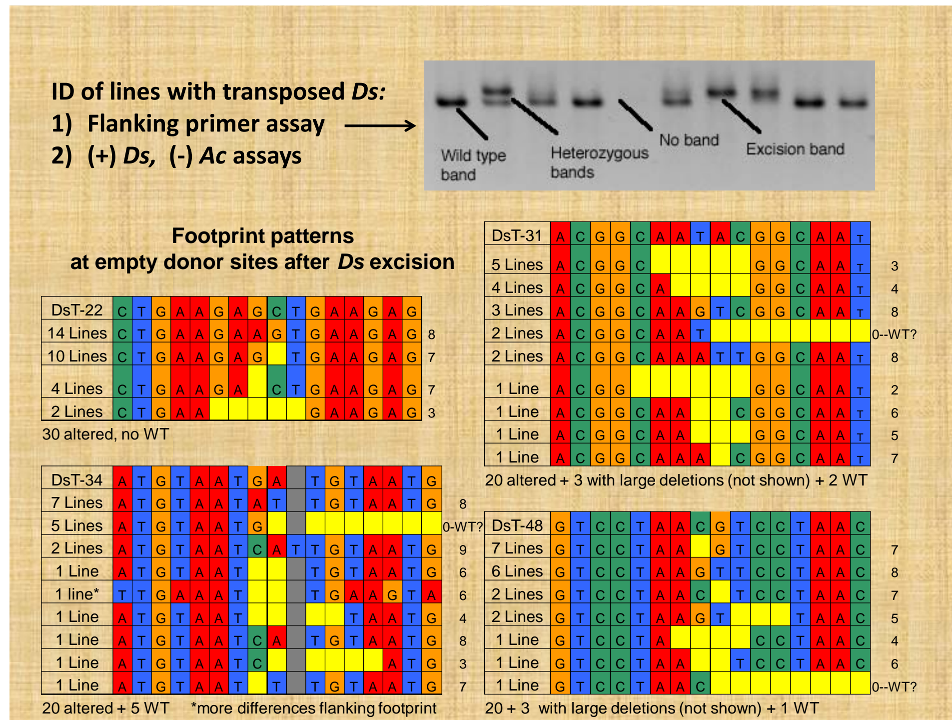
1H	1HS	2HS	2HL	3HS	3HL	4HS	4HL	5HS	5HL	6HS	6HL	7HS	7HL	Total
Distribution by Chromosome Arm														
2	1				4			1	3		2	2	1	16
Position (IPK FPC)														
12.0	7.0				71.1				111.3		119.1	12.7	161.0	
					95.5				140.0					
					141.9				151.9					

PDS 2 derivatives site 6HL 135 cM OWB map

1H	1HS	2HS	2HL	3HS	3HL	4HS	4HL	5HS	5HL	6HS	6HL	7HS	7HL	Total
Distribution by Chromosome Arm														
			1	1	2	3		1	2	1	4	2	1	18
Position (IPK FPC)														
			73.7	39.0	141.8	26.4			111.1	56.0	108.3	17.6	98.3	
					155.0	48.0			159.5		117.5	37.6		
											154.7			

Slide 5. Pattern of transpositon from lines identified at Berkeley, CA, using a Southern-based method to identify new lines, where the Ds-bar probe hybridized to unique restriction bands.

Another well-known aspect of the *Ac-Ds* system is that insertion of a transposon creates a duplication of the target site, typically but not always an 8 bp site. Following retransposition, this "footprint" is left behind. We looked at the footprints in selected lines (slide 6), and found that this expectation was met. It also appears that different patterns of footprints are formed, depending on where the *Ds-bar* was before re-transposition.



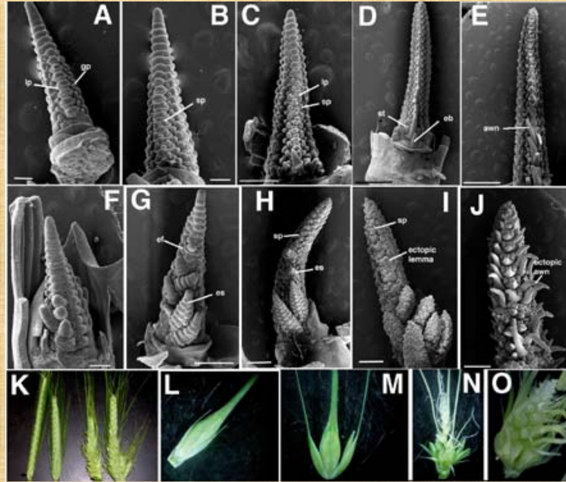
Slide 6. Top: PCR-based scheme for identifying plants with new transposition sites. Under appropriate conditions, primers specific to regions flanking the insertion site will not produce a product if Ds-bar is still there, but if it has excised will produce a band that is the expected size of the native region plus a few extra base pairs that represent the target site duplication footprint. Plants with both the native and the footprint bands that have a positive reaction when using primers specific to Ds-bar must have Ds-bar at a new location. Bottom: pattern of footprints from four different lines following transposition.

For most of our lines, no obvious abnormalities were seen and the plants grew well and were fully fertile. Some may have been less vigorous, however. In one of our lines, we recovered a line in which *Ds-bar* has inserted into a barley *miR172* ortholog (that is, the barley version of *miR172*), which functions in floral development. Plants with this mutation have abnormal spike development (Slide 7).

Another expectation if that the *Ds-bar* loci are transposed as intact, single-copy genes without rearrangement. Slide 8 shows the results of various PCR assays that show only single bands of expected sizes that are amplified using various primers for full-length (top gel) and partial sections (bottom gels) of the *Ds-bar* transposon, consistent with this expectation.

Generation of Phenotypic Changes

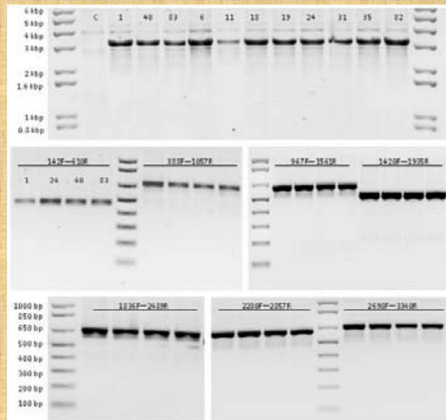
1) Majority of plants have no obvious changes, although many have reduced vigor (reduced seed size, fewer tillers, later maturity)
2) Insertion of *Ds* into *miR172* ortholog (a regulator of floral development) caused major alteration of spike morphology



Slide 7. Normal sdevelopment (a-e; left spikes in k; and l) and mutant development (f-j, right spikes in k; and m-o).

***Ds* as a tool to generate transgenic loci**

*less than 600bp of DNA from maize
*intensively studied, biology well-understood
*capable of moving 12 kb of sequence between terminal sequences
*proposed as a means of eliminating selectable markers, vector sequences
*should be capable of resolving complex loci



Expectation:
"clean" transgene loci without cues that induce RNAi-mediated silencing

PCR with 5' and 3' primers shows a specific product of expected length

"Walking" from the 5' to 3' regions of *Ds-bar* insert using a series of primers show specific products of expected lengths

Slide 8. PCR assays using primers to specific regions of the Ds-bar transposon. Multiple bands and bands of unexpected sizes would indicate rearrangement, duplication, or other modifications of the Ds-bar transposon.

The integrity of transposed genes seen above is a useful tool for producing useful transgenic plants. Another requirement for efficient use of this system for transgene delivery is that transposition must occur at reasonable frequencies. Transposition frequencies vary from 5 to 41%, depending on the site of excision (slide 9).

_Ds_ as a tool to generate transgenic loci

Important Considerations

1) **Integrity of transgene--avoid silencing**
2) **Stable, heritable expression—as with any native allele**
3) **Site of transposed loci—insertion into native genes or sequences affecting native gene expression**
4) **Frequency of transposition—determining population sizes**

DsT-	n	Excision + WT	Excision + WT + Ds	Excision	WT	Ds (+)	exc. frequency	Tranposition frequency	Frequency of new insertions
19	924	63	49	63	912	626	0.07	0.78	0.05
22	722	122	121	255	539	611	0.35	0.99	0.35
31	1173	222	142	443	909	791	0.38	0.64	0.24
34	371	139	74	201	300	160	0.54	0.53	0.29
48	1016	408	275	622	767	614	0.61	0.67	0.41
67	474	119	107	187	377	418	0.39	0.90	0.35
79	234	44	36	73	191	169	0.31	0.82	0.26
93	40	2	2	9	16	25	0.23	1.00	0.23

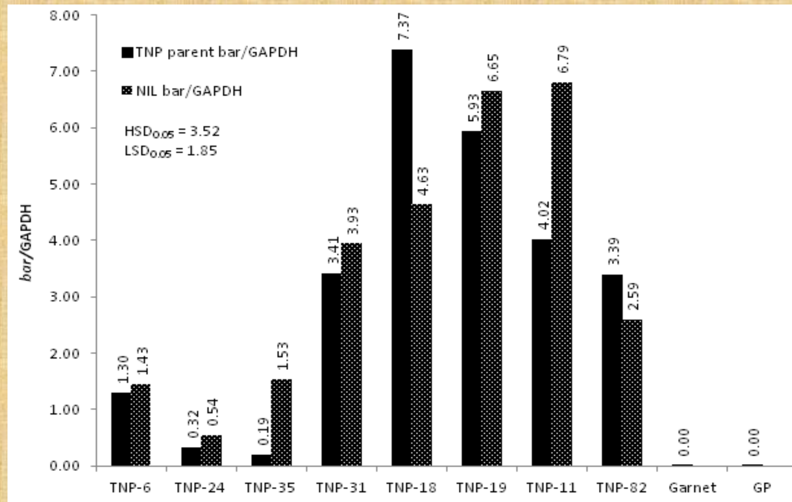
Slide 9

As noted on slide 9, stable and heritable expression of transgenes is desirable. Examination of the expression of the *bar* gene from transposed *Ds-bar* loci was examined in donor parents with various levels of expression and in near-isogenic lines developed by backcrossing. The heritability of expression is high (slide 10).

Another point on slide 9 is that useful transgenic plants should not have a transgene inserted into a native gene. This is in contrast to the desired result for tagging genes, where insertion into a gene is the desire result. To determine how often transposition into genes occurs, native sequences flanking *Ds-bar* elements were searched against non-redundant sequence and protein databases, and against EST (expressed sequence) databases. The results of these searches are shown in slide 11.

Overall, these data suggest that the use of *Ds*-mediated transposon delivery can result in "good transgenes in good locations" (slide 12). Future work will concentrate both on direct delivery of transgenes of interest, and for the delivery of sites that facilitate a site-specific recombination strategy known as recombinase-mediated cassette exchange (RMCE). RMCE facilitates integration of transgenes at a pre-determined location, enabling the development of multiple lines each expressing a different transgene from the same site (slide 13).

Inheritance of *bar* expression in BC_8 near-isogenic lines
***Garnet as the recurrent parent**
***DsT lines (Golden Promise) with various levels of *bar* expression as donor parents**



Slide 10. Heritability of bar expression in parent and near-isogenic line derivatives when delivered by Ds transposition, as determined by qRT-PCT Taqman assays.

Determining locationof *Ds* insertions relative to known genes/ESTs

BLAST searches (EST, NR) of native sequences flanking *Ds* insertions

Location of *Ds*	n
exon	34
intron	12
intergenic	132
intergenic < 1kb from gene/EST	34
intergenic > 1kb from gene/EST	98
total # of insertions	178
# w/in 1kb or in gene or intron	80
% not around/in known genes/ESTs	55.00

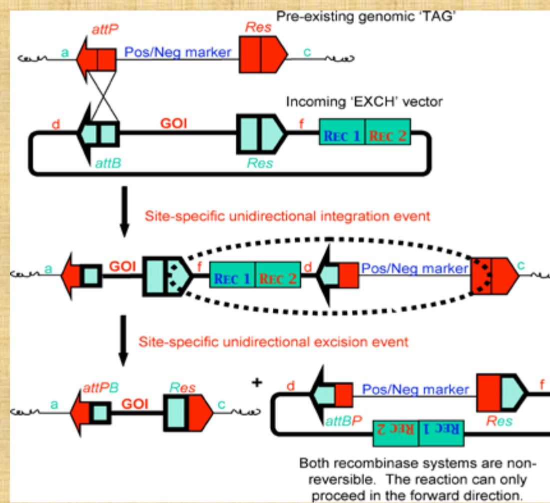
Slide 11

Conclusion:
***Ds* transposition delivers good transgenes to good places!**

Slide 12

What's Next?

***Ds*-mediated delivery of transgenes targeting FHB**
***Ds*-mediated delivery of 'TAG' sites for site-specific recombination**
Bregitzer; Thomson, ARS-Albany; Dahleen, ARS-Fargo; Trail, MSU



Slide 13

References/Acknowledgements

Bregitzer P, Cooper LD, Hayes PM, Lemaux PG, Singh J, Sturbaum AK (2007) Viability and *bar* expression are negatively correlated in Oregon Wolfe barley dominant hybrids. Plant Biotech J 5:381-388.

Brown RH, Bregitzer, P (2011) A *Ds* insertional mutant of a barley *miR172* gene results in indeterminate spikelet development. Crop Sci 51:1664-1672

Brown RH, Dahleen LS, Bregitzer P (2012) An efficient method for flanking sequence isolation in barley. Crop Sci 52:1229-1234.

Koprek T, McElroy D, Louwerse J, Williams-Carrier R, Lemaux PG (2000) An efficient method for dispersing *Ds* elements in the barley genome as a tool for determining gene function. Plant J 24:253-263.

Cooper LD, Marquez-Cedillo L, Singh J, Sturbaum AK, Zhang S, Edwards V, Johnson K, Kleinhofs A, Rangel S, Carollo V, Bregitzer P, Lemaux PG, Hayes, PM (2004) Mapping Ds insertions in barley using a sequence-based approach. Mol Gen Genomics 272:181-193.

Koprek T, Rangel S, McElroy D, Louwerse JD, Williams-Carrier RE, Lemaux PG (2001) Transposon-mediated single-copy gene delivery leads to increased transgene expression stability in barley. Plant Physiol. 125:1354-1362.

Singh J, Zhang S, Chen C, Cooper L, Bregitzer P, Sturbaum A, Hayes P, Lemaux, PG (2006) High-frequency *Ds* remobilization over multiple generations in barley facilitates gene tagging in large genome cereals. Plant Mol Biol 62:937-950.