UNIVERSIDADE DE BRASÍLIA
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

IDENTIFICAÇÃO DE GENES RESPONSIVOS À SECA EM RAIZ DE ARROZ DE SEQUEIRO (*Oryza sativa* L.)

ALINE RODRIGUES RABELLO

DISSERTAÇÃO DE MESTRADO EM AGRONOMIA

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DISSERTAÇÃO DE MESTRADO SUBMETIDA À FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA DA UNIVERSIDADE DE BRASÍLIA, COMO PARTE DOS REQUISITOS NECESSÁRIOS À OBTENÇÃO DO GRAU DE MESTRE EM AGRONOMIA NA ÁREA DE CONCENTRAÇÃO DE DISCIPLINAS DE PRODUÇÃO SUSTENTÁVEL.

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BRASÍLIA/DF, 3 de dezembro de 2008.

À minha mãe Maria Rodrigues e ao
meu pai Willer Larry, pelo esforço, suor
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LISTA DE ABREVIATURAS E SIGLAS

ABA – Absisic Acid;

ABRE - ABA-responsive element;

AP2 – Transcription factor AP2;

bZIP - basic-region leucine zipper;

CAT – Catalase;

CDPK- Calcium-dependent protein kinase;

CE - Coupling element;

DRE/ CRT – Dehydration responsive element / C-repeat;

DREB - DRE-binding protein;

erd1 – Early responsive to dehydration;

ERF- Ethylene-responsive factor;

EST- Expressed sequence tag;

LEA- Late embryogenesis abundant;

MAPK - Mitogen-activated protein kinase;

MYB e MYC- Transcriptions factor;

NACR - NAC recognition site;

PEG – Polyethylene glycol;

PTP - Protein tyrosine phosphatase;

ROS - Reactive oxygen species;

rps1 - 1-like sequence;

RT-PCR – Reverse transcriptase PCR;

SOD - Superoxide dismutase;

SSH - Suppression Subtractive Hybridization;

ZF-HD - zinc-finger homeodomain.

RESUMO GERAL: IDENTIFICAÇÃO DE GENES RESPONSIVOS À SECA EM RAIZ DE ARROZ DE SEQUEIRO (*Oryza sativa* L.)

O arroz é cultivado sob diferentes formas, no entanto seu cultivo em condições de sequeiro apresenta perdas consideráveis de quantidade e qualidade dos grãos produzidos. A ocupação de novas áreas como o Cerrado, aliado à preferência de grãos, tem exigido o desenvolvimento de novas cultivares mais adaptadas e resistentes a estresses bióticos e abióticos. A disponibilidade da sequência do genoma de arroz torna os estudos de genômica funcional sob condições de estresse hídrico incontestavelmente necessários. Neste estudo, bibliotecas subtrativas de cDNA de raiz de arroz de genótipos contrastantes para a tolerância à seca foram construídas. Foi realizada também uma análise proteômica para a identificação de proteínas diferencialmente expressas. Os resultados obtidos revelaram vários genes possivelmente envolvidos com a tolerância à seca, principalmente os relacionados com a manutenção da integridade da célula, além de proteínas expressas sob estresse hídrico. A identificação desses genes e proteínas contribui para a compreensão do funcionamento global de tolerância a seca em arroz de sequeiro. Atualmente, as variedades de sequeiro têm sido submetidas a intensos trabalhos de melhoramento com o objetivo de transformá-las em variedades adaptáveis e altamente atrativas para o cultivo sob condições aeróbicas. A compreensão dos mecanismos de tolerância a seca em arroz de sequeiro contribuem para auxiliar os programas de melhoramento visando a obtenção de genótipos melhor adaptados a condições de restrição hídrica.

Palavras-chave: *Oryza sativa*, biblioteca de cDNA, proteoma, expressão diferencial, arroz de sequeiro

ABSTRACT: IDENTIFICATION OF DROUGHT-RESPONSIVE GENES IN ROOTS OF UPLAND RICE (*Oryza sativa* L)

Rice is cultivated under different systems, and when it is cultivated in dry conditions, considerable losses in terms of quantity and quality of grain produced are obtained. The occupation of new areas such as Cerrado, combined with grain preferences, has called attention for the need to develop new varieties more adapted and resistent especially to biotic and abiotic stresses. The availability of the genome sequence of rice makes the study of functional genomics under conditions of water stress unquestionably important. In this study, cDNA subtractive libraries of rice roots of genotypes contrasting for the tolerance to drought were constructed. A proteomic analysis to identify proteins differentially expressed was also performed. The results revealed several genes possibly involved in the tolerance to drought, especially those related to maintainance of cell integrity, and proteins expressed under water stress. The identification of these genes and proteins contribute to a better understanding of the global functioning of tolerance to drought in upland rice. Currently the uplands varieties have been subjected to intense genetic improvement aiming to obtain more adapted varieties for the cultivation under aerobic conditions. The understanding of the mechanisms of drought tolerance in upland rice can contribute to the genetic improvement programs to obtain genotypes better adapted to conditions of water restriction.

Key words: *Oryza sativa*; cDNA library; proteome; differential expression; upland rice

INTRODUÇÃO GERAL

O arroz é um cereal que tem relevante papel na nutrição humana. O crescimento da população e a escassez mundial de água apontam para a necessidade de se aumentar a produção através de inovações tecnológicas.

A maior parte do arroz (90%) é cultivada na Ásia (BERNIER et al., 2008). No Brasil, a rizicultura ocupa posição de destaque no agronegócio, sendo que as regiões brasileiras de maior produção de arroz são as regiões Sul e Centro-Oeste, com mais de 80% da produção nacional (CONAB, 2008). Na região Centro-Oeste, destaca-se o cultivo sob sequeiro, que é caracterizado por ser pouco exigente em insumos, e representou um importante impacto na ocupação pioneira da região do Cerrado, iniciada na década de 1960.

O arroz tem, evolutivamente, a particularidade de ser planta semi-aquática. Como resultado, apresenta relativamente pouca adaptabilidade para condições hídricas limitantes e é extremamente sensível ao estresse de seca (LAFITTE et al., 2004). A seca, a alta salinidade e baixas temperaturas são os principais fatores de estresse ambiental que influenciam o crescimento das plantas de arroz e limitam a produtividade no mundo. A redução global na produção de arroz devido à seca é de, em média, 18 milhões de toneladas anualmente (O'TOOLE, 2004). No Brasil, a produção e a produtividade do arroz de sequeiro são comparativamente menor que a de arroz irrigado.

O conceito de tolerância à seca é bastante amplo e está relacionado à capacidade da planta de produzir grãos mesmo sob condições de estresse hídrico em alguma fase do seu desenvolvimento (PRICE et al., 2002). A planta pode utilizar mecanismos fisiológicos e/ou anatômicos para evitar o efeito do estresse hídrico ou para recuperar-

se rapidamente (ZHENG et al., 2000; PRICE et al., 2002). Estudos do germoplasma do arroz indicam a ocorrência de uma escala diversa de mecanismos geneticamente complexos de resistência à seca, incluindo escape (duração curta do ciclo), tentativas de evitar seção estresse (aprofundamento de raízes) e de tolerância (ajuste osmótico) (VENUPRASAD et al., 2002; PASSIOURA, 2006; RANATHUNGE et al., 2004; PRICE et al., 2002; HAZEN et al., 2005; PANTUWAN et al., 2002). As respostas à seca são provavelmente complexas devido a essa multiplicidade de processos físicos ou bioquímicos afetados diretamente.

O controle genético de tolerância à seca é quantitativo, aparentemente envolvendo vários loci distribuídos em diferentes regiões do genoma do arroz, cujos genes são difíceis de identificar por simples análise de segregação. A identificação e o isolamento desses genes de tolerância à seca são fundamentais para o conhecimento do controle genético desta característica e para o desenvolvimento de linhagens capazes de tolerar diferentes níveis de estresse hídrico. O maior desafio relacionado à estresses abióticos reside no alto grau de interação entre genótipo e ambiente, dificultando a seleção de fenótipos que reflitam as diferenças genotípicas (LEUNG, 2008).

O arroz é considerado um organismo modelo para estudos de genética e genômica funcional, uma vez que a sequência completa do genoma está disponível para análise (INTERNATIONAL RICE GENOME SEQUENCING PROJECT, 2005), além de grandes coleções de linhagens mutantes (HIROCHIKA et al., 2004; WU et al., 2005) e de muitos mapeamentos especializados de populações. Este cenário viabiliza o uso de ferramentas que visam caracterizar as funções dos genes relacionados com determinadas condições biológicas, como a tolerância à seca (MACKILL, 2007; COLLARD e MACKILL, 2008).

Várias técnicas têm sido utilizadas para o estudo da genômica funcional, incluindo macro e microarranjos, RT-PCR, SAGE (*Serial Analysis of Gene Expression*), MPSS (*Massive Parallel Signature Sequencing*) e Proteômica, entre outras. Em arroz submetido a condições de estresse abiótico, tem sido identificada uma maior expressão de diversos genes, incluindo genes codificando as proteínas LEA, Glutathione S-transferase, S-adenosylmethionine decarboxylase, S-adenosylmethionine synthetase, proteínas de canais de água, CDPK, peroxidase, calmodulin entre outras (REDDY et al., 2002; WANG et al., 2004; ZENG et al., 2006). Algumas proteínas também têm sido identificadas em arroz como superexpressas sob estresse hídrico e incluem serine hydroxymethyltransferase I, 2Cys peroxiredoxina, *actin depolymerizing factor*, *photosystem II oxygen*, todas envolvendo complexos de proteínas (BERNIER et al., 2008). Embora uma relevante quantidade de genes e proteínas tenha sido reportada, os mecanismos de tolerância à seca ainda não são bem compreendidos.

Atualmente grande ênfase tem sido dada na busca do entendimento dos mecanismos de respostas e de tolerância à seca especialmente para variedades de sequeiro, buscando combinar a maior capacidade de resposta ao estresse aliada ao potencial produtivo de variedades irrigadas.

REVISÃO DE LITERATURA

1 Os diferentes sistemas de produção de arroz

O plantio de arroz pode ser feito sob uma variada gama de condições climáticas, ainda que seja o cereal mais exigente em umidade do solo. Segundo Poehlman e Sleper (1995), os ecossistemas de arroz são classificados em quatro tipos: irrigado, sequeiro de terras baixas (várzeas), inundado e sequeiro de terras altas (*upland*). Os cultivos irrigados e inundados são os predominantes, correspondendo a 55% da área de plantio global, sendo responsáveis por 75% da produção mundial. Se localizam em áreas de solos férteis, não sujeitos à adversidades climáticas e, além disso, recebem o maior investimento (KHUSH, 1997).

O arroz de terras altas é geralmente o sistema mais propenso à seca (BABU et al., 2003) Segundo Stone (1986), sob condições de déficit hídrico, a cultura apresenta reduções no número de grãos cheios por panícula e no seu peso, no rendimento total de matéria seca, na altura da planta e no índice de colheita, com aumento na porcentagem de grãos vazios. Esses efeitos adversos podem variar de acordo com a idade da planta e a interferência de outras condições ambientais

No Brasil, o arroz é plantado, predominantemente, sob duas formas distintas: sequeiro, que ocorre de outubro a dezembro em terras altas, não irrigadas artificialmente e dependente da precipitação pluvial, com alto risco climático; e sob inundação, com elevada exigência em relação à quantidade de água.

A rizicultura irrigada é responsável por 65% da produção nacional, porém, com alto custo de produção. O cultivo de sequeiro tem sido relevante na região Centro-Oeste, no entanto se apresenta sujeito à restrição hídrica. No período de 1986-2001, a

área de sequeiro no Brasil declinou de 4.8 para 1.9 milhões de hectares (61%), enquanto a produção diminuiu de 5,4 para 3,3 milhões de toneladas, representando uma redução de 40% (PINHEIRO et al., 2006). Para a safra de 2008/09 a maior rentabilidade de outros produtos e a diminuição da abertura de novas áreas, reduziu a expectativa da área plantada com arroz nesta safra. Usando variedades tradicionais de arroz de sequeiro sob manejo adequado, rendimentos superiores a 4 t / ha, foram alcançados desde a década de 1980 (SEGUY, 1988, SEGUY et al., 1989).

Novas variedades com alto potencial produtivo e superior qualidade de grãos estão sendo estudadas e especificamente para áreas de sequeiro (BRESEGHELLO et al., 1998). Melhoramento genético tem resultado no desenvolvimento de variedades com maior índice de colheita e capacidade de respostas, apresentando maior potencial produtivo. Atualmente, esforços têm sido realizados no Brasil e em vários países Asiáticos, para o melhoramento do "arroz aeróbico" (ATLIN et al., 2006).

2 O sistema radicular de arroz durante o estresse hídrico

O arroz é uma cultura notoriamente suscetível à seca, em parte devido ao seu pequeno sistema radicular, rápido fechamento estomatal e senescência foliar durante um leve estresse hídrico (HIRASAWA, 1999).

Ao comparar o sistema radicular do arroz com o de outros cereais, é evidente que suas raízes são muito mal adaptadas às condições limitantes de água (FUKAI e INTHAPAN, 1988). As raízes apresentam uma menor condutividade radial de água do que a maioria das espécies herbáceas devido à existência de um extenso aerênquima, de barreiras apoplásticas, e de uma endoderme restritiva (MIYAMOTO et al., 2001; RANATHUNGE et al., 2004).

Vários fatores que contribuem potencialmente para a resistência à seca em arroz foram relatados (FUKAI e COOPER, 1995; NGUYEN et al., 1997, PRICE e COURTOIS, 1999) enfatizando as raízes. Entretanto, suas respostas ao ambiente são ainda mal compreendidas, uma vez que as raízes são intrinsecamente difíceis de estudar (PRICE et al., 2002).

Durante a restrição hídrica, as raízes podem apresentar tentativas de evitar a seção do estresse, realizando um aprofundamento no perfil do solo, aumentando água disponível à cultura. A habilidade de manutenção do crescimento é uma resposta importante da raiz sob condições de estresse hídrico e representa um benefício para a planta, pois consiste em uma forma de melhorar a aquisição de água em situações restritivas. Esse crescimento da raiz encontra-se sob controle genético (O' TOOLE e BLAND, 1987; SPONCHIADO et al., 1989).

Em situações onde a divisão e a expansão das células são inibidas diretamente pelo estresse de água, outro mecanismo utilizado pelas raízes consiste na alocação de fotoassimilados, antes utilizados para o crescimento, em estratégias de proteção da célula (ZHU, 2002)

A raiz é a primeira a detectar as condições restritivas de seca e sintetizar sinais químicos para a resposta antecipada da planta ao estresse (WILKINSON e DAVIES, 2002). Isso porque, as respostas nas folhas devem ser provocadas rapidamente para impedir que a estrutura fotossintética seja danificada irreversivelmente. Reddy et al. (2002) através da construção de bibliotecas de raiz, encontrou a expressão de muitas classes de kinases, corroborando com o fato de a raiz ser um importante órgão na percepção e na sinalização do estresse.

Alguns estudos visando à identificação de genes e proteínas expressas em raiz durante o estresse hídrico têm sido realizados. Ozturk et al. (2002) identificou

transcritos específicos de raiz, entre eles glioxalases (HB102H12; AB107042), non-LTR retroelemento da transcriptase reversa (HC104F03; AC006300), a subunidade regulatória da fosfatase 2A e proteínas de canal de água, entre outros. Além disso, diversos transcritos superexpressos em raiz constituem possíveis intermediários na transdução de sinal ou produtores de mensageiros secundários.

Segundo Cho et al. (2007), proteínas antioxidantes, de respiração celular, relacionadas à defesa, modificadoras e chaperoninas, além de proteína de biossíntese de membrana representam 74% do total encontrado na raiz durante o estresse hídrico.

Ozturk et al. (2002) em seu estudo, mostrou que a maior parte do perfil de transcritos de raiz de cevada (aproximadamente 30%), está na categoria de proteínas "não classificadas". Suas homologias são desconhecidas, ou correspondem a ESTs hipotéticos com função desconhecida em outros organismos. Além disso, uma alta porcentagem (10,6%) correspondem a ESTs sem homólogos no banco de dados.

A maior quantidade de genes categorizados como "*no Hit*" encontrado em raízes em relação às folhas, reflete a relativa escassez de estudos com raízes de plantas (OZTURK et al., 2002).

3 Mecanismos envolvidos nas respostas ao estresse hídrico

3.1 Percepção do sinal

O déficit hídrico, em plantas, inicia um complexo de respostas, começando com a percepção do estresse, o qual desencadeia uma seqüência de eventos moleculares que é finalizada em vários níveis de respostas fisiológicas, metabólicas e

de desenvolvimento (BRAY, 1993). O caminho genérico da transdução do sinal, segundo Xiong et al. (2002), inicia-se na percepção.

Uma planta submetida ao déficit hídrico apresenta uma mudança no volume das células individuais da raiz, alterando, assim, o potencial de pressão (tensão física; turgor) e o potencial osmótico (concentração) dessas células.

As alterações no potencial de pressão inicial induzem alterações na membrana celular e em vários de seus componentes, como os fosfolipídeos (MUNNIK et al., 1998). Ademais, modificações na conformação de proteínas sensoriais críticas embebidas nas membranas celulares, alteram a continuidade entre a parede celular e a membrana celular (HARE et al., 1996; SHINOZAKI e YAMAGUCHI-SHINOZAKI, 1997, 1999).

A mudança no potencial osmótico durante a deficiência hídrica provoca um acúmulo de uma variedade de componentes osmoliticamente ativos, provocando a entrada de água na célula a fim de se manter o turgor e a sobrevivência celular sob condições de baixo status de água (ZHANG et al., 1999).

No caso de seca, a percepção do estresse hídrico ocorre por sensoriamento da tensão da membrana. A membrana plasmática tem, portanto, um papel chave na percepção e na transmissão da informação externa, devido ao fato de sensores capazes de detectar alterações em sua conformação estarem nela localizados. Diante da complexidade da percepção do sinal, é importante que sensores múltiplos percebam a condição de estresse e sinalizem todas as etapas subseqüentes (XIONG et al., 2002).

3.2 Transdução do sinal

A percepção do estresse pela raiz induz a produção de compostos químicos que serão transportados para a parte aérea onde se tornam ativadores de resposta. O conhecimento da transdução do sinal requer a coordenação espacial e temporal apropriada de todas as moléculas sinalizadoras envolvidas. Segundo Jia et al. (2002), a sinalização celular de deficiência hídrica envolve diversas moléculas e íons, em especial o hormônio Ácido Abscísico (ABA), com duplo papel na regulação fisiológica. Além de sinalizador e ativador de genes, em altas concentrações ajuda na sobrevivência da planta pela inibição da abertura estomática e crescimento e em baixas concentrações tem importante papel na manutenção do crescimento em vários órgãos, como na raiz primária (CHENG et al., 2002; FINKELSTEIN et al., 2002; SHARP et al., 2000; SPOLLEN et al., 2000).

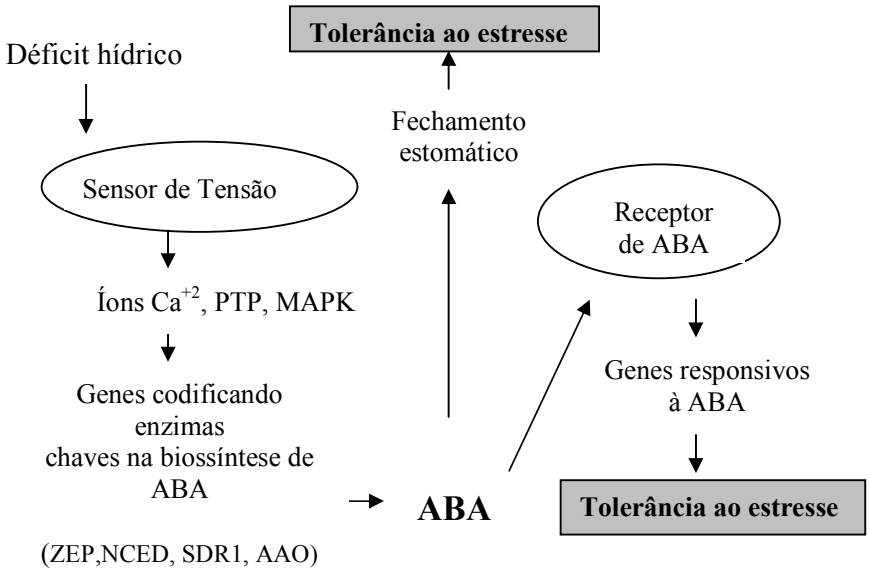

Figura 1: Sinalização celular de deficiência hídrica (modificado a partir de Jia et al., 2002).

Os mecanismos de sensoriamento do estresse resultam na produção de Ácido Abscícico (ABA), o qual ativa as vias de sinalização (Fig. 1) (ZHANG et al., 2006). Íons Ca^{2+}, a proteína tirosina phosphatases (PTP) e proteínas kinases ativadas por mitógeno (MAPKs), são possíveis componentes sinalizadores no início da percepção da desidratação.

Em condições de déficit hídrico, alterações na conformação da membrana celular provocam mudanças em canais de transporte ativados por pressão, como por exemplo, os canais responsáveis por influxo de Ca^{2+} (XIONG et al., 2002). O aumento de Ca^{2+} citosólico facilita a geração das moléculas sinalizadoras secundárias. Pandey et al. (2004) demonstrou que o sensor de cálcio calcineurin B-like 9 modula a sensibilidade e a biossíntese de ácido abscísico em *Arabidopsis*.

ZEP, NCED, SDR1 e AAO são genes codificando enzimas chaves na biossíntese de ABA (CHENG et al., 2002; BITTER et al., 2001).

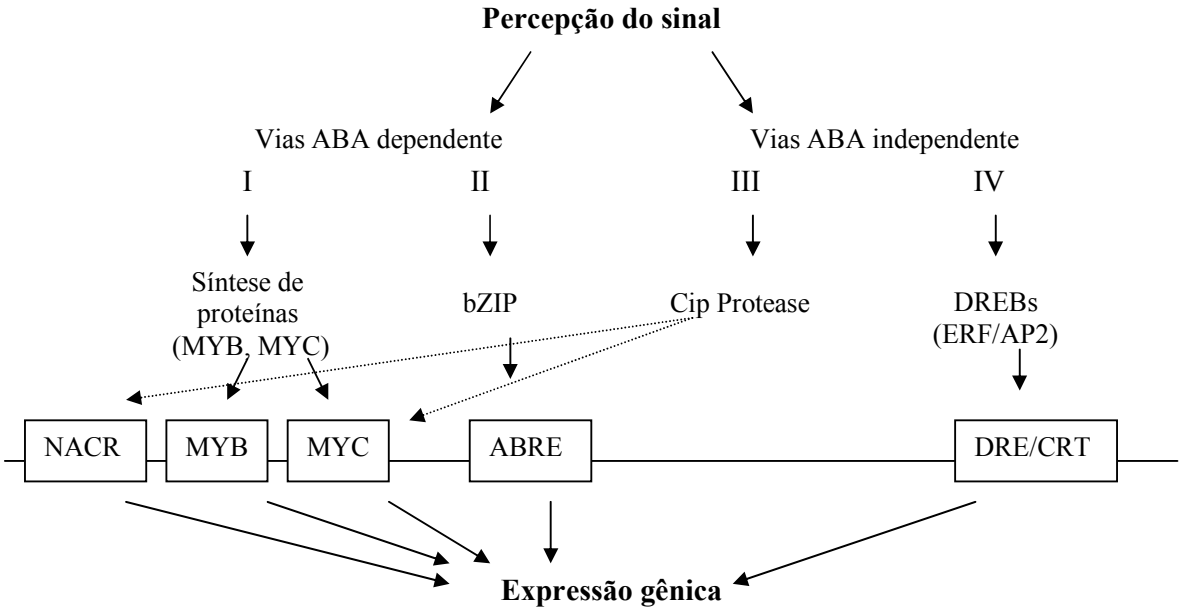

Figura 2: Via de transdução de sinais ao estresse hídrico desde a percepção até a expressão gênica (adaptado de YAMAGUCHI-SHINOZAKI E SHINOZAKI, 2005).

Até o momento foram descritas quatro vias de transdução de sinal envolvidas na resposta da planta ao déficit hídrico (Fig. 2): duas vias são ABA dependentes (I e II) e as outras duas ABA independentes (III e IV) (SHINOZAKI e YAMAGUCHI-SHINOZAKI, 2000; SEKI et al., 2003;YAMAGUCHI-SHINOZAKI e SHINOZAKI, 2005). O ABA é, sem dúvida, o fitohormônio mais diretamente envolvido com a transdução do sinal.

Na sinalização ABA dependente, pode ocorrer duas diferentes rotas, requerendo ou não a síntese de novas proteínas (BRAY, 2002). Na rota onde não é exigida a síntese de novas proteínas, o promotor de todos os genes responsivos a ABA possui o domínio ABRE, que funciona como um elemento em *cis*, que se liga aos fatores de transcrição bZIP para a expressão do gene (UNO et al., 2000). A seqüência ABRE foi primeiramente identificada como um elemento em *cis*, no gene *RAB*16 de arroz, expresso em tecidos desidratados e em sementes em fase de maturação (MUNDY et al., 1990).

Na rota onde a síntese de novas proteínas é um pré-requisito, os genes não possuem o domínio ABRE e o elemento responsivo a ABA combinam-se com fatores de transcrição da família MYC. A ativação de genes de fatores de transcrição da família MYC e a síntese de fatores de transcrição devem preceder a ativação de qualquer gene induzido por ABA (BRAY, 2002).

A principal rota ABA independente apresenta um elemento em *cis* conhecido como *DRE/C-repeat element* (LEUNG e GIRAUDAT, 1998). A região DRE induz a transcrição na presença de proteínas da família DREB1 (em resposta a estresse de frio) ou da família DREB2 (em resposta a salinidade ou seca) (NEBRASKA et al., 2003). Transgênicos de Arabidopsis superexpressando a proteína DREB1/CBF ligada ao

DRE/CRT, apresentaram mudança na expressão de mais de 40 genes induzidos por estresse como frio, seca e salinidade (FOWLER e THOMASHOW, 2002; MARUYAMA et al., 2004). Plantas de arroz com expressão constitutiva de CBF3/DREB1A (CBF3) mostraram tolerância à seca, bem como à alta salinidade, ativando genes que parecem estar envolvidos na aclimatação às condições de estresse (OH et al., 2005).

O ABA apresenta muitas outras funções em plantas sob estresse hídrico. Quando ocorre alcalinização do xilema, o ABA é transportado e absorvido pelas células-guarda induzindo o fechamento estomático (DAVIES et al., 2002; BECKER et al., 2003). Os movimentos estomáticos são induzidos por mudanças na turgescência das células-guarda, que são mediados por seus conteúdos de íons e solutos orgânicos (ISRAELSSON et al., 2006). O controle estomático impede que a planta perca altos índices de água, mantendo seu turgor e contrabalanceando a inibição do seu crescimento.

O acúmulo de ABA também é requerido para a manutenção da taxa de alongamento da raiz em condições de baixo potencial de água do solo. Segundo Sharp e Lenoble (2002), a interferência do ABA no crescimento da raiz e da parte aérea é indireta através do seu efeito inibitório na síntese de etileno. Desta forma, o crescimento da parte aérea é interrompido devido à ação do etileno, uma vez que a concentração de ABA nessa área é insuficiente para impedir a síntese do etileno. Nas raízes, a alta concentração de ABA previne a inibição do crescimento mediada pelo etileno.

Christmann et al. (2005) em experimentos em *Arabidopsis* utilizando um sistema de gene repórter em plantas submetidas à estresse hídrico induzido por ABA,

revelou a expressão do gene repórter em brotos, supondo que o estresse hídrico poderia induzir a síntese de ABA em folhas e não apenas no interior de raízes.

Outros hormônios também têm um papel importante na sinalização do estresse, como citocininas, etileno e ácido jasmônico (GAZZARRINI e MCCOURT, 2001; CHAVES et al., 2004). Esses diferentes hormônios podem interagir na sinalização e na regulação da tolerância ao estresse nas plantas. Por exemplo, foi demonstrado que o etileno realça a ação do ABA nas sementes (GAZZARRINI e MCCOURT, 2001), mas pode neutralizar efeitos do ABA em tecidos vegetativos sob o estresse da seca (SPOLLEN et al., 2000).

Espécies reativas de oxigênio (*ROS*) são elementos que, frequentemente iniciam uma cascata de eventos moleculares. Entre as *ROS* induzidas pela seca, estão, o superóxido, o peróxido de hidrogênio, e os radicais de hidroxila (HASEGAWA e BRESSAN, 2000). Pesquisas realizadas com duplos mutantes em *Arabidopsis* evidenciaram o papel de *ROS* como mensageiras secundárias na sinalização por ABA em células-guarda (PEI et al., 2000; KWAK et al., 2003). Foi reportado que o ABA e o Metil Jasmonato (MJ) promoveram o fechamento estomático através de um mecanismo dependente da produção de *ROS* e da alcalinização citoplasmática (SUHITA et al., 2004).

Fosfatos de inositol e espécies reativas de oxigênio induzem também, a fosforilação de proteínas. A fosforilação estimula a transcrição de proteínas alvo envolvidas diretamente nos fatores celulares de proteção. Além disso, as *ROS* podem estar relacionadas a fatores de transcrição controlando a regulação de genes específicos induzidos pelo estresse.

3.3 Ativação dos genes alvo

A ativação transcricional de alguns dos genes de resposta ao estresse ocorre, na maior parte das vezes, através de promotores que contêm o ABRE - *ABA-Responsive Element* e o DRE/CRT - *Drought Response Element/C-repeat* (YAMAGUCHI-SHINOZAKI E SHINOZAKI, 1994; STOCKINGER et al., 1997) (Fig. 3).

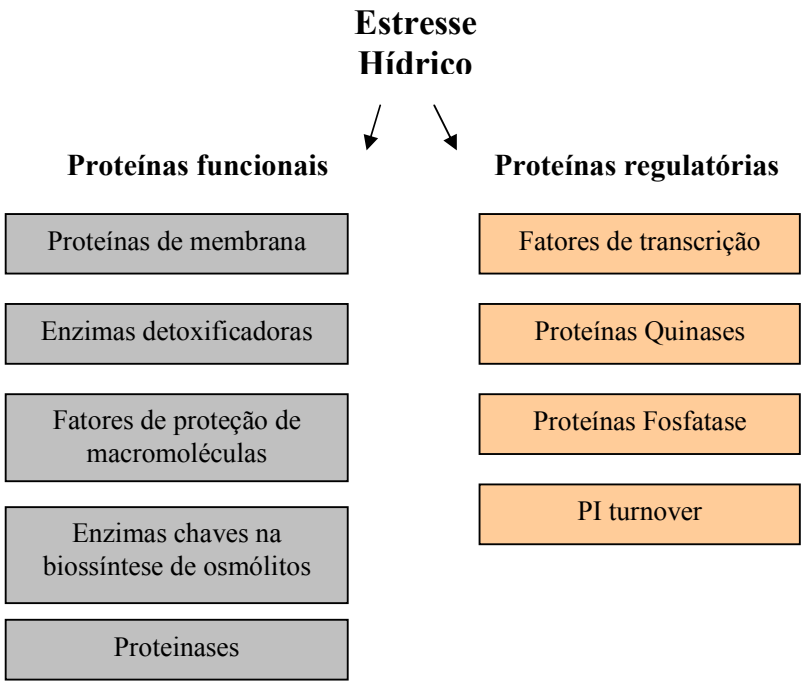

Figura 3: Genes induzidos durante o estresse hídrico e suas possíveis funções na resposta e tolerância ao estresse (Adaptado SHINOZAKI E YAMAGUCHI-SHINOZAKI, 2000).

Os genes induzidos em condições de restrição hídrica podem estar envolvidos na regulação da expressão de outros genes ou apresentar função direta na tolerância ao estresse. De uma forma geral, esses genes ativados estão ligados a uma resposta adaptativa à uma condição de restrição hídrica, promovendo sinalização, tolerância da

célula à desidratação, funções de proteção no citoplasma, alterações no potencial osmótico celular para aumentar a absorção de água, controle da acumulação de íons e metabolização de compostos gerados pelo estresse (BRAY, 1993, 1997; NEPOMUCENO et al., 2000).

Genes com função relacionada ao movimento de água na célula e com o transporte de íons, como os que codificam aquaporinas e transportadores de íons ligados à membranas, são induzidos em arroz (BLUMWALD et al., 2000). Estudos da proteína de canal de água RWC3 em arroz submetido ao tratamento de estresse hídrico utilizando polietileno glicol (PEG) revelou uma maior expressão do mRNA da RWC3 em cultivares de arroz de sequeiro sob estresse, ao passo que em cultivares de arroz irrigado, a mudança de expressão não foi significativa. Além disso, a superexpressão de RWC3 em cultivares transgênicas de arroz irrigado, levou a uma maior condutividade hidráulica na raiz, maior potencial de água na folha e transpiração relativa acumulada, evidenciando o papel do RWC3 na prevenção da seca em arroz (LIAN et al., 2004). Zhang et al. (2008) isolou seis novos genes de aquaporinas em trigo (*TaAQP1-TaAQP6)* (*Triticum aestivum* aquaporinas *AQP)* propondo que estas participem da redistribuição interna de água em trigo durante o estresse osmótico.

Entre os genes que apresentam a função de proteção das membranas e das proteínas, destacam-se os que codificam as proteínas de choque térmico (*Hsps*), as chaperoninas, as proteínas LEA (*late embryogenesis abundant*) (INGRAM e BARTELS, 1999; BRAY et al., 1997), os osmoprotetores, e os detoxificadores (BOHNERT e SHEVELEVA, 1998).

Babu et al. (2004) em estudo de plantas transgênicas de arroz expressando o gene LEA de trigo, *HVA1*, sob prolongado ciclo de estresse hídrico, demonstrou que as plantas transgênicas mantiveram maior conteúdo relativo de água na folha e menor

diminuição no crescimento das plantas sob o estresse em comparação com plantas não-transgênica. Isto indica um melhor desempenho de plantas transgênicas de arroz, protegendo a membrana celular de lesões durante o estresse hídrico.

Um estudo recente de proteínas de choque térmico, revelou que a superprodução de *sHSP17.7*, uma pequena proteína de choque térmico de arroz, pode aumentar a tolerância à seca em plântulas transgênicas (SATO e YOKOYA, 2008).

Entre os genes codificando enzimas de detoxificação (*ROS scavenging)* expressos durante o estresse hídrico, estão os que codificam as superóxido dismutases (SODs), primeiros antioxidantes de defesa enzimática. Em arroz dois genes de Cu/Zn – SODs (citosólico e plasmático), um gene de Mn-SODs (mitocondrial) e Fe-SODs (cloroplástico) foram descritos e estudados extensivamente (SAKAMOTO et al., 1995; KAMINAKA et al., 1997, 1999)

Junto com SODs, CATs (Catalases) constituem a linha de frente de defesa contra *ROS*, convertendo H_2O_2 em água. Em arroz, os genes *Cat* A, *Cat* B e *Cat* C têm sido associados à proteção contra oxidação (BENAVENTE-MENEZES et al., 2004). Recentemente, foi reportado que a PHGPx like, enzima envolvida na detoxificação, é induzida por peróxido de hidrogênio e alumínio em arroz (LI et al., 2000).

O balanceamento osmótico, essencial para a sobrevivência das células durante o estresse hídrico, é realizado por osmólitos que se acumulam nas células. (MANSCHADI et al., 2006; SHARP et al., 2004). Osmólitos compatíveis incluem aminoácidos como a prolina, compostos quaternários de amônio (glicina betaína, prolina betaína, B-alanina betaína, e cholina-*0*-sulfato) e o composto terciário de sulfato, 3-dimetilsulfoniopropionato (DMSP) (KISHOR et al., 2005). A enzima *P5CS(*Δ^1-pirrolina-5-caboxilato Sintetase) é limitante para a síntese de prolina, sendo sensível à inibição por retroalimentação. O gene *P5CS* de *Vigna aconitifolia* foi

introduzido em arroz sob o controle de um promotor ABA-induzido. Plantas transgênicas de arroz acumularam 2,5 vezes mais prolina que as plantas controle sob condição de estresse. Resultados preliminares mostraram que a expressão induzida do gene *P5CS* na segunda geração de plantas transgênicas de arroz proporcionou um aumento da biomassa, refletindo no aumento do peso fresco de raiz e parte aérea sob condições de estresse hídrico e salino (ZHU et al., 1998).

Entre os genes que codificam enzimas regulatórias, destacam-se os envolvidos na regulação transcricional. Fu et al. (2007) através da técnica de SSH (Supressão da Hibridização Subtrativa) e de RT-PCR de folhas de plântulas de arroz observou que o percentual de 4.6% dos 316 clones únicos de cDNA candidatos identificados estavam relacionados com a regulação transcricional, entre eles WRKY12 TF, Zinc Wnger protein, HD-zip transcription factor.

Membros de DREB ou CBF, MYB, bZIP, e família de dedos-de-zinco tem sido bem caracterizados com papel na regulação de defesas de plantas e respostas a estresse (ZHU, 2002, SEKI et al., 2003). Numerosos estudos sugerem que a superexpressão de alguns fatores de transcrição induzidos pelo estresse como *DREB1A, CBF4*, *SCOF*, *Tsi*, e *OSISAP1* podem aumentar a tolerância a seca, salinidade e a baixa temperatura em *Arabidopsis* ou outras espécies de plantas *(*KASUGA et al., 1999, HAAKE et al., 2002).

Hu et al. (2006) relatou que a superexpressão de SNAC1, gene NAC responsivo ao estresse, regula a expressão de outros genes relacionados ao estresse, aumentando significativamente a resistência à seca em arroz transgênico em áreas sob condições severas de estresse hídrico durante o estágio reprodutivo.

4 Metodologias utilizadas para a identificação de genes e proteínas envolvidas na tolerância a seca

Diante da grande velocidade com que os genomas de diferentes organismos são seqüenciados, ocorre um acúmulo exponencial de seqüências gênicas depositadas em bancos de dados públicos mundiais. Entretanto, essa grande quantidade de seqüências disponíveis tem levado a uma demanda por metodologias que permitam a identificação funcional dos genes, além da elucidação dos padrões de expressão.

O arroz foi o primeiro cereal a ser seqüenciado (YU et al., 2002), o que representa um grande impacto na agricultura. Esta cultura também tem emergido como uma espécie modelo para o estudo do genoma de outras plantas como milho e sorgo, que são maiores e mais complexos (INTERNATIONAL RICE GENOME SEQUENCING PROJECT, 2005). Isto se deve a sua colinearidade genômica com outras gramíneas que compartilham um ancestral comum.

A genômica funcional aparece então, como uma abordagem extremamente poderosa na identificação de funções de genes novos, na reconstrução de redes de controle genético e no entendimento de processos biológicos no nível molecular (LIVESEY e HUNT, 2002). Diversas metodologias têm sido aplicadas atualmente neste propósito.

A tecnologia de Microarray baseada no RNA, por exemplo, está sendo utilizada para analisar os caminhos de resistência à seca em arroz, através da comparação dos níveis de expressão entre genótipos resistentes e suscetíveis (KATHIRESAN et al., 2006). Esta tecnologia permitiu a identificação de 351 genes em *Arabidopsis* que são induzidos por estresse de seca ou frio ou alta salinidade, e também a identificação de um grupo de genes induzidos pelos três estresses ambientais (SEKI et al., 2002). Genes envolvidos na resistência à múltiplos estresses podem ser utilizados para o

desenvolvimento de variedades vegetais com resistência múltipla (VALLIYODAN e NGUYEN, 2006).

Estudos proteômicos em arroz também têm sido frequentemente realizados para analisar respostas a estresses bióticos e abióticos. Salekdeh et al. (2002) identificaram proteínas que apresentaram um perfil de expressão diferente durante o estresse hídrico. GSH (Glutationa)-dependente dehidroascorbato redutase (DHA), apresentou um acréscimo de 60% em abundância, *S-like ribonuclease*, teve a expressão até 4.5 vezes maior em variedades tolerantes quando o arroz foi submetido a déficit hídrico. Entre as proteínas que tiveram um aumento da concentração durante a seca, encontram-se enzimas do metabolismo de carbono, fator de despolimerização da actina (células envolvidas na forma de regulação), rubisco activase e proteína EF-Tu, além de várias outras proteínas que correspondem a pontos de convergência observados na resposta a vários estresses abióticos, como a peróxido dismutase (KOMATSU e TANAKA, 2005).

Os programas de melhoramento de arroz, entretanto, fizeram poucos progressos em relação à tolerância à seca. Isto pode ser explicado pelo fato de que esta é uma característica controlada por muitos genes com diferentes efeitos, e apresenta muita plasticidade. Além disso, a seca envolve uma interação entre os genes associados ao potencial produtivo e os genes de resistência ao estresse (PRICE et al., 2002). No entanto, segundo Salekdeh et al. (2002) o melhoramento para tolerância à seca tem sido mais complexo devido a grandes diferenças entre cultivares “de terras altas” e “de terras baixas” no que se refere a características de raiz, mecanismos de tolerância e adaptação a condições aeróbicas e anaeróbicas, tornando assim, inevitável o conhecimento específico de cada uma dessas circunstâncias.

OBJETIVO GERAL

O objetivo deste trabalho foi identificar genes e proteínas do sistema radicular de arroz de sequeiro, expressos sob condições de estresse hídrico, em dois genótipos contrastantes para a tolerância à seca.

OBJETIVOS ESPECÍFICOS

1. Construção de bibliotecas subtrativas de cDNA de raízes dos genótipos suscetível e tolerante a seca;
2. Identificação de genes diferencialmente expressos nos dois genótipos;
3. Análise proteômica de raízes dos genótipos suscetível e tolerante a seca através de 2-DE e espectrometria de massa;
4. Identificação de genes e proteínas potencialmente envolvidos na tolerância à seca em arroz.

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CAPÍTULO ÚNICO: **"IDENTIFICATION OF DROUGHT-RESPONSIVE GENES IN ROOTS OF UPLAND RICE (*Oryza sativa* L.)"**

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Identification of drought-responsive genes in roots of upland rice (*Oryza sativa* L.)

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Abstract

Background: Rice (*Oryza sativa* L.) germplasm represents an extraordinary source of genes that control traits of agronomic importance such as drought tolerance. This diversity is the basis for the development of new cultivars better adapted to water restriction conditions, in particular for upland rice, which is grown under rainfall. The analyses of subtractive cDNA libraries and differential protein expression of drought tolerant and susceptible genotypes can contribute to the understanding of the genetic control of water use efficiency in rice.

Results: Two subtractive libraries were constructed using cDNA of drought susceptible and tolerant genotypes submitted to stress against cDNA of well-watered plants. *In silico* analysis revealed 463 reads, which were grouped into 282 clusters. Several genes expressed exclusively in the tolerant or susceptible genotypes were identified. Additionally, proteome analysis of roots from stressed plants was performed and 22 proteins putatively associated to drought tolerance were identified by mass spectrometry.

Conclusions: Several genes and proteins involved in drought-response, as well as genes with no described homologs were identified. Genes exclusively expressed in the tolerant genotype were, in general, related to maintenance of turgor and cell integrity. In contrast, in the susceptible genotype, expression of genes involved in protection against cell damage was not detected. Several protein families identified in the proteomic analysis were not detected in the cDNA analysis. There is an indication that the mechanisms of susceptibility to drought in upland rice are similar to those of lowland varieties.

Background

Rice *(Oryza sativa* L.*)* is a cereal of high economic and social value, which is used as a staple food by more than half of the world's population. It is the only cereal which is solely produced for human consumption. The production of rice must increase 20% in the next 15 years in order to keep pace with population growth. One of the main constraints that affect yield in rice production is water deficit. The increasing worldwide water shortage and uneven rainfall distribution limit the use of irrigated agriculture, typical of rice production. Irrigation costs are increasingly high worldwide. There is, therefore, a need to develop rice varieties, which are more efficient in the use of water [1, 2]. A major challenge for the research community is the relatively limited progress made so far in improving the drought tolerance of high yielding rice varieties [3].

Rice is a highly diverse species, which can be grown in many types of soil moisture regimes, ranging from aerobic upland to permanently flooded lowland. Although upland rice constitutes a relatively small proportion of the total rice area worldwide, it is the predominant method of rice cultivation in Latin America and West Africa (about 75% and 50% of rice area, respectively) [4]. In Brazil, upland rice responds for approximately 40% of the total rice production. In some areas of the country, upland rice is a subsistence crop planted by farmers who apply limited inputs to their crops. The cultivation of upland rice in marginal areas with low soil fertility and threatened by severe abiotic stresses, such as periods of drought during the cropping season, has a significant impact on rice production [5, 6]. Due to exposure to many environmental constraints, some local varieties of the tropical *japonica* rice developed high adaptability to drought stress, hot and dry climatic conditions of regions in Latin America and Africa. Therefore, these varieties may show high levels

of water usage efficiency and constitute an excellent material for studying drought tolerance mechanisms in rice. In Brazil, for example, EMBRAPA maintains a germplasm bank enriched with traditional upland rice landraces collected in areas where cultivated rice has been grown since its introduction in the country, centuries ago, and may represent an extraordinary source of genes that control traits of economic importance such as drought tolerance [7].

The determination of the mechanisms directly involved in drought tolerance remains a challenging task since drought is a complex trait that involves several metabolic pathways [3]. The identification and isolation of genes associated with drought tolerance is of major importance in order to better understand this trait and increase the efficiency in developing drought tolerant varieties [8-10]. At the molecular level, the response of roots to water limiting conditions seems to be crucial to trigger drought tolerance mechanisms, since roots are one of the primary sites for stress signal perception in which a signaling mechanism initiates a cascade of gene expression responses to drought. These transcriptional changes can result in successful adaptations leading to stress tolerance by regulating gene expression and signal transduction in the stress response (regulatory proteins) or directly protecting the plant against environmental stress (functional proteins) [11].

Several functional genomic studies of rice have been performed using different approaches such as macro and microarray [12, 13], RT-qPCR, SAGE (*Serial Analysis of Gene Expression*), MPSS (*Massive Parallel Signature Sequencing*) and more recently oligoarray using the transcriptome of rice to evaluate responses to abiotic stresses [14]. Proteome analyses have also been increasingly employed to complement genomic studies [15-18], however in a lower rate. Although numerous genes and proteins, which potentially contribute to drought tolerance in rice, have been reported

[19-22], most of these studies have focused on lowland rice genotypes. Currently, very little is known about gene and protein expression in upland rice [22-25]. Moreover, most ESTs from drought stressed plants available were obtained from libraries constructed using seedlings [26]. There are very few reports on gene expression of drought-stressed plants in the reproductive stage and using root tissue of plants growing under defined field capacity.

The comprehension of drought responses in upland rice is important for designing breeding strategies to develop varieties more tolerant to water constraints. Recently, the tolerance of ten traditional upland varieties of rice submitted to drought stress has been evaluated as part of an effort to identify new sources of drought tolerance in rice [27]. Concomitantly, the root system of two of the above mentioned upland rice genotypes, characterized as susceptible and tolerant to drought stress, have been analyzed at the reproductive stage using genomic and proteomic approaches. Several genes and proteins were identified, which may play important roles in drought tolerance.

Methods

1. Plant material and phenotypic evaluation

Plants of traditional upland rice (*O. sativa* L. var. *japonica*) varieties were grown on PVC pipe columns (25 cm of diameter; 80 cm of height) filled with fertilized Oxisol under screenhouse conditions [27]. The experimental design was a split-plot design with two watering regimes as main plots, ten traditional upland varieties as subplots and three replications. The watering regimes were (a) control, consisting of a main plot of well-watered plants throughout the experiment, which received 100% reposition of the water lost daily and a minimum soil humidity of - 0,025 MPa at 15

cm of depth, and (b) drought stress, which consisted of 50% reposition of the water lost daily from anthesis on. Water reposition was calculated based on daily weighting of columns with a mechanical scale. Twenty-one days after initiating the drought stress treatment (at anthesis), roots of each treatment (control and drought stress) were collected from each rice variety. All root samples were immediately frozen in liquid nitrogen and maintained at – 80ºC until their use for RNA and protein extractions. At harvest, grain yield and yield components of each genotype were evaluated, including root and shoot dry weight, harvest index, spikelet sterility, grains per panicle and weight of 100 grains. Drought tolerance parameters were estimated based on calculations of drought severity, drought tolerance index and drought susceptibility index [28]. The genotypes submitted to the drought stress showed differences in most of the yield parameters analyzed, which were significantly influenced by the drought severity applied to the experiment [27]. These parameters were then used to classify the genotypes according to their reaction to stress. Among them, two contrasting genotypes for drought stressing conditions were selected for the present study: Prata Ligeiro, as the tolerant, and IRAT20, as the susceptible variety. The RNA and protein analyses proceeded only with root tissue extracted from these two varieties.

2. RNA extraction and subtractive library construction

For each genotype, a bulk of approximately 250 mg of plant roots from the three replications were homogenized in liquid nitrogen and total RNA was extracted using the Concert^{TM} Plant RNA Reagent (Invitrogen, USA), according to manufacturer's instructions. This procedure was followed for roots harvested from drought stressed as well as unstressed plants. mRNA was then isolated from total RNA by using PolyATtract mRNA Isolation System (Promega, USA). Quantity and quality

of the isolated mRNA was evaluated by spectrophotometry and electrophoresis in agarose gel 1%, respectively.

Isolated mRNAs were used for cDNA synthesis and suppression subtractive hybridization (SSH) library construction by using the PCR Select Subtraction Kit (Clontech, USA). Subtractive hybridizations were performed using cDNA from stressed plant roots (as tester) against cDNA from well-watered unstressed plant roots (as driver) of each genotype, in order to identify genes involved in drought response. The subtractive PCR products obtained were cloned into pGEM T-Easy (Promega, USA) and sequenced in ABI Prism 3700 DNA Analyser (Applied Biosystems Inc., USA). A minimum insert size of 30 bp and at least 20 bp with quality of phred> 20 were considered for the analysis. Sequences were deposited in GenBank under the accession numbers of FG124418 through FG124880 and sequence homologues were identified using the Blast program [29]. An *in silico* subtraction was performed by clustering all sequences from both cDNA libraries according to the methodology described by Telles and da Silva [30], allowing the identification of genes exclusively found in each library.

3. Protein extraction and 2-DGE

Total protein was extracted from roots of the drought tolerant (Prata Ligeiro) and susceptible (IRAT20) genotypes according to procedures described by de Mot and Vanderleyden [31] Plant material of the three replications were pooled, pulverized and mixed with extraction buffer (0.7 M sucrose, 0.5 M TrisHCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM DTT) and phenol (100%) in the same volume (750 µl). Proteins were precipitated with ammonium acetate 0.1 M in methanol, washed with acetone 80% (v/v), dried and stored at -20ºC. Protein quantification was performed

using the Bradford Reagent (Invitrogen, USA). Isoelectric focusing was conducted using 11-cm immobilized pH gradient (IPG) strips with a pH range of 4–7 and a Multiphor II electrophoresis system (GE). Strips containing approximately 220 μg of protein were rehydrated with 2% (v/v) CHAPS, 8 M urea, 7 mg dithiothreitol (DTT) and 2% IPG buffer. Second dimension analysis was performed in 10% gels by SDS-PAGE as described by Laemmli [32] and at least five replications of each genotype were performed. Protein spots were visualized after silver [33] or Comassie blue staining.

4. Image analysis

The 2D gel images were evaluated using the Platinum software (GE Healthcare, UK) and three high quality gels obtained for both genotypes were analyzed. First, a calibration with a grey scale was performed to transform grey levels into OD values for each pixel (px) of the gel image. The wizard detection method proposed by the software was used to detect the spots with the following parameters: 15 px for estimated spot size, 50 px for minimum spot area and a spot contrast enhancement of 75%. Automatically detected spots were checked and some of them were manually added or removed. Following the detection procedure, the normalization step was carried out to attribute a common spot identity for the same spots derived from different images utilizing the reference gel construct and automatically matching options. A synthetic gel from each genotype was constructed by using the mean value of volume percentage of each protein spot present in the three replicates, according to the Platinum software's (GE Healthcare, UK) instructions. The two obtained synthetic gels were then overlapped using the molecular marker as well

as several protein spots present in both profiles as landmarks. The overlapped images were based on landmark spots showing same pI and Mw.

5. Trypsin digestion and mass spectrometry analysis

Protein spots were excised manually from 2D gels and in-gel digested with sequencing grade trypsin (Promega, Madison, WI) according to Schevchenko et al. [34]. Briefly, each protein spot was placed in a 0.5 mL polypropylene (Eppendorf) tube and destained by washing 5-8 times with 200 μL of 50% (v/v) acetonitrile/10 mM ammonium bicarbonate solution. The gel pieces were subsequently dehydrated by washing with 200 μL of 100% acetonitrile and completely dried in a Speedvac concentrator. Ten microliters of 50 mM ammonium bicarbonate/10% (v/v) acetonitrile solution containing 100 ng of trypsin were added, and the sample incubated at 37 °C for 16 h. Aliquots of each tryptic digest (1 μL) were mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid, spotted onto a MALDI target plate, and allowed to air dry.

Mass spectra were acquired using a MALDI-TOF/TOF Autoflex II spectrometer (Bruker Daltonics, Bremen, Germany) operating at a laser frequency of 50 Hz. MS analysis were performed in a positive ion reflection mode. Voltage parameters were set as IS1 19kV, IS2 16.8kV, Lens 8kV, Reflector 20kV, Reflector2 9.54kV. The delay time was 70 ns and acquisition mass range 700–3200 Da. External calibration was performed using a peptide mix contaning ACTH (1-24), ACTH (18-39), Somatostatin, Angiotensin I and Angiotensin II, all from Sigma. MS/MS analysis were performed in a positive ion LIFT reflection mode. Voltage parameters used were IS1 6kV, IS2 5.3kV, lens 3.15kV, Reflector 23.5kV, Reflector2 9.7kV, LIFT1 19kV

and LIFT2 4kV. The delay time was set as zero and acquisition mass range 40-2400 Da.

Peak lists were generated using the FlexAnalysis 3.0 software (Bruker Daltonics). The sophisticated numerical annotation procedure (SNAP) algorithm was used to detect the monoisotopic peak values, with a quality factor threshold of 30 and 6 as S/N threshold. Database searches were performed in February 2008 using the MASCOT search engine (Matrix Science, UK) with the NCBInr protein database and *Oryza sativa* taxonomy. The mass tolerance was 100 ppm and one missed cleavage was allowed. Carbamidomethylation of cysteines, oxidation of methionine, and acrylamide-modified cysteines were considered for PMF searches. For accepting the identification, the cutoff value for the Probability Based Mowse score calculated by MASCOT (at $p < 0.05$) was used. For MS/MS data, the peptide mass tolerance was 0.5 Da, MS/MS ion mass tolerance at 0.5 Da, allowance of 1 missed cleavage, and charge state +1. When the pI and MW of matched proteins were not available, these values were calculated using ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

Results and Discussion

1. Experimental design and sampling

Plants were submitted to drought stress after anthesis for twenty-one days. Flowering is the period in which the plant is most sensitive to water deficit and several tolerance mechanisms need to be activated at this stage in order to guarantee grain filling and production [6]. During root sampling, a clear visual difference in Prata

Ligeiro and IRAT20 plants could be observed. An intense leaf rolling was noticed in the susceptible genotype as opposed to the tolerant. In addition, a more pronounced aerial biomass loss could be visualized in IRAT20. At harvest, yield and yield component parameters were measured [27]. The variety IRAT20, a high yielding variety under irrigated controlled conditions, showed a 51% reduction in grain yield when submitted to drought stress. On the other hand, Prata Ligeiro, a low yielding variety under well watering conditions, had a 23% reduction in grain yield under drought stress. The drought susceptibility index based on yield was estimated as 0.73 for Prata Ligeiro (tolerant) and 1.57 for IRAT20 (susceptible).

Collected roots of both genotypes were then used for cDNA library construction and proteome studies. In the cDNA library study, stressed plants were contrasted with well-watered plants, whereas in the proteome analysis, stressed plants from both genotypes were compared.

Water reposition, based on the evapotranspiration rate, has been used to determine an impartial and consistent response of plants to drought stress, during long periods of drought in the soil [35]. Several studies have tried to define the critical limit of water in the soil after which crop development and production are significantly affected [36]. According to Rosenthal *et al.* [37], the symptoms of water deficit occur when water availability is around 50% of the field capacity.

The response of plants to drought stress is also dependent on the extension and rate of water loss [38]. Fukai *et al*. [39] reported that when a rapid water deficit occurs, the morpho-physiological mechanisms are severely affected. When the deficit is prolonged for a few days, plants are allowed to adapt to the stress, enabling the identification of variability in drought tolerance within different genotypes, since plants can respond differently to the same stress condition [38]. Therefore, the

sampling time used in this study (21 days of drought stress) may have allowed the analysis of adaptive responses of the plant to tolerate water deficit.

Several studies reported the response of rice seedlings to drought stress [13, 26, 40] however, little attention has been given to the expression of genes in water-stressed plants at the reproductive stage (flowering, grain filling) in which a higher yield impact is observed [6].

2. cDNA library analysis

Roots are one of the primary sites responsive to restrictive conditions of water availability and, as a result, synthesize chemical signals for a rapid response of the plant to drought stress [41]. This occurs since the response in leaves must be stimulated rapidly to avoid irreversible damage to the photosynthetic machinery. In this work, two subtractive cDNA libraries were constructed using mRNA from roots of tolerant and susceptible upland rice genotypes subtracted from their respective unstressed well-watered controls. The subtracted PCR products obtained after primary and secondary PCR ranged from 0,1 - 1,5 kb.

The SSH libraries of the tolerant (Prata Ligeiro) and susceptible (IRAT20) genotypes were concluded with a novelty index of 66% and 55%, respectively. The general analysis of the two libraries revealed a total of 463 valid sequences (230 from Prata Ligeiro and 233 from IRAT20) and the average fragment size was of 300 bp. Several genes commonly expressed in both genotypes were identified and are probably not directly involved in drought tolerance.

In order to determine the genes exclusively expressed in the tolerant and susceptible genotypes, an *in silico* subtraction was performed using sequences of both libraries. The results for the *in silico* subtraction revealed that the 463 sequences

represented 282 different transcripts: 127 were found in both genotypes, 84 were exclusively expressed in the Prata Ligeiro library (Table 1) and 71 were observed only in the IRAT20 library (Table 2).

2.1. Putative drought-tolerance genes identified in Prata Ligeiro

Drought tolerance is a complex trait and involves mechanisms that act in isolation or combined to avoid or tolerate periods of water deficit. It is expected that genotypes responding differently to drought stress show differences in gene expression, and that a portion of the differences is related to drought tolerance. Therefore, the analysis of the genes found exclusively in the tolerant genotype is of interest to identify genes associated with water usage efficiency.

Among the 84 transcripts uniquely reported in the tolerant genotype, 14 did not present known homologs (no hits) and 17 showed similarities to proteins with unknown function (hypothetical proteins). Three sequences showed similarity to non-plant proteins and probably represent contaminating sequences (Table 1). The other transcripts showed similarity to several proteins previously reported as associated to drought stress and some of them are discussed below.

Genes involved in signaling routes were exclusively identified in Prata Ligeiro and include serine/threonine kinase, ethylene-responsive factor and calcium-transporting ATPase/ calmodulin binding sequences. Serine/threonine kinases are Ca^{2+} dependent proteins kinase (CDPKs), involved in the phosphorylation cascade of proteins. Several studies have shown that CDPKs are induced or activated by abiotic stresses, suggesting that they may be involved in drought signaling [42-45]. Another identified gene associated to signal transduction was an ethylene-responsive factor. Ethylene is a well characterized phytohormone that may act alone or in combination

with ABA in regulating gene expression under abiotic stress [46]. Calcium-transporting ATPase/ calmodulin binding are also stress-signaling proteins and are responsible for regulation of the osmotic potential of the cell.

Some genes that participate in metabolism alterations as a result of the limitation caused by low levels of intracellular CO_2 observed during drought stress were also identified only in Prata Ligeiro. Among these genes are those coding for Phosphoenolpyruvate carboxykinase, an enzyme that has a key role in nocturnal fixation of CO_2; malato dehydrogenase, which is an enzyme particularly important for the assimilation of carbon in C4 plants; Glutamate-1-semialdehyde aminotransferase and glucose-1-fosfato adenililtransferase [47-49], both involved in carbohydrate metabolism.

It has been proposed that the mechanism involved in drought tolerance in upland rice is a result of a higher expression of genes involved in oxidative stress protection [23]. Indeed, in the present study some genes associated to the protection of the cell were expressed only in the tolerant genotype. Among them, we found a Methionine sulfoxide reductase A and a Respiratory burst oxidase homolog, which act in the recognition of reactive oxygen species (ROS) in biotic and abiotic stresses [50]. Other interesting genes identified are Metallothionein, a superfamily of low molecular weight proteins involved in metal detoxification [51] and scavenging of oxygen-free radicals, which can decrease injury in oxidative tissue, and Ferredoxin, regulated by different environmental stresses including biotic and abiotic conditions.

Genes associated to maintenance of cell turgor were also identified such as IQ calmodulin-binding and Calcium-transporting ATPase/calmodulin binding. These genes were previously reported to participate in typical defense mechanisms in upland varieties [23].

In this study we have also identified genes which have not yet been directly related to drought tolerance, such as B12Dg1 protein, Nuclear protein SET domain containing protein and Putative pollen specific protein C13 precursor, as well as genes with unknown function. Further studies need to be performed in order to assign biological function, since these genes may play important roles in plant adaptation during drought stress conditions.

2.2. Drought-responsive genes identified in IRAT20

Regarding the response of the susceptible genotype to drought stress, 71 transcripts were exclusively expressed in this genotype. As in Prata Ligeiro, a high number of genes (14) with no known homologs (no hits) were identified (Table 2). Moreover, a total of 23 genes encoding hypothetical or unknown proteins were also observed. Further expression studies of these genes may reveal important genes associated to drought stress response, which have not been explored so far. This information may contribute to a better understanding of the mechanisms related to drought susceptibility in upland rice varieties.

As in Prata Ligeiro, three transcripts showed similarity to non-plant proteins and were not considered in the analysis since they probably represent contaminating sequences (Table 2). The other transcripts showed similarity to genes associated to different functions including the transport of small molecules or inorganic ions, such as HCO_3-transporter and Vacuolar H+ pyrophosphatase. The expression of these genes was previously reported by Wang *et al.* [23] in a lowland variety. These results suggest that upland genotypes susceptible to drought may present similar responses to those of lowland varieties, which are naturally more susceptible to water deficit.

Interestingly, the well-known transcription factor WRKY was uniquely identified in IRAT20. WRKY mediates plant stress responses [52-54] and the increased expression of this protein has been frequently associated to drought stress response in rice [23, 55].

3. Proteome analysis

In order to complement the genomic studies, protein maps of roots from water-stressed plants of the susceptible (Figure 1A) and tolerant (Figure 1B) genotypes were compared. Triplicates of the gels from each genotype were compared and revealed a total of 463 proteins in the Prata Ligeiro profile and 522 in IRAT20. The two obtained synthetic gels were overlapped and this procedure allowed the identification of 307 overlapped spots, 156 proteins exclusive to the tolerant genotype and 215 proteins exclusive to the susceptible genotype. These results show a higher diversity in the protein pattern of the susceptible genotype.

A total of 50 intense proteins observed in the tolerant genotype profile after Coomassie blue staining was excised from the gel, digested and analyzed by mass spectrometry. By using the Mascot program, 22 proteins could be identified with a significant score (Table 3), including 16 up- and 4 down-regulated, 1 new and 1 equally expressed in both genotypes (Figure 2). The other proteins were in insufficient amounts for the identification analysis or did not return reliable matches when using the Mascot program. This probably occurs due to a low protein quantity and/or low ionization capacity of molecular components present in the samples analyzed. It is also possible that, considering the high amount of "no hits" obtained in the genomic analysis, protein sequences matching the peptides searched were not available in

public databases. The peptide sequences obtained were also analyzed using the Blastp program.

Spots PL1 and PL2 (up-regulated in Prata Ligeiro) were identified as hypothetical proteins which contain Ricin B-related lectin domain. Other up-regulated hypothetical proteins were also identified and include protein spots PL34, PL45 and PL51. Spot PL45 and PL51 were expressed 2.6 and 4.5 fold, respectively, in the tolerant genotype (Figure 2), indicating that these proteins may play an important role in drought tolerance. Spot PL57 was another protein identified as hypothetical and was exclusively expressed in Prata Ligeiro. These proteins are interesting candidates for futures studies aiming at the determination of biological function.

Spots PL3 and PL60 were identified as the same protein chitinase and spot PL11 as a Chain A, Crystal Structure of Class I Chitinase. Chitinases are pathogenesis-related proteins expressed in response to biotic and abiotic stresses and have been studied in grasses such as rye in response to cold and drought stress [56]. Spot PL60 was highly induced in the tolerant genotype, which confirms the up-regulation of this protein during drought stress. Chitinases have also been reported as being induced in tomato plants tolerant to drought when compared to the susceptible genotype [57].

Two other pathogenesis-related proteins were identified: one was up-regulated (spot PL33) and the other repressed (PL30) in the tolerant genotype (Figure 2). The expression of these proteins has been previously reported in roots of rice in drought stress conditions and although the role of proteins of this family is not well established, they have been associated to hypersensitive reaction in response to biotic and abiotic factors [58]. In drought stress conditions, pathogenesis-related proteins as well as the salt stress-responsive SalT protein have been reported in rice roots [59].

As observed in the constructed cDNA libraries, several proteins involved in oxidative stress protection were induced in the tolerant genotype and were identified as a superoxide dismutase [Cu-Zn] (PL20), L- ascorbate peroxidase 1 (PL23), ascorbate peroxidase (PL38) and cytosolic malate dehydrogenase (PL63) (Table 3). Peroxidases are anti-oxidative enzymes, described in varieties of rice tolerant to high salinity conditions [25, 60] and in upland rice roots in response to osmotic stress [24]. These proteins are involved in cellular detoxification and it is possible that this is a general defense mechanism in response to water deficit in upland rice. According to Wang *et al.* [23, 24] tolerance to drought stress observed in upland varieties includes detoxification mechanisms, limiting the accumulation of reactive oxygen species. These authors reported that these proteins were up-regulated in upland cultivars when comparing tolerant lowland and upland rice. Unexpectedly, proteins identified as superoxide dismutase (PL7) and GSH-dependent dehydroascorbate reductase (PL13) were down-regulated in the tolerant genotype. These proteins were not identified in the genomic analysis, highlighting the importance of proteomics studies to complement the results obtained.

Another down-regulated protein (PL24) identified in the Prata Ligeiro genotype was triosephosphate isomerase (Table 3), involved in carbohydrate metabolism. According to Wang *et al*. [23], genes related to metabolism are more expressed in lowland than in upland genotypes. It is possible that susceptibility to drought in upland rice may occur in a similar way as in lowland rice.

Spots PL43 and PL46 were both identified as enolase, a glycolytic enzyme, which participates in metabolic processes. The up-regulation of enolase has been previously reported in rice roots in response to salt stress [61] and to PEG treatment [24]. Unexpectedly, PL46 was equally expressed in Prata Ligeiro and IRAT20, while

spot PL43 was up-regulated in Prata Ligeiro. The existence of multiple enolase isoforms in plants has been reported [62] and it is possible that the enolases identified in this study represent different isoforms, which respond differently to drought stress conditions. Indeed, difference in the expression of enolase isoforms was observed in maize in response to anaerobiosis [63].

A highly induced protein (15 fold) in the tolerant genotype (PL40) showed identity to a hypothetical protein as well as a salt stress induced protein (Table 3). Similarly, spot 27 (2.6 fold higher in Prata Ligeiro) also presented identity to the salt stress induced protein. It is possible that these spots represent new rice proteins, not identified so far that contain a conserved region present in both matching proteins. The induction of proteins involved in tolerance to salt stress, during water deficit conditions, shows that osmotic stress is an important aspect during drought. Similar mechanisms are activated in response to different abiotic stresses, as previously reported [10].

Conclusions

Several genes and proteins involved in drought-response as well as genes with no described homologs were identified in this work. Genes exclusively expressed in the tolerant genotype were, in general, related to maintenance of turgor and cell integrity. In contrast, in the susceptible genotype, expression of genes involved in protection against cell damage was not detected, indicating that there may be a higher degradation of cellular components in these genotypes. Similar results were obtained by Wang *et al.* [23] when comparing tolerant upland and lowland varieties. These results indicate that the mechanisms of susceptibility in upland rice are similar to those

of lowland varieties, considering that the upland rice is naturally more tolerant to drought stress.

The proteomic analyses were complementary to the genomic data obtained. The expression of genes associated with cell protection against oxidative damage is considered important to cope with water deficit in upland rice. In this study, genes and proteins related to this function showed a higher expression in the tolerant genotype. Interestingly, in the proteomics analysis, the susceptible genotype showed a higher diversity in the protein profile, revealing more uniquely expressed proteins than the tolerant genotype. On the other hand, in the genomic study, the number of exclusively expressed transcripts in the susceptible genotype was lower. It is well known that transcript levels do not always reflect protein amounts [64, 65]. Therefore, it is possible that the transcripts related to the proteins exclusively present in IRAT20 2D maps were in low amounts, and not detected by the genomic analysis, or they were subtracted from the control condition in the hybridization process. Differences in translation efficiency may have occurred, resulting in a higher amount of the corresponding proteins, further detected by 2-DGE. These results clearly show that proteomics studies can reveal important additional information and that the use of complementary approaches is useful for a better understanding of complex biological traits, such as drought tolerance.

Overall, due to the low amount of information regarding upland rice gene and protein expression in response to water deficit, this study sheds some light over the comprehension of this complex mechanism. However, the high amount of transcripts and proteins with unknown function obtained is still intriguing. These genes and proteins need to be further investigated in order to assign their biological function and advance our knowledge regarding drought tolerance in upland rice.

Authors' contributions

AM, ARR, CMG, MEF and PHNR designed and performed the research. FRS analyzed the sequence data and EMS and DS analyzed the mass spectrometry data. ARR and AM drafted the manuscript. ACMB and CRS critically revised the article. All authors approved the final version.

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Table 1. Genes detected exclusively in roots of the tolerant genotype (Prata Ligeiro) SSH library.

Encoded protein	Homologous organism	Accession number
Proteins of known function		
Glutamate-1-semialdehyde 2,1 aminomutase	*Oryza sativa*	NM_001068872
Metallothionein-like protein	*Oryza sativa*	NM_001056317
Malate dehydrogenase	*Oryza sativa*	NM_001062924
Methionine sulfoxide reductase A	*Oryza sativa*	NM_001063272.1
Phosphatidylinosytol 3 and 4 kinase	*Oryza sativa*	NM_001060732
Ubiquitin-conjugating enzyme	*Oryza sativa*	NM_001048429
Nuclear protein SET domain containing protein	*Oryza sativa*	NM_001067672
Splicing factor 3B subunit 5-like protein	*Oryza sativa*	dbj|BAD10044.1|
PEP carboxikinase	*Oryza sativa*	gb|ABF95034.1|
Putative malate dehydrogenase	*Oryza sativa*	gb|AAT69584.1|
Eukaryotic translation initiation factor 5A-2 (eIF-5A) (eIF-4D)	*Oryza sativa*	NC_008405
Metallothionein-like protein type 1	*Oryza sativa*	NP_001068544.1
ADP glucose pyrophosphorylase	*Oryza sativa*	EF122437
CBL-interacting protein kinase 1	*Oryza sativa*	NM_001049327
ADP-ribosylation factor	*Oryza sativa*	NM_001051134
DSS1 /SEM1 family protein	*Oryza sativa*	NC_008394
Ankyrin repeat containing protein	*Oryza sativa*	NM_001054582
Pathogenesis-related transcriptional factor and ERF domain containing protein	*Oryza sativa*	NC_008402
E-class P450, group I family protein	*Oryza sativa*	NM_001074239
FAR1 domain containing protein	*Oryza sativa*	NM_001057341
Tubulin alpha-1 chain	*Oryza sativa*	NM_001074145
Putative ubiquitin conjugating enzyme	*Oryza sativa*	dbj|BAB89662.1|
DEAD/DEAH box helicase domain containing protein	*Oryza sativa*	NM_001069156
Putative pollen specific protein C13 precursor	*Oryza sativa*	gb|AAM08621.1|
IQ calmodulin-binding	*Oryza sativa*	NM_001061046
HAD superfamily hydrolase 5' nucleotidase protein	*Oryza sativa*	NM_001057956
SAM biding motif domain containing protein	*Oryza sativa*	NM_001070787
Peptidase aspartic family protein	*Oryza sativa*	NM_001063168
Nonaspanin (TM9SF) family protein	*Oryza sativa*	NM_001056027
Ethylene responsive element binding factor 5	*Oryza sativa*	NM_001063579
TMS membrane protein	*Oryza sativa*	NM_001054899
Heat shock protein DnaJ family protein	*Oryza sativa*	NM_001060020
Ferredoxin III, chloroplast precursor (Fd III)	*Oryza sativa*	NC_008396
Anther ethylene-upregulated protein ER1 (Fragment)	*Oryza sativa*	NM_001055765
Chaperone protein DNA-J-related like	*Oryza sativa*	dbj|BAD27799.1|
Isoflavone reductase family protein	*Oryza sativa*	NM_001068997

U box domain containing protein	*Oryza sativa*	NM_001071339
Ribossomal protein L	*Curculio glandium*	AM049038
Short chain dehydrogenase tic32	*Oryza sativa*	NM_001048577
Arabinogalactan protein	*Oryza sativa*	NC_008394
Ribonuclease T2 family protein	*Oryza sativa*	NM_001070328
HvB12D protein (B12Dg1 protein)	*Oryza sativa*	NM_001063815
Respiratory burst oxidase homolog	*Oryza sativa*	NM_001049555
Phosphatidylinositol-4-phosphate 5-kinase family protein	*Oryza sativa*	NM_001068386
Nodulin-like	*Oryza sativa*	NM_001070322
Cathepsin B-like cysteine protease form 2	*Ixodes ricinus*	gb|ABO26563.1|
Cathepsin L-like cysteine proteinase precursor	*Acanthoscelides obtectus*	gb|AAQ22984.1|
Calcium-transporting ATPase/calmodulin binding	*Arabidopsis thaliana*	NP_188931.1
Myb, DNA biding domain containing protein	*Oryza sativa*	NM_001062445
TGA-type basic leucine zipper protein	*Phaseolus vulgaris*	gb|AF402607.1|
Tocopherol O-methyltransferase, choroplast precursor	*Oryza sativa*	NM_001054379
ATP-dependent Clp protease ATPbiding subunit Clpx-like mitochondrial precursor	*Oryza sativa*	dbj|BAD15818.1|
HvB12D protein (B12Dg1 protein)	*Oryza sativa*	NM_001063815
Uncharacterized protein family containing protein	*Oryza sativa*	gb|ABA91393.1|
Protein of unknown function		
Protein of unknown function	*Oryza sativa*	NC_008397
Protein of unknown function	*Oryza sativa*	NC_008403
Unknow function	*Oryza sativa*	NM_001067277
Hypothetical protein	*Oryza sativa*	AP008208
Hypothetical protein	*Oryza sativa*	gb|EAY93896.1|
Conserved hypothetical protein	*Oryza sativa*	NM_001065538
Hypothetical protein	*Oryza sativa*	gb|EAY84091.1|
Hypothetical protein	*Oryza sativa*	CT836006
Hypothetical protein	*Oryza sativa*	NC_008394.1
Hypothetical protein	*Oryza sativa*	NC_008394.1
Hypothetical protein	*Oryza sativa*	AP008208
Hypothetical protein	*Oryza sativa*	NM_001057688
Hypothetical protein	*Oryza sativa*	NM_001066910
Hypothetical protein	*Oryza sativa*	NM_001053573
Hypothetical protein	*Oryza sativa*	CT829595
Hypothetical protein	*Oryza sativa*	CT834076

Table 2. Genes detected exclusively in roots of the susceptible genotype (IRAT20) SSH library.

Encoded protein	Homologous organism	Accession number
Proteins of known function		
T complex 11 family protein	*Oryza sativa*	NM_001059402
Protein kinase domain containing protein	*Oryza sativa*	NM_001071926
Protein disulphide isomerase family protein	*Oryza sativa*	AP008208
TPR-like domain containing protein	*Oryza sativa*	NM_001058028
Protein kinase	*Oryza sativa*	NM_001074788
Pinoresinol-lariciresinol reductase TH1	*Oryza sativa*	NM_001073059
Smr protein; MutS2 c- terminal domain containing protein	*Oryza sativa*	NM_001048992
SIPL protein (Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1)	*Oryza sativa*	NM_001055581
Similar to CG 9092- PA	*Tribolium castanum*	XP_967647.1
Putative ATP-dependent Clp protease ATP-binding subunit ClpX1 (CLPX)	*Oryza sativa*	dbj|BAD15818.1|
Cytocrome P450 family protein	*Oryza sativa*	NM_001071591
Preprotein translocase subunit sec Y, chloroplast precursor	*Oryza sativa*	NM_001067916
Vacuolar H+ pyrophosphatase	*Oryza sativa*	NM_001063501
Similar to UPF 0139 protein CGI-140	*Tribolium castaneum*	XP_971064.1|
60 kDa inner membrane insertion protein family protein	*Oryza sativa*	NM_001055291
Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	*Oryza sativa*	NM_001055382
Similar to splicing coativator subunit SRm 300	*Monodelphis domestica*	XP_001371550.1|
Cysteine synthase, mitocondrial precursor	*Oryza sativa*	NM_001052112
TPR-like domain containing protein	*Oryza sativa*	NM_001056953
HCO3-transporter	*Oryza sativa*	NM_001073581
Banched chain amino-acid aminotransferase-like protein 3	*Oryza sativa*	NM_001049072
Beta tubulin (fragment)	*Oryza sativa*	NM_001049296
HAT dimerisation domain containing protein	*Oryza sativa*	NC_008402
Urease accessory protein G	*Oryza sativa*	NM_001062872
Glycoside hydrolase, family 47 protein	*Oryza sativa*	NM_001054615
WRKY transcription factor 82	*Oryza sativa*	DQ298186
Tubby family protein	*Oryza sativa*	NM_001062568
Ribosomal protein L41 family protein	*Oryza sativa*	NC_008400
Granule-bound starch synthase I, chloroplast precursor	*Oryza sativa*	NM_001065985
Putative RNA polymerase I transcription factor RRN3	*Oryza sativa*	dbj|BAD45608.1|
Aconitate hydratase, cytoplasmic (Citrate hydro-lyase) (Aconitase)	*Oryza sativa*	NM_001055433
Short chain alcohol dehydrogenase-like	*Oryza sativa*	NM_001056212
Putative ubiquitin-conjugating enzyme E2	*Oryza sativa*	dbj|BAD25096.1|
Peptidase s26A signal peptidase I family protein	*Oryza sativa*	NM_001074823

Protein of unknown function		
Unknown protein	*Oryza sativa*	NM_001068742
Hypothetical protein	*Oryza sativa*	AC119292
Hypothetical protein	*Oryza sativa*	AP008208
Hypothetical protein	*Oryza sativa*	AK243578
Hypothetical protein	*Oryza sativa*	NC_008395.1
Hypothetical protein	*Oryza sativa*	AP008208
Hypothetical protein	*Oryza sativa*	NM_001057104
Hypothetical protein	*Oryza sativa*	NC_008395
Hypothetical protein	*Oryza sativa*	NM_001074804
Hypothetical protein	*Oryza sativa*	NM_001057688
Hypothetical protein	*Oryza sativa*	NC_008401.1
Hypothetical protein	*Oryza sativa*	NC_008395.1
Hypothetical protein	*Oryza sativa*	CR855113
Hypothetical protein	*Oryza sativa*	AC145477
Hypothetical protein	*Oryza sativa*	AC092556
Hypothetical protein	*Oryza sativa*	AK242616
Hypothetical protein	*Oryza sativa*	AP008209
Hypothetical protein	*Oryza sativa*	NC_008398.1
Hypothetical protein	*Oryza sativa*	AC099401
Hypothetical protein	*Oryza sativa*	NM_001050487
Hypothetical protein	*Oryza sativa*	CT831698
Hypothetical protein	*Oryza sativa*	CT828847
Hypothetical protein	*Oryza sativa*	CT832865

Table 3. Proteins identified by peptide mass fingerprinting or *de novo* sequencing.

Spot n°	Peptide sequence	Protein identification	Accession #	Score	Mr (gel)	pI (gel)	Mr (cal)	pI (cal)
PL 1		Hypothetical protein	gi|115452789	138	38.0	6,7	39	6.3
PL2		Hypothetical protein	gi|115452789	65	39.0	6.6	39	6.3
PL 3	WAPSPADAAAGR	Chitinase	gi|407472	56	36.0	6.6	35.5	7.3
PL 7	EHGAPQDENR	Zinc-superoxide dismutase	gi|22296339	26	15.0	6.4	14.7	5.9
PL 11	GPIQLSFNFNYGPAGR	Chain A, Crystal Structure Of Class I Chitinase	pdb|2DKV|A	30	37.0	6.2	32.6	5.8
PL 13	AAVGHPDTLGDCPFSQR	GSH-dependent dehydroascorbate reductase 1	gi|6939839	43	26.0	6.1	23.5	5.6
PL 20	GTSQVEGVVTLTQDDQGPT TVNVR	Putative superoxide dismutase [Cu-Zn]	gi|42408425	72	17.0	5.5	20.5	5.7
PL 23		L-ascorbate peroxidase 1,	P93404	99	28.0	5.5	27	5.4
PL 24	VATPDQAQEVHDGLR	Triosephosphate isomerase	gi|553107	49	28.0	5.4	27.5	6.6
PL 27	EFSIPLQDSGHVVGFFGR	Salt stress-induced protein	gi|158513205	88	11.0	5.0	15.1	5.1
PL 30	MIEDYLVAHPAEYA	Pathogenesis-related protein Bet v I	gi|9230755	55	18.0	4.9	16.6	4.9
PL 33	ADVGVGPVSWDDTVAAYA ESYAAQR	Acidic PR-1 type pathogenesis-related protein PR-1	gi|12005673	182	17.5	4.2	17.5	4.5
PL34	WWDTFPANVDGAR	Hypothetical protein	gi|115461070	87	29.0	4.7	27.2	5.0
PL 38		Ascorbate peroxidase	NP_001060741	74	32.0	5.2	27	5.2
PL 43	MTAEIGEQVQIVGDDLLVT NPTR	Enolase	gi|780372	88	60.0	5.4	47.9	5.4
PL 46		Enolase	Q42971	74	50.0	5.4	47.9	5.4
PL 45		Hypothetical protein	gi|115465323	98	60.0	5.2	58.8	5.9
PL 51	KADATVAGDDR	Hypothetical protein	gi|125557770	37	45.0	5.7	95.7	8.0
PL 57	AGYAPPHWVQPGQGDR	Hypothetical protein	gi|125532459|	73	25.0	4.2	24.5	4.6
PL 60	ELFEQLLLHR	Chitinase	gi|561873	51	36.5	4.2	34.3	4.4
PL 63	ELVADDEWLNTEFISTVQQR	Cytosolic malate dehydrogenase	gi|115482534	66	37.5	5.9	35.5	5.75
PL 40	EFSIPLQDSGHVVGFFGR	Salt stress-induced protein Hypothetical protein	gi|158513205 EAY73933	104	39	5.3	15.1 40.6	5.1 8.6

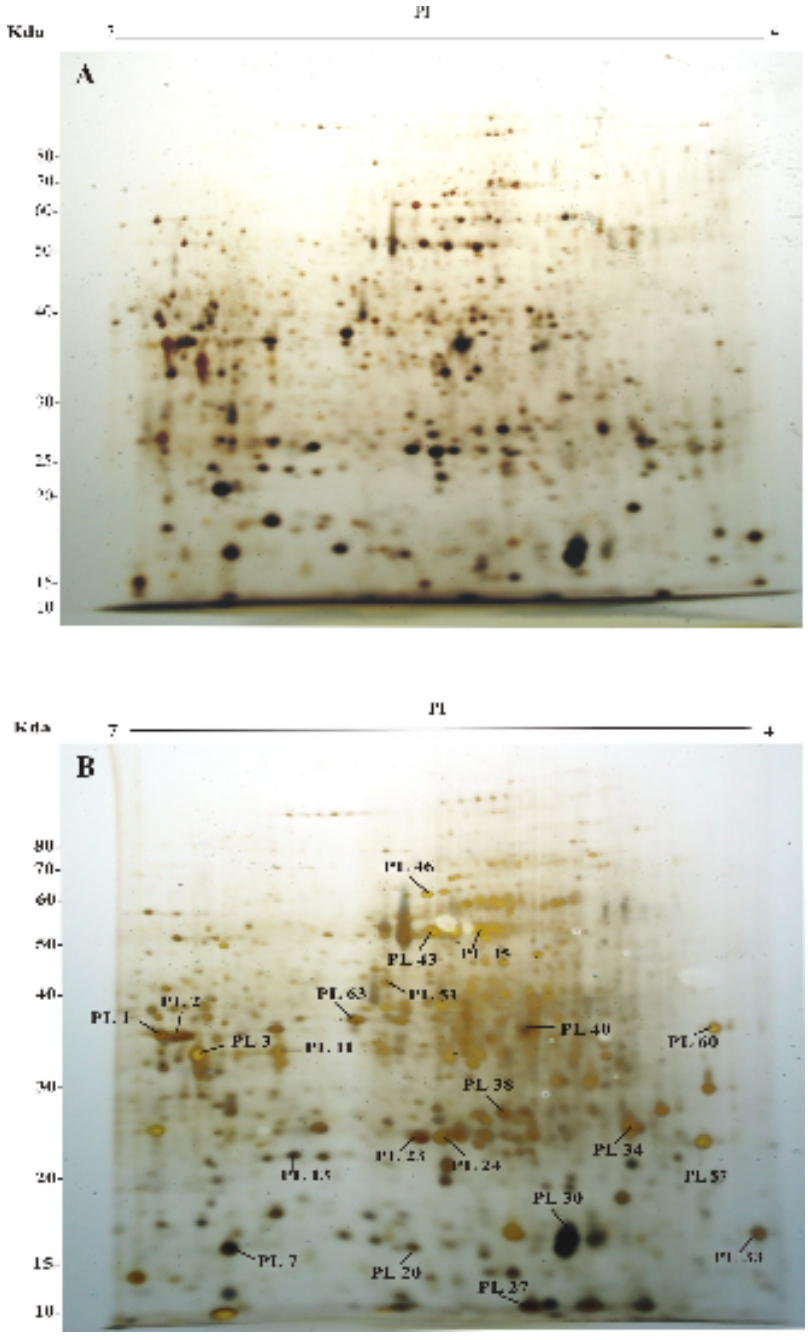

Figure 1. Root protein profiles by 2-DGE of the susceptible (A) and tolerant (B) genotypes. Total soluble protein (ca. 220 µg) was separated by 2-DGE and the spots were visualized after silver staining. Numbers indicate the protein spots successfully identified by mass spectrometry. Benchmark Protein Ladder (Invitrogen, USA) was used to estimate the molecular mass of the proteins visualized.

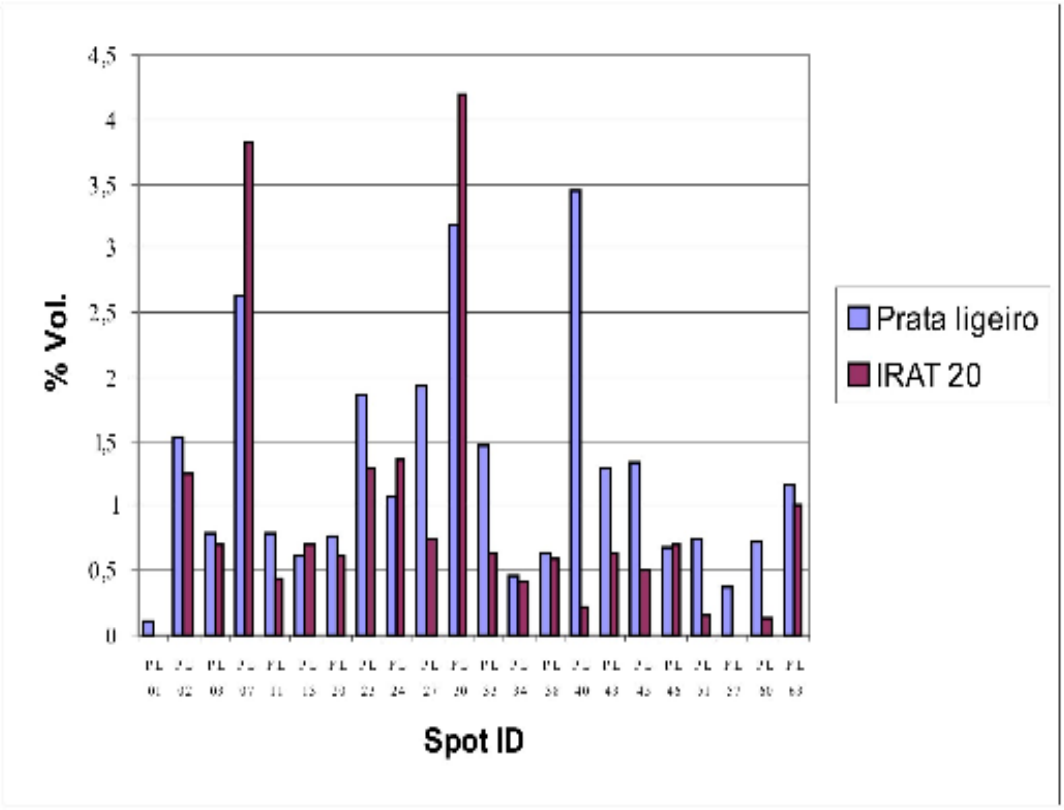

Figure 2. Histogram representing expression levels of up- and down-regulated proteins identified in the tolerant (Prata Ligeiro) and susceptible (IRAT20) genotypes, as determined by the Platinum software (GE Healthcare, UK).

Perspectivas futuras

Os resultados apresentados neste trabalho sugerem como pesquisas futuras a validação da expressão de alguns genes encontrados, por meio de RT-PCR, bem como a investigação da função fisiológica dos genes desconhecidos.

Os genes identificados como responsivos à seca podem, ainda, ser utilizados como marcadores moleculares em programas de melhoramento baseado em seleção assistida.