Supplementary online material:

Assessment of transgene stability and transmission:

A small subset of the transgenic events produced significantly more than the 50% expected herbicide resistant plants. There are multiple explanations for the transgenic events that have altered frequencies of resistant plants when crossing hemizygous transgenics to non-transgenic plants. The lines that show a higher than expected frequency of resistant plants are likely due to the presence of multiple unlinked copies of the transgene. Several examples of multiple transgenic loci segregating were observed by the Southern blot analysis. This type of aberrant segregation ratio was resolved by selecting one of the two transgenic loci, thus restoring the expected segregation ratio for the transgenic line.

A significant proportion (~1/4) of the transgenic events generated by this project displayed lower than expected frequencies of resistant plants upon outcrossing (SOM Figure 1B). By separately plotting the number of resistant plants per family, it became obvious that there were some individual families that were all sensitive and may have skewed the summary data (SOM Figure 2A). By separately visualizing the resistance data for all of the rows grown for each transgenic event (multiple rows, representing multiple crosses grown over multiple generations and locations), it was possible to determine the relatively stability of each event and the contribution of different rows to the total. For any given construct, there was variation in the stability among different transgenic events (SOM Figure 2B-D). For example, events 3832.004 or 3832.007 are quite stable and never produced rows with all susceptible plants while other events, such as 3832.001 or 3832.014, are unstable and frequently produced rows with lower than expected frequencies of resistant plants.

One source of variability could be the directionality of outcrossing if the transgene is inherently more stable when transmitted through the male or female parent. Across all events, results indicated that the transgenes are less stable when transmitted through the male (41.4% resistant plants in 39,855 plants) than when transmitted through the female parent (45.0% resistant plants in 43,230 plants). In some events, such as 3385.016 (SOM Figure 2C), the transgene appeared stable when transmitted through one parent but not the other. There are a number of potential sources of variability in the combined data represented in SOM Figure 2: the sex of the transmitting parent varied in different families, and the data were compiled from multiple field locations (with different personnel scoring the resistance phenotypes), multiple generations of transgenic plants, and variable numbers of plants in each family in each planting.

To standardize the screen for parent-or-origin effects on transgene stability, another study was conducted. In the summer of 2003, resistant plants from 123 transgenic events were crossed as females by non-transgenic B73. A total of 223 ears were generated (including 27 events with a single ear and 95 events with multiple ears derived from sibling transgenic female parents). Sixty seeds from each ear were grown in standard greenhouse conditions and the seedlings were screened for herbicide resistance after 14 days of growth (SOM Figure 3A). A number of the ears (42/223) produced a lower than expected percentage of resistant seedlings ($X^2 < 0.05$). Only 5/223 ears produced significantly higher than expected resistant seedling; this is approximately the number of statistical false-positives expected for this population size.

Another experiment was directed at asking whether transmission from male *vs.* female parent influenced the proportion of resistant progeny (Figure 5 and SOM Table II, Experiment 1). For each of 22 randomly selected events, a single hemizygous transgenic plant was crossed reciprocally (as male and female) with a non-transgenic B73 plant generating 44 ears. Sixty seeds from each reciprocal-cross ear were planted during the summer of 2004 and scored for herbicide resistance. For 16 of the 22 events, parental origin of the transgene did not significantly affect the proportion of resistant progeny (Figure 5). However, for five events, transmission of the transgene through the male led to a significantly lower proportion of resistant progeny than when the same transgene was transmitted from a female parent (indicated by black arrows in Figure 5). For one event, 3571.022, the opposite was true, *e.g.,* transmission from a female parent led to lower resistance in the progeny than when the transgene was inherited from a male parent (indicated by open arrow in Figure 5). In general, there were more instances of lower than expected resistant progeny in male transmitted rows than in female transmitted rows (SOM Table II).

This apparent bias against male transmission could be the result of reduced transmission of transgenic pollen or preferential silencing in the male germline. Because the male inflorescence (tassel) is formed later in development than the female inflorescence (ear), it is possible that progressive transgene silencing during later stages of development contributes to the bias against male transmission. For Experiment 1 (SOM Table II), the transgenic parent plants had been screened by performing a leaf painting assay on seedlings to identify resistant plants. To evaluate the potential effect of progressive silencing through development, we conducted a second experiment in which the parental transgenic plants were tested for resistance by spraying the entire plant at three different times during plant development: at the 5-6 leaf stage, at the 10-12 leaf stage and finally just prior to tassel emergence. Some plants that survived the first or second herbicide application exhibited sensitivity at the third application, which likely indicates silencing of the transgene late in development. In the absence of late herbicide application, crossing of such a plant would lead to transmission of a silent transgene to resulting progeny. However, by applying selection for resistance late in development, we reasoned that plants undergoing progressive transgene silencing would be killed and would not be used for crossing. To test this idea, for a set of 73 independent events (including most of the 22 events used for the first experiment), we performed reciprocal crosses to B73 for two sibling plants that survived the third herbicide application. Thirty seeds from each resulting ear were grown in the greenhouse and the seedlings were screened for resistance by whole-plant spraying after 14 days of growth (SOM Table II, Experiment 2). A total of 9,382 seedlings were screened. The proportion of resistant plants was higher in this experiment (48.0% compared to 38.2% in the first experiment). We attribute this to the more rigorous screening for resistance in the previous generation, which probably selected against plants in which progressive transgene silencing was occurring. Although fewer lines displayed unexpected segregation ratios in this experiment (7.5% of lines, compared to 39% in the first experiment), there were still some lines with lower than expected resistance frequencies. Again, this was more common in progeny where the transgene was transmitted through the male.

A skewed proportion of herbicide resistant progeny, depending upon the transmitting parent, could be due either to erratic transmission of the transgene or to poor expression of the *bar* gene, perhaps through transgene silencing. To distinguish between these possibilities, we isolated DNA from herbicide susceptible individuals in 78 families, including the events that yielded lower than expected frequencies of resistant plants in the experiments above, and used PCR to look for the presence of the transgene (SOM Table III). If the low frequency of resistance was due to transgene silencing, then some of the susceptible plants should have the transgene, whereas if the low frequency of resistance was due to problems in transmission, then the transgene should not be detected in susceptible plants. Of the 78 families tested, 24 families had no resistant plants and 54 families had a lower than expected number of resistant plants (SOM Table III). In 44 of the 78 families, the transgene was not detected in any of the herbicide susceptible plants, suggesting that the cause of low proportions of resistant plants was due to poor transmission of the transgene. In the other 34 families, the transgene was detected in at least one of the susceptible plants, suggesting that the cause of low proportions of herbicide resistant plants in these events is attributable to low levels of expression of the *bar* transgene, silencing of the transgene or a combination of these mechanisms. In some cases (6 of these 34 families) the low proportion of susceptible plants that exhibit presence of the transgene suggests that both reduced transmission, in addition to transgene silencing, may contribute to the reduced frequency of resistant plants (Table S3).

SOM Figure 1. Analysis of transgene stability by construct and by event. (A) The data from all field seasons were grouped based on the construct used to perform transformation. The percent resistant plants for multiple generations and constructs was tabulated for each event. (B) The percent resistant plants was determined for each event that included at least 8 plants. The data for each event can include scoring for multiple field seasons and multiple generations of transgenic plants.

SOM Figure 2. Analysis of transgene stability by event. (A) The percent resistant plants for all rows with at least eight plants was determined and plotted on the y-axis. Each row was plotted along the x-axis according to the transgenic event number. The red points indicate rows in which the transgene was transmitted through the female parent and the blue points indicate rows in which the transgene was transmitted through the male parent. A small amount of “jitter” was applied to the plot to allow visualization of overlapping data points. (B) An expanded version of this plot was produced for the empty vector control and three different constructs. The event number is indicated along the bottom of the x-axis.

SOM Figure 3. Transgene stability in a controlled set of events. (A) Sixty seeds were planted and screened for herbicide resistance. The data are sorted according the percent resistance and plotted with a separate bar for each packet of seeds that were analyzed. The lines indicate the point at which the data are significantly different from expected at a chi-square value <0.05. (B) The correlation between the multiple generations or planting from the same event was assessed. Each vertical line along the X axis represents a different transgenic event. The y-axis indicates the percentage of plants that displayed BAR resistance for each row that was planted in the field. The blue spots indicate a chi-square significant value ($P<0.05$). Note that in many cases there is correlation between different rows of plants derived from the same event.

SOM Table I. Transformation pipeline and efficiences for producing transgenic lines used in this study.

Target gene name	Unique MGC number	Embryo plates shot (25 embryos/plate)	Resistant callus events	T0 plantlets regenerated and transferred to greenhouse	Plants that made T1 seed	% effiency from callus to T1 seed
brd101	5013	20	8	5	2	25
brd102	5912	18	9	5	2	22
brd103	5297	20	20	8	7	35
chb101	5812	20	25	12	10	40
chc101	3348	13	8	8	4	50
che101	6271	17	13	3	1	8
chr101/106	2758	57	15	15	10	67
chr101	3882	49	36	17	14	39
chr106	3768	25	10	2	0	0
chr110	2960	61	13	4	3	23
chr112	3385	63	32	18	13	41
chr113	3928	38	5	2	0	0
chr115	6432	23	49	27	17	35
chr118	3832	40	24	16	11	46
chr119	3491	29	22	8	4	18
chr120	3353	46	20	6	3	15
chr122	4191	24	19	19	15	79
chr124	4943	14	5	2	0	0
chr125	5211	34	26	14	11	42
chr126	5305	30	9	4	3	33
chr131	6091	20	7	3	2	29
crd101	5752	53	23	6	3	13
dmt101	2685	86	11	10	8	73
dmt102	3471	59	11	9	5	45
dmt103	2822	27	10	10	8	80
dmt104	3944	24	8	8	5	63
dmt106	5943	15	32	18	6	19
epl101	5652	44	14	6	3	21
fie102	5663	42	10	2	2	20
gta101	4618	20	9	3	2	22
gta107	5936	17	12	4	3	25
gtb101	5321	21	4	2	1	25
gtc102	4761	20	12	5	4	33
gtc102	4992	62	18	7	4	22
gte101	3932	24	9	6	3	33
gte102	5231	20	37	17	12	32
hac101	3826	36	11	10	7	64
hag101	4681	32	35	17	14	40
hag102	4291	41	26	11	10	38
hag103	4361	31	20	6	4	20

ham101	4202	26	37	17	12	32
hda101	3751	33	34	12	7	21
hda102	4571	40	43	21	13	30
hda108	4671	33	11	3	3	27
hda109	4162	24	9	7	6	67
hda110	3534	67	18	5	5	28
hdt101	3955	30	42	12	7	17
hdt103	3361	13	5	5	3	60
hmga102	4977	20	18	5	4	22
hmgb101	4301	20	21	9	6	29
hmgb102	5924	21	9	5	2	22
hmgb103	4963	20	22	14	11	50
hmgb104	4172	26	24	8	4	17
hmgb106	5071	25	33	17	13	39
hmgb108	6281	20	33	8	5	15
hmgb110	6102	20	2	1	0	0
hmgb113	6151	21	45	23	12	27
hon101	5736	20	9	2	0	0
hon102	5065	54	90	49	35	39
hon103	6082	21	29	18	10	34
hon104	5094	20	7	2	2	29
hon110	6123	19	25	14	12	48
hxa102	3544	71	45	13	9	20
mbd101	3818	46	44	13	11	25
mbd105	5052	40	28	12	3	11
mbd108	4585	40	13	5	5	38
mbd109	5832	45	70	38	27	39
mbd113	6311	20	21	10	7	33
nfa101	3772	24	33	14	8	24
nfa102	4707	20	10	6	4	40
nfa104	3322	11	7	7	5	71
nfc102	3480	36	10	8	7	70
nfc103	3331	17	6	6	4	67
nfc104	4271	31	24	10	9	38
nfe101	6071	20	39	21	9	23
nfe101	6199	20	22	9	5	23
sdg101	4795	38	15	6	3	20
sdg102	4268	44	36	20	13	36
sdg103	4322	25	28	12	9	32
sdg104	4281	38	23	18	11	48
sdg105	5171	33	36	15	13	36
sdg106	4716	16	10	3	3	30
sdg107	5104	24	20	8	7	35
sdg108	6238	18	52	19	8	15
sdg110	4621	16	20	6	5	25
sdg111	4721	37	15	6	3	20
sdg113	4633	16	8	2	2	25
sdg115	4985	20	13	5	3	23
sdg116	4731	16	9	4	2	22
sdg117	4656	36	24	10	7	29
sdg118	5314	65	17	3	3	18

sdg119	5511	20	36	9	5	14
sdg122	5801	20	42	22	14	33
sdg123	5825	20	24	14	12	50
sdg124	5641	53	48	22	14	29
sdg125	6321	19	10	6	3	30
sdg129	6180	20	22	7	4	18
sga101	6468	20	10	4	4	40
sgb101	3846	112	149	51	45	30
sgb102	4187	42	36	14	12	33
smh101	6114	19	19	9	5	26
smh104	4784	20	3			0
srt101	3571	36	41	22	16	39
vef101	4747	20	5	2	1	20
Empty vector[a]	161	94	71	20	10	14
B-Peru [a]	2973	56	27	21	8	30
		3382	2494	1144	766	

[a] These are control constructs. pMCG161 contains no IR insertion and pMCG2973 contains an IR targeting the

SOM Table II. Parental effects on transgene stability

	Sex of transmitting parent	Events	Segregating families analyzed	Resistant	Sensitive	Total	# Families with high frequency of resistant plants (P < 0.05)*	# Families with low frequency of resistant plants (P < 0.05)*
Experiment 1	Male	22	22	358	705	1063	0	10
	Female	22	22	444	594	1038	2	5
Experiment 2	Male	73	146	2225	2503	4728	1	18
	Female	73	146	2283	2371	4654	3	10

*indicates values significantly different than expected based on chi square analysis

SOM Table III. Herbicide resistance frequencies in transgenic plants.

Event	Transgenic parent	Total plants	% BAR resistant plants	Transgene presence in S plants	Likely cause of reduced frequency of BAR resistant plants
3544.16	Female	11	0%	3 / 8	Silencing
5511.12	Female	12	8%	2 / 8	Silencing
6238.33	Female	15	0%	2 / 8	Silencing
5171.08	Female	16	0%	2 / 8	Silencing
5825.01	Female	17	24%	2 / 8	Silencing
3571.22	Female	47	0%	3 / 9	Silencing
6321.01	Female	48	0%	2 / 10	Silencing
2822.01	Female	54	37%	1 / 10	Silencing
4322.05	Female	56	41%	4 / 9	Silencing
3882.21	Female	26	8%	3 / 15	Silencing
4281.13	Female	26	27%	2 / 15	Silencing
3882.16	Female	28	18%	10 / 15	Silencing
4281.26	Female	29	17%	4 / 15	Silencing
3882.21	Female	30	3%	9 / 15	Silencing
4162.03	Female	30	13%	5 / 15	Silencing
4621.06	Female	30	17%	9 / 15	Silencing
5171.01	Female	30	7%	9 / 15	Silencing
4621.06	Male	11	0%	4 / 8	Silencing
5832.33	Male	14	0%	4 / 8	Silencing
5171.08	Male	19	0%	4 / 8	Silencing
6321.03	Male	38	0%	6 / 10	Silencing
4322.05	Male	48	33%	7 / 10	Silencing
6432.27	Male	48	33%	2 / 10	Silencing
6321.01	Male	56	0%	4 / 10	Silencing
3882.03	Male	20	25%	6 / 15	Silencing
2822.03	Male	29	34%	7 / 15	Silencing
3882.03	Male	30	50%	4 / 15	Silencing
4268.04	Male	30	10%	4 / 15	Silencing
2758.04	Female	11	0%	1 / 8	Silencing and transmission
3491.13	Female	29	0%	1 / 15	Silencing and transmission
5297.15	Female	29	3%	1 / 15	Silencing and transmission
3491.13	Female	30	7%	3 / 15	Silencing and transmission
4187.11	Female	30	17%	3 / 15	Silencing and transmission
4202.05	Male	29	17%	3 / 15	Silencing and transmission
6180.10	Female	10	20%	0 / 7	Transmission
4268.04	Female	17	24%	0 / 8	Transmission
4721.12	Female	18	0%	0 / 8	Transmission
6432.27	Female	47	40%	0 / 10	Transmission
4268.14	Female	49	67%	0 / 10	Transmission
4281.19	Female	50	24%	0 / 10	Transmission
4281.18	Female	54	43%	0 / 10	Transmission
4281.18	Female	28	21%	0 / 15	Transmission
4322.05	Female	28	4%	0 / 15	Transmission
4268.23	Female	29	7%	0 / 15	Transmission
4268.23	Female	29	24%	0 / 15	Transmission
4271.20	Female	29	14%	0 / 15	Transmission
4281.03	Female	29	7%	0 / 15	Transmission

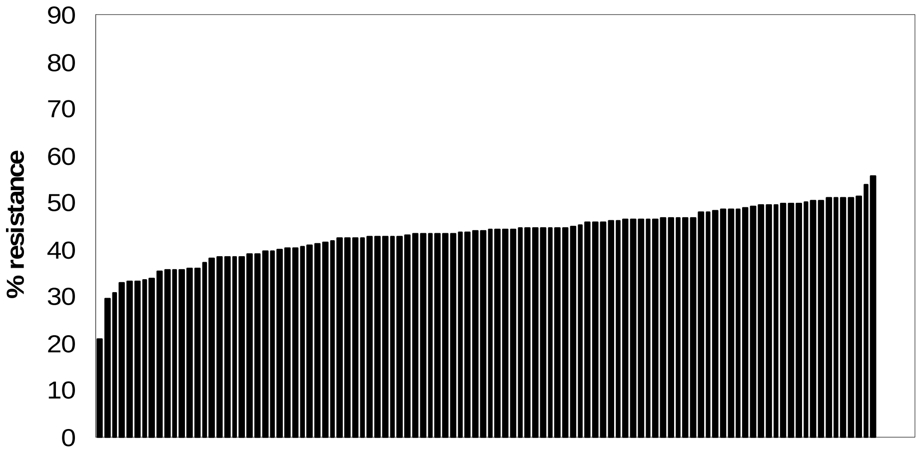
4281.03	Female	29	31%	0 / 15	Transmission
4281.19	Female	29	10%	0 / 15	Transmission
4291.18	Female	29	0%	0 / 15	Transmission
3944.17	Female	30	20%	0 / 15	Transmission
4271.15	Female	30	7%	0 / 15	Transmission
4271.15	Female	30	7%	0 / 15	Transmission
4281.19	Female	30	7%	0 / 15	Transmission
4322.05	Female	30	7%	0 / 15	Transmission
5104.04	Female	30	10%	0 / 15	Transmission
5104.13	Female	30	3%	0 / 15	Transmission
4301.09	Male	11	0%	0 / 7	Transmission
5812.29	Male	12	0%	0 / 8	Transmission
2758.09	Male	13	0%	0 / 8	Transmission
4268.22	Male	13	0%	0 / 6	Transmission
4795.12	Male	14	0%	0 / 8	Transmission
2822.10	Male	15	0%	0 / 8	Transmission
3944.17	Male	15	0%	0 / 8	Transmission
6238.07	Male	15	0%	0 / 8	Transmission
5171.09	Male	17	24%	0 / 8	Transmission
3361.08	Male	18	17%	0 / 8	Transmission
4716.02	Male	18	28%	0 / 8	Transmission
4281.06	Male	44	5%	0 / 10	Transmission
4281.18	Male	49	2%	0 / 10	Transmission
4268.20	Male	53	42%	0 / 10	Transmission
4281.19	Male	54	0%	0 / 10	Transmission
3385.16	Male	57	0%	0 / 9	Transmission
4268.23	Male	28	25%	0 / 15	Transmission
4268.23	Male	29	10%	0 / 15	Transmission
4268.23	Male	30	13%	0 / 15	Transmission
4291.10	Male	30	3%	0 / 15	Transmission
4291.10	Male	30	20%	0 / 15	Transmission

SOM Table IV. Impact of selfing transgenic lines on herbicide resistance frequency.

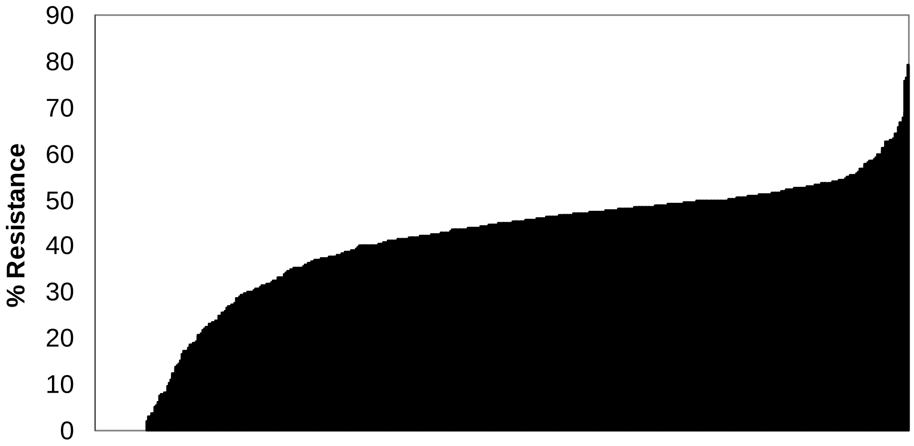
	Self-pollinated progeny (1:2:1)		Out-crossed progeny (1:1)	
Line	# R plants / total	% R	#R / total	% R
3571.004	15/19	79%	8/20	40%
3571.009	14/20	70%	8/20	40%
3571.003	13/19	68%	11/19	58%
2822.006	38/60	63%*	8/17	47%
4268.004	12/20	60%	7/16	44%
3361.005	9/19	47%*	9/19	48%

*indicates values significantly lower than expected based on chi square analysis

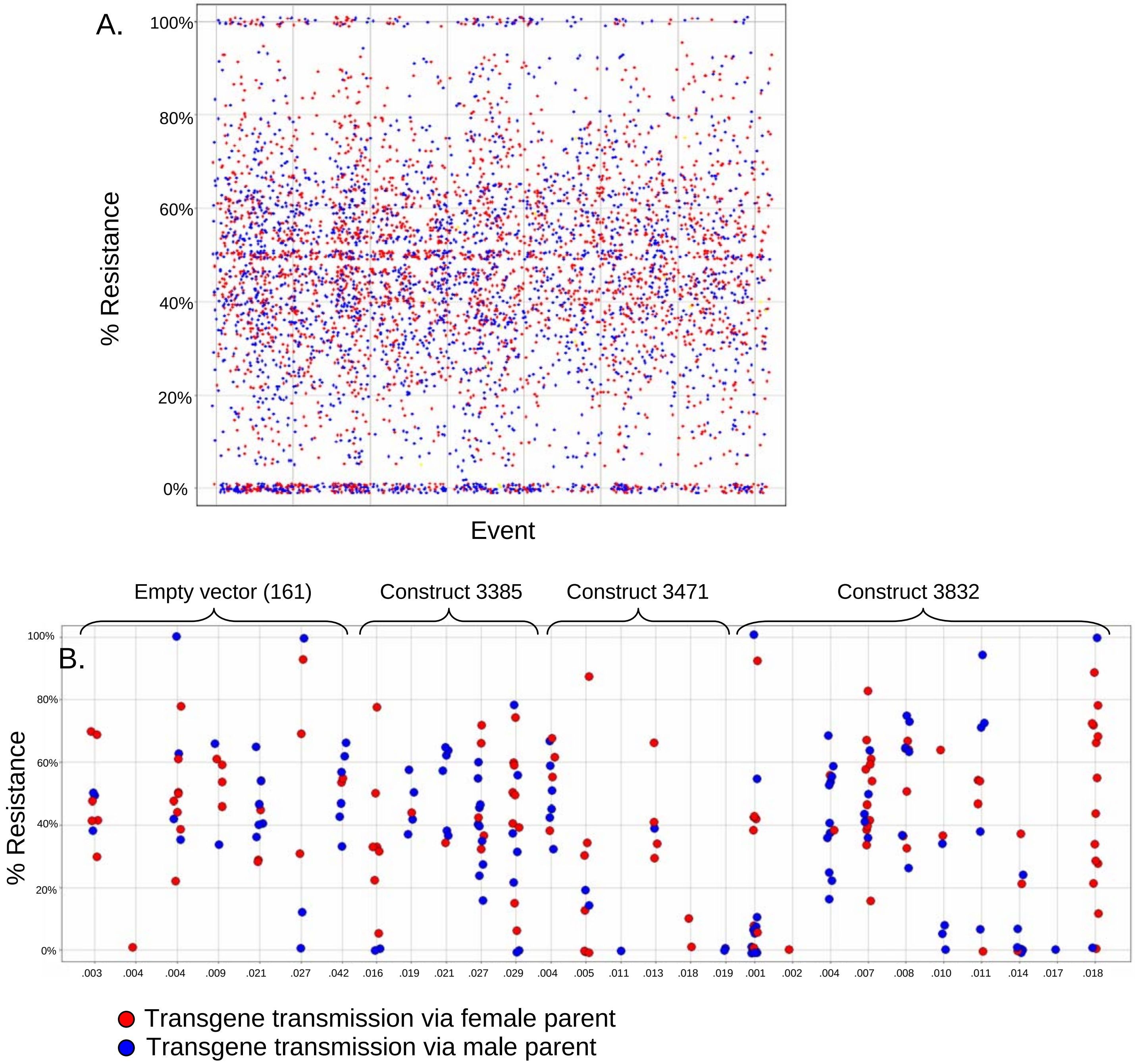
A. % Resistance by construct



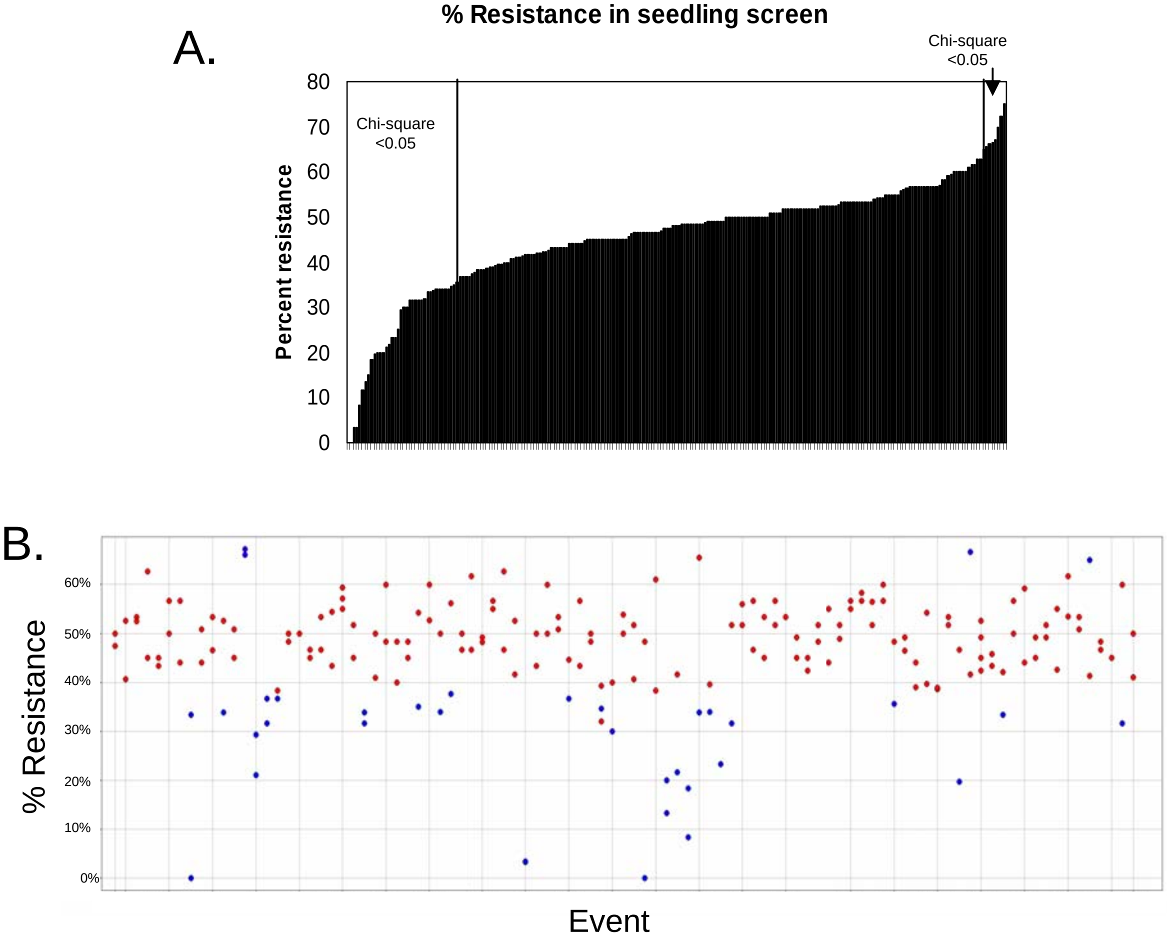
B. % Resistance by event



SOM Figure 1. Analysis of transgene stability by construct and by event. (A) The data from all field seasons was grouped based on the construct used to perform transformation. The percent resistant plants for multiple generations and constructs was tabulated for each event. (B) The percent resistant plants was determined for each event that included at least 8 plants. The data for each event can include scoring for multiple field seasons and multiple generations of transgenic plants. Note that some events show higher than expected frequencies of resistant plants while other events show less than expected frequencies of resistant plants.



SOM Figure 2. Analysis of transgene stability by event. (A) The percent resistant plants for all rows with at least eight plants was determined and plotted on the y-axis. Each row was plotted along the x-axis according to the transgenic event number. The red points indicate rows in which the transgene was transmitted through the female parent and the blue points indicate rows in which the transgene was transmitted through the male parent. A small amount of "jitter" was applied to the plot to allow visualization of overlapping data points. (B) An expanded version of this plot was produced for the empty vector control and three different constructs. The event number is indicated along the bottom of the x-axis.



SOM Figure 3. Transgene stability in a controlled set of events. (A) Sixty seeds were planted and screened for herbicide resistance. The data are sorted according the percent resistance and plotted with a separate bar for each packet of seeds that were analyzed. The lines indicate the point at which the data are significantly different from expected at a chi-square value <0.05. (B) The correlation between the multiple generations or planting from the same event was assessed. Each vertical line along the X axis represents a different transgenic event. The y-axis indicates the percentage of plants that displayed BAR resistance for each row that was planted in the field. The blue spots indicate a chi-square significant value (P<0.05). Note that in many cases there is correlation between different rows of plants derived from the same event.