Protocol for creating unigene set and single seeded decent lines

Plant selection and seed production

Fifty seeds were sown on GM selected media using R7402 20ng/ml and PPT 10μg/ml, after 10-14 days 24 plants per pool were transferred to soil and each plant assigned a unique SM number. Tissue was harvested after 10-14 days and DNA preparations performed using a Qiagen Dneasy 96 plant extraction kit (catalogue number 69181). Seeds were harvested and cleaned after approximately 6 weeks and sent to the Nottingham Arabidopsis Stock Centre (NASC) and assigned a NASC number in addition to a SM number.

Plant selection on soil

Seeds were sown directly on soil and allowed to grow to second leaf stage then sprayed twice at a 7 day interval with BASTA (375mg/ml) and R7402 (1μg/ml or 2μg/ml) and the growth of plants assessed after 7 days.

DNA sequencing and sequence output

DNA samples (2μl) were digested with Apo1 (2U per reaction) for 90 minutes at 50 C and the enzyme inactivated at 75 C for 20 minutes . The samples were ligated overnight (ligase 0.2U at 4 C and 4μl of the ligated DNA aliquoted for the inverse PCR (iPCR) which was performed using two transposon specific primers spm 31 (5'GCT TGT TGA ACC GAC ACT TTT AAC ATA AG 3') and inv6-2 (5' GCT AAG CAC ATA CGT CAG AAA CCA TTA TT 3') at 5 pmoles each and the following cycles, 2 minutes denaturation step at 94 C (94 C 10 seconds 60 C 30 seconds 68 C 5 minutes repeated 38 cycles) and a final step 68 C for 5 minute. iPCR products (20μl) were cleaned up using 2U Exo1 and 2U SAP per reaction at 37 C for 30 minutes and a 10 minute denaturation step at 80 C. The products (5μl) were sequenced using Applied Biosystems Big Dye Terminator kit (catalogue number 4390244) and a nested transposon primer spm 32 (5' TAC GAA TAA GAG CGT CCA TTT TAG AGT GA 3') cycled 25 times at 96 10 seconds, 50 C 5 seconds, 60 C 4minutes. Ethanol precipitated samples were run on a Perkin Elmer 3700 sequencer and results assessed for sequence quality, length (>16 bases) , the presence of gene/transposon junction and alignment with Arabidoposis sequence using a p value <0.001.

iPCR protocol

Digestion

DNA	2.0μl (~200ng)
10X Buffer 3 (NEB)	1.0μl
ApoI (4 U/μl)	0.2μl
100XBSA (10mg/ml)	0.1μl
dH_2O	6.7μl
TOTAL	10.0μl

Digest at 50°C for 1 hour 30 minutes
Inactivate enzyme at 75°C for 20 minutes

Ligation

Digested DNA	10.0µl
10X Ligase Buffer	4.0µl
Ligase (1 U/µl)	0.2µl
dH_2O	25.8µl
TOTAL	40.0µl

Ligate overnight at 4°C

PCR

DNA (from ligation)	4.0µl
10X PCR Buffer	2.0µl
dNTPs (2mM)	2.5µl
Primer Spm31 (10µM)	0.5µl
Primer inv6-2 (10µM)	0.5µl
Taq (Qiagen) (1U)	0.2µl
dH_2O	10.3µl
TOTAL	20.0µl

Cycling parameters:

1.	94°C	2 min
2.	94 °C	10 sec
3.	60 °C	30 sec
4.	68 °C	5 min

go to 2 for a further 37 cycles

5.	68 °C	5 min

(extension is 45''-1' per kb)

<u>Cleanup</u>

PCR products	20µl
SAP (1 U/µl)	2µl
ExoI (10 U/µl)	1µl

Incubate 30 mins at 37 °C, 10 mins at 80 °C)

Sequencing reactions

template DNA	5µl
dSpm 32 primer (10µM)	1µl
terminator mix (v3)	3µl
dH_2O	1µl

Cycling parameters:

1.	96 °C	10 sec
2.	50 °C	5 sec
3.	60 °C	4 min

go to 2 for a further 24 cycles

Precipitate sequencing reactions

Add 60µl EtOH (95%) and 1µl EDTA (250mM) to each tube and gently mix
Incubate on ice for 30 mins
Centrifuge at 3500rpm for 30 mins

Inverted spin to remove ethanol at 300rpm for 1-2 mins
Add 150µl cold 70% EtOH to each tube and mix gently
Centrifuge at 3500rpm for 20 mins
Inverted spin to remove ethanol at 300rpm for 1-2 mins
Air-dry DNA pellets in tubes (NOTE: if white precipitate is visible, it is salt. Wash sample again in 70% EtOH)
Store samples at -20 ^{O}C

Sequence of dSpm (5'-3')showing positions of iPCR and sequencing primers

	Start	length	tm	gc%	primer sequence 5′ to 3′
spm31	5326	29	64.93	37.93	gcttgttgaaccgacacttttaacataag
Spm32	5377	29	65.10	37.93	tacgaataagagcgtccattttagagtga
Inv6-2	4182	29	64.51	37.93	gctaagcacatacgtcagaaaccattatt

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 3901 cgccggcatgtccgcctcggtggcacggcggatgtcggccgggcgtcgttctgggctcat

 3961 ggtaattgtaaatagtaattgtaatgttgtttgttgtttgttgttgttggtaattgttgt

 4021 aaaaatagtcgagttgagagtgaatatgagactctaattggataccgaggggaatttatg

 4081 gaacgtcagtggagcattttgacaagaaatatttgctagctgatagtgaccttaggcga

 4141 cttttgaacgcgcaataatggtttctgacgtatgtgcttagctcattaaactccagaaac
                     <<<<<<<<<<<<inv6-2<<<<<<<<<<<<

 4201 ccgcggctcagtggctccttcaacgttgcggttctgtcagttccaaacgtaaaacggctt

 4261 gtcccgcgtcatcggcgggggtcataacgtgactcccttaattctccgctcatgatcgcg

 4321 gcagatggatcacaggtacaatcctctactaaacatcatttgtctgataatcattggttg

 4381 cttgctctctgtctttctacatgtttgcttgtacatgatctattttaaacttttgtcatt

 4441 cgcatgatctatagttacttttattgttgatcaccgtaacttcttaatcataaacaagag

 4501 tgtgtcttctgtttcagggttctgcaacttctgtccaagaccaattgatgccattgggtg

 4561 tgataggagggcaaatgatgccgtgggcacctcgccagccaggcatttggccaccgatgc

 4621 aaacacagatgccaccgccgatgccgtggggatttcctcctcgtgggcagtcacaatcac

 4681 caggattgccctcacactcaccaggatcagtacgttaagttgatatcctttgcatctcta
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4741 tttgcttcgttgtttaagcagttactagaaaacatgcatgtatatgttgcagtctatgta

4801 tatgtttaattagttactcggtaaactaacaaatgtttgtttcttttaaagggttcaggc

4861 tcacatcatgctagtccgcctccggatcagagcacgtttatggacttattgatgaacaca

4921 agtggcggcggctccaatgacccaccaacagaatgaattaatatggaggcttgtgtggaa

4981 cttactatgattgcgttttgtatggactttaacttgttttagatggatttgaacttcttt

5041 cgtatggacttgaacttgtatgaatattgaatatggtgcttgtgttatgttatgttgaat

5101 atggtgcttgtgttgtgatatattgaatgttgtgcttatattgtgctgttatggaggctt

5161 cccatccggggagggagaaaaataaaattggatattaaaaaaaattattcactaagagtg

5221 tcggcccccacactcttatatgcgcccaggtagcttactgatgtgcgcgcagtaagagtg

5281 acggccacggtactggccgacacttttaacataagagtgtcggttgcttgttgaaccgac
                                                  >>>>>spm31>>>>>
5341 acttttaacataagagcgtcggtccccacacttctatacgaataagagcgtccattttag
     >>>>>>>>>>>>>>                      >>>>>>>>>spm32>>>>>>>>>>
5401 agtgacggctaagagtgtcggtcaaccgacactcttatacttagagtgtcggcttatttc
     >>>>>

5461 agtaagagtgtggggttttggccgacactccttaccttttttcttgtagtg
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