# EcoTILLING Stress Tolerance Genes in Cultivated and Wild Rice

EcoTILLING diverse germplasm allows SNP and haplotype discovery at loci of interest (Comai *et al* 2004 *Plant J* **37**:778-786). At IRRI, we are using this technique to characterize natural variation in diverse cultivated and wild germplasm selected from the International Rice Genebank Collection at candidate genes for abiotic and biotic stress tolerance. Candidate genes putatively involved in stress tolerance were identified through converging evidence taking into account functional annotation, altered expression, co-localization with QTLs, and/or allelic shifts under selection. Target loci include DREB1, trehalose 6-phosphatase (TPP) for drought tolerance and oxalate oxidase and 14-3-3 protein for blast tolerance (see poster #50 for biotic stress candidate genes) among others. We have designed locus-specific primers for upstream regulatory and coding sequence regions using the Nipponbare genomic sequence. Our initial studies have used either a panel of cultivated *Oryza sativa* with representatives from the indica, aus, deepwater types III and IV, aromatic, and japonica variety groups or a panel of wild *Oryza* accessions from all species and genome types. Reference lines for contrasts are Nipponbare (japonica type) and IR64 (indica type).

## EcoTILLING Methodology

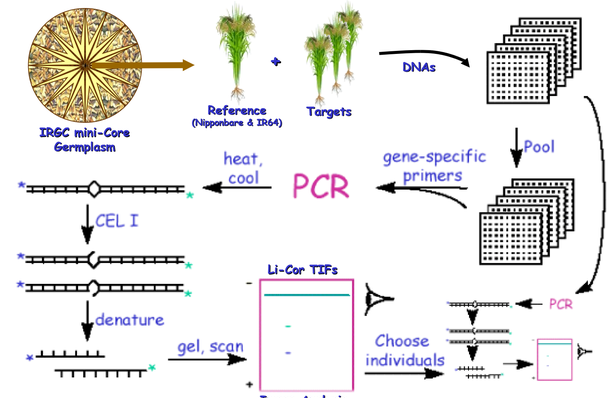
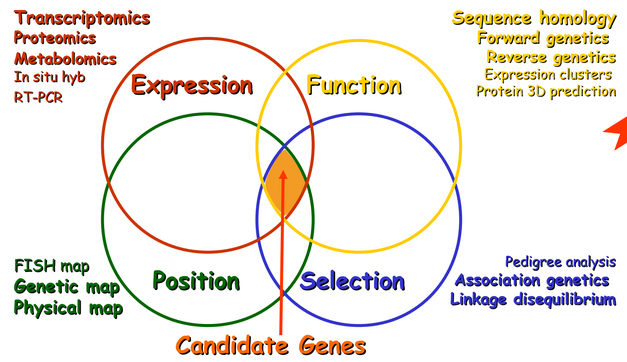



Figure 1. Cartoon depicting EcoTILLING procedure (adapted from Colbert *et al.* 2001).

## Choosing Candidate Genes

Figure 2. Converging multiple lines of evidence to support candidate gene choices. Those having more support are given higher priority



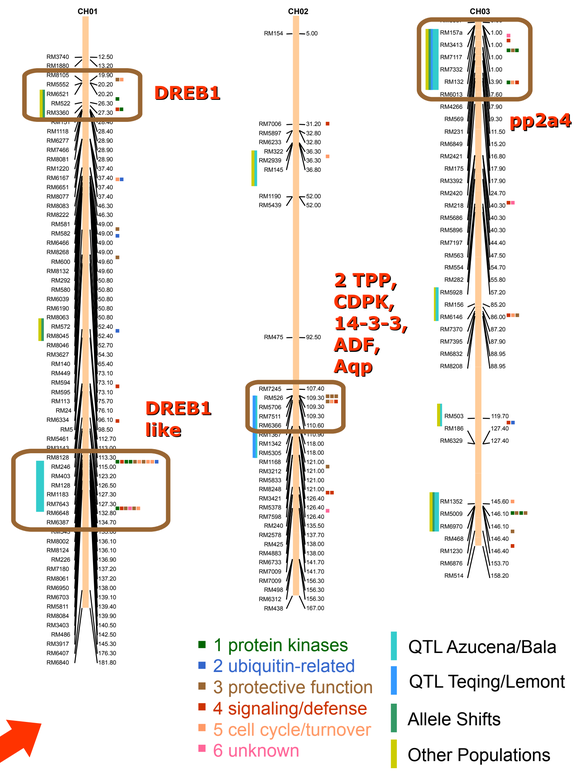




Figure 3. Bin EST data with QTL data for yield components under stress, cross-reference to gene annotations, identify homologs/paralogs in gene family. Up or down regulated ESTs and other candidate genes cluster in QTL bins – evidence for regulons in rice?

## *Germplasm and Candidate Gene Primers*

**Table 1. *Oryza sativa* micro-core panel**

| Code | GERMPLASM | IRGC Accno | VG |
|---|---|---|---|
| 1 | CT9993-5-10-1-M | | jap/ind |
| 4 | IR62266-42-6-2 | | ind/jap |
| 8 | CAROLINA GOLD | 1723 | |
| 13 | KALUKANTHA | 7755 | |
| 17 | VARY VATO 462 | 10964 | |
| 18 | FANDRAPOTSY 104 | 10984 | |
| 33 | MILYANG 23 | 34393 | jap/ind |
| 36 | BLACK GORA | 40275 | |
| 37 | PACHOLINHA | 50531 | |
| 2 | APO (IR55423-01) | | ind |
| 6 | DEE-GEO-WOO-GEN | 123 | ind |
| 10 | SINTANE DIOFOR | 5418 | ind |
| 14 | KUN MIN TSIEH HUNAN | 8195 | ind |
| 15 | RTS 12 | 8234 | ind |
| 16 | POKKALI | 8948 | ind |
| 23 | KHAO KAP XANG | 23423 | ind |
| 26 | KHAO DAWK MALI 105 | 27748 | ind |
| 27 | LEUANG PRATEW | 27762 | ind |
| 32 | PETA | 32571 | ind |
| 35 | IR36 | 39292 | ind |
| 38 | BALA | 50927 | ind |
| 39 | CO 39 | 51231 | ind |
| 45 | IR64 | 66970 | ind |
| 46 | JING XI 17 | 67676 | ind |
| 48 | TE-QING | 81093 | ind |
| 3 | VANDANA | | aus |
| 11 | N 22 | 6264 | aus |
| 20 | ARC 10177 | 12386 | aus |
| 25 | JAGLI BORO | 27516 | aus |
| 31 | DULAR | 32561 | aus |
| 44 | AUS JOTA | 66767 | aus |
| 24 | ASWINA | 26289 | dp3 |
| 34 | MATIA AMAN 53-13 | 37764 | dp4 |
| 47 | RAYADA | 77210 | dp4 |
| 12 | BASMATI 370 | 6426 | aro |
| 42 | CHHOTE DHAN | 58930 | aro |
| 30 | TCHAMPA | 32368 | aro |
| 5 | IR60080-46A | | trop jap |
| 7 | AZUCENA | 328 | trop jap |
| 9 | IGUAPE CATETO | 4122 | jap |
| 19 | MOROBEREKAN | 12048 | trop jap |
| 21 | NIPPON-BARE | 12731 | temp jap |
| 22 | YANCAOUSSA | 16071 | trop jap |
| 28 | RATHAL | 31524 | jap |
| 29 | GERDEH | 32301 | temp jap |
| 40 | CHODONGJI | 55471 | temp jap |
| 41 | PATBYEO | 55607 | temp jap |
| 43 | LEMONT | 66756 | temp jap |

Extended to 1536 diverse *O. sativa* accessions.

**Table 2. Wild *Oryza* spp. panel**

| Code | Species | IRGC Accno | Genome |
|---|---|---|---|
| MC1 | *O. barthii* | 105507 | AA |
| MC2 | *O. barthii* | 104142 | AA |
| MC3 | *O. barthii* | 104103 | AA |
| MC4 | *O. barthii* | 105612 | AA |
| MC5 | *O. barthii* | 104102 | AA |
| MC6 | *O. glumaepatula* | 100894 | AA |
| MC7 | *O. glumaepatula* | 82034 | AA |
| MC8 | *O. glumaepatula* | 101960 | AA |
| MC9 | *O. longistaminata* | 92636 | AA |
| MC10 | *O. longistaminata* | 92623 | AA |
| MC11 | *O. longistaminata* | 92607 | AA |
| MC12 | *O. meridionalis* | 103321 | AA |
| MC13 | *O. meridionalis* | 105279 | AA |
| MC14 | *O. meridionalis* | 105306 | AA |
| MC15 | *O. nivara* | 104612 | AA |
| MC16 | *O. nivara* | 106051 | AA |
| MC17 | *O. nivara* | 106329 | AA |
| MC18 | *O. nivara* | 105740 | AA |
| MC19 | *O. nivara* | 106041 | AA |
| MC20 | *O. nivara* | 105386 | AA |
| MC21 | *O. rufipogon* | 81900 | AA |
| MC22 | *O. rufipogon* | 104624 | AA |
| MC23 | *O. rufipogon* | 80781 | AA |
| MC24 | *O. rufipogon* | 106169 | AA |
| MC25 | *O. rufipogon* | 106523 | AA |
| MC26 | *O. rufipogon* | 105948 | AA |
| MC27 | *O. rufipogon* | 106157 | AA |
| MC28 | *O. rufipogon* | 106386 | AA |
| MC29 | *O. rufipogon* | 106515 | AA |
| MC30 | *O. alta* | 100161 | CCDD |
| MC31 | *O. australiensis* | 100882 | EE |
| MC32 | *O. eichingeri* | 105407 | CC |
| MC33 | *O. eichingeri* | 105414 | CC |
| MC34 | *O. grandiglumis* | 105144 | CCDD |
| MC35 | *O. meyeriana* | 106474 | GG |
| MC36 | *O. meyeriana/granulata* | 104989 | GG |
| MC37 | *O. minuta* | 83825 | BBCC |
| MC38 | *O. minuta* | 105130 | BBCC |
| MC39 | *O. officinalis* | 80733 | CC |
| MC40 | *O. officinalis* | 80755 | CC |
| MC41 | *O. officinalis* | 100949 | CC |
| MC42 | *O. punctata* | 89163 | BBCC |
| MC43 | *O. punctata* | 105153 | BBCC |
| MC44 | *O. rhizomatis* | 105443 | CC |
| MC45 | *O. rhizomatis* | 105445 | CC |
| MC46 | *O. ridleyi* | 100877 | HHJJ |
| MC47 | *O. schlechteri* | 82047 | HHKK |
| Refs. | *O. sativa* (IR64, Nipponbare) | 66970, 12731 | AA |

Extended to 96 diverse Oryza spp. accessions.

**Table 3. Candidate gene targets for drought tolerance and cds and/or upstream primers.**

| Gene | Bioprocess | Functional Evidence | Pimer_Name | Primer_sequence | TIGR_pm2_chr | Start | Stop | strand | Amplicon |
|---|---|---|---|---|---|---|---|---|---|
| Pp2a4 | protein phosphatase | Up-regulated under drought on Syngenta array | pp2a4L | ggTTggggCATATCTCCTCgTggT | 9631_chr03_osa1 | 3640223 | 3640247 | - | 619 |
| | | | pp2a4R | TCCTAggAgCTggTTCAAACTgCAA | 9631_chr03_osa1 | 3639579 | 3639604 | + | |
| | | | pp2a4L | | 9638_chr10_osa1 | 13756718 | 13756742 | + | 928 |
| | | | pp2a4R | | 9638_chr10_osa1 | 13757621 | 13757646 | - | |
| DREB1 | drought responsive element binding protein | Transformation of homologue results in enhanced survival under stress; cM 137 locus is closest hit to 16.1 cM locus | DREB1upL | ACgTCAAAACCCAACCCCAACCAT | 9629_chr01_osa1 | 3353301 | 3353325 | + | 988 |
| | | | DREB1upR | gATTgAATCAgggCCATCgCTTTTC | 9629_chr01_osa1 | 3354264 | 3354289 | - | |
| | | | DREB1-16cdL | CCgTTgATTgCTgATAgCCTCCTTgA | 9629_chr01_osa1 | 3354092 | 3354118 | + | 969 |
| | | | DREB1-16cdR | TgAAATATTCCTATTgACCCgCAgCA | 9629_chr01_osa1 | 3355035 | 3355061 | - | |
| | | | DREB1-137cdL | ACACCCAAACCCAACCTCCCAAAAC | 9629_chr01_osa1 | 33545491 | 33545516 | + | 984 |
| | | | DREB1-137cdR | CATCgCCggCATgATCTgTTCTAAT | 9629_chr01_osa1 | 33546450 | 33546475 | - | |
| TPP | Trehalose 6-phosphate phosphatase | Up-regulated under drought on IR64 panicle arrays; 2 loci on CH2 are best top hits to E.coli sequence that on transformation enhances survival | TPPL | ggCACACTgTCgCCTATTgTggATg | 9630_chr02_osa1 | 26336946 | 26336971 | - | 947 |
| | | | TPPR | gTTTACgAgCCgTgCgACCAgTTTC | 9630_chr02_osa1 | 26335974 | 26335999 | + | |
| | | | TPP5082uxL | CgAgCACACACACTCACTCCCTgTC | 9630_chr02_osa1 | 31222704 | 31222729 | + | 998 |
| | | | TPP5082uxR | gTTggAgCTTggTCCCTTgATgTCC | 9630_chr02_osa1 | 31223677 | 31223702 | - | |
| | | | TPP5082aL | ACCgCCTgggTggTgAgTgTTgTA | 9630_chr02_osa1 | 31223053 | 31223077 | + | 1001 |
| | | | TPP5082aR | gTggACggACAggCAgAACTTgTTg | 9630_chr02_osa1 | 31224029 | 31224054 | - | |
| | | | TPP5438uxL | CCTCgTCCgTTCTTATCCACCTCCA | 9630_chr02_osa1 | 33128991 | 33129016 | + | 1014 |
| | | | TPP5438uxR | TgTAgCCgCgCTTggACTggTAATC | 9630_chr02_osa1 | 33129980 | 33130005 | - | |
| | | | TPP5438aL | CAAggTCggTTTCTTCCTCCACTCg | 9630_chr02_osa1 | 33129811 | 33129836 | + | 1001 |
| | | | TPP5438aR | TCATCCCAAACCCAATACCCCAATg | 9630_chr02_osa1 | 33130787 | 33130812 | - | |
| | | | TPP4712aL | CgCACTACATTTCAgCCCACgAgAA | 9635_chr07_osa1 | 27905484 | 27905509 | + | 815 |
| | | | TPP4712aR | TgAAAACTgCTgCTgACCTCCAgACA | 9635_chr07_osa1 | 27906273 | 27906299 | - | |
| VP14 | viviparous14 (9-cis-epoxycarotenoid dioxygenase); ABA biosynthesis | Last-step in ABA synthesis; QTL at same position | VP14-1L | gCCCATTgCAAAgCCCAggTTgTAT | 9640_chr12_osa1 | 25844561 | 25844586 | + | 1064 |
| | | | VP14-1R | CCCATCCTTCTTCTTgCCACACCTC | 9640_chr12_osa1 | 25845600 | 25845625 | - | |
| | | | VP14-2L | TggCAAgAAgAAggATgggCTgAAC | 9640_chr12_osa1 | 25845606 | 25845631 | + | 1013 |
| | | | VP14-2R | TCCACAggTggAAgCAgAAgCAgTC | 9640_chr12_osa1 | 25846594 | 25846619 | - | |
| 14-3-3 | Membrane associated signal cascade | | CG18upL | CCATggCAgTTgCCCTATTgTTCAC | 9630_chr02_osa1 | 21952232 | 21952257 | + | 1009 |
| | | | CG18upR | CTCAgCAggCTgCgACATCTTCACT | 9630_chr02_osa1 | 21953216 | 21953241 | - | |
| | | | CG18-1L | ggCATgCTTgATTTgggACATgAgA | 9630_chr02_osa1 | 21952300 | 21952325 | + | 1005 |
| | | | CG18-1R | TCTCCTCATACCTCTCggCCTgCTC | 9630_chr02_osa1 | 21953280 | 21953305 | - | |
| | | | CG18-cd2L | TgAgCTTTCCCgTgAggAgAATgTg | 9630_chr02_osa1 | 21953237 | 21953262 | + | 838 |
| | | | CG18-cd2R | CAAgATTgCAAgCACggTCAggAgA | 9630_chr02_osa1 | 21954050 | 21954075 | - | |
| MAPK | Transcriptional control of stress response | | MAPk-1L | CACCATCTCCTTCAgCCTCCgTTTC | 9635_chr07_osa1 | 23069803 | 23069828 | + | 975 |
| | | | MAPk-1R | CACACCTCCACCCCAATCAAATTCC | 9635_chr07_osa1 | 23070753 | 23070778 | - | |
| | | | MAPk-2L | ATgggCATgAAgATgAgCCTCTTgg | 9635_chr07_osa1 | 23071388 | 23071413 | + | 1006 |
| | | | MAPk-2R | gggTgCCggCTATggTACgCTAgAT | 9635_chr07_osa1 | 23072369 | 23072394 | - | |
| Extensin | Cell-wall protein for growth expansion | | Ext-L | AggAgAAgATggCgATggCCAATAA | 9638_chr10_osa1 | 17603398 | 17603423 | - | 927 |
| | | | Ext-R | gAgCTCgAgATgCCTgATgATgTCC | 9638_chr10_osa1 | 17602446 | 17602471 | + | |
| TRAB1 | ABA responsive TF | | TRAB1-L | gCAggggTCgATCTACTCACTgACg | 9636_chr08_osa1 | 23061210 | 23061235 | + | 982 |
| | | | TRAB1-R | TCAACACAgCAAggACCCAATCTCg | 9636_chr08_osa1 | 23062167 | 23062192 | - | |
| CDPK | Signal transduction, regulatory cascade | | CDPK-L | ATTTTgATTCCgATCCCTggCCTgT | 9630_chr02_osa1 | 27647309 | 27647334 | + | 1000 |
| | | | CDPK-R | CAAggAACCAggTggCAgACTTTCA | 9630_chr02_osa1 | 27648284 | 27648309 | - | |
| FIERY1 | Inositol polyphosphate phosphatase | $Ca^{+2}$ dependent signal cascade | FIERY1-L | CCTCTCTTgCCACCggCCTTATCTT | 9631_chr03_osa1 | 34051918 | 34051943 | - | 947 |
| | | | FIERY1-R | CAgggAAgAAATTggAAgggCAgTg | 9631_chr03_osa1 | 34050946 | 34050971 | + | |
| SucSase | sucrose synthase | | Suc-L | CgCTCAgCgAgTgCgTgAgACTATT | 9635_chr07_osa1 | 25355903 | 25355928 | + | 1004 |
| | | | Suc-R | ggCgATTgTACACTgCACATgATgg | 9635_chr07_osa1 | 25356882 | 25356907 | - | |
| BZIP | Transcriptional control | | Bzip-L | ATggCATCggAgATgAgCAAgAACg | 9629_chr01_osa1 | 36901422 | 36901447 | + | 991 |
| | | | Bzip-R | TTCggAgCAACATgTgTACCTgCTTT | 9629_chr01_osa1 | 36902387 | 36902413 | - | |
| ADF | Actin depolymerizing factor | up-regulated in proteomics of drought QTL on CH7 is for leaf rolling | ADFCH02aL | CCTCCTTgCAgggCCTgTCTgT | 9630_chr02_osa1 | 26490266 | 26490288 | - | 954 |
| | | | ADFCH02aR | TggCAgTgAggCATTCTCATAAAA | 9630_chr02_osa1 | 26489288 | 26489312 | + | |
| | | | ADFCH02bL | AAggAACAATgCTgCTTTTggTCA | 9630_chr02_osa1 | 26489168 | 26489192 | - | 878 |
| | | | ADFCH02bR | ACCATATgACgCCACggCCTTT | 9630_chr02_osa1 | 26488268 | 26488290 | + | |
| | | | ADFCH07IaL | CTTTggggTggCgACTTgCTTg | 9635_chr07_osa1 | 11676840 | 11676862 | - | 1170 |
| | | | ADFCH07IaR | gCCCggTTTCCCCTCAAAACCT | 9635_chr07_osa1 | 11675648 | 11675670 | + | |
| | | | ADFCH07Ib4L | TgACCCAggTTTTgAggggAAACC | 9635_chr07_osa1 | 11675652 | 11675676 | - | 881 |
| | | | ADFCH07Ib4R | AAgCCgTAgggCACggTgAACA | 9635_chr07_osa1 | 11674749 | 11674771 | + | |

## *Oryza sativa* Panel – TPP cds

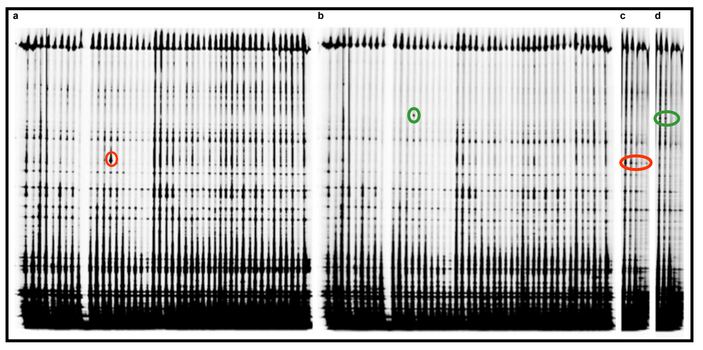



**Fig. 4. EcoTILLING of TPP on *Oryza sativa*.** Trehalose 6-phosphatase (TPP) primers tested on panel of 46 *Oryza sativa* germplasm accessions contrasted to IR64. Circles indicate a clear SNP detected in the IRD700 (**a**) and IRD800 (**b**) channels for KunMin Tsieh Hunan. This germplasm was pooled with IR64 and one or more others at 1:2, 1:4, 1:8, 1:12, and 1:16. Mismatch detected in 1:16 pool for IRD700 (**c**) and in 1:8 pool for IRD800 (**d**).

## Wild Species Panel - DREB1 cds

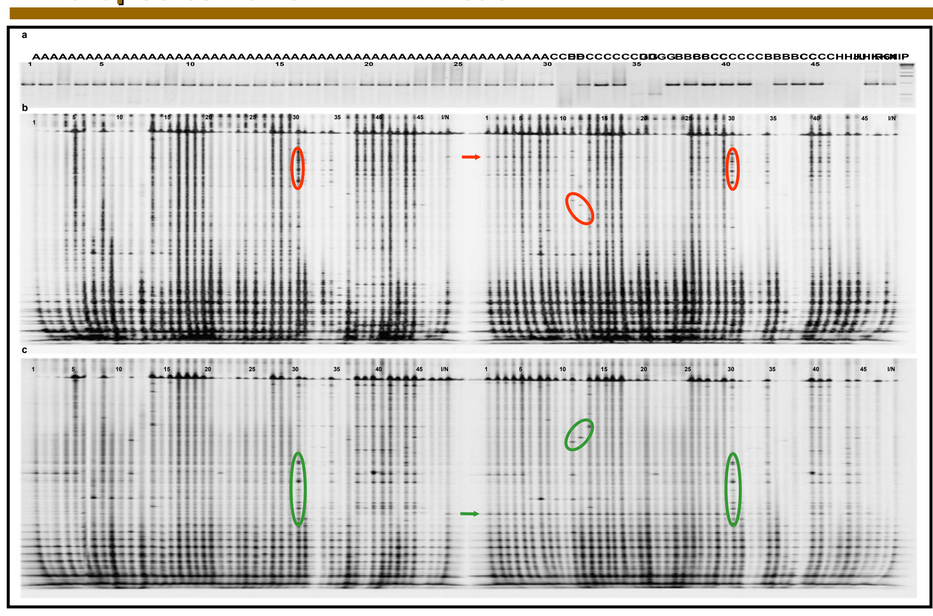



**Fig. 5. EcoTILLING of DREB1 on wild *Oryza*.** DREB1 amplicons of a similar size were produced from 47 wild *Oryza* species using primers designed from Nipponbare (**a**). Mixtures of these products were combined with either IR64 (left set of lanes) or Nipponbare (right set of lanes). The IRD700 channel is shown in panel **b** and the IRD800 in **c**. Circles indicate clear difference present in both channels. Arrows indicate a mismatch present in japonica but absent from most wild and indica.

## Analysis of Distant Contrasts

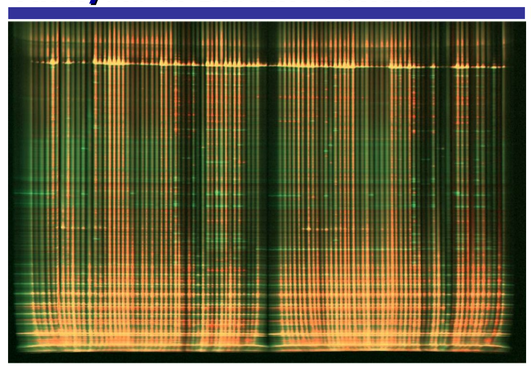


**Fig 6. Strategy for interpreting profiles of distant contrasts (wild/sativa).** Replace RGB colors (IR700 white to red, IR800 white to green), balance intensities for the channels, overlay as objects, merge by difference.

## Sequence Validation of Mismatches

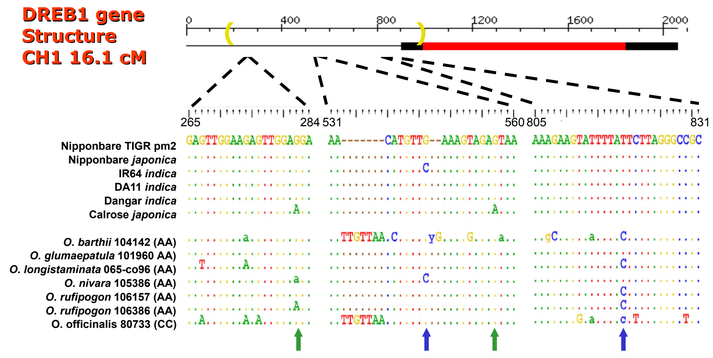



**Fig 7. Sequence verification of putative SNPs in DREB1 upstream region.** In the limited set sequenced so far, every mismatch detected by EcoTILLING has been confirmed.

## Summary of Mismatch detection on up to 192 accessions tested

PP2a4 cds: Tested on 22 Korean *japonica* and tongil varieties contrasted to Nipponbare. Common SNP detected in Milyang30, Taebaek, Samgang, Sindongjin and Milyang23. A second was found in Milyang30 and Milyang23 while a third occurred in Suwon345. SNP detected in SHZ2 by LTH, the parental lines of a breeding population for blast.

TPP cds: Tested on 96 cultivated germplasm by Nipponbare and IR64. Distinct SNPs between Kun Min Tsieh Hunan and Mudgo for either Nipponbare and IR64.

On wild germplasm, amplicons were only obtained for AA genome accessions. A number of haplotypes occurred.

DREB1 cds: Tested on 96 accessions of cultivated rice contrasted to IR64 and Nipponbare. A SNP was detected in IR64 by Nipponbare, occurs in all *indica/japonica* contrasts. Other SNPs were found in Mimidam by IR64 and in Taichung Native 1 by Nipponbare.

Amplicons were obtained from wild relatives as distant as GG genome but not in EE, HHJJ, or HHKK genomes. Many putative SNPs including one present in *japonica* but not in most wild and *indica*.

DREB1 up: Tested on 192 accessions of cultivated rice. SNP detected in IR64 by Nipponbare, occurs in *indica/japonica* contrasts (verified). Mismatch detected in Dagozan by Nipponbare and Dangar by IR64 (verified).

For wilds, amplicons obtained for AA and CC genomes. Contrasts of these to IR64 or Nipponbare identified many putative mismatches (possibly more than for cds region). All samples sequenced verified multiple SNPs

Pooling: Pools up to 1:8 definite are achievable allowing analysis of 768 germplasm per gel. Pools up to 1:16 may be possible with further optimization.

## Conclusions and Future Work

EcoTILLING in rice germplasm across the cultivated and wild gene pools promises to deliver a broad range of putative SNPs. Validation of mismatches by sequencing will play an important role; we will routinely sequence one or more lines having a similar haplotype. Additional reference lines for the different genome types will facilitate scoring of these contrasts. Furthermore, 600 of the 1536 sativa accessions are being phenotyped under severe vegetative stage stress for drought. This data will allow association tests to be performed between DREB1 cds or upstream SNPs and survival, a similar type of screening as done for DREB1 transformants in rice and wheat. We are extending the candidate gene targets to include all AP2 domain transcription factors that co-localize with QTL for yield under drought components.

## References


Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S. High-throughput screening for induced point mutations. *Plant Physiology* **126**:480-484.

Comai L, Young K, Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Henikoff S. (2004). Efficient discovery of DNA polymorphisms in natural populations by ecotilling. *Plant J* **37**:778-786.



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