BASES MOLECULARES DA RESPOSTA À SECA E CARACTERIZAÇÃO DO POTENCIAL ANDROGENÉTICO A CULTIVARES BRASILEIRAS DE TRIGO

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RESUMO

O trigo (*Triticum aestivum* L.) é uma importante cultura no Brasil. Poucas cultivares são recomendadas para produção do tipo sequeiro no Bioma Cerrado onde a escassez de água limita o rendimento de grãos. Aqui reportamos uma análise de transcriptoma do MGS1 Aliança (cultivar de trigo adaptada ao Cerrado) sob estresse de seca. Um grupo de 4.422 transcritos diferencialmente expressos foi encontrado em raízes e folhas. O número de transcritos reprimidos em raiz (1.102) foi menor que os transcritos induzidos (1.706), enquanto o oposto ocorreu em folhas (1,017 induzidos e 647 reprimidos). O número de transcritos comuns entre ambos órgaõs foi 1.249, enquanto 2.124 foram específicos para raíz e 1.049 específicos para folhas. Análises de RT-qPCR de 35 transcritos selecionados ao acaso revelou uma correlação de 0,78 com os dados de transcriptoma. Os transcritos diferencialmente expressos foram distribuídos por todos os cromossomos e componentes do genoma. O número de transcritos no genoma B foi maior do que nos genomas A e D. Ainda, um grande número de transcritos relacionados à seca foi mapeado nos cromossomos 3B, 5B e 2B. Quando consideramos ambos órgãos, 116 diferentes rotas metabólicas foram alteradas. Uma rota em comum, entre as três mais alteradas em ambos órgãos, foi o metabolismo do amido e da sacarose. A comparação de transcritos derivados de raiz e de folha permite a identificação de transcritos importantes relacionados à respota ao estresse de seca em cada um destes órgãos. Os dados obtidos, também, abrem caminho para o desenvolvimento de futuros marcadores e seleção de genes candidatos ligados à característica. Estes resultados são úteis para o entendimento de rotas metabólicas envolvidas na tolerância à seca em trigo. A informação gerada será usada, a mais longo prazo, para propósitos de transgenia. Para isto, a metodologia de duplo-haploides é desejável e uma primeira investigação sobre a eficiência de protocolo se mostrou necessária. Micrósporos são células gaméticas com capacidade de dar origem a uma nova planta via embriogênese *in vitro*. Plantas duplo-haploides geradas pela cultura de micrósporos isolados são completamente homozigotas e representam uma importante ferramenta para estudos genéticos e melhoramento de plantas O processo androgenético é desencadeado por diferentes pré-tratamentos de estresse, os quais são empregados para mudar os micrósporos da rota gametofítica para a rota esporofítica. Embora a cultura de micrósporos isolados tenha inúmeras vantagens, importantes limitações tem impedido sua apliação em larga escala. Diferenças genotípicas na resposta androgenética e na formação de plantas albinas ainda constituem desafios. Embora o albinismo seja principalmente uma característica genética, pré-tratamentos e meios de cultura apropriados podem evitar este fenômeno até certo ponto. A resposta androgenética de cinco genótipos de trigo brasileiro foi avaliada no presente estudo. Dois pré-tratamentos foram testados: frio (4°C) e ácido 2-hidroxinicotinico (100 mg/L). O frio foi melhor que o pré-tratamento químico, produzindo mais plantas verdes em quatro de cinco genótipos. Somente dois genótipos brasileiros tratados com ácido 2-hidroxinicotinico produziram plantas, e um deles apenas uma única planta albina. Nossos reultados mostram, também, que o meio semilíquido (contendo 10% de Ficoll) promoveu uma maior resposta androgenética que o meio líquido, aumentando o número de embriões e plantas regeneradas.

Palavras-chave: Sequenciamento 454 • Estresse abiótico • Expressão gênica diferencial • RNA-seq • RT-qPCR • Androgênese • Duplo-haploides • *Triticum aestivum*.

ABSTRACT

Wheat (*Triticum aestivum* L.) is an important crop cultivated in Brazil. Few cultivars are recommended for rainfed production in the Cerrado Biome where water scarcity limits grain yield. Here we report a transcriptome analysis of MGS1 Aliança (a wheat cultivar adapted to the Cerrado) under drought stress. A set of 4,422 differentially expressed transcripts was found in roots and leaves. The number of down-regulated transcripts in roots (1,102) was lower than the up-regulated transcripts (1,706), while the opposite occurred in leaves (1,017 induced and 647 repressed). The number of common transcripts between the two tissues was 1,249, while 2,124 were specific to roots and 1,049 specific to leaves. Quantitative RT-PCR analysis of 35 randomly selected transcripts revealed a 0.78 correlation with the transcriptome data. The differentially expressed transcripts were distributed across all chromosomes and component genomes. The number of transcripts on the B genome was greater than on the A and D genomes. Additionally, a greater number of drought related transcripts was mapped on chromosomes 3B, 5B and 5D. When considering both tissues, 116 different metabolic pathways were changed. One common pathway, among the top three changed pathways in both tissues, was starch and sucrose metabolism. The comparison of root- and leaf-derived transcripts allows the identification of important transcripts related to water stress response in each of these tissues. It also paves the way for future marker development and selection of candidate genes linked to that trait. These results are useful for understanding the metabolic pathways involved in wheat drought response. The information generated will be used for transgenic wheat purposes. For this the doubled-haploid method is desirable and an investigation about the protocol eficiency is needed. Microspores are gametic cells with capacity to give rise to a new plant via *in vitro* embryogenesis. Doubled haploid plants generated by isolated microspore culture are completely homozygous and represent an important tool for plant genetics and breeding research. This process is triggered by different stress pretreatments, which are employed to switch microspores from gametophytic to a sporophytic pathway. Although isolated microspore culture has innumerous advantages, important limitations have prevented its application on a large scale. Genotypic differences in androgenic response and the formation of albino plants remain great challenges. Although albinism is a major genetic characteristic, appropriated pretreatments and culture medium can avoid this phenomenon to some extent. The androgenic response of five Brazilian wheat genotypes was evaluated in the present study. Two pretreatments were tested: cold (4°C) and 2-hydroxynicotinic acid (100 mg/L). Cold was better than chemical pretreatment, producing more green plants in four out of five genotypes. Only two Brazilian genotypes treated with 2-hydroxynicotinic acid produced plants, and one of them produced a single albino plant. Our results also show that semi-liquid medium (containing 10% Ficoll) promoted a higher androgenic response than did liquid medium, increasing the number of embryos and regenerated plants.

Key-words: 454 Sequencing • Abiotic stress • Differential gene expression • RNA-seq • RT-qPCR • Androgenesis • Doubled-haploid • *Triticum aestivum*.

LISTA DE ABREVIATURAS E SIGLAS

2-HNA: ácido 2-hidroxinicotinico / *2-hydroxynicotinic acid*

ABA: ácido abscísico / *abscisic acid*

CSIRO: *Commonwealth Scientific and Industrial Research Organisation*

DE: diferencialmente expressa / *differentially expressed*

DET: transcritos diferencialmente expressos / *differentially expressed transcipts*

DH: duplo-haploide / *doubled haploid*

GO: ontologia gênica / *gene ontology*

H: haploide / *haploid*

IMC: cultura de micrósporos isolados / *isolated micropore culture*

KEGG: Kyoto Enciclopédia de Genes e Genomas / *Kyoto Encyclopedia of Genes and Genomes*

LEA: embriogênese tardia abundante / *late embryogenesis abundant*

NGS: sequecniamento de nova geração / *next generation sequencing*

QTL: locus de características quantitativas / *quantitative trait loci*

RIN: número de integridade do RNA / RNA *integrity number*

RT-qPCR: PCR quantitativo precedido de transcrição reversa / reverse transcription quantitative PCR

RWC: conteúdo relative de água / *relative water content*

SNP: polimorfismo de nucleotídeo único / *single nucleotide polymorphism*

TF: fator de transcrição / *transcription factor*

TFDB: banco de dados de fator de transcrição / *transcription factor database*

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INTRODUÇÃO

INTRODUÇÃO

O trigo e sua relação com o Brasil

Estudos indicam que o trigo se originou de gramíneas silvestres provenientes da Ásia, entre 10.000 e 15.000 anos A.C. (Scheeren, 1986), sendo a cultura chave para o desenvolvimento da civilização ocidental. A espécie autógama *Triticum aestivum* L. pertence à família Poaceae, faz parte do grupo dos hexaplóides (2n=6x=42 cromossomos) e possui conjuntos completos de cromossomos de três outras espécies. É um alopoliplóide que surgiu naturalmente após dois rounds de hibridações entre três espécies diploides diferentes (cada uma com um conjunto de sete pares de cromossomos). A primeira hibridação ocorreu entre a espécie diploide doadora do genoma A (*T. urartu*) e espécie também diploide doadora do genoma B (esta ainda não descoberta ou extinta, próxima à *Aegilops speltoides*) a cerca de 0,5 milhões de anos atrás dando origem ao trigo tetraploide *T. turgidum* (tendo seu genoma simbolizado por AABB). A terceira espécie (*T. tauschii*) doadora do genoma D se hibridizou com o trigo tetraploide a cerca de 8.500 anos atrás dando origem então ao trigo hexaplóide *T. aestivum* (tendo seu conjunto de três genomas representando por AABBDD). (Feldman e Levy, 2012; Leach et al., 2014). Estas hibridações ocorreram de forma natural e conferiram grande resistência a fatores ambientais e estresses bióticos e abióticos (Brammer, 2003).

O trigo foi introduzido no Brasil por imigrantes europeus (Federezzi et al., 1999; Ambrosi et al., 2000; Cunha, 2005), no ano de 1534, na capitania de São Vicente, durante a colonização (Scheeren, 1986; Ambrosi et al., 2000; Cunha, 2005). Este cereal foi incorporado para satisfazer o consumo europeu, começando no estado de São Paulo de onde foi se espalhando para as terras do sul, as quais possuíam melhores condições ambientais para seu desenvolvimento (Ambrosi et al., 2000; Cunha, 2005), apesar de possuir uma grande adaptabilidade agronômica, sendo cultivado em regiões subtropicais e tropicais (Silva e Andrade, 2001; Dalmago et al., 2009). É considerado um dos itens de fundamental segurança alimentar para o Brasil (Guarienti, 2009).

O Brasil é o segundo maior importador mundial de trigo, estando atrás apenas do Egito. Estimativas da *Food and Agriculture Organization of the United Nations* (FAO - http://faostat.fao.org/site/339/default.aspx) indicam que desde o ano de 2008 o Brasil manteve-se entre a 16ª e 20ª posição mundial na produção de trigo. A média da produção entre 2008 e

2013 ficou ao redor de 5 a 6 milhões de toneladas, representando cerca de 54% do consumo interno (de Mori e Só e Silva 2013; FAO, 2013) que chega a dez toneladas por ano (Scheeren et al, 2002; Scheeren, 2005). Já no quesito produtividade, o Brasil ficou classificado em 56º, com 2.480 kg/ha, enquanto a Irlanda ocupou a primeira posição com 9.063 kg/ha (FAO, 2013). No entanto, em condições de campo para trigo irrigado, já foi relatado rendimento de 8.000 kg/ha (Silva e Andrade, 2001), demonstrando que o trigo brasileiro tem potencial genético próximo ao nível dos países que lideram neste quesito. Portanto, a ação de fatores ambientais (estresses bióticos e abióticos) tem papel fundamental na menor produtividade deste cereal.

O trigo possui amplas possibilidades de expansão no país. À medida que novas tecnologias foram se desenvolvendo, a produção de trigo também foi aumentando (Federezzi et al., 1999). Mas, apesar deste aumento, a produção ainda é pequena quando comprada ao seu potencial, existindo a possibilidade de expansão do cultivo no país para somar ainda mais na produção total (Cunha, 2005).

No Brasil, a produção de trigo está dividida em três grandes áreas, as regiões Sul (RS, SC e sul do PR), Centro-Sul (PR, MS e SP), que juntas representam 98% da produção, e Central (GO, DF, MG, MT, BA) (Cunha et al., 2011). Cerca de 90% da produção se dá apenas na região sul, onde tem-se um clima predominantemente frio e úmido (Scheeren et al., 2008; Cunha et al., 2011). Essa região caracteriza-se por solos ácidos com alumínio, e clima úmido, não há uma estação seca e a precipitação pluvial excede a da necessidade da cultura, causando, assim, estresses associados ao excesso de umidade; além disso, a presença de geadas também afeta negativamente a cultura (Scheeren et al, 2002). A região Central ainda é pouco expressiva, mas com um grande potencial para o crescimento de produção (Scheeren et al, 2002). Esta última incluindo uma parte do estado de SP, caracteriza-se por ser quente e seca, características que influenciam bastante na produção (Cunha et al., 2011). A região Central é constituída principalmente do bioma Cerrado (Fig 1), o qual é considerada a nova fronteira para a expansão da triticultura (Silva e Andrade 2001). Cerca de 25% (ftp://geoftp.ibge.gov.br/mapas_tematicos/mapas_murais/biomas.pdf) da área do território brasileiro (8,5 milhões de km^2) (http://www.ibge.gov.br/home/geociencias/cartografia/default_territ_area.shtm) é ocupada pelo bioma Cerrado. Neste bioma, a temperatura varia de 22 °C na região mais ao sul a 27 °C na região mais ao norte, apresentando estações secas e chuvosas (Yamada, 2005). Contudo mesmo na estação chuvosa (80% do total da precipitação) pode ocorrer escassez de água, com duração de uma a três semanas (Embrapa-Cerrados, 2005).

Na safra de 2012/13, um total de 105 genótipos de trigo foram recomendados para plantio no Brasil. Contudo, somente três destes eram para cultivo no Cerrado com sistema sequeiro (BR18 Terena, MGS1 Aliança e MGS Brilhante), e outros quatro para sistema sequeiro e irrigado (CD 105, CD 111, CD 116 e CD117) (IAPAR, 2012). Isto demonstra a falta de genótipos disponíveis para esta área tão promissora. Ainda, os três genótipos disponíveis para sistema sequeiro são cultivares "antigas", em outras palavras, com características não apropriadas para as adversidades atuais. A produção de trigo no Cerrado contribui apenas com 5%, sendo a maior parte desta sob o plantio

Figura 1. Localização do bioma Cerrado no Brasil. Fonte: Documentos 190 ISSN 1517 – 5111, novembro 2007 – Mapeamento de cobertura vegetal do bioma cerrado: estratégias e resultados, EMBRAPA, Edson Eyil Sano, Roberto Rosa, Jorge Luis Silva Brito, Laerte Guimarães Ferreira, p. 15

irrigado (Embrapa-Cerrados, 2005; Fischer, 2009). Embora no sistema irrigado a média possa chegar a 4.000 kg/ha (de Mori e Só e Silva, 2013), é o sistema sequeiro que tem o grande potencial de aumentar a produção de trigo, uma vez que requer menos investimentos e possui uma grande área de expansão. Contudo, para que isto seja possível, tem-se de lidar com três principais estresses abióticos: acidez do solo, calor e seca (Scheeren et al., 2008).

No Cerrado brasileiro, o trigo também é uma alternativa importante para prevenir as consequências negativas da monocultura. O monocultivo de tomate e de leguminosas aumenta a incidência de doenças como esclerotínia, rizoctoniose e fusariose. O trigo não é hospedeiro

desses fitopatógenos, constituindo-se na principal alternativa para romper o ciclo biológico dos mesmos, por meio da rotação com estas culturas no Cerrado (Silva e Andrade 2001; Tibola et al., 2009).

Seca

A seca é amplamente aceita como o estresse ambiental mais importante na agricultura e o maior obstáculo para a sobrevivência da planta, sua produtividade e qualidade (Tuberosa e Salvi 2006; Cattivelli et al., 2008; Harb et al., 2010; Nezhadahmadi et al., 2013). De um modo geral, o déficit de água no solo e atmosfera por um período prolongado de tempo, afeta o metabolismo e o desenvolvimento das plantas. Agronomicamente, a seca ocorre quando a capacidade/necessidade da planta é maior que a água disponível, levando à prevenção do crescimento (Wang et al., 2013) e restringindo a expressão total do potencial genético da planta (Swindale e Bidinger 1981; Boyer, 1982).

A seca já constitui um importante obstáculo e espera-se que esta condição piore devido a aumentos de temperatura e mudanças no perfil de precipitação (Budak et al., 2013; Comas et al., 2013; Wang et al., 2013). De acordo com o Painel Intergovernamental sobre Mudanças no Clima (IPCC – AR4), no fim deste século, a temperatura global deverá aumentar 1,1-6,4 °C com intensificação da escassez de água em várias regiões do globo. No Brasil, o rendimento de trigo deverá reduzir de 1,3% a 30% (Siqueira et al., 1994; Siqueira e Salles, 2001; Jaggard et al., 2010). Previsões indicam que em pouco mais de 10 anos, 1,8 bilhões de pessoas sofrerão com absoluta falta de água e 65% do mundo viverá em ambientes com escassez de água (Nezhadahmadi et al., 2013). Uma vez que a produção de trigo brasileira no Cerrado está sendo realizada no sistema irrigado e espera-se que os recursos de água diminuam num futuro próximo, o melhoramento do trigo para ser usado no sistema sequeiro terá importância ambiental e sócio-econômica.

As plantas desenvolveram diferentes mecanismos para lidar com estresses relacionados à água, sendo a tolerância à seca uma característica quantitativa envolvendo uma complexa resposta em níveis molecular, metabólico e fisiológico (Pennisi, 2008; Rosales et al., 2013), bem como a interação dos genes entre si e com o meio ambiente (Reynolds e Tuberosa, 2008; Budak et al., 2013). No trigo, o tempo dos estádios fenológicos, metabolismo de carboidratos, condutância de estômatos, ajuste osmótico, senescência retardada da folha bandeira, tempo de

florescimento, aumento na razão parte aérea:raiz, valores elevados de carboidratos solúveis na haste pouco depois da antese e aumento da cobertura do solo, entre outros fatores, tem sido associados com a resposta de tolerância à seca (Morgan, 1983; Dorion et al., 1996; Fischer et al., 1998; Slafer e Whitechurch, 2001; Verma et al., 2004; Moinuddin et al., 2005; Foulkes et al., 2007; Reynolds et al., 2007). De modo a compreender os mecanismos subjacentes a esta resposta, estudos de expressão gênica diferencial tem sido realizados em trigo usando genes-alvo, bibliotecas subtrativas de cDNA e microarranjos (Zhang et al., 2004; Houde et al., 2006; Rampino et al., 2006; Xue et al., 2006; Mohammadi et al., 2007; Diab et al., 2008; Mohammadi et al., 2008; Xue et al., 2008; Ergen e Budak, 2009; Ergen et al., 2009; Li et al., 2012).

Embora a tecnologia e o melhoramento insistente tenham feito progresso na investigação da tolerância à seca, as bases genéticas da tolerância em trigo ainda são elusivas. Isto pode ser demonstrado pela diminuição da produtividade quando plantas de trigo são submetidas a ambientes secos (Budak et al., 2013; Wang et al., 2013a,b). Para desenvolver genótipos com aumentada tolerância à seca, a identificação de moléculas relacionadas a estresse e a determinação de seus papéis e localização em diversas redes fisiológicas, bioquímicas e gênicas torna-se necessária (Budak et al., 2013). Para que estas metas sejam alcançadas, genes podem ser identificados usando a tecnologia de sequenciamento de nova geração (NGS – do inglês *Next Generation Sequencing*) para a análise de transcritomas.

Uma das técnicas que utiliza o NGS é o RNA-seq. Esta tecnologia permite a obtenção do perfil de expressão baseado na presença e quantidade de RNAs transcritos a partir de um genoma em um determinado momento ou condição específica. Esta metodologia pode melhorar a compreensão da resposta à seca em trigo, revelando genes candidatos, fornecendo perfis de expressão e anotação funcional de genes presentes em redes transcricionais complexas (Budak et al., 2013; Oono et al., 2013), sendo assim possível amostrar transcritomas inteiros de uma maneira mais eficiente e econômica (Varshney et al., 2009; Thakur et al., 2013). Também permite a detecção de transcritos raros ou aqueles instáveis em bactérias, diminuindo erros associados à clonagem (Vera et al., 2008), evitando regiões não-codificantes e repetitivas e possibilitando sequenciamento em organismos não-modelos (Hou et al., 2011). O NGS permite, ainda, uma descrição mais completa do transcritoma, uma vez que produz um grande número de sequências, discrimina variantes de splicing, alelos e outras isoformas e não é limitado pelo número de transcritos pré-definidos como sondas em microarranjos (Deyholos, 2010).

Em espécies-modelo, estudos de transcritoma geralmente são realizados mapeando os *reads* a um genoma de referência. Infelizmente, até o momento, esta estratégia não está disponível para trigo. O sequenciamento e a anotação gênica ainda estão incompletos, embora em fase final. Enorme progresso tem sido feito pelo Consórcio Internacional do Genoma do Trigo (IWGSC – do inglês *International Wheat Genome Sequencing Consortium*). Este projeto tem um grande obstáculo, sequenciar um genoma hexaploide altamente repetitivo, 40 vezes maior que o do arroz (Oono et al., 2013). Tendo isto em vista, a tecnologia de RNA-seq apresenta-se como boa alternativa para prospectar o genoma do trigo (Duan et al., 2012).

A complexidade do genoma do trigo deve ser considerada no uso de NGS. O genoma do trigo hexaploide - *T. aestivum* - possui um tamanho de 17 gigabases, com 124.121 genes anotados, sendo mais de 80% composto por sequências repetidas de transposons (Brenchley et al., 2012). Até o momento existem poucos relatos de RNA-seq em trigo, incluindo estudos relacionados ao conteúdo proteico de grãos (Cantu et al., 2011), eventos de poliploidização (Pont et al., 2011), desenvolvimento do amido do endosperma (Pellny et al., 2012), SNPs (*Single nucleotide polymorphism*) (Lai et al., 2012), desenvolvimento de ferramentas para montagem *de novo* de genomas (Duan et al., 2012),tolerância ao estresse biótico causado por *Fusarium graminearum* (Kugler et al., 2013), regulação nutriente-responsiva (Oono et al. 2013), padrão de expressão de genes homeólogos (Leach et al., 2014), transcriptoma à mudança climática no estágio reprodutivo (Kumar et al. 2015).

A identificação de sequências relacionadas à tolerância à seca em trigo, por meio de RNA-seq, abre grandes oportunidades para a elucidação da resposta da planta a este estresse. Após a identificação, tais sequências poderão ser usadas em diversas abordagens e projetos como, por exemplo, transformação genética, desenvolvimento de marcadores moleculares, assistência ao melhoramento genético tradicional, seleção assistida, entre outros.

Melhoramento, Transgenia e Interação da cultura de tecidos com programas de melhoramento genético

O melhoramento genético das plantas cultivadas, tem como alguns de seus objetivos contribuir com o aumento do rendimento, expansão do potencial agrícola, estabilidade da produção e aumento de resistência contra fatores adversos (Moraes-Fernandes, 1985; Borém, 2005). Um desafio contínuo para os programas de melhoramento vegetal no Brasil é gerar

genótipos altamente produtivos, que tenham tolerância ou resistência aos diversos estresses bióticos e abióticos. As doenças fúngicas, tais como a ferrugem da folha, a giberela e a brusone, o estresse hídrico e o alumínio tóxico no solo, fazem parte dos principais fatores limitantes da produção de grãos (Darkó et al., 2004; Lobato et al., 2007; Passioura, 2007; Bolton et al., 2008).

A cultura de tecidos vegetais (parte significativa da biotecnologia) pode interagir de várias maneiras com os programas de melhoramento genético. Alguns exemplos podem ser citados: a) conservação e avaliação de germoplasma; b) aumento da variabilidade genética para fins de seleção; c) introgressão de genes de interesse em espécies-alvo (polinização *in vitro*, cultura de embriões, fusão de protoplastos, haploidização, transformação genética) e d) aceleração de programas de melhoramento (germinação de sementes e cultura de frutas *in vitro*, clonagem de genótipos para teste de capacidade de combinação, cultura de anteras ou micrósporos para obtenção de haploides, limpeza clonal) (Ferreira et al., 1998).

A transformação genética ou transgenia é uma das ferramentas biotecnológicas atualmente disponíveis que pode auxiliar os programas de melhoramento de maneira única, especialmente em situações de difícil solução por técnicas convencionais. O domínio da técnica pode auxiliar na introdução de genes específicos, oriundos de outros organismos, em genótipos de trigo (sem a co-integração de genes indesejáveis), além de genes não existentes na natureza e modificados artificialmente, levando ao aumento do *pool* gênico disponível para os programas de melhoramento.

Um fator limitante para a melhoria de genótipos de trigo via engenharia genética é a recalcitrância da espécie (Ji et al., 2013). Além disto, como já mencionado, as características do genoma (grande tamanho e poliploidia) representam desafios adicionais (Bhalla et al., 2006). Vários métodos já foram descritos para a geração de plantas de trigo transgênicas, dentre eles a transferência direta de DNA para protoplastos (Lörz et al., 1985), eletroporação (He et al., 1994), método do tubo-polínico (Chong et al., 1998) e vórtex (Serik et al., 1996), contudo estes não são facilmente reproduzíveis. Os métodos mais usados são os de bombardeamento e sistema *Agrobacterium*, os quais são mais reprodutíveis e têm apresentado melhores resultados (Jones et al., 2005; Bhalla et al., 2006; Ji et al., 2013).

Descrições das primeiras transformações de trigo obtidas com sucesso por meio da técnica de bombardeamento ocorreram no ano de 1992 (Jones et al., 2008). Este método, apesar de eficaz, traz desvantagens como fragmentação do DNA no momento do bombardeio, inserção de partes do vetor e inserção de várias cópias do gene (Hu et al., 2003). Além disso, estas

múltiplas cópias frequentemente sofrem rearranjos em trigo (Janakiraman et al., 2002), sendo um agravante para as etapas posteriores de cruzamento e segregação.

O sistema de transformação por *Agrobacterium* primeiramente foi considerado não funcional para espécies de monocotiledôneas. Contudo, este impasse foi superado com uso de cepas bacterianas super-virulentas (McCormac et al., 1998), bem como a adição de determinados compostos que induzem a virulência (acetoseringona por exemplo) (Wu et al., 2003) e o uso de vetores binários contendo genes adicionais de virulência (Khanna e Daggard, 2003). Em alguns estudos, a eficiência do sistema *Agrobacterium* ultrapassa a do bombardeamento em trigo (Hu et al., 2003).

No Brasil, há poucos registros de uso da transgenia em trigo, até o momento. Vendruscolo et al. (2007) descreveram a obtenção de um trigo mais tolerante à seca, produzido pelo bombardeamento de partículas, usando como alvo embriões imaturos. Outro estudo, com participação da Embrapa Trigo, mas realizado no SCIRO (*Commonwealth Scientific and Industrial Research Organisation* - Austrália), refere-se à transformação de trigo (bombardeamento de partículas) objetivando plantas mais resistentes ao alumínio ácido do solo (Pereira et al., 2010).

Alguns prerequesitos para o sucesso nos processos de transformação são: genótipo, tipo de explante, condições de cultura e genes marcadores e de seleção. Em termos de genótipo, a cultivar Bobwhite tem sido uma das mais utilizadas e recomendada para experimentos de transformação (Weeks et al., 1993; Pellegrineschi et al., 2002; Pereira et al., 2010; Brunner et al., 2011) devido a sua alta frequência de regeneração e potencial de transformação (Liu et al., 2002; Pellegrineschi et al., 2002; Bhalla et al., 2006; Shariatpanahi et al., 2006).

Além da notável e consagrada aplicação de plantas duplo-haploides (DH) no melhoramento genético vegetal, acelerando a formação de populações para os mais variados fins (Zhang et al., 2008), a cultura de micrósporos isolados (IMC – do inglês: *insolated microspore culture*), também, vem sendo usada como uma ferramenta para o desenvolvimento e estudos de organismos geneticamente modificados. Os tecidos originados da cultura de micrósporos, assim como os próprios micrósporos, são excelentes explantes para a transformação genética, visto que a transferência de genes para estas células darão origem a plantas haploides transformadas e que poderão ser diploidizadas tornando-se homozigotas diploides (para maiores detalhes ver próximo tópico). O método de bombardeamento mostrou ser eficiente, resultando na obtenção de plantas transgênicas de cevada a partir de embriões

derivados de micrósporos (Wan e Lemaux, 1994) e de trigo (Folling e Olesen, 2001) e cevada a partir de micrósporos isolados (Yao et al., 1997).

Apesar da IMC já estar estabelecida para algumas culturas, continua sendo uma técnica difícil de realizar com baixa eficiência. Apesar de muitas revisões e trabalhos destacarem a importância dos micrósporos como alvos de transformação genética, há poucos trabalhos neste sentido. Alguns mostrando apenas expressão transiente outros regenerando plantas quimeras e pouquíssimas plantas homozigotas. Ainda há a necessidade de estabelecimento de um protocolo otimizado de transformação usando os micrósporos como alvos para regenerar plantas DH.

Androgênese

Formação do gameta masculino

O aparelho reprodutor masculino, denominado androceu, é composto pelos estames. Estes por sua vez dividem-se em filete, tecido conectivo e antera. A antera é formada por quatro microsporângios ou sacos polínicos. Cada saco polínico contém as células mãe do pólen, que estão envoltas por um tecido nutritivo mais interno denominado tapete, camadas médias, endotécio e epiderme da antera (Bedinger, 1992; Vidal e Vidal, 2000; Zanettini e Lauxen, 2003).

Ocorrendo o desenvolvimento normal, as células mãe de pólen ($2n$) irão sofrer meiose. Cada célula mãe dará origem a quatro células haploides (n), denominadas micrósporos, que estarão reunidos em uma tétrade e serão liberados pela ação da enzima calase. Os micrósporos liberados irão aumentar de volume, formar um vacúolo e as duas membranas (exina e intina) que apresentam um poro. Também conterão muitos ribossomos em seu citoplasma e um grande vacúolo central que desloca o núcleo para a periferia da célula, no sentido oposto ao poro (Bedinger, 1992; Vidal e Vidal, 2000; Zanettini e Lauxen, 2003).

O micrósporo sofrerá sua primeira mitose, caracteristicamente assimétrica, resultando na formação de uma célula vegetativa (grande) e outra célula generativa (menor). Numa segunda mitose, a célula generativa dividir-se-á em duas, formando as duas células espermáticas ou gametas masculinos. Portanto o grão de pólen maduro é tricelular (Bedinger, 1992; Kaltchuk-Santos e Bodanese-Zanettini, 2002).

Haploides e Duplo-haploides

Indivíduos haploides são aqueles que possuem apenas metade de seu conjunto cromossômico, ou seja, são plantas com *n* cromossomos ao invés de *2n* (diploides). O grande significado da existência de plantas haploides tanto para o melhoramento como para a pesquisa genética foi reconhecido desde a primeira observação natural destas em 1921 por Bergner em *Datura stramonium* (Blakeslee et al., 1922). Mas sua origem foi descoberta somente após quatro décadas quando Guha e Maheshwari (1964) observaram que estas plantas surgem de pólens imaturos em condições *in vitro*, como um produto imediato da meiose. Em um experimento onde colocaram anteras de *D. innoxia* em condições *in vitro*, não intencionalmente, recuperaram plantas haploides representando o conjunto cromossômico paterno pós-meiose. E a partir desta "redescoberta" das plantas haploides, muitos trabalhos têm sido desenvolvidos.

Plantas haploides possuem um desenvolvimento "normal", porém por conterem apenas metade do conjunto cromossômico, são estéreis. Na ausência de pareamento homólogo, o processo meiótico resultará em gametas desbalanceados que contêm menos do que o conjunto necessário (*n*) de cromossomos. Contudo, a fertilidade porde ser restaurada por meio da duplicação do conjunto cromossômico (espontaneamente ou induzida), dando origem então às plantas denominadas DH. Em outras palavras, plantas completamente homozigotas geneticamente normais e fenotipicamente estáveis representando toda a variação gamética devido ao processo de recombinação da meiose (Islam e Tuteja, 2012).

As plantas DH são uma ferramenta valiosa para os programas de melhoramento genético uma vez que permite a produção de plantas completamente homozigotas em uma única geração. Esta última característica faz com que a liberação de cultivares seja acelerada em cerca de cinco anos, quando comparada ao melhoramento tradicional (Barkley e Chumley, 2012). O uso destas plantas DH traz ainda como vantagem melhor eficiência de seleção, uma vez que um número menor de plantas é necessário para a busca de características selecionadas, bem como a ausência do mascaramento da heterozigose reduz os custos; e permite uma seleção induzida de algumas características ainda *in vitro*. Por exemplo, a seleção *in vitro* tem sido usada para desenvolver genótipos resistentes a patógenos e tolerantes a desequilíbrios minerais e à seca (Liu et al., 2002), sem mencionar seu uso na pesquisa básica e avançada (Abdollahi et al., 2007) e na detecção de QTLs (do inglês, *Quantitative Trait Loci*) (Seymour et al., 2012).

Os DH representam um grande avanço na manipulação genética *in vitro*, constituindo-se em uma importante ferramenta dentro da biotecnologia. Existem várias técnicas para a geração destes indivíduos incluindo a gimnogênese, eliminação de cromossomos em embriões híbridos interespecíficos, a cultura de anteras e de micrósporos isolados (Forster et al., 2007). Contudo, a IMC vem ocupando espaço significativo nas pesquisas da área de biologia celular (Rodrigues et al., 2004), sendo reconhecida como a de maior potencial para produzir grandes números de haploides e DH (Kasha et al., 2001; Zheng, 2003; Forster et al., 2007), especialmente em situações em que não se obtém resultado satisfatório pelas outras técnicas de haploidização (Kasha et al., 2001; Zheng et al., 2002). Ao utilizar o micrósporo, há a vantagem de isolar milhares de células de uma vez (Jähne e Lörz, 1995; Peters et al., 1999; Li et al., 2005), já que há cerca de mil a dois mil grãos de pólen por antera (Peters et al., 1999). Em trigo a IMC permite a regeneração de um maior número de plantas férteis por espiga (Patel et al., 2004; Li et al., 2005; Shariatpanahi et al., 2006; Labbani et al., 2007). Tem sido mostrado, também, que esta técnica resulta em maior frequência de plantas verdes regeneradas (Ritala et al., 2001; Liu et al., 2002; Labbani et al,. 2007). Uma vantagem adicional constitui na maior proporção de plantas DH espontaneamente geradas na IMC, quando comparada à cultura de anteras, para trigo e cevada (Jähne e Lörz, 1995; Chugh e Eudes, 2007), podendo ser possível, em alguns casos, a eliminação da etapa de duplicação induzida de cromossomos.

Embora a haploidização tenha tido sucesso em culturas de anteras e de micrósporos em várias espécies, existem algumas vantagens importantes associadas à IMC sobre a cultura de anteras: abundância de micrósporos por espiga, ausência da parede da antera que pode prejudicar o processo da cultura, segurança de que todos os embriões desenvolvidos são derivados de micrósporos, melhor disponibilidade de nutrientes para as células em cultura e facilidade e possibilidade de rastrear o desenvolvimento e a observação individual de células (Liu et al., 2002; Ferrie e Caswell, 2011). Diversos protocolos de IMC têm sido estabelecidos para diversas espécies de plantas. Contudo, a cultura de micrósporos de cereais ainda precisa de alguns ajustes para ser usada em grande escala, especialmente focando na taxa de produção de embriões, regeneração de plantas verdes e duplicação espontânea de cromossomos (Castillo et al., 2009).

O processo de androgênese é um dos mais notáveis exemplos de totipotência celular. Entretanto, alguns requisitos são necessários para que uma célula gamética se desenvolva numa nova planta. Condições fisiológicas da planta doadora e o estádio de desenvolvimento do micrósporo são muito importantes, uma vez que podem modificar a resposta androgenética até

em genótipos responsivos (Germanà, 2011). Pretratamentos de estresses também são considerados cruciais para desencadear a totipotência e a rota esporofítica no micrósporo (Shariatpanahi et al., 2006; Islam e Tuteja, 2012). O princípio básico da regeneração de plantas a partir da célula reprodutiva é a mudança da rota gametofítica do futuro grão de pólen para a rota esporofítica, na qual o micrósporo sofre sucessivas mitoses e forma uma estrutura embriogênica, a qual se desenvolverá em uma planta (Peters et al., 1999; Seguí-Simarro e Nuez, 2008).

OBJETIVOS

OBJETIVOS

Objetivo geral

O objetivo geral deste trabalho é investigar respostas baseadas em cultura de tecidos e análises transcritômicas para obtenção de tolerância à seca em trigo (*Triticum aestivum* L.).

Objetivos específicos

a) Identificar genes relacionados à resposta da planta ao estresse hídrico;

b) Identificar genes responsivos à seca órgão específicos (folha e raíz)

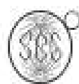
c) Testar dois diferentes pretramentos (4 °C e 2-HNA) para a produção de plantas haploides/duplo-haploides;

d) Testar o efeito da presença/ausência do agente gelificante Ficoll no meio de cultura de indução na técnica de micrósporos isolados;

b) Identificar genótipos com potencial androgenético, para obtenção de plantas haploides/duplo-haploides a partir de micrósporos isolados.

CAPÍTULO I

Gene expression analysis reveals important pathways for drought response in leaves and roots of a wheat cultivar adapted to rainfed croppring in the Cerrado Biome



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Gene expression analysis reveals important pathways for drought response in leaves and roots of a wheat cultivar adapted to rainfed cropping in the Cerrado biome

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Abstract

Drought limits wheat production in the Brazilian Cerrado biome. In order to search for candidate genes associated to the response to water deficit, we analyzed the gene expression profiles, under severe drought stress, in roots and leaves of the cultivar MGS1 Aliança, a well-adapted cultivar to the Cerrado. A set of 4,422 candidate genes was found in roots and leaves. The number of down-regulated transcripts in roots was higher than the up-regulated transcripts, while the opposite occurred in leaves. The number of common transcripts between the two tissues was 1,249, while 2,124 were specific to roots and 1,049 specific to leaves. Quantitative RT-PCR analysis revealed a 0.78 correlation with the expression data. The candidate genes were distributed across all chromosomes and component genomes, but a greater number was mapped on the B genome, particularly on chromosomes 3B, 5B and 2B. When considering both tissues, 116 different pathways were induced. One common pathway, among the top three activated pathways in both tissues, was starch and sucrose metabolism. These results pave the way for future marker development and selection of important genes and are useful for understanding the metabolic pathways involved in wheat drought response.

Keywords: 454 sequencing, candidate genes, RT-qPCR, Triticum aestivum, *water deficit.*

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Introduction

The central part of Brazil, consisting mostly of the Cerrado biome, is considered to be the new frontier for increasing Brazilian wheat production. Although the wheat harvest in that region can reach more than 4,000 kg.ha^{-1} on average in irrigated areas (De Mori and Só e Silva, 2013), rainfed cropping has great potential to improve production once it requires little investment and has a large area for expansion. To follow this path, rainfed wheat production in the Brazilian Cerrado must cope with three major abiotic stresses: soil acidity, heat and drought (Scheeren *et al.*, 2008).

Drought is broadly accepted as the most important environmental stress in agriculture and is a major constraint on plant survival, productivity and quality (Nezhadahmadi *et al.*, 2013). Drought is forecast to be exacerbated by incremental increases in temperature and changes in precipitation profiles. For instance, each degree °C of increase in global mean temperature is projected to reduce global wheat grain production by approximately 6% (Asseng *et al.*, 2015). In Brazil, wheat yield is theorized to be reduced up to 31% with temperature increases of 3–4 °C, offsetting the positive effects of increased CO_2 levels on wheat grain yield (Siqueira *et al.*, 2000; Streck and Alberto, 2006). Because water is largely used in irrigated agriculture (70–90% of global water use), this sector will be heavily affected by climate change (Bär *et al.*, 2015). In this context, improving drought tolerance of wheat cultivars is essential for yield increases in rainfed farming.

Plants have developed several mechanisms to address drought stress, and drought tolerance is a quantitative trait with a complex response at molecular, metabolic and physiological levels (Nezhadahmadi *et al.*, 2013). In wheat, sev-

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eral traits, such as the timing of phenological stages, carbohydrate metabolism, stomatal conductance, osmotic adjustment, late senescence of the flag leaf, flowering time, increased root:shoot ratio, high values of soluble stem carbohydrate shortly after anthesis, and increased early ground cover, among others, have been linked to the drought tolerance response (Fischer *et al.*, 1998; Foulkes *et al.*, 2007; Reynolds *et al.*, 2007; Nezhadahmadi *et al.*, 2013). To understand the mechanisms underlying this response, gene expression analysis using subtractive cDNA libraries and microarrays have been performed in wheat (Zhang *et al.*, 2004; Way *et al.*, 2005; Xue *et al.*, 2006, 2008; Mohammadi *et al.*, 2008; Ergen *et al.*, 2009; Li *et al.*, 2012; Reddy *et al.*, 2014). However, nowadays, the most preferred technique to evaluate gene expression is high-throughput cDNA sequencing (RNA-Seq) based on next-generation sequencing technology. Up till now, the use of RNA-seq, which is not limited to the number of transcripts predefined in probes, to study the drought response in bread wheat (*Triticum aestivum*) has been rare (Okay *et al.*, 2014; Liu *et al.*, 2015; Budak *et al.*, 2015). One obstacle to that type of study in bread wheat is the complexity of its hexaploid genome, which is estimated to be 17 gigabases in size and encoding more than 124,000 genes, of which approximately 76% of the assembled sequences contain repeats (IWGSC- International Wheat Genome Sequencing Consortium, 2014).

In the present study, a gene expression analysis was performed aiming at the identification of candidate genes involved in the drought responses in a wheat cultivar adapted to the Brazilian Cerrado region. A set of 4,422 candidate genes was obtained, with 2,124 specific to roots, 1,049 specific to leaves, and 1,249 sequences that were common between both tissues. A strong correlation between RNA-seq and RT-qPCR (quantitative reverse transcription polymerase chain reaction) data was observed. The importance of specific chromosome regions and genomes, as well as the most activated pathways, are reported. These results are also applied to the understanding of the metabolic pathways involved in wheat drought response.

Materials and Methods

Plant material, drought stress and RNA extraction

The Brazilian wheat cultivar MGS1 Aliança (*Triticum aestivum*) was used in this study due its good productivity in rainfed farming in the Brazilian Cerrado. This cultivar showed the highest yield across different sowing dates among 152 wheat genotypes tested under drought conditions in the Cerrado (Ribeiro Júnior *et al.*, 2006). MGS1 Aliança was released in 1990 by EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais) and it is still recommended for wheat production in the Cerrado (Comissão Brasileira de Pesquisa de Trigo e Triticale (2016). Seeds of MGS1 Aliança were surface-sterilized in NaClO (0.2% of active chlorine) for 1 min, washed three times with sterile distilled water (1 min each) and germinated at 23 °C in the dark for two days. Germinated seeds were transferred to pots (3 seeds per pot) containing 6.5 kg of a mixture of soil, sand and vermiculite (2:1:1) and incubated in a glasshouse with natural light at 22 ± 4 °C. Plants were watered daily. Control plants were grown for five weeks at 100% of field capacity while, in the stress treatment, plants were watered for 2 weeks at 75% of field capacity followed by 3 weeks of water deprivation. The water status of the plants was monitored by measurement of the leaf relative water content (RWC) (Barrs and Weatherley, 1962) and the water potential (Scholander pump). All three plants from one pot were pooled and the leaves and roots were collected separately, immediately frozen in liquid nitrogen, and stored at -80 °C. Total RNA was extracted with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions, and purified using an RNeasy Mini Kit (Qiagen). During the purification, a DNase digestion step was performed with an RNase-free DNase Set (Qiagen). RNA quality was assessed using a Bioanalyzer (Agilent) and samples with an RIN (RNA integrity number) > 7.5 and rRNA ratio > 1.5 were used in subsequent analyses.

454 Sequencing

Total RNA was sent to Macrogen Inc. (South Korea) for sequencing of four libraries (control root, treated root, control leaf, and treated leaf) on a Genome Sequencer FLX Titanium instrument (Roche) according to standard protocols.

Sequence data analysis, *de novo* assembly and functional annotation

The sequence data analysis, assembly and annotation followed the protocol available from Macrogen. Briefly, raw data were processed using the Roche GS FLX software v 2.8. The reads were assembled using GS De Novo Assembler software v 2.6. The assembly parameters were kept at default values for both the assembly and cDNA option. Singleton cleaning (elimination of contaminants, low quality, low-complexity and vectors) was performed in SeqClean (http://sourceforge.net/projects/seqclean/), with a minimum length of 100 bp and Lucy (http://lucy.sourceforge.net/). Similarity analysis was performed using BLAST (1.0e-3 cutoff) and the Gene Ontology (GO) (http://www.geneontology.org/) database to obtain sequence annotations. The data discussed in this study have been deposited in NCBI Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE81833 (www.ncbi.nlm.nih.gov/geo).

Statistical analysis

Statistical analyses of differentially expressed transcripts between the control and stressed treatments were

performed with the DEGseq v 2.6 R package (http://www.bioconductor.org/packages/2.6/bioc/html/DEGseq.html), using the MARS model. Isotigs with a p-value < 0.001 were considered significantly different. The compared samples were: control leaf assembled sequences (isotigs) versus drought-stressed leaf assembled sequences (isotigs), and control root assembled sequences (isotigs) versus drought-stressed root assembled sequences (isotigs).

CAP3 assembly, Blast2GO, genome assembly and functional annotation

A second round of assembly was performed with two aims: (1) to group sequences lacking previous significant identity that could belong to the same transcript but may have come from different genomic regions; and (2) to compare the expression of transcripts in each tissue (leaf and root). All the isotigs and singleton sequences from roots and leaves, as well as the quality sequence files, were used as input. The analysis was performed with CAP3 (Huang and Madan, 1999) software using default parameters, except for a 40 overlap length cutoff and a 90 overlap percent identity cutoff. Assembled sequences that contained one or more differentially expressed transcript in their composition and had previously been determined (by DEGseq) were considered as differentially expressed (DE) as well. These DE sequences (contigs and singletons) from CAP3 assembly were annotated using Blast2GO software (Götz *et al.*, 2008) with default parameters. Blast2GO performs searches against the Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Interpro databases in order to determine the metabolic pathways they belong to. After annotation, the sequences obtained from the Cap3 assembly were mapped against the available Ensembl genomic sequences of *Triticum aestivum* (v. 1.26; http://plants.ensembl.org/Triticum_aestivum/Info/Index) using BWA (Li and Durbin, 2010) and SAMtools (Li *et al.*, 2009) to analyze the distribution of these sequences over the wheat chromosomes and genomes. Mapping was carried out using BWA-SW "-t 6" or 6 threads. A chi-square test was used to determine if the distribution among the *T. aestivum* component genomes was statistically different. To identify transcriptions factors (TFs) encoding transcripts among the genes differentially expressed under drought, the sequences were compared by similarity search (BlastP cutoff 1e-100) against the Plant Transcription Factor Database version 3.0 (PlantTFDB) (http://www.bmicc.org/web/english/search/planttfdb) (Jin *et al.*, 2014).

RT-qPCR

Drought stress treatment was similar to the procedure described previously, except that five plants were cultivated per pot and incubated in a growth cabinet with controlled conditions (22 °C with 16/8 hours light/dark and humidity at 60%). Root and leaf samples, from a pool of five plants belonging to the same pot, were collected at two time points: after two weeks of growth and after five weeks of growth. The experiment was performed in triplicate biological samples. RNA extraction and purification were performed as described above. RNA quality and quantity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and 1.5% agarose gels. Synthesis of cDNA was done with the Thermo Script™ RT-PCR System (Invitrogen) using 2 µg of DNA-free RNA and Oligo $(dT)_{20}$ primers. Gene-specific primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). RT-qPCR assays were conducted in technical triplicates using a 7500 Real Time PCR System (Applied Biosystems) with 7500 Software v2.0.6. The cycles and reactions were as follows: 10 min at 95 °C, followed by 40 cycles for 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and a final melting curve analysis protocol consisting of heating to 95 °C for 15 s, 60 °C for 1 min and heating to 95 °C. Reactions were performed in a final volume of 25 µL, containing 12.5 µL of SYBR® Green PCR Master Mix (Applied Biosystems), 10 µL of diluted cDNA (1:100), 0.25 µL of primers (10 µM each) and 2.25 µL of water. Relative expression data analyses were performed by comparative quantification of the amplified products using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The reference genes used for normalization of expression were those encoding ATPase, Ribosylation Factor, RNAseL (Paolacci *et al.*, 2009), Ta10105, Ta14126 and Ta27922 (Long *et al.*, 2010). The geNorm v3.5 software (http://medgen.ugent.be/ ~jvdesomp/genorm/) was used to select the two best reference genes for the respective experimental condition.

Results

Sequencing analysis

In order to search for candidate genes and metabolic pathways associated to drought stress in wheat, high-throughput sequencing was done using 454 sequencing technology with cDNA originating from drought-stressed and control roots and leaves. When harvested, the mean values for leaf water potential and for RWC were, respectively, -0.38 MPa and 98% in the control plants and -2.12 MPa and 50.1% in the stressed plants, indicating that, based on the parameters detailed by Hsiao (1973), the treated plants were severely drought-stressed. The sequencing analyses yielded 1,225,527 reads from the four libraries (control and treated roots, control and treated leaves). Among these, 305,731 reads were obtained for the root control sample and 300,665 for roots under drought stress. From the total 606,396 reads, 453,218 reads (74.7%) were fully assembled and 32,085 isotigs were identified, with an average size of 1,085 bases and an N50 of 1,299. Fifteen percent (90,933 reads) were partially assembled,

and 6.6% (40,377 reads) remained as singletons, with 37,457 reads considered valid. Additionally, 17,257 reads were anchored to repeat regions, 4,179 were considered outliers and 345 were too short to be used in the computational analysis (Table 1).

Regarding the leaf-derived sequences, 619,131 reads were used in the assembly computation (319,997 from leaves in control sample and 299,134 from leaves under drought stress). From the total, 519,150 reads (83.8%) were fully assembled and 19,899 isotigs were identified, with an average size of 952 bases and an N50 isotig size of 1,115. Approximately 10% of the reads (65,475 reads) were partially assembled and 5% (31,374 reads) were singletons, with 28,880 reads considered valid. Furthermore, 189 reads anchored to repeat regions, 2,806 were considered outliers and 135 were too short to be used in the computational analysis (Table 1).

Search for candidate genes

After assembly and annotation, we searched for candidate genes differentially expressed between control and treated samples. The homogeneous distribution of the four libraries is presented in Figure S1. A total of 4,422 candidate genes was identified in both tissues ($p < 0.001$) (Table S1). Among those, 2,808 isotigs were obtained from roots, with 1,100 up-regulated and 1,708 down-regulated isotigs under stress conditions. Statistical analysis showed that 1,614 isotigs in leaves were significantly different (p-value < 0.001). Up-regulation occurred in 1,017 isotigs, while down-regulation was observed in 597.

Gene Ontology (GO) categories of the candidate genes are shown in Figure 1. The functional annotation of the root and leaf isotigs revealed that 41% and 40% of the sequences were, respectively, involved in biological process, 25% and 24% in molecular function, 33% and 36% were cellular components, while the remaining sequences were no-hits. The comparison of GO terms among the four main categories revealed that the distribution of candidate genes was similar between root and leaf. Among the sequences annotated in biological processes, cellular and metabolic processes were highly represented. Among molecular functions, sequences related to binding and catalytic activity were the most represented GO terms. Regarding cellular component, the most represented category was cell part.

Table 1 - Analyses of the reads obtained from the four libraries (root control, root stressed, leaf control, and leaf stressed)

Reads	Root	Leaf
Number of reads	606,396	619,131
Number of bases	366,686,703	345,617,595
Reads in control condition	305,731	319,997
Number of bases in control condition	184,485,642	177,132,266
Average read length	603,425	553,544
Reads in drought condition	300,665	299,134
Number of bases in drought condition	182,201,061	168,485,369
Average read length	605,994	563,244
Fully assembled	453,218	519,150
Partially assembled	90,933	65,475
Singletons	40,377	31,374
Repeat	17,257	189
Outlier	4,179	2,806
Too short	345	135
Number of isogroups	24,900	16,585
Average contig count	1.5	1.4
Number of isogroups with one isotig	20,969	14,592
Number of isotigs	32,085	19,899
Average isotig size	1085,508	952.03
N50 isotig size	1,299	1,115
Valid singletons	37,457	28,880

Isogroup is the collection of contigs containing reads that imply connections between them. Isotig is analogous to an individual transcript.

Expression profile validation

For validation of the gene expression analysis, a second and independent experiment was performed where plant samples were collected after two and five weeks of growth. For the two-week-old plants, mean leaf water potential and RWC values were -0.37 MPa and 96.7% for the control plants and -0.39 MPa and 95.8% for the treatment. After five weeks of growth, the mean values for leaf water potential and RWC were -0.42 MPa and 95.6% for the control plants and -2.04 MPa and 54.6% for the drought-stressed plants. This indicates that the plants had a similar water status before water was withheld but a different status after five weeks of growth. The analyses of the expression profile in two-week-old control (just before the irrigation withholding) as well as in five-week-old control and treated plants, allowed for the comparison of candidate gene expression not only after the drought period but also before the stress.

The relative expression of 15 root- and 20 leaf-derived transcripts (Table S2) was measured by RT-qPCR for experimental validation of the RNA-seq data. These 35 transcripts were chosen for validation because they showed different levels of expression (up- or down-regulated), are associated to different enzymes from the same pathway or belong to different metabolic pathways (Table S1). The expression of four root isotigs was not validated because the control and the drought-stressed samples after five weeks of growth were statistically similar. On the other hand, the expression of five root isotigs (R15, R24, R28, R36 and R22) were significantly different between treated and control plants after five weeks of growth (Figure 2A). When comparing the samples collected from two-week-old plants, only the R15 sequence was significantly different

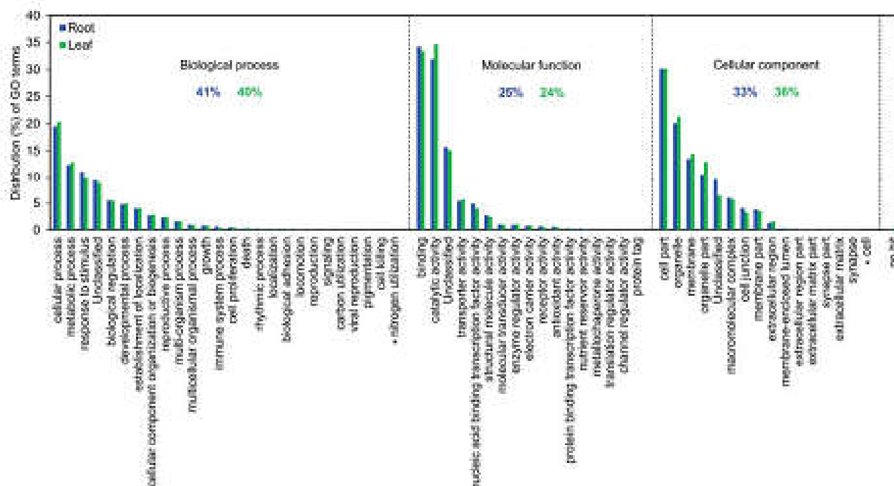

Figure 1 - Functional annotation of the 4,422 candidate genes detected in root and leaf tissues of the wheat cultivar MGS1 Aliança under drought stress. Gene Ontology (GO) analysis was performed for three main categories (biological process, molecular function and cellular component). Asterisk indicates GO terms present in root tissue only. No-hit sequences correspond to 0.1% and 0.3% of the leaf and root sequences, respectively. Note that a sequence may have multiples terms associated to it.

between treatments. For the other six isotigs, expression in control samples was not detected, but was detected in the drought-treated samples, indicating that their expression changed in response to water deprivation. Because of this change, the mean Cq values are presented (Figure 2B). A set of 20 transcripts from leaves was also evaluated by RT-qPCR. The expression of 12 isotigs (L1, L4, L8, L9, L10, L11, L15, L16, L17, L23, L25 and L29) were significantly different between the control and treated samples of five-week-old plants (Figure 3). Excluding the six root sequences with non-detected Cq values, the Pearson's correlation between the RNA-seq and RT-qPCR data for the other 29 transcripts was 0.78 (Figure S2).

Genome localization and tissue-specificity of the candidate genes

After comparing the candidate genes between root and leaf samples (2,808 and 1,614, respectively), 2,124 sequences were found to be specifically expressed in roots, 1,049 specifically in leaves, and 1,249 sequences were common to both tissues (Figure 4A). One sequence specific for each tissue (isotig06719, the same as the one used to design the primer R33 listed on Table S2), and isotig05306 (annotated as "AT1G47890 - defense response - kinase activity") for root and leaf, respectively, were used for RT-qPCR analyses. The positive amplification of these sequences in specific tissues (Figure 4B) corroborates our *in silico* analysis. Searches against KEGG failed to detect pathways for these specific sequences.

An additional assembly (performed with the CAP3 software) allowed for the comparison of transcript expression between the two tissues. A total of 118,321 sequences were used (32,085 isotigs and 37,457 singletons from roots and 19,899 isotigs and 28,880 singletons from leaves). After the assembly, 11,746 contigs and 69,407 singlets were obtained with 1,393 and 2,594, respectively, considered as differentially expressed and, consequently, as candidate genes. The 3,987 candidate genes (1,393 contigs and 2,594 singlets) were analyzed for functional annotation, with 96.4% of sequences annotated and 4.5% showed mapping results (Figure S3).The highest similarity rate corresponded to sequences from *Aegilops tauschii* (30%), followed by *Hordeum vulgare* (29.5%), *Triticum urartu* (15%), *T. aestivum* (13%) and *Brachypodium distachyon* (5.5%) (Figure S3). With regards to the GO distribution of the sequences assembled by CAP3 (Figure 5), the categories with the most abundant sequences in biological processes were metabolic processes, cellular processes, response to stimulus, single-organism processes, localization and biological regulation; for molecular function the most prevalent categories were catalytic activity and binding; and for cellular components the categories were cell, organelle and membrane.

To identify biological pathways that are active in wheat drought response, the 3,987 candidate genes de-

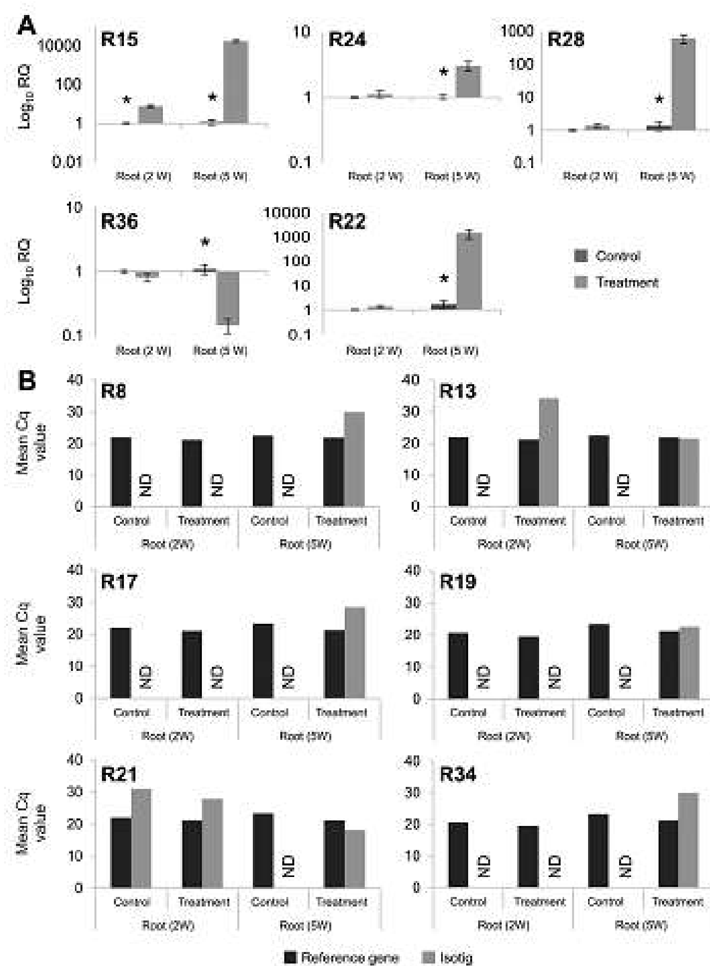

Figure 2 - RT-qPCR analysis of selected root transcripts. Expression is shown for 11 transcripts that were validated by RT-qPCR. Wheat roots were sampled from two-week-old (2 W) plants, where control plants were irrigated at 100% of field capacity (FC) and treated plants were irrigated at 75% FC. Five-week-old (5 W) plants are represented by control plants (irrigated at 100% FC) and treated plants, where irrigation was withdrawn for three weeks. The experiment was performed in triplicate biological samples and in technical triplicates. (A) Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Asterisks represent significantly different (Student's *t*-test, $p \leq 0.05$) transcript levels. (B) Mean of the Cq value. "ND" means not detected. For more details on primers see Table 2 and Table S2.

scribed above were analyzed using Blast2GO software against KEGG pathways. The results revealed 116 different pathways (Table S3) involved in wheat drought response. The top 20 pathways (with the highest number of sequences) for root and leaf tissues are presented in Figure 6.

Among the top 20 pathways in both tissues, 28 different pathways were detected, with 12 pathways in common but ranked in different positions. Starch and sucrose metabolism pathway-related transcripts had the highest ranking in roots but were the third most commonly identified ones in

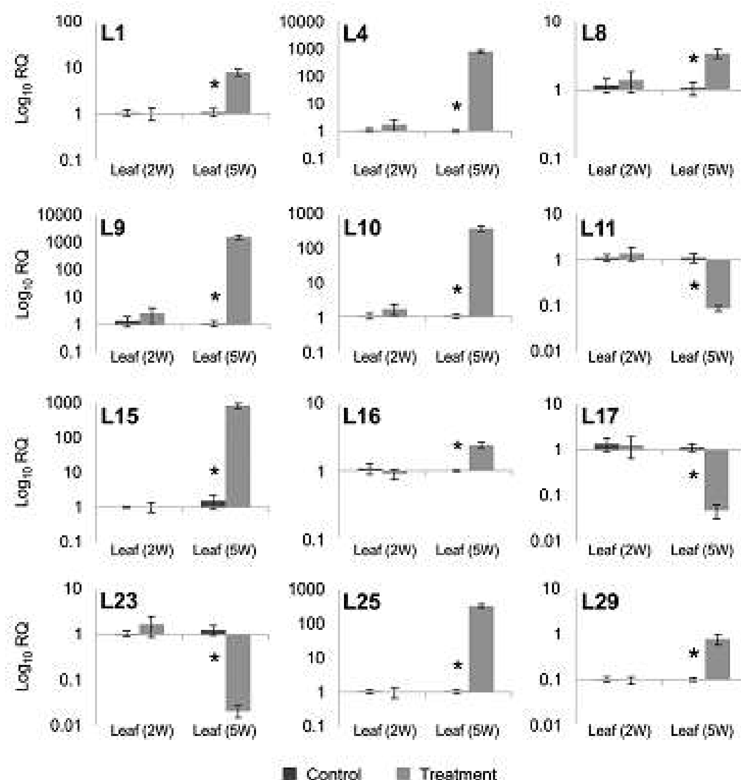

Figure 3 - RT-qPCR analysis of selected leaf transcripts. Expression is shown for the 12 transcripts that were validated by RT-qPCR. Wheat leaves were sampled from two week-old (2 W) plants, where control plants were irrigated at 100% of field capacity (FC) and treated plants were irrigated at 75% FC. Five-week-old (5 W) plants are represented by control plants irrigated at 100% FC, and treated plants, where irrigation was withdrawn for three weeks. The experiment was performed in triplicate biological samples, with technical triplicates for each. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Asterisks represent significantly different (Student's *t*-test, $p \leq 0.05$) transcript levels. For more details on primers see Table 2 and Table S2.

leaves. In addition, the number of sequences for the fructose and mannose pathways was 3.5 times higher in leaves, while the arginine and proline metabolism pathway presented a similar number of sequences for both tissues. We also analyzed putative pathways related to the 22 up-regulated sequences with annotation that were tested by RT-qPCR (Table S2). Among the 22 transcripts, 10 generated results when searched against KEGG, revealing 13 different pathways (Table 2). With the exception of L4, whose function was not linked to a specific pathway, all isotigs remained in the same enzyme classes and pathways as identified before the assembling with CAP3.

An analysis of the distribution of candidate genes across the wheat genome was done by BLAST searches against the sequenced *T. aestivum* cv. Chinese Spring genome (Figure 7, Figure 8). Among the 3,987 candidate genes assembled by CAP3, 158 transcripts could not be mapped. More candidate genes were located in the B genome ($p < 0.001$ by the chi-square test) compared to the A and D genomes (Figure 7A). In addition, chromosomes 3B, 5B and 2B had more sequences related to drought response (Figure 7B). Candidate genes specific to roots or leaves and in common between the two tissues were detected in all genomes and chromosomes (Figure 7C-E). Although chromosomes 3B, 5B and 2B showed the highest number of candidate genes, most of the transcripts mapping to these chromosomes were down-regulated. In fact, only chromosomes 5A, 6B, 7B and 3D presented at least 10% more up-regulated transcripts than down-regulated ones. The chromosomes with more up-regulated sequences were 3B,

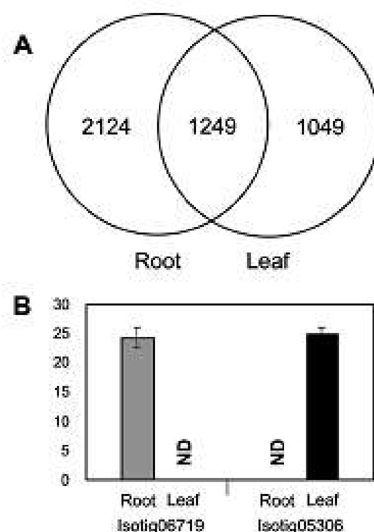

Figure 4 - Root or leaf specific candidate genes identified in response to drought stress in the wheat cultivar MGS1 Aliança. (A) Venn diagram representing the number of candidate genes obtained after DEGseq analysis. The candidate genes are distributed in root and leaf according to CAP3 assembly. (B) RT-qPCR analysis of two randomly selected specific transcripts, showing that isotig06719 (see primer sequences on Table S2) is specifically expressed in root tissue and the isotig05306 (primers CCGTGTCACTTCCCTTGATT and GGAGAGGTTGAGATGGGTGA) is expressed in leaves only. "ND" means not detected.

5B and 2A. The CAP3 assembly also allowed for the identification of no-hits candidate genes per chromosome (expression only in root or leaf and expression in both tissues), where 88 no-hit sequences were detected (Figure 8). The two chromosomes with the highest numbers of no-hit sequences were chromosomes 2B and 3B. In these chromosomes, most of the no-hit sequences was specific to roots (Figure 8B).

Transcription factors

Transcription factors (TFs) play a central role in the plant response to drought (Tuberosa and Salvi, 2006). Thus, we searched for TFs among the differentially expressed sequences. To achieve this, the similarity of the sequences was evaluated against a plant transcription factor database (PlantTFDB) with an E-value cutoff of e-100. Several TFs, such as E2F/DP, SRS, WOX, M-type, NF-YB, GRF, LBD, CPP, GeBP, STAT, BBR-BPC, Whirly, BES1, NF-YA, NF-YC, HB-PHD, GATA, DBB, NF-X1, VOZ, CO-like, AP2, B3, SBP, Dof, ARR-B, HB-other, MIKC, EIL, Nin-like, Trihelix, G2-like, HD-ZIP, CAMTA, MYB, HSF, ERF, TALE, WRKY, C2H2, FAR1, bHLH, NAC, bZIP, MYB related, C3H, ARF, GRAS and DREB were found (Figure S4). The species with the greatest numbers of hits were *Oryza sativa japonica*, *Sorghum bicolor*, *T. aestivum* and *B. distachyon* (data not shown).

Discussion

Rainfed wheat plants growing in the Cerrado biome need to cope with different abiotic stresses, with drought being one of the most important factors. In this context, a wheat cultivar adapted to that region represents an excellent model to study drought response mechanisms. Here, we identified 4,422 candidate genes associated to severe drought response in both root and leaf tissues during the tillering stage of the wheat cultivar MGS1 Aliança. Although the early stages of pollen development are the most vulnerable to drought in cereals (Fischer, 1973), seed germination and early seedling growth are also considered critical stages for wheat establishment (Zhang *et al.*, 2014). Therefore, the early phase of wheat development is an important stage to evaluate the effect of drought. Moreover, for wheat farming in the Cerrado, dry spells can occur during the tillering stage (Ribeiro Júnior *et al.*, 2006).

The functional annotation of the transcripts reported here (Figure 1) is in agreement with other reports (Deokar *et al.*, 2011; Li *et al.*, 2012; Zhou *et al.*, 2012). However, one important difference is the technique used here (454 sequencing technology) in comparison to the one used to evaluate the gene expression in previous studies. The 454 technology is an 'open' system in which gene expression can be accurately measured by counting the detected identical transcripts, potentially capturing all the transcripts in a sample (Coram *et al.*, 2008). Although the Blast2GO analysis showed similarity of the bread wheat expressed sequences with *A. tauschii* and *H. vulgare* (Figure S3), sequences with unknown function or no-hits were also found (Figure 8B). The no-hit sequences are an important contribution of high-throughput sequencing techniques because they represent a more complete description of gene expression and should be important to understand drought stress response in wheat. In our survey, the distribution of the no-hit sequences was higher on chromosome 2B (38, considering both chromosome arms). Regarding the three wheat genome components, the B genome harbored the highest number of no-hit sequences (44) when compared to the D genome (27) and A genome (17) (Figure 8B).

The total number of identified repressed transcripts in response to drought (2,305 for roots and leaves) was higher than the number of induced transcripts (2,117 for both tissues). However, when considering each tissue separately, the number of repressed transcripts was lower than the induced transcripts in leaves (597 repressed and 1,017 induced) but higher in roots (1,708 repressed and 1,100 induced). A higher number of repressed transcripts under drought conditions in hexaploid wheat was also reported by

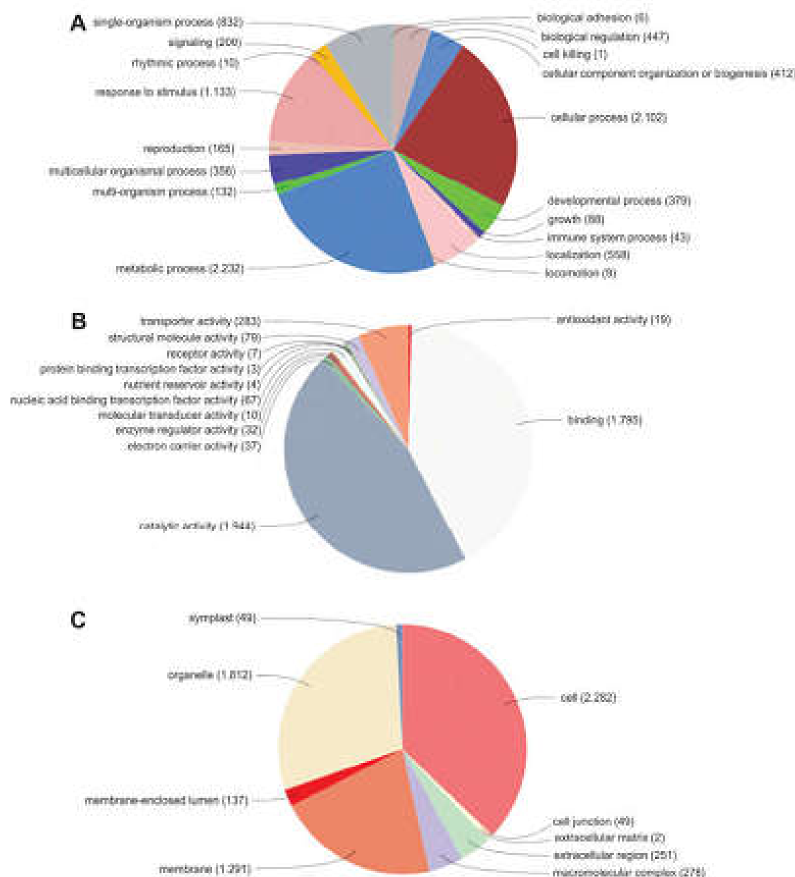

Figure 5 - Pie diagrams demonstrating the percentage of contigs and singlets generated in CAP3 within the functional categories of Gene Ontology. Results are based on Blast2GO data mining. (A) Biological process, (B) molecular function, and (C) cellular component.

other authors (Mohammadi *et al.*, 2008; Li *et al.*, 2012). To validate the repression/induction detected by the RNA-seq experiment, we used RT-qPCR to confirm the expression profile of 35 candidate genes (15 from roots and 20 from leaves). These candidate genes are representative of different pathways or code for different enzymes in the same pathway (Table S2), providing a broad validation of the RNA-seq experiment. The RT-qPCR assays revealed statistically significant differences for 73.3% and 60% sequences from root and leaf, respectively (Figure 2, Figure 3). Significant differences were not detected for the remaining sequences, but the direction of the expression profile was generally the same, and the Pearson's correlation between the RNA-seq and RT-qPCR data was 0.78 (Figure S2). It is important to note that the RT-qPCR assays were done as a second and independent experiment for confirmation of the gene expression. For RNA-seq experiments reported previously by others, the RT-qPCR correlation coefficients varied from 0.58 to 0.98 (Nagalakshmi *et al.*, 2008; Kogenaru *et al.*, 2012). The RT-qPCR technique was also used to confirm the expression of two tissue-specific sequences found among the candidate genes (Figure 4).

An important mechanism used by plants to tolerate drought is osmotic adjustment (Nezhadahmadi *et al.*,

Table 2 - Biological pathways related to 10 sequences validated by RT-qPCR and the respective contigs where they assemble in CAP3.

Primer*[1]	Pathway*[2]	#Seqs	#Enzs
R8	Fructose and mannose metabolism	Contig607LR	ec:1.1.1.21 - reductase
R8	Galactose metabolism	Contig607LR	ec:1.1.1.21 - reductase
R8	Glycerolipid metabolism	Contig607LR	ec:1.1.1.21 - reductase
R8	Glycosphingolipid biosynthesis - globoseries	Contig607LR	ec:3.2.1.22 - melibiase
R8	Pentose and glucoronate interconversions	Contig607LR	ec:1.1.1.21 - reductase
R8	Pyruvate metabolism	Contig607LR	ec:1.1.1.21 - reductase
R24	Arginine and proline metabolism	Contig3704LR	ec:2.7.2.11 - 5-kinase; ec: 1.2.1.41 - dehydrogenase
R28	Galactose metabolism	Contig575LR	ec:2.4.1.82 - galactosyltransferase, ec:3.2.1.22 - melibiase
R28	Glycerolipid metabolism	Contig575LR	ec:3.2.1.22 - melibiase
R28	Sphingolipid metabolism	Contig575LR	ec:3.2.1.22 - melibiase
L4	Purine metabolism	Contig506LR	ec:3.6.1.3 - adenylpyrophosphatase
L8	Arginine and proline metabolism	Contig5346LR	ec:1.2.1.19 - dehydrogenase
L8	Beta-Alanine metabolism	Contig5346LR	ec:1.2.1.19 - dehydrogenase
L8	Glycine, serine and threonine metabolism	Contig5346LR	ec:1.2.1.8 - dehydrogenase
L9	Pyruvate metabolism	Contig178LR	ec:1.1.1.38 - dehydrogenase (oxaloacetate-decarboxylating)
L10	Starch and sucrose metabolism	Contig472LR	ec:2.4.1.12 - synthase (UDP-forming)
L15	T cell receptor signaling pathway	Contig149LR	ec:3.1.3.16 - phosphatase
L25	Arginine and proline metabolism	Contig132L	ec:2.7.2.11 - 5-kinase; ec: 1.2.1.41 - dehydrogenase
L29	Arginine and proline metabolism	Singlet	ec:2.7.2.11 - 5-kinase; ec: 1.2.1.41 - dehydrogenase

Analysis was performed with Blast2GO against the Kyoto Encyclopedia of genes and Genomes (KEGG). #Seqs means the number of sequences in that pathway; #Enzs indicates the number of enzymes corresponding to the sequences.
*[1] One primer can correspond to more than one pathway.
*[2] Alphabetical order based on the primer name. See more detail of the primers in Table S2.

2013). In this process, accumulation of solutes in cells allows to decrease the osmotic potential and to maintain the cell turgor as drought stress develops. Osmoprotectants synthesized in response to drought stress include low molecular weight and highly soluble compounds, such as sugars, proline, polyols, and quaternary ammonium (Pintó-Marijuan and Munné-Bosch, 2013). In wheat, osmotic adjustment is positively associated with higher yield under drought stress and could partly explain the genotypic variation in stomatal response of wheat cultivars that differ in their responses to drought (Morgan and Condon, 1986; Izanloo *et al.*, 2008). Here, we identified the sucrose metabolism as an important pathway for drought response in the cultivar MGS1 Aliança (Figure 6). When considering both tissues separately, the sucrose metabolism pathway was still found to be among the three most important ones. There are four enzymes that play a key role in starch metabolism: EC 2.4.1.13 - Susase, EC 2.7.7.27 - AGPase, EC 2.4.1.21 - STSase and EC, 2.4.1.18 - SBE (Yang *et al.*, 2004), and all these enzymes, except for AGPase, were activated during the water stress evaluated in this study. These enzymes also played an important role when previously evaluated in wheat plants grown under water stress conditions (Ahmadi and Baker, 2001). In addition, starch and sucrose metabolism, phenylpropanoid biosynthesis, and glyoxylate and dicarboxylate metabolism were also the most frequently detected KEGG pathways in a transcriptome analysis of *Paulownia australis* grown under drought conditions (Dong *et al.*, 2014). Furthermore, proline is a solute that plays a role as a protective agent for cells under osmotic stress, performing an important function in the drought stress response (Nezhadahmadi *et al.*, 2013). In our study, the P5CS1 and DELTA-OAT transcripts, related to proline biosynthesis, were up-regulated in leaf and root tissues (Table S4). In contrast, the ALDH12A1 and P5CS2 transcripts were up-regulated only in leaves, and the ALDH1 transcript was up-regulated in roots only. In fact, the arginine and proline metabolism pathway is among the top 20 pathways found to be induced in leaves (Figure 6).

Other transcripts already linked to the drought response were found among the candidate genes (Table S4). These transcripts include glutathione *S*-transferase and others related to glutathione biosynthesis and catabolism (GGT1, GSTU25, AT1G65820 and GSTU18 down-regulated in roots; GSH1 and OXP1 up-regulated in roots; GR1 up-regulated in leaves; GSTL3 up-regulated in leaves and roots, and ERD9 down-regulated in roots and up-regulated in leaves); dehydrins (DHN1 up-regulated in leaves and roots); and other late embryogenesis abundant (LEA) proteins (LEA7 was down- and up-regulated in leaves, while up-regulation was observed for AtLEA4-1, LEA14, LEA

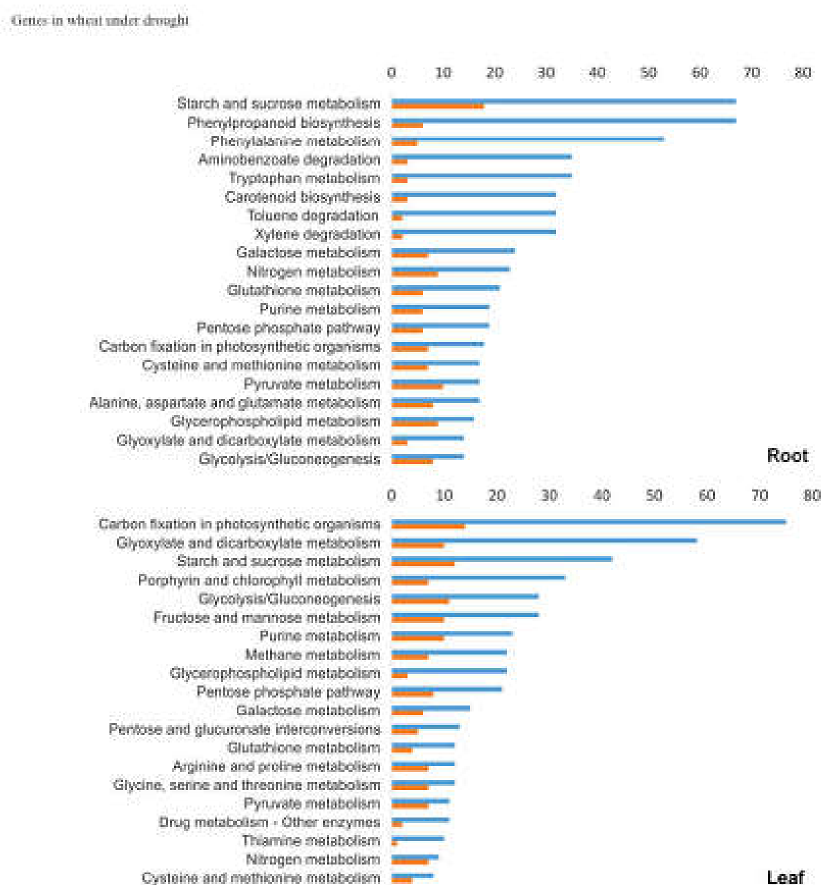

Figure 6 - Top 20 biological pathways of root and leaf tissues activated in the wheat cultivar MGS1 Aliança under drought conditions. Sequences were analyzed by Blast2GO against the Kyoto Encyclopedia of Genes and Genomes (KEGG). Blue bars represent the number of sequences and orange bars represent the number of enzymes.

and LEA4-5; in roots, LEA14, AT2G46140, AtLEA4-1 and LEA7 were up-regulated, while ACC1 was down-regulated). These proteins are important enzymes involved in stress responses, helping to cope with detoxification and reducing cellular damage by recovering denatured proteins and stabilizing membranes (Koag *et al.*, 2003; Umezawa *et al.*, 2006). For example, the wheat LEA genes PMA1959 and PMA80 improved water deficit resistance in rice (Cheng *et al.*, 2002), and the wheat dehydrin, DHN-5, improved drought tolerance when overexpressed in *Arabidopsis thaliana* (Brini *et al.*, 2007). Moreover, these proteins are among the differentially expressed transcripts detected in hard red winter wheat cultivars submitted to water-deficit conditions (Reddy *et al.*, 2014).

Another strategy to decrease the effects of drought is to retard leaf senescence (a process that is accelerated in drought-sensitive genotypes). In practical terms, leaf senescence leads to reduced yield, meaning that the suppression of drought-induced leaf senescence is desirable (Jewell *et al.*, 2010). In the MGS1 Aliança genotype analyzed here, candidate genes with GO terms related to leaf senescence were found in both tissues (RCA, HAI1 and LTI65 in leaf and SAG12, SAG29, LTI65, ARF1, WRYK70 and OPR1 in root) (Table S4). Moreover, many candidate genes related to the biosynthesis of the hormone abscisic acid

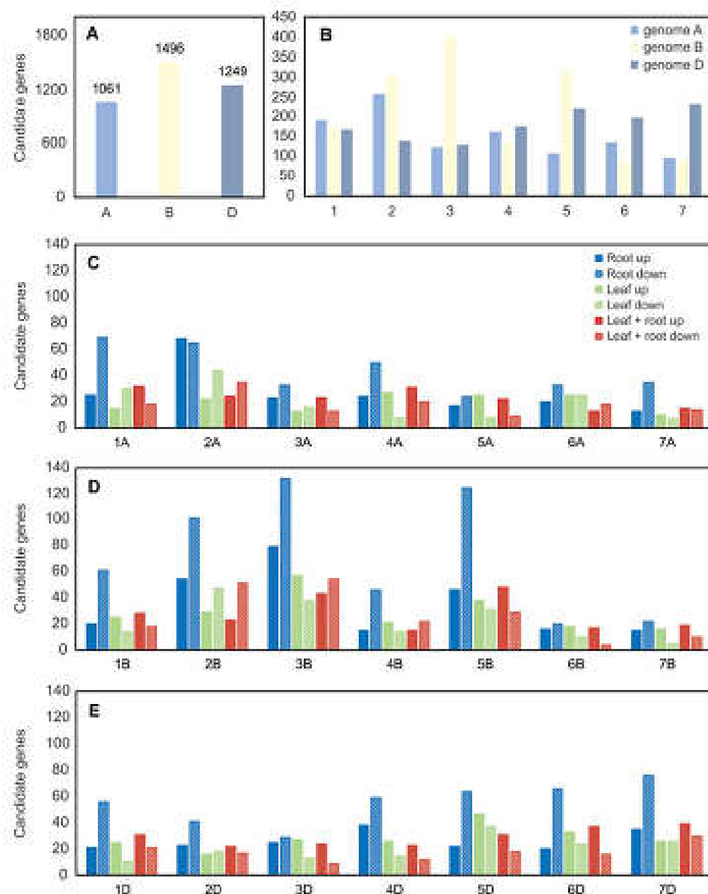

Figure 7 - Genome distribution of the candidate genes associated to drought response in the wheat cultivar MGS1 Aliança. Sequences were retrieved by BLAST against the *Triticum aestivum* cv. Chinese Spring genome at an E-value cutoff 1e-100. (A) Number of candidate genes per genome. (B) Total number of candidate genes per chromosome. (C), (D) and (E) Number of candidate genes that are up- or down-regulated and that are specific to roots, leaves, in common between both tissues for genome A, B and D, respectively.

(ABA) were also found in our study; for example, the AAO3 and NCED3 (9-cis-epoxycarotenoid dioxygenase) transcripts, which code for important enzymes in the ABA biosynthesis pathway (Table S4). Overexpression of the NCED3 transcript in Arabidopsis leads to tolerance of drought (Iuchi *et al.*, 2001). ABA synthesis increases in plants under water stress, inducing stomatal closure, reducing water loss via transpiration, and shaping transcript expression, which is also important for response to salinity and cold (Shinozaki and Yamaguchi-Shinozaki, 1997; Mahajan and Tuteja, 2005; Guóth *et al.*, 2009). In addition, transcripts related to ABA transduction signaling were also identified; for example, the OST1 transcript, which responds to ABA stimulus controlling stomatal closure (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002).

The candidate genes induced by drought stress and classified as "transcription factors" were less numerous than reported by Li *et al.* (2012). In this category, TFs such as bZIP, CBF, EREBP, WRKY, MADS, NAC and Myb were found (Figure S4). Some of these TFs have been ana-

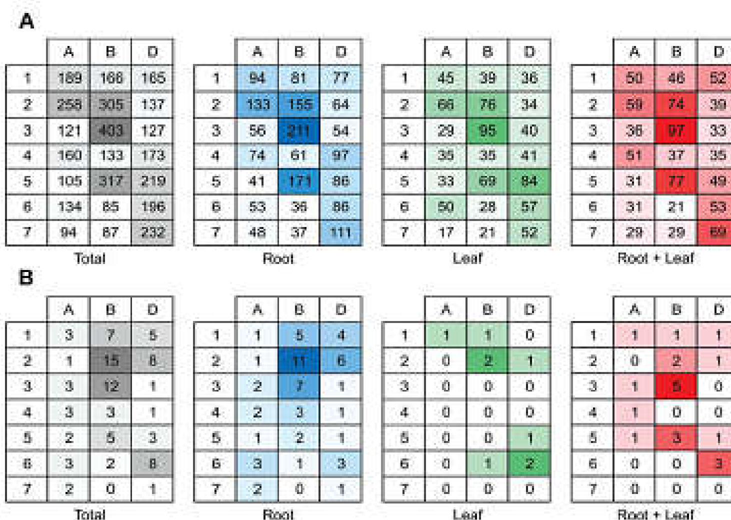

Figure 8 - Heat map showing the localization of the candidate genes associated to drought response in the wheat cultivar MGS1 Aliança. Hit distribution by tissue was obtained by BLAST against the genome of *Triticum aestivum* cv. Chinese spring at an E-value cutoff 1e-100. Numbers represent the hit distribution for each chromosome. "Total" means hit distribution of all sequences. "Root" means hit distribution of transcripts specifically expressed in roots. "Leaf" means hit distribution of transcripts specifically expressed in leaf. "Root + Leaf" means hit distribution of transcripts expressed in common between root and leaf. Darker colors mean higher number of reads. (A) All the candidate genes. (B) Only the candidate genes classified as no-hit.

lyzed by others, being up-regulated in roots of a drought-tolerant genotype (Okay *et al.*, 2014) and induced by drought stress in different species of *Triticum* (Baloglu *et al.*, 2014). A large number of TFs has also been found to be differentially regulated in response to heat, drought and their combination (Liu *et al.*, 2015). In addition, an increase in drought tolerance has been demonstrated in transgenic plants over-expressing some of those TFs, such as transgenic Arabidopsis expressing NAC TF or TaMYB2A (Mao *et al.*, 2011; Li *et al.*, 2014), rice expressing the DREB1A from Arabidopsis (Ravikumar *et al.*, 2014), wheat plants overexpressing MYB-TF (TaPIMP1) or TaERF3 (Zhang *et al.*, 2012; Rong *et al.*, 2014) and tobacco expressing TaABP1 (bZIP-TF) or TaWRKY10 (Cao *et al.*, 2012; Wang *et al.*, 2013).

One of the practical applications of the isolation of drought-related genes is the development of transgenic plants that are more tolerant to drought stress. So far, several papers have reported on that approach, using transcripts belonging to some of the functional groups discussed above. Examples in transgenic wheat include osmoprotectant genes (Abebe *et al.*, 2003; Vendruscolo *et al.*, 2007), LEA proteins (Sivamani *et al.*, 2000; Bahieldin *et al.*, 2005), a gene from the C_4 pathway (Qin *et al.*, 2015) and TFs (Morran *et al.*, 2011; Xue *et al.*, 2011; Saint Pierre *et al.*, 2012; Zhang *et al.*, 2012). In these reports, the experiments were performed with genes obtained from *A. thaliana* (*DREB*), *Atriplex hortensis* (*BADH*), *Escherichia coli* (*mtlD* or *betA*), barley (HVA1), cotton (*GhDREB*), rice (*SNAC1*), or *Vigna aconitifolia* (*P5CS*). Only a few studies have been performed with genes isolated from wheat, such as *TaDREB2*, *TaDREB3*, *TaNAC69-1*, or *TaPIMP1* (Morran *et al.*, 2011; Xue *et al.*, 2011; Zhang *et al.*, 2012). So far, field data regarding the performance of these transgenic plants have not been conclusive, with the transgenic lines not outperforming the controls or showing unstable performance along the years (Bahieldin *et al.*, 2005; Saint Pierre *et al.*, 2012). Nonetheless, it should be interesting to evaluate the production of these plants in the Cerrado region.

The candidate genes found here are distributed across all component genomes and chromosomes of the wheat genome (Figure 7, Figure 8). The number of sequences belonging to the B genome was higher in comparison with the A and D genomes. During evolution, the diploid genomes A and B (from wild species related to *T. urartu* and *Aegilops speltoides*, respectively) underwent an alloploydization event to form the tetraploid wheat *T. turgidum*, followed by another alloploydization with the D genome (*Aegilops tauschii*) (Leach *et al.*, 2014). It has been shown that there is a tendency of B genome homoeoloci to contribute more to gene expression in wheat than A or D

genome homoeoloci (Leach *et al.*, 2014). Moreover, the wild *T. turgidum* spp. *dicoccoides* (AABB genome), which is the ancestor of cultivated *T. turgidum* ssp. *durum* and *T. aestivum* (Budak *et al.*, 2013), contains a gene pool enriched for various agronomic traits, including drought tolerance (Peleg *et al.*, 2008; Ergen *et al.*, 2009). That information could encourage investigations on drought response in tetraploid wheat, and that tolerance could be incorporated into synthetic lines. However, it is important to note that the interaction among the A, B and D genomes could activate or silence homeologous genes (Wang *et al.*, 2011), making the introduction of genes from the B genome into the hexaploid genome a laborious task. Figures 7 and 8 also show that chromosomes 3B, 5B and 2B contribute with a greater number of drought-related transcripts in both roots and leaves. In wheat, quantitative trait loci (QTL) identified under different water regimes have been reported for traits like, for example, canopy temperature, carbon isotope discrimination, photosynthetic parameters and yield or yield components (Sheoran *et al.*, 2016). Virtually all wheat chromosomes and component genomes contain QTL for drought tolerance, most of them explaining a small fraction of the observed phenotypic variation. When focusing on chromosomes 3B, 5B and 2B, the major regions identified in our study, QTL for a number of traits correlated to drought tolerance have been described like, for instance, abscisic acid, canopy temperature, carbon isotope discrimination, chlorophyll content, coleoptile length, flag leaf rolling index, flag leaf senescence, grain number, grain size, grain weight, normalized difference vegetation index, water soluble carbohydrates, phenology (anthesis, heading, maturity), plant height, and yield (Sheoran *et al.*, 2016). The co-localization of the QTL with some of the candidate genes obtained in this survey could be an interesting target for future work.

In conclusion, the present study allowed for the identification of genes related to important pathways for drought response in the wheat cultivar MGS1 Aliança, a well-adapted cultivar for rainfed farming in the Cerrado biome. Clearly, our results showed that the main pathways activated under water deprivation differ for roots and leaves. Increments in drought tolerance through conventional and biotechnological approaches should take this difference into consideration. The drought stress-related transcripts described here will be further characterized to provide targets of interest for breeders. They are also important to elucidate the complex regulatory network(s) of the drought response. The characterization of candidate genes that are differentially expressed among drought-tolerant and -sensitive genotypes can help identify useful molecular markers and candidate genes. In the long run, the interesting targets and molecular markers can be used to achieve more sustainable wheat production.

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Supplementary Material

The following online material is available for this article:

Figure S1 - Gene expression distribution between control and drought-stressed (treated) roots and leaves of the wheat cultivar MGS1 Aliança.

Figure S2 - Correlation of transcript levels between RNA-seq and RT-qPCR data.

Figure S3 - Results of the Blast2GO analysis with the 3,987 candidate genes.

Figure S4 - Transcription factor distribution by family.

Table S1 - Set of 4,422 candidate genes identified in root and leaf tissues of the wheat cultivar MGS1 Aliança under drought.

Table S2 - Group of transcripts used in validation by RT-qPCR.

Table S3 - Biological pathways activated in wheat under drought (Blast2GO and KEGG analysis)

Table S4 - Details of selected candidate genes.

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CAPÍTULO II

Androgenic response of Brazilian wheat genotypes to different pretreatment of spikes and to a gelling agent

Androgenic response of Brazilian wheat genotypes to different pretreatment of spikes and to a gelling agent

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Abstract – The objective of this work was to analyze the androgenic response of Brazilian wheat genotypes to different pretreatments of the spikes, prior to the culture of isolated microspores, and to the effect of a gelling agent in the induction culture medium. Five genotypes were evaluated for embryo formation, green plant regeneration, and spontaneous chromosome duplication. Wheat spikes were subjected to two pretreatments: cold, at 4°C for 21 days; and 2-hydroxynicotinic acid, at 32°C for two days. Culture media were evaluated with or without Ficoll as a gelling agent. Cold produced more embryos and green plants than the chemical pretreatment in four out of five genotypes. Only two genotypes treated with 2-hydroxynicotinic acid were able to produce plants, and one of them produced a single albino plant. Medium containing Ficoll produced more embryos than liquid medium, and promoted a higher number of plants. Spontaneous chromosome duplication varied between genotypes and pretreatments, and showed high variability.

Index terms: *Triticum aestivum*, albinism, androgenesis, doubled haploid, isolated microspore culture, recalcitrance.

Resposta androgênica de genótipos brasileiros de trigo a diferentes pré-tratamentos das espigas e a um agente gelificante

Resumo – O objetivo deste trabalho foi analisar a resposta androgênica de genótipos brasileiros de trigo a diferentes pré-tratamentos das espigas, antes da cultura de micrósporos isolados, e ao efeito de um agente gelificante no meio de cultura de indução. Cinco genótipos foram avaliados quanto à formação de embriões, regeneração de plantas verdes e duplicação espontânea dos cromossomos. Espigas de trigo foram submetidas a dois pré-tratamentos: frio, a 4°C por 21 dias; e ácido 2-hidroxinicotínico, a 32°C por dois dias. Os meios de cultura foram avaliados com ou sem Ficoll como agente gelificante. O frio produziu mais embriões e plantas verdes do que o pré-tratamento químico, em quatro dos cinco genótipos testados. Apenas dois genótipos tratados com ácido 2-hidroxinicotínico foram capazes de produzir plantas, e um deles produziu uma única planta albina. O meio com Ficoll produziu mais embriões do que o meio líquido e gerou maior número de plantas. A duplicação espontânea dos cromossomos variou entre os genótipos e os pré-tratamentos e apresentou alta variabilidade.

Termos para indexação: *Triticum aestivum*, albinismo, androgênese, duplo-haploide, cultura de micrósporos isolados, recalcitrância.

Introduction

The significance of haploid (H) plants for plant breeding and genetic research was first recognized in 1921, when Bergner observed this natural phenomenon in *Datura stramonium* (Blakeslee et al., 1922). Four decades later, Guha & Maheshwari (1964) observed that these plants could arise from immature pollen in vitro as an immediate product of meiosis, representing all the diversity of the parental post-meiotic haploid chromosome set. Haploid plants have normal development cycle; however, one notable exception is related to their reproductive structure because most haploid plants have defective anthers and are sterile. In the absence of homologous pairing, meiosis produces gametes with fewer than the necessary complement of chromosomes. The duplication of chromosomes – spontaneous or induced – overcomes this impasse to yield doubled haploid (DH) and fertile plants. In other words, it can produce completely homozygous plants, genetically normal, phenotypically

stable, and representing all gametophytic variation due to the recombination process of meiosis.

The fact that DH can yield completely homozygous plants in a single year is a valuable asset for breeding programs, since it can accelerate the development of new cultivars by up to five years, in comparison to conventional breeding. The use of DH plants can also increase the selection efficiency because fewer plants are necessary for the screening of selected traits, which reduces costs, allowing even in vitro selection of specific traits. Liu et al. (2002) used in vitro selection in studies of resistance to pathogens, mineral imbalances, and drought tolerance. However, this tool is also used in basic and advanced research (Germanà, 2011), such as genetic analysis, induction of mutation, genome mapping, gene transfer (Abdollahi et al., 2007), and QTL detection (Seymour et al., 2012).

Although haploidization has been successfully achieved in anther and isolated microspore cultures (IMC), some important advantages are associated with IMC over anther culture, such as: abundance of microspores per spike, absence of anther walls that could damage the culture process, assurance that all developed embryos are microspore-derived, better nutrient availability to cells in culture medium, and the possibility for easily tracking the development of individual cells (Liu et al., 2002). IMC protocols have been improved for many plant species; however, cereal microspore culture requires some adjustment to be used in large-scale, especially the rate of embryo induction, green plant regeneration, and spontaneous chromosome doubling (Castillo et al., 2009).

Androgenesis is one of the most remarkable examples of cellular totipotency. However, some conditions should be met for gametes to develop into a new plant. Stress pretreatments are considered crucial for triggering totipotency and activating the sporophytic pathway of the microspore cell (Shariatpanahi et al., 2006). The physiological conditions of donor plants and the microspore development stage are also very important because they can modify the androgenic response even in responsive genotypes (Germanà, 2011).

The objective of this work was to evaluate the androgenic response of five Brazilian wheat genotypes to the effects of different pretreatments of the spikes and of a gelling agent in the induction culture medium.

Materials and Methods

The experiment was carried out at Embrapa Trigo, in Passo Fundo, RS, Brazil, in 2011/2012. Six genotypes were used to test androgenesis: five Brazilian wheat genotypes with unknown androgenic responses – 'MGS 1 Aliança', 'MGS Brilhante', 'BR 18 (Terena)', PF020037, and PF020062 –, and the responsive genotype 'Pavon 76' which was used as a control.

Seed of donor plants were treated with an insecticide and a fungicide solution – 1:1 Gaúcho (Imidacloprid 600 g L^{-1}) : Baytan (Triadimenol 150 g L^{-1}). Treated seed were distributed on a moistened germitest paper with distilled water, and kept in the dark, at 4°C, for four days. After this period, seed were transferred to a chamber (24°C, 16 hours light) for approximately one week. The germinated seed were transferred to 6.5 kg pots containing a mix of soil, vermiculite, and substrate (1:1:1), and moved to a Conviron growth chamber (20°C/15°C, 16 hours light). Each pot received two germinated seeds. Twenty plants were grown per genotype. Half-strength Hoagland nutrient solution was applied once a week (100 mL per pot) until the end of tillering.

Tillers containing spikes were sampled when microspores were in the early to mid-uninucleated stage (confirmed by acetocarmine staining), and their stems were immediately placed in a flask containing distilled water. Tillers were wrapped in aluminum foil and stored in flasks with distilled water.

Two pretreatments were applied to the spikes before microspore extraction: cold, tillers were kept in distilled water and stored at 4°C for 21±3 days; and chemical, tillers were transferred to 50 mL of 100 mg L^{-1} 2-hydroxynicotinic acid (2-HNA) solution, and kept at 32°C for two days.

After pretreatments and prior to extraction, awns were removed with scissors, and the surface of donor spikes were sterilized for 15 min in 15% bleach (0.038% active chlorine) solution, containing one drop of Tween 20 to eliminate external bacterial or fungal contaminants. Following disinfection, spikes were rinsed five times with sterile, distilled water. The outer glumes of each spikelet were discarded, and the flowers were removed from the rachis and transferred to a sterile miniblender cup (Waring, Thomas Scientific, Model 3392, Swedesboro, NJ, USA) containing 50 mL of refrigerated NPB-99 extraction medium (Liu et al.,

2002). Nine to 12 spikes were used for each extraction. The total number of spikes is described in Table 1.

Flowers were blended twice at low speed for 7–8 s, and the solution was transferred to a cold beaker. The solution was filtered through a 100 µmol L^{-1} sterile membrane into two 50 mL sterile centrifuge tubes, then it was centrifuged for five min at 100 *g* (4°C) using a swinging bucket rotor. Supernatant was discarded, and the pellets were resuspended in 35 mL of NPB-99 and centrifuged again. Two more cycles of centrifugation were repeated at the same speed. The final pellets were resuspended in 300–1,000 µL NPB-99, and the microspore concentration was verified using a hemocytometer. Cells were adjusted to a final concentration of approximately 80,000 cells mL^{-1}.

Microspore suspension (250 µL) was plated on 40x11 mm diameter Corning plastic Petri dishes containing 2,750 µL NPB-99 and 0.001% arabic gum from the acacia tree, with or without 10% Ficoll PM400 as a gelling agent: half of each extraction was plated on medium with Ficoll, and the other half, in a medium without Ficoll. One immature ovary per milliliter was added to each plate; ovary donor spikes were sterilized under the same conditions as those of the microspore donor spikes. The plates were sealed with Parafilm and placed into a 150 mm Petri dish containing an open 35 mm Petri dish with sterile distilled water.

The Petri dishes were placed in the dark inside an incubator at 27°C for approximately 3–4 weeks. Embryos with at least 2 mm in size were transferred to 90x15 mm Petri dishes containing 20 mL of GEM medium (Eudes et al., 2003). The Petri dishes were stored in a growth room and exposed to 16 hours of light per day, at 24°C.

Once embryos had regenerated into green plants with roots and leaves, each plantlet was transferred to 14 mL rooting medium (Eudes et al., 2003) and maintained in the same room. When plantlet roots were well developed, they were transplanted into 500 mL PVC cups containing vermiculite (weekly fertilized) and covered with a beaker. After acclimation, plants were ready to be transplanted in the soil (two plants per 6.5 kg pot). They were grown in a controlled environment room, under the same conditions as those of the donor plants, until the end of the cycle. Colchicine treatment was not applied to plants, and chromosome doubling was verified by checking fertile spikes.

Results and Discussion

Visible differences in the total number of cells (cell yield) and stage of microspore development were observed between the two types of pretreatment. The number of uninucleated cells per spike was markedly higher in plants pretreated with cold, varying among genotypes from 41,672 to 107,410 cells, in comparison to 2,982 to 33,527 cells using 2-HNA (Table 1). The application of 2-HNA to tillers accelerated the development cycles of both spikes and cells, and the remaining leaves of tillers rapidly yellowed. The optical microscopy images indicated that most of the microspore cells were at the early binucleated stage after 2-HNA pretreatment, and some of these cells were identified as pollen grains.

In general, most of microspore cells continue along their normal gametophytic pathway, even after stress pretreatment. Only a small percentage of competent cells will follow the sporophytic pathway. Therefore, increasing the number of purified uninucleated cells per spike (yield) would likely increase the number of competent cells that would follow a sporophytic pathway. Thus, the cold pretreatment applied to the spikes generated better results (higher yield of cells), which increased the number of embryos and regenerated plants in four out of five tested Brazilian

Table 1. Yield of microspore cells obtained from six wheat genotypes in response to different types of pretreatment.

Genotype	2-HNA			Cold		
	Number of spikes	Number of cells	Number of cells/spike	Number of spikes	Number of cells	Number of cells/spike
'Pavon 76'	20	373,998	18,700	31	2,561,184	82,619
PF020037	54	1,810,443	33,527	42	3,037,956	72,332
'MGS 1 Aliança'	32	312,198	9,756	29	2,209,734	76,198
'MGS Brilhante'	23	193,332	8,406	30	3,222,315	107,410
'BR 18 (Terena)'	40	119,265	2,982	42	1,750,243	41,672
PF020062	56	1,120,194	20,003	30	2,551,092	85,036

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genotypes. However, the application of 2-HNA yielded lower number of cells and embryos, and plants were obtained only from two Brazilian genotypes (PF020037 and 'MGS 1 Aliança'), and from 'Pavon 76' (responsive control).

Regarding embryo and plant development, cold treatment also triggered microspore embryogenesis more effectively than heat associated with 2-HNA. Shirdelmoghanloo et al. (2009) found similar results. These authors compared heat associated with chemical treatment (2-HNA) and cold, alone or combined with mannitol. In their study, a short period of cold (seven days at 4°C) combined with mannitol promoted higher number of embryos and green plants. However, Konzak et al. (2000) reported opposite results regarding the benefits of 2-HNA as a chemical pretreatment for triggering embryogenesis. They observed that 2-HNA significantly increased the androgenic response in wheat microspore culture.

The application of high temperatures to spikes has been suggested to accelerate the rate at which 2-HNA triggers androgenesis in microspores (Liu et al., 2002). 2-HNA was used as a pretreatment in the present study to verify these effects. However, our results significantly differed from those reported by Konzak et al. (2000), and Liu et al. (2002). Regenerated plants were obtained only from one Brazilian genotype (PF020037), and it was a single albino plant from 'MGS 1 Aliança'.

In our study, wheat tillers were maintained in 2-HNA solution for two days at 32°C, which resulted in yellowish leaves and accelerated spike development. One flower of the spike, removed after pretreatment and observed under an optical microscope, showed that the microspore cells were in a very advanced stage of development, and most were bicellular or pollen-like. None of the cells showed a "star-like" morphology, a feature generally associated with the onset of androgenesis (Maraschin et al., 2005). Considering the severe effect of the stress treatment with 2-HNA, less aggressive treatments to trigger microspore embryogenesis would be preferable. Collecting tillers during an earlier stage of microspore development, and reducing the time or the temperature of this type of pretreatment may also be useful for triggering the response in recalcitrant wheat genotypes.

Although widely used, the mechanism by which low temperatures elicit an androgenic response remains unclear. Cold has been shown to repress the gametophytic pathway by inducing symmetrical microspore mitotic divisions and delaying pollen development (Shirdelmoghanloo et al., 2009). Cold shock is also thought to ensure the survival of a greater number of cells (Shariatpanahi et al., 2006). According to these authors, the understanding of an accurate mechanism of stress, with respect to microspore embryogenesis, is far to be elucidated, regardless of the chosen stress type (cold, heat, chemical or others). Based on these information, some authors believe that cold is not a stress "per se", but an anti-stress that is actually protecting microspores more than harming them, and that the real stress is caused by starvation (Zoriniants et al., 2005).

Because of its importance for plant breeding and genetic studies, many protocols for producing DH plants via microspore embryogenesis have been reported for different crops and species. However, a positive response of the method is largely dependent on the genotype, and improvements are required for routine uses (Ferrie & Caswell, 2010; Asif, 2013). Understanding the mechanisms involved in the microspore embryogenic process is important to identify a less genotype-dependent protocol.

Three important steps are primarily responsible for androgenic development: the acquisition of embryogenic capacity, the initiation of several cell divisions, and organ formation and differentiation (Maraschin et al., 2005). The acquisition of embryogenic potential (first step) is largely affected by the genotype, and specifically elicited by stress treatments. Each unique isolated microspore culture step equally contributes to achieve microspore embryogenesis. However, the "real trigger" of the process is based on reprogramming the original route of microspores to sporophytic development.

After microspore cells are triggered to follow the sporophytic route, a multiple step process initiated by several cell divisions takes place leading to the formation of an embryo-like structure. In our study, microspore-derived embryos began to emerge after three to four weeks in the culture medium. Embryos were either floating on the surface of the medium or sinking.

In addition to pretreatment-derived differences, the presence of a gelling agent (Ficoll) in the culture medium also yielded clear differences in the number

Table 2. Embryo induction and plant regeneration from isolated microspore culture of six wheat genotypes.

Genotype	Culture medium with Ficoll								Culture medium without Ficoll							
	Cultured cells	Embryos	Total[1]	Albino[2]	Green				Cultured cells	Embryos	Total	Albino	Green			
					Total[3]	H[4]	DH[5]	Dead[6]					Total	H	DH	Dead
	2-HNA treatment															
'Pavon 76'	252,498	1,405	177 (13%)	55 (31%)	122 (69%)	70 (57%)	36 (30%)	16 (13%)	121,500	393	181 (46%)	121 (67%)	60 (33%)	30 (50%)	24 (40%)	6 (10%)
PF020037	1,058,637	3	0	0	0	0	0	0	751,806	186	43 (23%)	23 (53%)	20 (47%)	11 (55%)	7 (35%)	2 (10%)
'MGS 1 Aliança'	265,200	46	1 (2%)	1 (100%)	0	0	0	0	46,998	-	-	-	-	-	-	-
'MGS Brilhante'	193,332	0	0	0	0	0	0	0	*	*	*	*	*	*	*	*
'BR 18 (Terena)'	106,665	0	0	0	0	0	0	0	12,600	0	0	0	0	0	0	0
PF020062	489,096	19	0	0	0	0	0	0	631,098	34	0	0	0	0	0	0
	Cold treatment															
'Pavon 76'	1,010,328	556	205 (37%)	136 (66%)	69 (34%)	19 (28%)	45 (65%)	5 (7%)	1,550,856	1355	525 (39%)	395 (75%)	130 (22%)	39 (30%)	85 (65%)	6 (5%)
PF020037	1,298,313	143	8 (6%)	8 (100%)	0	0	0	0	1,739,643	1062	199 (19%)	115 (58%)	84 (42%)	34 (41%)	44 (52%)	6 (7%)
'MGS 1 Aliança'	1,104,867	532	88 (16%)	77 (87,5%)	11 (12,5%)	1 (9%)	10 (82%)	1 (9%)	1,104,867	1107	196 (18%)	179 (91%0)	17 (9%)	9 (53%)	7 (41%)	1 (6%)
'MGS Brilhante'	1,503,858	471	11 (2%)	6 (55%)	5 (45%)	1 (20%)	4 (80%)	0	1,718,457	1022	32(3%)	13 (41%)	19 (59%)	6 (32%)	12 (63%)	1 (5%)
'BR 18 (Terena)'	975,121	0	0	0	0	0	0	0	775,122	122	41 (34%)	27 (66%)	14 (34%)	9 (64%)	5 (36%)	0
PF020062	1,282,956	0	0	0	0	0	0	0	1,268,136	7	0	0	0	0	0	0

[1]Total regenerated plants (% embryos). [2]Albino plants (% total regenerated plants). [3]Total green plants (% total regenerated plants). [4]Haploid plants (% green plants). [5]Doubled haploid(% spontaneous DH plants/green plants). [6]Total dead plants (% green plants). *Not enough cells.

of embryos and regenerated plants (Table 2). Both embryos and regenerated plants were far more abundant when Ficoll was added to the medium. Moreover, the culture could be more easily manipulated because of the higher viscosity of the medium, which reduces injury to the embryos.

The use of Ficoll in the culture medium increases the viscosity and is associated with medium density and osmolality. Eudes & Amundsen (2005) compared the presence of Ficoll (10%) in NPB-99 medium for the IMC of triticale, and they observed that the medium supplemented with Ficoll increased the total number of floating embryos, which are considered to be more embryogenic than the sinking ones. This difference was attributed to better gas exchange on the medium surface, which is important for embryo development. The authors also observed that floating embryos were more likely to develop into green plants, and the opposite was true for sinking embryos (produced more albino plants). We observed that the presence of 10% of Ficoll in the culture medium increased the androgenic response, which resulted in a higher number of embryos and regenerated plants. However, as an exception, the responsive genotype 'Pavon 76' produced a higher number of embryos when 2-HNA was applied to the spikes and combined with the induction medium without Ficoll. For this genotype/pretreatment, 13% of the embryos converted into plants in response to this condition. Taken together, our results indicate that cold (4°C) and induction medium with Ficoll (10%) improved embryo formation and plant regeneration.

Three Brazilian genotypes ('MGS 1 Aliança', PF020037, and 'MGS Brilhante') stood out and produced a high number of embryos, in response to cold pretreatment and induction medium containing Ficoll, similarly to 'Pavon 76' (Table 2). Two genotypes ('BR 18 (Terena)' and PF020062) produced a lower number of embryos. 'BR 18 (Terena)' also generated a low number of cells per spike (yield). The low number of embryos could be associated with the low yield of cells. However, PF020062 exhibited high yields and produced only seven embryos.

As expected, 'Pavon 76' produced the highest total percentage of regenerated plants (46% of embryos converted into plants). Two Brazilian genotypes were moderately responsive: PF020037 (19%) and 'MGS 1 Aliança' (18%). 'MGS Brilhante' produced a substantial amount of embryos (1,022), but only 3% of them regenerated into plants. Conversely, 'BR 18 (Terena)' produced very few embryos (122), but 34% converted into plants. PF020062 was highly recalcitrant, producing the lowest number of embryos, and no plants.

Albino plants were observed in most genotypes (except for PF020062). 'MGS 1 Aliança' and 'Pavon 76' produced the highest percentages of albino plants (91 and 75%, respectively, Table 2). In addition to genotypic limitations, the high frequency of albino plants has largely affected the production of DH plants in cereals (Jacquard et al., 2009; Kumari at al., 2009). In many cases, albinism can be observed in up to 100% of regenerated plants (Labbani et al., 2005). Diverse factors could influence the rate of albinism: the genotype and physiological conditions of donor plants, pretreatment temperatures, meiotic abnormalities, hormonal imbalances, mutations, and incompatibility between plastid and nuclear genomes (Kumari et al., 2009). Albino plants could also be a result of the maternal inheritance of plastids, a hypothesis first proposed by Vaughn et al. (1980). However, other studies have shown that not all albino plants carry plastid DNA deletions (Torp & Andersen, 2009).

Although deletions and the reorganization of plastid genomes were observed in wheat, barley (Day & Ellis, 1984, 1985) and rice (Harada et al., 1991, 1992), albinism is also related to altered transcript patterns and translation levels (Ankele et al., 2005).

A lack of chlorophyll in albino plants is usually governed by one or two recessive genes, although more genes control this genetic trait in some species, including additive and nonadditive genes (Kumari et al., 2009). Because it is a recessive trait, albinism is difficult to eradicate from a population; the allele will persist at a low frequency in heterozygous plants. However, some approaches could minimize the frequency of albino phenotypes. The addition of copper sulfate to the pretreatment and culture medium of barley anthers increased the anther response by 15%, and the number of green plants by 400% (Wojnarowiez et al., 2002). Jacquard et al. (2009) showed that this modification increased the number of DH plants and reduced the number of albino plants in barley anther culture, including the presence of green plants in a genotype that regenerated only albino plants.

The total number of green plants (H, DH, and dead plants) and the percentage of green plants (total)

were recorded for all genotypes, varying from 9 to 59% among evaluated genotypes. Spontaneous chromosome doubling was recorded by checking the seed set. The overall efficiency of chromosome doubling ranged from 36% in 'BR 18 Terena' to 63% in 'MGS Brilhante'.

Three types of regenerated plants were observed: plants containing sterile spikes only, plants having fertile spikes only, and plants presenting fertile and sterile spikes (Figure 1 A). Additionally, four different types of spikes were found: fertile, partially fertile, sterile, and atypical (always sterile and with the same phenotype) (Figure 1 B). Phenotype alterations of spikes did not differ between genotypes.

Genome duplication is a very important step to recover the fertility of the H plant. The spontaneous

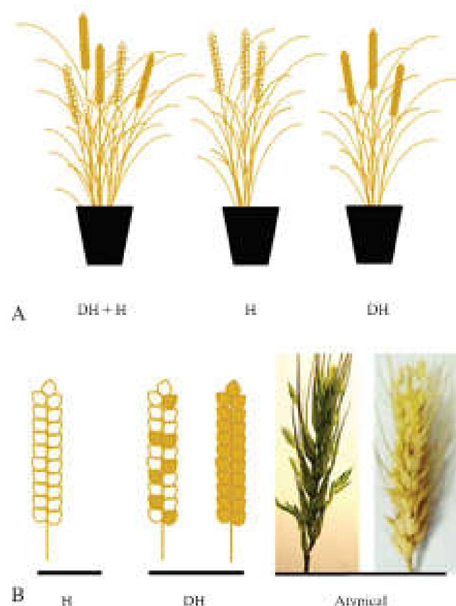

Figure 1. Types of plants and spikes obtained from microspore culture. A) Three types of regenerated plants: DH + H, containing fertile and sterile spikes; H, containing only sterile spikes; and DH, containing only fertile spikes. B) Four types of spikes: H, completely sterile spike; DH, partially fertile or completely fertile spike; and atypical, spikes with different phenotypes, always sterile.

doubling of chromosomes in anther or microspore culture is highly desirable because it avoids artificial chromosome doubling, which is time-consuming, costly, and inefficient. Induced chromosome doubling by chemical agents, such as colchicine, results in high mortality rates, which decreases the process efficiency (Castillo et al., 2009). Four different mechanisms were identified as responsible for spontaneous chromosome doubling: endoreduplication, a type of cell cycle in which nuclear chromosomal DNA replication occurs without cell division; nuclear fusion; endomitosis; and c-mitosis (Testillano et al., 2004). However, the precise mechanism is not well understood, and the factors leading to these processes remain unknown, but are highly susceptible to the in vitro conditions (Testillano et al., 2004). Differences between genotypes can also influence the doubling of the chromosome set (Indrianto et al., 1999). Castillo et al. (2009) reported doubling rates of chromosomes varying from 25 to 75% for bread wheat.

Our results corroborate the hypothesis that spontaneous genome duplication is genotype-dependent, ranging from 35%, for 'BR 18 (Terena)', to 63% for 'MGS Brilhante'; besides 65% for the responsive genotype 'Pavon 76'. Types of pretreatments and the presence of Ficoll in the culture medium also affected chromosome duplication exhibiting high variability for this trait. Indrianto et al. (1999) reported similar results for spontaneous chromosome doubling. The authors observed that cold pretreatment resulted in a higher number of spontaneous diploids than heat or starvation. Kasha et al. (2001) also observed that cold or cold plus mannitol had the same effect, that is: higher spontaneous doubling rates compared with other pretreatments. In general, chromosome doubling will mostly occur at the beginning of cell division. The low frequency of chimeric plants observed in our study suggests that chromosome doubling took place very early during in vitro cultivation, most likely at the time of induction, as suggested by Kasha et al. (2001). However, even at low frequencies, the presence of mixoploid plants indicates that chromosome doubling did not occur in all tissue cells. Lantos et al. (2006) also reported chimeric wheat plants with different chromosome numbers that exhibited fertile, partial fertile, and sterile spikes obtained from IMC. According to the authors, spikes abnormalities were likely due to the intrinsic in vitro

culture process, and they are not caused by artificial chromosome duplication, as in our study.

Conclusions

1. Cold shock triggers androgenesis more effectively than chemical treatment with 2-HNA.
2. Induction culture medium containing 10% Ficoll produces more embryos and green plants than induction medium without Ficoll.
3. Albinism is the restraining factor for all tested genotypes, since its incidence ranged from 41% for 'MGS Brilhante') to 91% for 'MGS 1 Aliança'.
4. The DH protocol via IMC should be substantially improved to be cost effective and efficient.

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DISCUSSÃO

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A região do Brasil, constituída em sua maior parte pelo bioma Cerrado é a nova fronteira para aumentar a produção de trigo no país. As características ambientais da região exigem que as plantas lidem com diferentes estresses sendo a seca o mais importante, quando se considera a agricultura do tipo sequeiro. Não existem ainda trabalhos que avaliem a expressão gênica de uma maneira global em genótipos brasileiros de trigo submetido a estresse de seca. Neste contexto a análise de transcriptoma desenvolvida neste estudo é um importante passo para caracterizar a resposta de plantas de trigo à seca. Atualmente o trigo hexaplóide (cultivar *Chinese Spring*) está tendo seu genoma finalizado pelo *International Wheat Genome Sequencing Consortium* (IWGSC), o qual tem como meta completar a sequência de referência do genoma até o final de 2017 (http://www.agri-pulse.com/Wanted-13-million-to-unlock-the-wheat-genome-03112015.asp).

As ferramentas de sequenciamento *high-throughput* e a bioinformática vêm permitindo uma análise global da expressão gênica, mudando o foco de um único gene para todo genoma ou, no nosso caso, para o transcriptoma. Por meio destas ferramentas, foi possível a identificação de 4.422 sequências diferencialmente expressas (DE) relacionadas à tolerância à seca no genoma do trigo hexaplóide tolerante à seca, MGS1 Aliança, na época de perfilhamento em ambos órgãos, raiz e folha, período em que pode ocorrer seca no Cerrado brasileiro (Ribeiro-Junior et al., 2006).

Quando organismos são submetidos a diferentes situações/ambientes eles percebem os estímulos ao seu redor e respondem da melhor maneira para se adaptarem às novas condições. Para que haja esta mudança, principalmente em ambientes com condições mais extremas, acontece uma reprogramação molecular na planta, onde haverá a indução de determinados genes e repressão de outros, os quais irão definir as respostas da planta. A identificação destas mudanças é um passo chave para o entendimento da resposta do trigo à seca. No presente estudo verificamos que o número de sequências reprimidas foi maior do que o de sequências induzidas sob estresse de seca. Resultado semelhante foi obtido por Mohammadi et al. (2008) e Li et al. (2012). Dentre todas as categorias, verifica-se a ocorrência predominante de genes relacionados a processos biológicos, nas classes: processo celular, processo metabólico, respostas a estímulos e não classificadas; dentro das funções moleculares as classes: ligação, atividade catalítica e não classificadas; já na categoria de componente celular as classes: parte celular,

organela, membrana, parte de organela e não classificadas. As categorias encontradas na anotação funcional de *Gene Ontology* das 4.422 sequências estão de acordo com outros estudos (usando outras tecnologias), por exemplo, o transcriptoma de *Ammopiptanthus mongolicus* (planta do deserto tolerante à seca), análises de microarranjo de grão-de-bico sob estresse de seca terminal e estudos de microarranjo no genótipo de trigo tolerante à seca 'Luohan No 2' em raiz na planta no estágio de duas folhas (Deokar et al., 2011; Li et al., 2012; Zhou et al., 2012).

Nos programas de melhoramento genético, frequentemente, a característica desejada tem que ser buscada em *backgrounds* de ancestrais ou em *landraces*. Para a característica de tolerância à seca a fonte seria a espécie diploide ancestral *Aegilops tauschii* (doadora do genoma D) e o *background* mais selvagem do tetraplóide *Triticum durum*, o *T. dicoccoides* (genoma AB) (Nevo & Chen, 2010). Alelos conferindo alto rendimento de grãos sob estresse de escassez de água foram identificados em dois locus do genoma B (2B e 7B) de *T. dicoccoides* (Nevo & Chen, 2010). É interessante destacar que, no presente estudo, as sequências DE estão localizadas preferencialmente no genoma B. Esta peculiaridade se observa, também, se considerarmos apenas as sequências classificadas como no-hits, as quais se localizam preferencialmente no cromossomo 2B. Porém, devemos manter em mente que ao se reunir os três genomas (A, B e D) a regulação de genes homeólogos pode ser modificada e criar-se um novo ambiente de contribuição de cada genoma no trigo hexaplóide, não refletindo necessariamente a maior contribuição de um parental ou outro.

Vários estudos têm relatado a análise de QTLs, que permite a identificação de regiões cromossômicas que explicam a variação fenotípica relacionada à tolerância à seca. Uma série de estudos tem registrado QTLs identificados em trigo sob diferentes regimes hídricos, onde diferentes características foram avaliadas, como por exemplo, temperatura do dossel, discriminação isotópica de carbono, parâmetros fotossintéticos, comprimento e peso da raiz, e rendimento ou componentes do rendimento (Maccaferri et al., 2008; Mathews et al., 2008; Rebetzke et al., 2008; McIntyre et al., 2010; Pinto et al., 2010; Czyczylo-Mysza et al., 2011; Sharma et al., 2011; Bennett et al., 2012; Bonneau et al., 2013; Edae et al., 2014; Zhang et al., 2014). Interessantemente, nossos dados mostram que as sequencias DE correspondem a genes localizados principalmente nos cromossomos 3B, 5B e 5B. Coincidentemente, outros trabalhos reportaram QTLs relacionados a rendimentos de grãos, componentes de rendimento, altura da planta, temperatura do dossel, discriminação isotópica de carbono e vigor inicial localizados no cromossomo 3B (Maccaferri et al., 2008; Rebetzke et al., 2008; Pinto et al., 2010; Bennett et al., 2012; Bonneau et al., 2013; Edae et al., 2014), rendimento de grãos, componentes de

rendimento, altura da planta e temperatura do dossel no cromossomo 5B (Mathews et al., 2008; Bennett et al., 2012; Edae et al., 2014) e componentes de rendimento e fluorescência de clorofila no cromossomo 5D (Czyczylo-Mysza et al., 2011). Isso sugere que genes DE encontrados no nosso trabalho podem estar localizados dentro de QTLs importantes para a tolerância à seca no trigo. No entanto análises mais detalhadas sobre locos candidatos são necessárias.

Um importante passo em estudos de RNA-seq é a validação dos dados obtidos pelo sequenciamento por outras metodologias. Neste estudo foi utilizado RT-qPCR (PCR quantitativo em tempo real) para este fim e ao final das análises foi possível validar estatisticamente 65,7% das sequências (23 de 35 sequências analisadas, representando diferentes rotas), sendo a validação para os dados de raiz superior (73,3%) aos de folha (60%). É importante ressaltar que este resultado apoia os dados obtidos no RNA-seq, uma vez que um experimento independente (nas mesmas condições) foi realizado para a validação.

Além da identificação de genes, o presente estudo permitiu a identificação de rotas metabólicas que estão envolvidas na tolerância à seca. Foram identificadas 116 rotas diferentes envolvidas na resposta à seca em trigo. Neste ranking, dentre as primeiras 20 rotas, 11 são comuns em folha e raiz, sendo as outras 29 diferem nos dois órgãos. Uma rota comum em folha e raiz, que se encontra dentre as três rotas metabólicas representadas por maior número de sequências relacionadas, está o metabolismo de amido e sacarose (primeira posição em raiz e terceira em folha). Existem quatro enzimas com papel chave no metabolismo do amido e sacarose (Yang et al., 2004), sendo que três destas foram identificadas como transcritos diferencialmente expressos em nosso sequenciamento (EC 2.4.1.13 – Susase, EC 2.4.1.21 – STSase e EC 2.4.1.18 – SBE). Resultados similares, no ranking das rotas, foram encontrados por Dong et al. (2014) em *Paulownia australis*. A identificação dessas rotas constitui mais uma possibilidade de escolha para próximos estudos mais específicos para se elucidar os mecanismos de tolerância à seca.

Os fatores de transcrição desempenham um papel importante na regulação da expressão gênica em resposta a estresses. Estes são alvos poderosos na engenharia genética, quando o objetivo é obter tolerância a estresses, pois a alteração de expressão de apenas um fator de transcrição pode levar à indução ou repressão de uma vasta gama de genes relacionados à resposta ao determinado estresse. Outra importante revelação de nosso estudo, que serve como ponto de partida para próximos estudos, foi a identificação, entre os genes DE, de fatores de transcrição tais como bZIP, CBF, EREBP, WRKY, MADS, NAC, MYB related e DREB, os

quais foram bem menos numerosos que outros genes. Estes mesmos fatores de transcrição foram identificados por Li et al. (2012) e também foram os genes menos numerosos em raízes da variedade tolerante de trigo 'Luohan No 2'. Trabalhos da literatura têm demonstrado aumento de tolerância à seca em plantas transgênicas expressando alguns dos fatores de transcrição encontrados. Este fenótipo foi observado em plantas transgênicas de Arabidopsis expressando o fator de transcrição NAC ou TaMYB2A (Mao et al., 2011; Li et al., 2014); de arroz expressando DREB1a de Arabidopsis (Ravikumar et al., 2014); de trigo superexpressando MYB-TF (TaPIMP1) ou TaERF2 (Zhang et al., 2012; Rong et al., 2014) e de tabaco expressando TaABP1 (bZIP-TF) ou TaWRKY10 (Cao et al., 2012; Wang et al., 2013).

Um dos próximos passos no estudo seria a caracterização de sequências classificadas como no-hits. Esta estratégia pode fornecer subsídios importantes para esclarecer a resposta do trigo ao estresse de seca. Num futuro próximo seria de grande valia, também, aplicar a metodologia deste trabalho a outros estádios fenológicos do trigo, a fim de analisar o comportamento destes genes ao longo do desenvolvimento. A co-localização de QTLs com alguns dos transcritos obtidos no experimento pode ser proveitosa em futuros trabalhos. Outro ponto interessante seria sequenciar o RNA de outras variedades de trigo com diferentes respostas à seca e fazer uma comparação das categorias dos genes entre as cultivares (primeiramente num aspecto mais amplo para depois focar em famílias específicas). A mais longo prazo seria importante, ainda, a comparação de sequências de espécies diferentes para uma melhor compreensão do papel dos genes envolvidos no processo de resposta da planta ao estresse hídrico.

Plantas transgênicas são ferramentas valiosas para estudos de expressão e função gênica. Entretanto, na maioria dos estudos têm sido utilizadas plantas modelos tais como Arabidopsis, tabaco e arroz. O presente trabalho iniciou trabalhos com o objetivo final de obter plantas transgênicas (via IMC) da espécie de interesse, ou seja, de cultivares de trigo, para a análise funcional de genes candidatos, envolvidos na resposta da planta ao estresse hídrico. A eficiência na obtenção de plantas transgênicas de trigo ainda é baixa. A maioria dos métodos para a geração destas plantas usam embriões imaturos como explante. Uma das desvantagens do uso deste tecido alvo é que são necessários vários anos para realizar as análises genéticas, caracterização molecular das plantas transgênicas e obtenção de plantas homozigotas (Zhou et al., 2003). Portanto, para tentar superar a baixa eficiência e as dificuldades na avaliação das plantas transgênicas, assim como a mais rápida utilização das mesmas nos programas de melhoramento, seria desejável o desenvolvimento de um protocolo de transformação usando

como alvo para a transferência gênica os micrósporos isolados. Para se alcançar este objetivo as seguintes etapas deveriam ser realizadas: (1) otimizar o protocolo existente de cultura de micrósporos isolados (IMC – do inglês *isolated microspore culture*)), (2) identificar genótipos com potencial androgenético e (3) estabelecer um protocolo de transformação genética de micrósporos isolados de trigo.

Trabalhos disponíveis na literatura usando como explantes micrósporos isolados para a transformação genética são escassos. Em cevada, Jähne et al. (1994), Yao et al. (1997) e Carlson et al. (2001) utilizaram a técnica de bombardeamento de partículas e obtiveram plantas transgênicas possivelmente hemizigotas para o transgene e possíveis plantas homozigotas. Em trigo foi registrado sucesso na regeneração de plantas transgênicas a partir de micrósporos isolados transformados pelo método *Agrobacterium* (Liu, 2004), método *Agrobacterium* e eletroporação (Brew-Appiah et al. (2013).

Em nosso estudo foram realizados experimentos preliminares com cultivares controle Bobwhite e Fielder, que possuem potencial para transformação (Weeks et al., 1993; Pellegrineschi et al., 2002; Haliloglu and Baenziger, 2003; Zale et al., 2004; Jones et al., 2005; Brunner et al., 2011), e Pavon 76 (alta resposta androgenética), para o estabelecimento de um protocolo de transformação de micrósporos isolados na Embrapa Trigo (dados não mostrados). Os experimentos basearam-se no protocolo utilizado por Liu (2004). Este protocolo usava como método de transformação o sistema *Agrobacterium tumefaciens*. A extração e o cultivo dos micrósporos isolados seguem o mesmo protocolo utilizado no experimento realizado para avaliar a resposta androgenética de diferentes cultivares. Para a transformação, foi adicionado o passo de co-cultivo com a agrobactéria. A inoculação da bactéria foi feita no momento da extração dos micrósporos, ou seja, no dia zero de cultivo. Vinte e quatro horas após o início do co-cultivo foram adicionados antibióticos ao meio de cultura, para a supressão da bactéria. Os resultados obtidos não foram satisfatórios. A adição de mais passos ao protocolo e a inoculação de bactérias ao meio líquido impediu a recuperação de plantas a partir dos embriões.Verificou-se a necessidade de otimizar o protocolo de IMC, buscando maior eficiência na regeneração de plantas, para então iniciar os experimentos de transformação. Devido a este impasse, o objetivo de transformar micrósporos isolados ficou para ser alcançado a mais longo prazo, após estabelecer as melhorias no protocolo de IMC.

Dados da literatura indicam que não existe um protocolo padrão para a androgênese, que é uma resposta altamente dependente do genótipo. Há três principais passos para o

desenvolvimento androgenético: aquisição da capacidade embriogênica, início de inúmeras divisões celulares e formação dos órgãos e diferenciação (Maraschin et al., 2005). A aquisição do potencial androgenético é amplamente afetada pelo genótipo e, especificamente, iniciada pelos pré-tratamentos de estresse, embora o real gatilho seja a reprogramação para que a célula siga a rota esporofítica ao invés da gametofítica. Diferentes tipos de estresse podem desencadear a mudança de rota. Para o trigo o estresse mais utilizado e que tem gerado melhores resultados é a aplicação de frio nas células alvo. Contudo, há alguns poucos trabalhos demonstrando que aplicação de ácido 2-hidroxinicotínico (2-HNA) pode render resultados muito mais eficientes (Zheng et al., 2003; Liu et al., 2002). Tentando aprimorar o protocolo de IMC este pretratamento químico foi testado em genótipos brasileiros selecionados. Nossos resultados indicam que o pré-tratamento de frio ainda permanece como melhor alternativa para desencadear o processo androgenético nos genótipos testados. Resultado similar foi obtido numa cultivar iraniana onde o pré-tratamento com 2-HNA a 33 °C também foi pior que a apliação de frio (Shirdelmoghanloo et al. 2009). É possível que melhores resultados com o pré-tratamento de 2-HNA possam ser alcançados se modificados alguns parâmetros, como por exemplo, o tempo de exposição.

Um obstáculo encontrado no desenvolvimento deste trabalho foi a alta produção de plantas albinas. Plantas albinas ocorreram em todos os genótipos testados. Sabe-se que diversos fatores podem afetar a produção de plantas albinas, sendo um deles o tipo de pretratamento ou a composição do meio de cultura. Baseado nos trabalhos de Wojnarowiez et al., (2002) e Jacquard et al, (2009), em cevada, um dos próximos passos na busca da otimização do protocolo de IMC em trigo seria testar a adição de sulfato de cobre ao meio de cultura na etapa de pré-tratamento.

Outra variável para tornar a IMC mais eficiente no que se refere à produção de plantas regeneradas consiste na modificação da composição do meio de cultura. A presença de agente gelificante no meio levou a uma melhora na resposta. A presença de Ficoll conferiu ao meio uma textura semissólida, fazendo com que as células se mantivessem na parte superior do meio de cultura, ajudando nas trocas gasosas e facilitando a manipulação dos embriões formados. Resultados semelhantes, mostrando que a presença de Ficoll (10%) melhorou a resposta androgenética em triticale, foram alcançados por Eudes e Amundsen (2005).

Uma vez que espécies e genótipos dentro de uma mesma espécie diferem no potencial de regeneração de tecidos *in vitro*, um dos objetivos do estudo foi avaliar a resposta

androgenética de diferentes genótipos de trigo, especialmente quanto à produção de plantas verdes com o genoma espontaneamente duplicado. A duplicação espontânea dos cromossomos é altamente desejável num protocolo de IMC. Para o trigo esta taxa de duplicação tem variado de 25% a 75% (Castillo et al., 2009) e é altamente dependente do genótipo (Brew-Appiah et al., 2013). A taxa de duplicação para os genótipos testados no presente estudo variou de 35% a 63%. A duplicação parece ter ocorrido no início das divisões, no momento da formação do embrião, uma vez que plantas totalmente DH, quimeras ou totalmente haploides foram regeneradas.

CONCLUSÃO

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A identificação de genes relacionados à resposta à seca em trigo foi realizada. Isto abre perspectivas para novas pesquisas que devem ser direcionadas para estudos da função de cada gene identificado e estudo do envolvimento destes genes em rotas metabólicas específicas. Além disso, esses resultados permitirão o desenvolvimento de marcadores moleculares e de estratégias para obtenção de plantas transgênicas, entre outros. O sequenciamento do transcriptoma de outros estádios fenológicos e outras cultivares, com diferentes respostas ao estresse hídrico, poderá contribuir para ampliar o entendimento dos mecanismos moleculares de tolerância à seca em trigo.

Além da identificação de genes de respostas à seca, este trabalho também permitiu a identificação de genótipos de trigo com potencial androgenético. Futuros trabalhos para otimizar o protocolo de IMC, visando sua aplicação para o estabelecimento de um protocolo de transformação eficiente, serão realizados.

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