Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion

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Controversy regarding genetically modified (GM) plants and their potential impact on human health contrasts with the tacit acceptance of other plants that were also modified, but not considered as GM products (e.g., varieties raised through conventional breeding such as mutagenesis). What is beyond the phenotype of these improved plants? Should mutagenized plants be treated differently from transgenics? We have evaluated the extent of transcriptome modification occurring during rice improvement through transgenesis versus mutation breeding. We used oligonucleotide microarrays to analyze gene expression in four different pools of four types of rice plants and respective controls: (*i*) a γ-irradiated stable mutant, (*ii*) the M1 generation of a 100-Gy γ-irradiated plant, (*iii*) a stable transgenic plant obtained for production of an anticancer antibody, and (*iv*) the T1 generation of a transgenic plant produced aiming for abiotic stress improvement, and all of the unmodified original genotypes as controls. We found that the improvement of a plant variety through the acquisition of a new desired trait, using either mutagenesis or transgenesis, may cause stress and thus lead to an altered expression of untargeted genes. In all of the cases studied, the observed alteration was more extensive in mutagenized than in transgenic plants. We propose that the safety assessment of improved plant varieties should be carried out on a case-by-case basis and not simply restricted to foods obtained through genetic engineering.

food safety evaluation | rice | genetically modified organisms | genetic engineering | γ-irradiation

Plant breeding started thousands of years ago, through the unconscious selection of seeds from plants with higher quality and productivity. After sexual plant reproduction was discovered, in the 17th century, people started to use deliberate interbreeding (crossing) of closely or distantly related species to produce new crops with desirable properties (1). With the discovery, in the beginning of the 20th century, that x-rays induced mutations in the fruit fly *Drosophila melanogaster* and barley, plant breeders and geneticists started to use mutagenesis to rapidly create and increase variability in crop species and ultimately change plant traits. The high efficiency of classical mutagenesis has been widely documented (2), and its global impact for crop improvement has also been evaluated (3). Since the establishment of the joint Food and Agriculture Organization/International Atomic Energy Agency, Division of the Nuclear Techniques in Agriculture (www-infocris.iaea.org/MVD), 1,916 crop and legume varieties were released worldwide (40% γ-irradiated).

Since the 1970s, advances in molecular biology have provided the basis for the development of genetic engineering, leading to the next level of genetic gain in crop cultivars. This technology permits the identification, isolation, and transfer of a gene of interest, originated from any type of organism, to plant cells. Transformed plants are then regenerated from these cells through tissue culture (4).

Contrasting with the readily acceptance of food products obtained through conventional plant breeding, the potential benefits of this new technology have been held largely at bay because of the enormous controversy regarding the food safety of the resulting products (5).

Despite the lack of universal methods for evaluating the potentially hazardous effects of genetic modification, Food and Agriculture Organization and the European Food Safety Authority recommendations call for targeted approaches to evaluate macro-, micro-, and anti-nutrients, toxins, allergens, and secondary metabolites. To increase the chances of detecting unintended effects, some molecular profiling methods have also been proposed (6). One of the mentioned profiling techniques is microarrays. This technology allows for monitoring the expression of thousands of genes simultaneously.

In this study, we used expression microarray analyses to monitor the extension of unexpected transcriptome modifications obtained in rice by conventional plant breeding by γ-irradiation as compared with the ones obtained through genetic engineering. We have analyzed four rice lines (two mutagenized and two transgenic ones) and further compared the stable lines against the recently modified ones.

Results and Discussion

Differentially Expressed Genes Increase with Genetic Instability and from Transgenic to Mutant Lines. Hierarchical clustering (Fig. 1) of the microarray data of transgenic, mutagenized, and control plants showed that duplicate samples always grouped together and modified genotypes always grouped with the respective unmodified controls [see supporting information (SI) Fig. 3 for Pearson's correlation between samples]. Despite the different type of breeding strategy used, genetically stable samples [transgenic single-chain variable fragment (ScFv) and mutant Estrela A] are more closely grouped with their corresponding controls than nonstable ones. Additionally, in nonstable lines, transgenic Nipponbare [Nip. genetically modified (GM)] is more closely related to its control than the line obtained through 100-Gy γ-irradiation. As visible in volcano plots (Fig. 2), 11,267 genes showed differential expression in the nonstable mutagenized rice line, whereas only 2,318 genes were detected in the nonstable transgenic line (despite the inserted gene being a transcription factor). The number of affected genes was strongly

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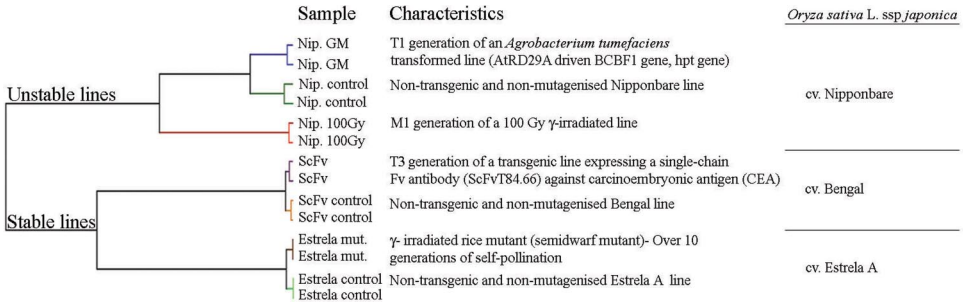

Fig. 1. Plant material used and hierarchical clustering dendrogram of the different samples.

reduced in stable lines (to 51 in the mutant and 25 in the transgenic).

The Analyzed Breeding Strategies Cause Stress, and Plants Respond to It by Modifying Transcription for Several Generations. The list of the differentially expressed genes with a cut-off $P < 0.05$ and with >2-fold change (>2 or <−2) after Log2 transformation (identified as high fold change) is shown in Table 1. For nonstable lines, only the top 50 differentially expressed genes are presented (Table 1). For those two experiments we also present a pie chart with all of the differentially expressed genes with a cut-off $P < 0.05$ and high fold change (we only considered genes whose function could be retrieved) separated by functional categories (SI Fig. 4). The genes listed in Table 1 were identified and analyzed for their functions by using Affymetrix, TIGR rice genome annotation, National Center for Biotechnology Information, UniProt, and Pfam internet resources. We found that in all of the experiments, the acquisition of the desired traits is accompanied by modifications in transcript levels of untargeted stress-related genes (genes whose altered transcription cannot be directly related with the introduced transgenes or desired traits are yellow-shadowed in Table 1).

We have also verified that the stressing event is memorized along several generations, although with a decreasing impact in the number of altered transcripts in each new generation (Table 1). This phenomenon of transgeneration memory of stress could be possibly attributed to epigenetic mechanisms and has been reported by others (7).

Although a complete understanding of plant stress response is far from being reached, various papers reporting molecular and biochemical studies suggest the involvement of at least six classes of genes (a–f): class a, genes implicated in stress/defense signaling-signal perception (several types of receptor-like protein kinases, two-component histidine kinases, G protein-coupled receptors, Ca^{2+}-releasing modules), and signal transduction (protein kinases, protein phosphatases, MAP kinases) (8–10); class b, second messengers, such as reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA), and ethylene, which are involved in the regulatory pathways (11); class c, genes implicated in stress response–ROS network (GST, peroxidases) (12) and the systemic acquired resistance (SAR) response (pathogenesis-related genes) (13); class d, genes implicated in protein modification (methylation, isoprenylation, lipidation, ubiquitination) and scaffolds or adapters [these molecules regulate the activity of stress signaling components (8)]; class e, genes encoding transcription factors that are involved in the temporal and spatial regulation of specific stress genes (14); and class f,

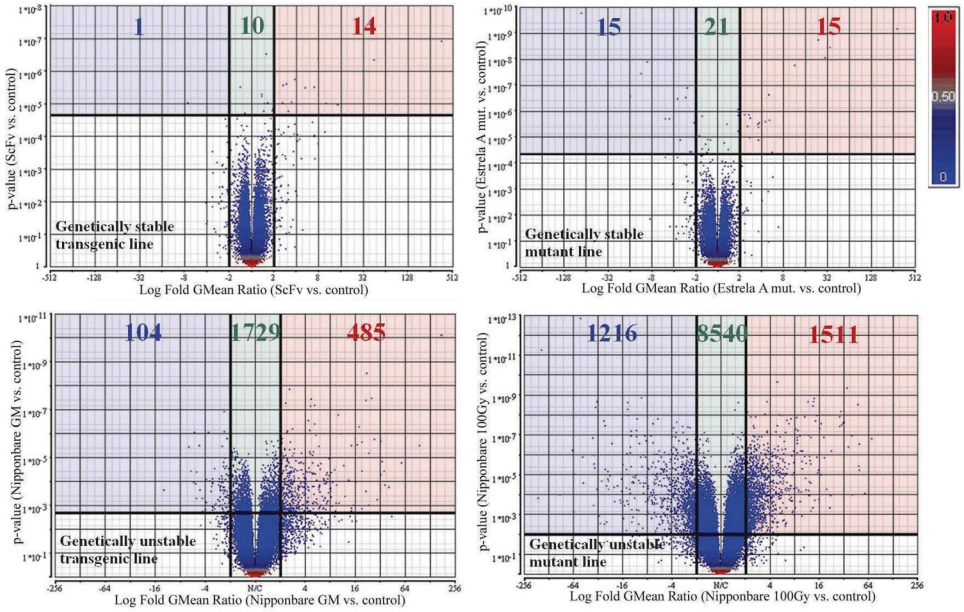

Fig. 2. Volcano plots for differentially expressed genes. Differentially expressed genes appear above the thick horizontal lines. Genes induced >2-fold are on the right of the right vertical lines, and the ones repressed >2-fold are on the left of the left vertical line. The numbers corresponding to the differentially expressed genes induced >2-fold for each experiment (red-shadowed area) are red, and those corresponding to the genes repressed >2-fold (blue-shadowed area) are blue. The green-shadowed area corresponds to differentially expressed genes that were up- or down-regulated <2-fold (green-colored numbers). Blue-colored genes are those with *P* between 0 and 0.5, and red-colored genes are those with *P* between 0.5 and 1.

Table 1. Significantly induced and repressed genes (fold change > 2 or < −2) for each experiment

Putative function	Affymetrix ID	Fold change	Putative function	Affymetrix ID	Fold change
Stable ScFv vs. control					
Signal transduction			**Unknown function**		
C2 domain containing protein	OsAffx.12493.1.S1_at	38.76	Hypothetical protein	OsAffx.26698.1.S1_at	5.24
Wall associated kinase	Os.15516.1.S1_at	-7.26	Hypothetical protein	Os.6148.1.S1_at	3.46
Transposons/ Retrotransposons			Hypothetical protein	Os.51535.1.S1_at	3.80
Retrotransposon gag protein family	Os.50472.2.S1_x_at	9.94	Hypothetical protein	Os.50472.2.S1_s_at	14.46
Retrotransposon protein	Os.21278.1.S1_at	2.66	Expressed protein	Os.57052.1.S1_at	7.81
Transposon protein, putative, Pong sub-class	OsAffx.8070.1.S1_x_at	2.62	Hypothetical protein	Os.27851.1.A1_a_at	7.05
Marker gene			Expressed protein	Os.55384.1.S1_s_at	3.12
Hygromycin B phosphotransferase	RPTR-Os-K01193-1_at	365.25	Expressed protein	Os.8480.1.S1_at	2.96
			Hypothetical protein	OsAffx.16638.1.S1_at	44.91
Stable Estrela A mut. vs. control					
Signal transduction			**Metabolism**		
Putative receptor-like kinase	Os.21180.1.S1_at	3.70	Pyruvate kinase-like protein	Os.18503.1.S1_s_at	289.6
Phosphatidylinositol 3- kinase	Os.19018.2.S1_at	-3.24	Flavonol 3-O-glucosyltransferase	Os.8143.1.S2_at	30.14
Transposons/ Retrotransposons			Adenylyl-sulfate kinase	OsAffx.7376.1.S1_at	3.24
Retrotransposon gag protein	Os.50755.1.S1_at	5.65	Phosphomannose isomerase type I	OsAffx.17841.1.S1_at	2.80
Retrotransposon gag protein	Os.35216.2.S1_x_at	5.14	Nitrilase-associated protein	OsAffx.31420.1.S1_at	-11.10
Retrotransposon gag protein	Os.35216.1.S1_at	3.66	**Unknown function**		
Retrotransposon protein	Os.27592.1.A1_at	-2.42	Expressed protein	Os.14913.1.S1_at	23.93
Stress/defense response/ apoptosis			Expressed protein	Os.22675.1.S2_at	3.66
Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily	Os.11753.1.S1_at	2.71	Expressed protein	Os.9028.1.S1_at	3.59
Acid phosphatase/vanadium-dependent haloperoxidases related	OsAffx.15718.1.S1_at	-2.08	Expressed protein	OsAffx.19321.1.S1_at	2.70
Regulation of transcription			Hypothetical protein	Os.50431.1.S1_at	2.55
Zinc finger, C2H2 type family	OsAffx.28219.1.S1_at	2.35	Expressed protein	Os.7793.1.S1_at	-2.02
AP2 domain	Os.55500.1.S1_at	-3.58	Hypothetical protein	Os.31112.2.S1_at	-2.32
Moving, modifying, storing and degrading proteins			Expressed protein	Os.7723.1.S1_at	-2.61
Leucine Rich Repeat family	Os.26855.1.A1_at	-2.09	Unknown	Os.26863.1.A1_a_at	-4.62
Zinc finger (C3HC4-type RING finger)	Os.51816.1.S1_at	-2.71	Transmembrane BAX inhibitor motif	OsAffx.31431.1.S1_at	-9.14
Ubiquitin carboxyl-terminal hydrolase	Os.7411.1.S1_at	-5.27	Hypothetical protein	Os.18313.1.S1_at	-73.37
F-box domain	Os.21626.1.S1_s_at	-15.98			
T1 Nipponbare GM vs. control					
Signal transduction			**Metabolism**		
EF hand- calcium ion binding	Os.27569.1.S2_at	8.37	Cytochrome P450	Os.51923.1.S1_at	58.25
Putative calcium-dependent protein kinase	Os.12642.1.S1_at	7.88	Cytochrome P450	Os.14105.1.S1_at	35.87
Protein tyrosine kinase	Os.53763.1.A1_at	-12.57	Hypothetical protein (may have a role in ATPase activation)	Os.32889.1.S1_at	32.45
Transposons/ Retrotransposons			Putative cytochrome P450	Os.9067.1.S1_at	9.66
Transposon protein, putative, CACTA, En/Spm sub-class	Os.3808.4.S1_x_at	19.42	Chalcone and stilbene synthases	Os.14616.1.S1_at	8.18
Stress/defense response/ apoptosis			Glycosyl hydrolases family	Os.47778.1.A1_s_at	8.08
S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	Os.11812.1.S1_at	19.20	NADP-dependent oxidoreductase	Os.15917.1.S1_at	7.47
Phenylalanine and histidine ammonia-lyase	Os.10930.1.S1_at	16.27	**Unknown function**		
Trehalose-6-phosphate phosphatase	Os.6092.1.S1_at	12.04	Expressed protein	Os.8823.1.S1_at	175.86
Cysteine proteinase precursor	Os.4181.1.S1_at	14.59	Expressed protein	Os.12498.2.S1_at	29.27
NB-ARC domain containing protein	Os.27538.1.S1_at	11.64	Hypothetical protein	Os.55261.1.S1_at	27.28
Putative pathogenesis-related protein	Os.50993.1.S1_at	8.56	Hypothetical protein	Os.10660.1.S1_at	25.10
Regulation of transcription			Expressed protein	Os.56018.1.S1_at	23.41
Helix-loop-helix DNA-binding domain	Os.6043.1.S1_at	95.47	Expressed protein	Os.10266.1.S1_at	22.11
ZIM motif family protein	Os.46849.1.S1_at	41.23	Expressed protein	Os.56053.1.S1_at	21.66
AP2 domain	OsAffx.17366.1.S1_at	36.24	Expressed protein	Os.51546.1.S1_at	21.65
AP2/ ERF domain	Os.54944.1.S1_at	34.03	Unknown protein	Os.45902.1.A1_at	19.33
CRT/ DRE AP2 domain	Os.51078.1.S1_at	32.59	Expressed protein	OsAffx.12383.1.S1_at	14.36
CBF1 AP2 domain	OsAffx.27442.1.S1_at	23.27	Expressed protein	Os.40325.1.A1_at	13.47
WRKY DNA binding domain	Os29987.2.S1_at	17.79	Hypothetical protein	Os.50587.1.S1_at	10.29
Putative no apical meristem (NAM) protein	OsAffx.20377.1.S1_x_at	17.01	Expressed protein	Os.53407.1.S1_at	10.03
Helix-loop-helix DNA-binding domain	Os.46956.1.S1_at	10.80	Expressed protein	Os.57343.1.S1_at	9.28
ZIM motif family protein	Os.9923.1.S1_s_at	8.34	**Cell structure and biogenesis**		
Zinc finger, C2H2 type	Os.54232.1.S1_at	8.11	Repetitive proline-rich cell wall protein precursor	Os.17946.1.A1_x_at	7.80
Myb-like DNA-binding domain	Os.10172.1.S1_at	7.62	**Moving, modifying, storing and degrading proteins**		
NB-ARC domain	Os.48915.1.S1_at	7.45	Germin-like protein subfamily	Os.51532.1.S1_at	9.05
Transport			Putative germin A	Os.23557.1.A1_at	8.25
Triose-phosphate transporter	Os.32298.1.s1_at	9.43	Subtilisin-like serine proteinase	Os.57054.1.S1_at	8.02
			RING/C3HC4/PHD zinc finger-like	Os.11365.1.S1_at	7.86

genes encoding retrotransposons that represent sensitive markers of plant stress (15).

Genes Whose Altered Transcription Could Be Directly Related to the Introduced Genes or Desired Traits. Some of the differentially expressed genes found in Table 1 can be directly associated with the transgenes introduced or with the desired new characteristics of the modified plant (green shadowed). One example of these differentially expressed genes is the hygromycin B phosphotransferase gene, used as a marker gene in the ScFv stable transgenic line (Table 1).

Some of the differentially expressed genes found in the stable

Table 1. (continued)

Putative function	Affymetrix ID	Fold change	Putative function	Affymetrix ID	Fold change
M1 Nipponbare 100Gy vs. control					
Signal transduction			**Metabolism**		
Apyrase	Os.27696.1.S1-at	47.48	Pyruvate, phosphate dikinase, Chloroplast precursor	OsAffx.13147.1.S1_s_at	21.50
Similar to S receptor kinase	Os.49584.1.S1_at	34.95	Photosystem II protein D2	OsAffx.32349.1.A1_at	-23.14
Putative serine/threonine kinase	Os.26226.1.s1_at	22.34	Endo-1,3;1,4-beta-D-glucanase precursor	Os.8582.1.S1_at	-23.68
Protein tyrosine kinase	Os.53763.1.A1_at	-62.99	ent-kaurene oxidase	Os.57506.1.S1_at	-30.18
Transposons/ Retrotransposons			chloroplast ATP synthase	OsAffx.32209.1.A1_at	-33.38
Transposon protein, putative, CACTA, En/Spm sub-class	OsAffx.22999.1.S1_at	-35.25	NADH ubiquinone oxidoreductase	OsAffx.32259.1.A1_at	-35.69
Stress/defense response/ apoptosis			**Unknown function**		
Glutathione S-transferase	Os.49030.1.A1_s_at	57.56	Hypothetical protein	Os.54340.1.S1_at	70.13
NB-ARC domain	Os.4192.1.S1_at	32.64	Expressed protein	Os.27342.1.S1_a_at	50.07
NB-ARC domain	Os.26992.1.S1_at	30.33	BURP domain	Os.37718.1.S1_at	49.60
Late embryogenesis abundant	Os.12551.1.S1_s_at	21.88	Expressed protein	Os.28200.1.S1_x_at	48.84
Phosphoethanolamine methyltransferase	Os.17921.1.S1_at	-25.28	Hypothetical protein	Os.36176.1.S1_at	40.71
Beta-glucosidase aggregating factor	OsAffx.23382.1.S1_at	-26.34	Hypothetical protein	OsAffx.18924.3.S1_at	36.60
Terpene synthase	Os.27751.1.S1_at	-26.92	Expressed protein	Os.52807.1.S1_at	31.56
Peroxidase family	OsAffx.4250.1.S1_s_at	-28.69	Hypothetical protein	Os.54340.1.S1_x_at	29.39
Putative thionin Osthi1	Os.8655.1.S1_at	-31.02	Hypothetical protein	Os.51266.1.S1_at	28.55
Peroxidase 2 precursor	Os.11556.1.S1_at	-33.15	Hypothetical protein	Os.4887.1.S1_at	28.26
Regulation of transcription			Expressed protein	Os.10760.1.s1_at	-20.57
NB-ARC domain	Os.52240.1.S1_at	40.39	Expressed protein	Os.10659.1.S1_at	-21.63
Myb-like DNA-binding domain	OsAffx.15178.1.S1_s_at	-26.16	Hypothetical protein	OsAffx.27752.1.S1_s_at	-27.03
Transport			Expressed protein	OsAffx.28294.2.S1_x_at	-34.29
Probable aquaporin TIP2.1	Os.12345.1.S1_at	-24.53	Expressed protein	Os.9180.1.S1_at	-36.55
Plastocyanin-like domain	OsAffx.5542.1.S1_s_at	-36.71	Expressed protein	Os.10659.1.S1_at	-49.63
ZIP Zinc transporter	Os.19632.1.S1_at	-37.26	Expressed protein	Os.7108.1.S1_at	-63.80
High affinity nitrate transporter	Os.49093.1.S1_at	-48.79	Hypothetical protein	Os.46728.1.S1_at	-66.79
Lipid transfer	Os.47380.1.S1_at	-93.91	Expressed protein	Os.10038.1.S1_s_at	-157.15
Cell structure and biogenesis			Expressed protein	Os.11315.1.S1_at	-174.92
Glycine-rich cell wall structural protein 2 precursor	Os.46669.1.A1_at	-20.16	**RNA processing**		
			Glycine rich RNA binding protein	Os.46902.1.S1_at	-52.90

For T1 Nipponbare GM vs. control and M1 Nipponbare 100 Gy vs. control, only the top 50 differentially expressed genes are presented. Yellow shading indicates the genes are directly or indirectly related with stress response. Green shading indicates the genes' altered expression can be associated with the introduced genes or desired traits. Red shading indicates up-regulated genes, and blue shading indicates down-regulated genes.

Estrela A line (Table 1) can be eventually related to a reduced indole-3-acetic acid (IAA) content, because the down-regulation of a nitrilase-associated protein was observed in the mutant/dwarfed line (SI Fig. 5*A*). Nitrilases are key enzymes in the biosynthesis of the plant hormone IAA (16), which belongs to the auxin class of plant growth regulators. The enzyme phosphatidylinositol 3-kinase, found in the signal transduction functional group, can also be related to this putative reduced IAA content because the phosphatidylinositol signaling pathway is also involved in plant responses to hormones, like auxins (17). We also found, in this experiment, a group of genes implicated in protein modifications whose altered transcription can be related to the hypothetical reduced IAA content. This group consisted of two proteins involved in ubiquitination: one F-box domain-containing protein and the ubiquitin carboxyl-terminal hidrolase. F-box proteins act as adaptor components of the modular E3 ubiquitin ligase SKP1-CUL1-F-box protein (SCF) complex that functions in phosphorylation-mediated ubiquitination. Protein ubiquitination is a precise strategy for regulating gene function, driving tagged proteins for degradation via the proteasome, and it is suggested as an important control system in desiccation tolerance (18). The down-regulation of these two proteins could be explained by the decreased auxin content because auxin regulates transcription by promoting the degradation of a family of transcriptional repressors known as Aux/IAA proteins, this degradation depending on a ubiquitin protein ligase named SCF(TIR1). In the presence of auxin, the F-box protein TIR1 binds to the Aux/IAA proteins, resulting in their ubiquitination and consequent degradation (19).

The unstable transgenic line Nipponbare GM contains one copy of the barley CBF1 gene (*BCBF1*). C-repeat binding factors (CBFs) specifically interact with the *cis*-acting dehydration-responsive element-DRE (core motif:G/ACCGAC) and control the expression of many stress-inducible genes (20). Although *BCBF1* gene is under the control of a stress-inducible promotor (*AtRD29A*), preliminary experimental results obtained within our team (unpublished data) reveal a leaky expression of the *BCBF1* gene in rice, even in the absence of stress conditions. For this reason, in this particular case, the differential expression of the stress-related genes found in our experiments may be either caused by the stress imposed by the *Agrobacterium*-mediated genetic modification or, at least in part, by the introduced *BCBF1* transcription factor. To clarify this point we decided to analyze the promoter (2 kb upstream of the ATG start codon) of the top 50 differentially expressed genes to search for DRE core motifs. From this study we found that almost all of the top 50 genes (90%) contain several DRE core motifs in their promoter regions (green shadowed in Table 1). Therefore, it seems that the differential expression of these genes may be related mainly to the specific transgene integrated. This result highlights the importance of carefully studying transformants carrying inserted genes coding for transcription factors.

Genes Implicated in Stress/Defense Signaling (Class A). All of the differentially expressed genes found in the signal transduction category, and not related to the transgene's introduction or desired traits, could be related with stress/defense. Thus, in Table 1 we observe in this functional group a wall-associated kinase and a C2 domain-containing protein. In plants, many protein kinases and phosphatases are involved in environmental stress responses (8–10). The C2 domain is a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins

involved in signal transduction or membrane trafficking and thought to be involved in binding calcium-dependent phospholipids (21). This domain has been correlated with stress signaling (22). In the stable mutagenized line we found two signal transduction-associated proteins, both also already characterized as stress/defense associated (Table 1): a receptor-like kinase (9, 10) and a phosphatidylinositol 3-kinase (17). Finally, concerning the unstable mutagenized line (Table 1) we found, in this category, an apyrase (23), a S receptor kinase (9, 10), a putative serine/threonine kinase (24), and a protein tyrosine kinase (25).

Genes Implicated in Stress/Defense/Apoptosis (Class C). In this functional group we found four genes potentially involved in the ROS network, (one GST and three peroxidases) (12), three NB–ARC domain-containing proteins (26), one glyoxalase (27), one late embryogenesis abundant protein (28), one phosphoethanolamine methyltransferase (29), one β-glucosidase (30), one terpene synthase (31), and one putative thionin (32).

Genes Implicated in the Regulation of Transcription (Class E). All of the differentially expressed genes found in this category, and not related with the transgenes' introduction or desired traits, could also be related with stress/defense. Thus, we found one AP2 domain, one zinc finger of the C_2H_2 type family, one WRKY DNA binding domain, a helix–loop–helix DNA-binding domain, a NB-ARC domain, and a Myb-like motif. All of these domain-containing proteins were previously associated with stress response (10, 14, 26, 33).

Transposons/Retrotransposons (Class F). All of the tested plants showed detected alteration in the transcription of genes encoding transposons/retrotransposons. As stated above, these genes are sensitive markers of plant stress (15).

Other Genes That Could also Be Indirectly Related to Stress. We could find in Table 1 some genes whose altered expression can be also indirectly related to stress. Thus, concerning the stable Estrela A mutagenized line (Table 1) the up-regulation of adenylylsulfate kinase can be related to gluthatione-based detoxification of methylglyoxal because this enzyme is involved in the sulfate assimilation pathway required for glutathione production (34). The up-regulation of a putative flavonol 3-O-glucosyltransferase could also be related to stress. This enzyme catalyzes the transfer of glucose from UDP-glucose to a flavonol, one of the last steps in anthocyanin pigment biosynthesis. Anthocyanins are produced by various plants as a result of stress and in senescing foliage as a consequence of the autumn hostile environment (35). Finally, the up-regulation of both pyruvate kinase and phosphomanose isomerase may also be related to stress. Pyruvate kinase is involved in glycolysis, and phosphomanose isomerase catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate, also a component of the glycolytic pathway. The stress induction of glycolysis transcripts has been reported in other studies (36). Regarding the unstable Nipponbare 100-Gy line (Table 1), we also found, in the different functional groups, some genes already associated with stress/defense responses, specifically to NaCl-stress response: several aquaporin and lipid transfer proteins (37), one high-affinity nitrate transporter (38), one glycine-rich cell wall protein (24), and one endo-1,3–1,4-β-D-glucanase (39). The altered expression of the photosynthesis-associated genes encoding photosystem II protein D2 and chloroplast ATP synthase is consistent with the already known effect of γ-irradiation on the photosynthetic activity (40). Pyruvate phosphate dikinase up-regulation, NADH ubiquinone oxidoreductase down-regulation, and down-regulation of the photosynthesis-related genes may be a response to oxidative stress and a way of limiting mitochondrial ROS production while keeping the electron transport chain relatively oxidized (41).

The pie charts we obtained for the genetically unstable lines (SI Fig. 4) are strikingly similar to the one obtained for *Arabidopsis* under various stress conditions (9). This similarity also supports our statement about the relation between genetic modification and stress response.

In conclusion, we have demonstrated that:

(*i*) DNA microarray technology should be considered as a powerful profiling tool for studying altered gene expression induced by different breeding strategies. However, changes in transcriptome do not necessarily correlate with risk. Proteomic studies should thus be performed to provide data on the nature of proteins.

(*ii*) Transcript profile of the stable lines was less altered than that of unstable ones and tested GM plants showed fewer genetic alterations than mutagenized ones. This last difference remains well known for the tested stable lines despite the higher number of self-pollinations for the mutant stable line as compared with the transgenic (10 vs. 3). Although these results may be specific to the particular mutagenized and transgenic plants examined here, they show that transgenic plants may have fewer changes than mutagenized ones.

(*iii*) The improvement of a plant variety through the acquisition of a new desired trait or modification of a previous one (either by genetic engineering or mutagenesis) causes stress and thus has a broad impact on gene expression.

(*iv*) Even several generations after the breeding event, the plant still maintains the "memory" of that incident and responds accordingly.

(*v*) Similar phenotypes do not obligatorily mean similar transcript profiles, which was evident for the unstable mutant line (SI Fig. 5*B*). However, we cannot rule out that under certain environmental conditions different morphology would not become evident.

Finally, we believe that safety assessment of improved plant varieties should be carried out on a case-by-case basis and not simply restricted to foods obtained through genetic engineering.

Materials and Methods

Plant Materials. Two genetically stable *Oryza sativa* L. ssp. *japonica* lines: a γ-irradiated rice mutant (cv. Estrela A) and a well characterized transgenic rice line (cv. Bengal) were used as well as controls (Fig. 1). The stable mutant was obtained in 1988 by γ-irradiation, had already gone >10 generations of self-pollination, and had a mature average height ≈45 cm lower than the wild type (SI Fig. 5*A*). The stable transgenic line, which was already in the third generation of self-pollination after transformation, expresses a ScFV antibody (ScFvT84.66) against carcinoembryonic antigen, a well characterized tumor-associated marker antigen (42).

We have also used two genetically unstable rice lines: the M1 generation of a 100-Gy γ-irradiated line (98% survival after mutagenesis) and the T1 generation of an *Agrobacterium*-transformed transgenic line (both cv. Nipponbare) containing one copy of the *BCBF1* gene driven by the *AtRD29A* promoter from *Arabidopsis* and one copy of the *hpt* II gene (Fig. 1). We used seeds from the same self-pollinated panicle for control and irradiation/transgenesis. The nonstable mutant line chosen for this experiment was the one showing a phenotype more similar to that of the nonirradiated control (SI Fig. 5*B*).

In the case of the transgenic lines, stability was based on the stable inheritance of the introduced transgenes in the homozygous progeny. Regarding the mutagenized plants we have defined as genetically stable plants those that, after mutagenesis, had already gone through several cycles of self-pollination while maintaining the desirable traits.

Seed Treatment and Seedling Growth. Seeds were manually peeled and immersed for 30 min at 50°C in 0.1% Benlate (fungicide). After washing in distilled sterilized water, seeds were surface-disinfected with 70% (vol/vol) ethanol for 1 min and then with a solution of 2% sodium hypochloride with traces of Tween 20, for 30 min, at room temperature. After thorough washing with distilled sterile water seeds were kept overnight in the final wash and then soaked in Yoshida's medium (43) for germination in the dark for 2 days at 28°C. Seedlings were further grown at 28°C for 10 days under a 12-h

photoperiod regime. Yoshida's medium used for the transgenic lines was supplemented with 30 mg/liter of hygromicin B. Twelve-day-old seedlings were frozen in liquid nitrogen and kept at −80°C until RNA extraction.

RNA Extraction and Microarrays. Two pools of six whole seedlings were prepared for each condition under test, and RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was kept at −80°C and sent to the Affymetrix core facility (Instituto Gulbenkian de Ciência, Oeiras, Portugal), where quality-control analysis was carried out before cDNA synthesis from the mRNA [with appropriate oligo(dT) primers], labeling (through synthesis of cRNA with incorporation of biotinylated ribonucleotide analogs), and hybridization to the GeneChip Rice Genome Array (Affymetrix). This array contains probes to query 51,279 transcripts representing two rice subspecies (48,564 *japonica* transcripts and 1,260 transcripts of *indica* subspecies).

Data Analysis. Microarrays data analysis was performed with Partek Genomics Suite software. Affymetrix CEL files were imported by using the Robust Multichip Average method, which involves four steps: background correction of the perfect match values, quintile normalization across all of the chips in the experiment, Log2 transformation, and median polish summarization. The logged data were used for hierarchical cluster analysis and statistical analysis. Hierarchical cluster analysis was performed by using Pearson's dissimilarity product moment correlation coefficient and Ward's algorithm.

For the identification of differentially expressed genes we used ANOVA and a false discovery rate with a 0.05 threshold.

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