**Daniela Feltrim**

# “Análise funcional e fisiológica de genes de milho induzidos pelo estresse por alumínio”

**CAMPINAS**
**2013**

# UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

**DANIELA FELTRIM**

## "ANÁLISE FUNCIONAL E FISIOLÓGICA DE GENES DE MILHO INDUZIDOS PELO ESTRESSE POR ALUMÍNIO"



Tese apresentada ao Instituto de Biologia da UNICAMP para obtenção do Título de Mestra em Genética e Biologia Molecular, na área de Genética Vegetal e Melhoramento.

Orientador: Prof. Dr. Marcelo Menossi Teixeira
Coorientadora: Dra. Agustina Gentile

**CAMPINAS,**
**2013**

FICHA CATALOGRÁFICA ELABORADA POR
MARA JANAINA DE OLIVEIRA – CRB8/6972
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

F347a

Feltrim, Daniela, 1983-
Análise funcional e fisiológica de genes de milho induzidos pelo estresse por alumínio / Daniela Feltrim. – Campinas, SP: [s.n.], 2013.

Orientador: Marcelo Menossi Teixeira.
Coorientador: Agustina Gentile.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Milho - Genética. 2. Plantas – Efeito do alumínio. 3. Fator de transcrição WRKY. I. Teixeira, Marcelo Menossi, 1968-. II. Gentile, Agustina. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações para Biblioteca Digital

**Título em Inglês**: Functional and physiological analysis of maize genes induced by aluminum stress
**Palavras-chave em Inglês**:
Maize - Genetics
Plants - Aluminum efects
WRKY transcription factor
**Área de concentração:** Genética Vegetal e Melhoramento
**Titulação:** Mestra em Genética e Biologia Molecular
**Banca examinadora:**
Marcelo Menossi Teixeira [Orientador]
Victor Alexandre Vitorello
Renato Atílio
**Data da defesa:** 27-02-2013
**Programa de Pós Graduação:** Genética e Biologia Molecular

Campinas, 27 de fevereiro de 2013

**BANCA EXAMINADORA**

Prof. Dr. Marcelo Menossi Teixeira (orientador) _____ Assinatura

Prof. Dr. Renato Atilio Jorge _____ Assinatura

Prof. Dr. Victor Alexandre Vitorello _____ Assinatura

Dra. Odalys Garcia Cabrera _____ Assinatura

Dr. Marcio Jose Da Silva _____ Assinatura

## Agradecimentos

Agradeço a Deus pela força e graça de ter galgado mais um degrau em minha formação pessoal e profissional. Aos meus pais por seu exemplo de vida e seu caráter. Aos meus familiares mesmo que de longe estarem presentes. Ao meu orientador Prof. Dr. Marcelo Menossi Teixeira por ter me dado a oportunidade de fazer pesquisa em seu laboratório e por todo o suporte e idealização deste projeto. A minha co-orientadora Agustina Gentile por todo apoio durante os experimentos, conversas e escrita desta dissertação e aos meus amigos de laboratório: Andrea, Bruna, Camila, César, Giane, Diego, Lucia, Kevin, Jennifer, Lara, José Pedro, José Sérgio, Thaís, Tiago, Valter, Vanessa e Wilson, por seu incentivo e atenção. Ao meu amigo Maicon, Rafael Tavares e sua esposa Camila, por seu exemplo de pesquisa e vida pessoal. A todos os professores da graduação por terem passado seu conhecimento. A pós-graduação pelo auxílio à pesquisa através da bolsa CAPES durante o mestrado.

“Há duas coisas infinitas:

O Universo e a tolice dos

Homens”.

Albert Einstein

# Índice

# Índice de ilustrações

## Capítulo 1.

# Índice de tabelas

## Capítulo I

Lista de abreviaturas

| | |
|---|---|
| WRKY | Fator de transcrição WRKY |
| SDR | Short chain reductase/desidrogenase |
| GFP | Green fluorescent protein |
| QDO | Quadruple dropout medium |
| X-α-GAL | X-α-D-Galactoside |
| SOD | Superóxido desmutase |
| PX | Peroxidase |
| *ALMT1* | Al-activated malate transporter |
| NJ | Neighbour joining |
| AP2 | APETALA2 |
| NAC | N-acetyl-L-cysteine |
| SPF1 | Sweet Potato Factor 1 |
| ZAP1 | Zinc-dependent Activator Protein-1 |
| ABF1 | Autonomously replicating sequence-binding factor 1 |
| SA | Salicylic acid |
| RT-qPCR | Reverse transcription–quantitative polymerase chain reaction |
| UTR | Untranslated region |
| X-Gluc | 5-bromo-4-chloro-3-indolyl glucuronide |
| BAP | 6-Benzylaminopurine |
| GUS | β-glucuronidase |

**Resumo**

O presente trabalho possui como objetivo caracterizar fisiológica e funcionalmente um gene de milho que têm expressão relacionada com a tolerância ao cultivo em solo ácido contendo níveis tóxicos de alumínio (Al). Este gene, denominado *Zmwrky69*, foi identificado na linhagem de milho Cat100-6, tolerante ao Al (Mattiello et al., 2010). A análise da sequência da proteína ZmWRKY69 indica que ela pertence ao grupo III dessa família de fatores de transcrição, apresentando o motivo WRKYGKQ na região amino terminal e o motivo dedo de zinco $Cx_7Cx_{23}HxC$ na região carboxi terminal. A proteína ZmWRKY69 está localizada no núcleo, conforme inferido por ensaio de localização subcelular em epitélio de cebola empregando uma fusão da proteína de milho com a proteína verde fluorescente (GFP). Ensaios de duplo híbrido em levedura indicam que a proteína ZmWRKY69 interage com um receptor de giberelina, uma proteína envolvida com a regulação por auxinas e uma proteína com função desconhecida. Plantas transgênicas de tabaco superexpressando *Zmwrky69* apresentaram menor inibição do crescimento radicular na presença de Al, comparativamente à plantas selvagens. Esses resultados indicam que o gene *Zmwrky69* codifica um fator de transcrição que apresenta um papel importante na tolerância ao Al em milho.

**Abstract**

The objective of the present work is to characterize a gene differentially expressed in the maize inbred line Cat100-6 (aluminum tolerant) grown in acidic soil containing toxic levels of aluminum. This gene called *Zmwrky69* was identified in the aluminum tolerant Cat100-6 inbred line (Mattiello et al., 2010). The sequence analysis of the ZmWRKY69 protein indicated that it belongs to the group III of this transcription factor family classification. The protein has the motif WRKYGKQ in the amino terminal region and a zinc finger motif $Cx_7Cx_{23}HxC$ in the carboxi terminal region. This protein is localized in the nucleus as inferred by subcellular localization in onion epidermis using a maize protein fused to the Green Fluorescent Protein (GFP). Yeast two-hybrid essays indicate that the protein ZmWRKY69 interacts with a gibberellin receptor, a protein involved in auxin regulation and a protein with an unknown function. Transgenic tobacco plants overexpressing *Zmwrky69* presented a lower degree of root growth inhibition in the presence of Al compared to wild type plants. These results indicate that *Zmwrky69* gene encodes a transcription factor that presents an important role in Al tolerance in maize.

# I. Introdução

## 1. Alumínio e solos ácidos

Como organismos sésseis, as plantas desenvolveram mecanismos diversos que as tornam tolerantes a muitas condições de estresse ambiental. Uma das mais proeminentes condições de estresse ambiental que afeta significantemente o desenvolvimento e produtividade das plantas é a condição ácida dos solos (Delhaize, 2004). Cerca de 30% dos solos mundiais são ácidos e 60% destes solos estão localizados nos trópicos e subtrópicos. Cerca de 70% do território brasileiro apresentam condições ácidas (Adámoli et al., 1985), especificamente a região do Cerrado, com mais de dois milhões de quilômetros quadrados.

A química do alumínio é extremamente complexa e ainda não completamente entendida. Isto é devido ao fato do alumínio ser capaz de se complexar com uma ampla gama de elementos como compostos inorgânicos polinucleares e complexos organometálicos (Poscherinder, 2008). Apesar destes fatores, a forma fitotóxica do alumínio para as plantas é sua forma iônica trivalente $Al^{3+}$ presente como $Al(H_2O)_6^{3+}$ encontrada em solos cujo pH decai para uma faixa abaixo de 5 (Kochian, 1995). Para propósitos de agricultura, o pH do solo é neutralizado utilizando-se $CaCO_3$, porém este processo pode levar anos para ser atingido completamente, o que acarreta problemas para que as plantas completem de maneira adequada seu desenvolvimento (Delhaize, 2004).

Economicamente, o Brasil é o terceiro maior produtor de milho totalizando 53,2 milhões de toneladas em 2009/2010. Para 2019-2020 a produção devera atingir 70,12 milhões de toneladas e o consumo interno 56,20 milhões de toneladas. Cerca de 12,6 milhões de toneladas serão destinadas a exportação. O principal destino do milho produzido é para indústria de rações para animais seguido de seus derivados como óleo, farinha,

amido, margarina, flocos e cereais. Devido a grande importância econômica do milho nacionalmente, os solos ácidos brasileiros apresentam um estresse ambiental que necessita ser atenuado. A geração de plantas transgênicas contendo genes que confiram tolerância ao metal permitindo aumentar a produção por área e desta maneira tornar o setor agrícola mais competitivo (Dados do Ministério da Agricultura, cultura milho).

## 2. Efeitos fisiológicos do alumínio

A principal causa das plantas não se desenvolverem em condições ácidas deve-se principalmente aos efeitos rizotóxicos do alumínio, sendo o ápice radicular a região mais sensível ao metal. Concentrações micromolares de Al causam na planta mudanças em sua estrutura radicular e redução na captação de água e nutrientes (Foy, 1988). Em estágios mais avançados, o Al também muda completamente a arquitetura da parede celular e inibe a divisão celular (Doncheva, 2004). A membrana celular é um sítio alvo para o alumínio uma vez que ele pode se ligar aos grupos fosfato e carboxil (Akeson, 1989). Uma vez em contato com a parede celular, o Al pode adentrar a célula, através de mecanismos e transportadores ainda não conhecidos e interagir com componentes celulares como ATP, GTP, ácido nucleico e citoesqueleto, bem como vias de sinalização de $Ca^{2+}$ (Jones, 1998). Ácidos orgânicos são compostos que podem quelar o alumínio no simplasto e impedir que ele interaja com os alvos (Ma, 2004).

Espécies reativas de oxigênio também são geradas, causando danos às membranas por mecanismo de peroxidação de lipídeos, sendo o estresse oxidativo uma provável causa da inibição do crescimento radicular (Kochian, 2004). Adicionalmente, Boscolo (2003), observaram a formação de reativos de oxigênio (ROS) e consequente oxidação proteica na

variedade de milho S1587-17 (sensível ao Al). Essa variedade também apresentou uma maior atividade enzimática de superóxido desmutase (SOD) e peroxidase (PX) na presença de Al, comparativamente aos níveis observados na linhagem Cat100-6 (tolerante ao Al).

## 3. Mecanismos de tolerância ao alumínio

O primeiro local de contato do alumínio com a célula vegetal ocorre na região da parede celular ou espaços intercelulares, chamado apoplasto. Uma parte do Al encontra-se complexado externamente à parede celular pelos ácidos orgânicos e parte entra na célula por canais ainda desconhecidos, ou através de um gradiente eletroquímico favorável, gerado pela quelação do $Al^{3+}$ por citrato ou malato citosólicos (Delhaize, 1995).

Um dos mecanismos fisiológicos de tolerância ao alumínio já bem estabelecido é a exclusão do íon. Neste processo o Al é imobilizado na parede celular pelos ácidos orgânicos exsudados pela célula, sendo impedido de entrar na raiz (Kochian, 2004). Estudos com trigo, um cereal amplamente estudado, mostraram que a variedade tolerante acumula menos alumínio em suas pontas radiculares do que as sensíveis devido à exsudação de ácidos orgânicos (OAs) capaz de quelar o alumínio depositado externamente à parede (Kochian, 2004). A superexpressão do gene da citrato sintase em plantas de *Nicotiana tabacum* e *Carica papaya* (de la Fuente, 1997) resultou em plantas tolerantes ao alumínio. Delhaize (2004) também mostrou que o gene transportador de malato *ALMT1* confere tolerância quando expresso constitutivamente em cevada. A despeito destes fatores, Maron (2008) utilizando a técnica de macroarray mostrou que a síntese de ácidos orgânicos não está relacionada à tolerância ao alumínio, mas que a regulação do transporte destes

compostos deve exercer um papel relevante nas linhagens de milho estudadas (Cat100-6 tolerante e L53 sensível).

O segundo mecanismo de tolerância é conhecido como detoxificação, sendo caracterizado pela fixação do alumínio da parede celular, complexação no simplasma por ligantes orgânicos e internalização nos vacúolos (Kochian, 2004).

## 4. Tabaco como planta modelo

Plantas modelo são utilizadas em engenharia genética como uma ferramenta que permite estudar genes e desenvolver novas tecnologias para geração de plantas mais tolerantes aos diversos tipos de estresses abióticos e bióticos, como baixo pH do solo, salinidade, seca, pragas e até a geração de antibióticos a fármacos especiais para uso na indústria e medicina (Ganapathi et al., 2004). *Nicotiana tabacum* é uma planta alotetraplóide formada pela hibridização de dois diploides *N. tomentosiformis* e *N. sylvestris*. Ela possui uma geração rápida em torno de três meses e produz uma ampla quantidade de sementes por cada geração. A primeira planta transgênica foi produzida em tabaco, assim como os primeiros avanços em engenharia genética foram majoritariamente obtidos através desta planta. O protocolo para transformação de tabaco está bem estabelecido permitindo gerar ao final uma planta contendo um gene de interesse para estudos genéticos com finalidades comerciais e econômicas (Ganapathi et al., 2004).

## II. Hipótese

A comparação de plantas cultivadas em solo corrigido com o de plantas cultivadas em solo ácido contendo níveis tóxicos de Al permitiu a identificação de diversos genes que têm expressão aumentada no último tratamento. Nossa hipótese é que alguns desses genes possa ser parte de uma via importante do mecanismo de defesa da planta sob estresse e que a superexpressão deles em plantas transgênicas de tabaco confere maior tolerância ao alumínio.

## III. Objetivos gerais

Ampliar o conhecimento dos mecanismos genéticos e fisiológicos que permitem que as plantas se desenvolvam em solos ácidos.

## Objetivos específicos

- Avaliar funcional e fisiologicamente um gene candidato à resistência/tolerância ao alumínio superexpressando-o em plantas de tabaco.
- Determinar o local onde a proteína de interesse é expressa, através do ensaio de localização subcelular.
- Determinar quais são as possíveis proteínas que interagem com o gene estudado neste trabalho.

## IV. Apresentação do trabalho

O primeiro capítulo descreve o gene *Zmwrky69*, que codifica uma proteína com alta identidade com fatores de transcrição da família wrky, presente em mono e dicotiledôneas, bem como alguns organismos unicelulares como *Giardia lamblia e Dictyostelium discoideum.* Foi realizado ensaio de localização subcelular utilizando a fusão com a proteína verde fluorescente (*Green Fluorescent Protein*) para verificar a localização desta proteína dentro da célula. Uma análise filogenética baseada no método de NJ (*Neighbour joining*) foi produzida para determinar o grupo ao qual pertence a proteína. Ensaios de interação com outras proteínas, utilizando a técnica de duplo híbrido em levedura, permitiram uma inspeção inicial das proteínas com as quais interage a proteína ZmWRKY69. Ensaios em hidroponia empregando plântulas de tabaco transgênicas super-expressando o gene *Zmwrky69* foram realizados para verificar se o referido gene confere tolerância ao alumínio.

O texto foi escrito em inglês, uma vez que pretendemos publicar os dados em revistas científicas de circulação internacional.

# V. CAPITULO I

## Molecular characterization of *Zmwrky69*, a member of the WRKY transcription factor family that is induced by aluminum in maize

Daniela Feltrim, Agustina Gentile, Marcelo Menossi [#]

Laboratório de Genoma Funcional, Departamento de Genética, Evolução e Bioagentes

Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

[#] Author to whom correspondence should be addressed:

menossi@unicamp.br

C. P. 6109

CEP 13083 -970

Campinas- São Paulo- Brazil

Phone: 0055 19 3521 6236

Fax: 0055 19 3521 6241

**Abstract**

The WRKY transcription factor members are involved in many biological processes such as pathogen defense, hormone signalization, seed development and abiotic stresses, like freezing, salinity, cold, heat and oxidative stress. This study is the first to access the role of a maize WRKY factor, *Zm*WRKY69, in aluminum tolerance. Sequence analysis showed that the *Zm*WRKY69 transcription factor shares high similarity to the *At*WRKY41 transcription factor and with others monocot factors, all members of the WRKY III group. Subcellular localization using *Zm*WRDKY69 fused to the green fluorescent protein showed that this protein is nuclear according to its biological role as a transcription factor. In addition, analyses of protein-protein interactions using the yeast two-hybrid system were done. By using the ZmWRKY69 transcription factor as bait against a cDNA library from Al-stressed roots, we identified 100 positive interacting clones. Among the proteins that interact with the WRKY protein we found a gibberellin receptor, a lipoxygenase, an auxin-repressed protein and a protein with unknown function. The root length from tobacco plants overexpressing *Zm*wrky69 was not affected by the presence of 50 and 150 µM Al in hydroponic medium, while the wild type plants presented reduced root. Taken all together, these results probably reflect the role of this transcription factor in conferring tolerance to aluminum in maize.

Keywords: WRKY transcription factor, aluminum, maize, yeast two hybrid

## 1. Introduction

The phytotoxicity of aluminum is one of the major abiotic stresses in tropical and subtropical area and represents impairment in plant development as well as productivity in acid soil (Yin et al., 2010). The primary symptom is reduced root area and consequently deficiency in water and nutrient uptake from the soil (Delhaize et al., 2004). Among the attempts to solve this problem is the overexpression of organic acid transport gene (*ALMT*1) from wheat in barley (Delhaize et al., 2004), citrate synthase from *Pseudomonas aeruginosa* in tobacco and papaya (*Carica papaya*); in both cases, transgenic plants presented increased tolerance to aluminum (de la Fuente et al., 1997). Another family associated to aluminum tolerance is the MATE family. They were first identified by fine mapping in sorghum and barley and are present in both eukaryotes and prokaryotes, where they function as secondary transporters. Maron et al. (2010) found that the maize gene *ZmMATE1* is localized in a telomeric region of chromosome 6 and that it is a functional homologous of sorghum *Sb*MATE, a major Al tolerant gene responsible for the exudation of citrate from root tips. The expression of *ZmMATE1* in Arabidopsis conferred aluminum tolerance due to increased citrate release from roots (Maron et al., 2010).

The constitutively greater expression of *TaALMT1* in an Al-tolerant wheat is associated to a series of *cis* mutations in its promoter and several alleles possess tandem repeats that enhance the expression of *TaAMLT1*, conferring tolerance to $Al^{3+}$ (Delhaize et al., 2012). Insertion of a transposable element in the 5'UTR upstream of the coding region of another protein involved in aluminum tolerance in barley (*HvAACT1*), increases transcript abundance and alters gene expression in the root apices, the principal location of $Al^{3+}$ activity, protecting plants to the toxic effects of the metal on acid soils (Delhaize et al.,

2012). For *TaMATE*, a transposable element inserted 11.1kb upstream of the start codon enhances the protein expression in root apices and the constitutively citrate efflux (Delhaize et al., 2012).

Nevertheless, plants like maize, possess a complex genetic structure concerning to aluminum tolerance, with more than one gene acting together in the tolerance mechanism (Kochian et al., 2004). This scenario makes aluminum stress a challenging area for plant breeding programs.

Transcription factors can be classified according to their characteristic DNA-binding domains (Maeo et al., 2001). There are many kinds of DNA-binding domains, such as the AP2 domain controlling plant development, the B3 domain involved in seed maturation, NAC domain important in meristem establishment (Jofuku et al., 1994; McCarty et al., 1991; Sablowski and Meyerowitz, 1998) and the WRKY domain involved in many physiological and developmental processes (Ulker and Somssich, 2004). The WRKY domain is identified in transcription factors which mediate gene expression in plant physiological process like plant hormones, response to pathogens and to light (Maeo et al., 2001). Generally, transcription factors function in network and controls directly or indirectly the gene expression in a time and spatial manner (Wyrick and Young, 2002).

Despite the fact that these family members are eminently found in plant kingdom, the WRKY transcription family members were also found in the protist *Giardia lamblia* and in the slim mold *Dictyostelium discoideum,* showing an early origin (Ulker and Somssich, 2004). The first WRKY cDNAs cloned were SPF1 from sweet potato (*Ipomoea batatas*), ABF1, 2 from wild oat (*Avena fatua*), *Pc*WRKY1, 2, 3 from parsley (*Petroselinum crispum*) and ZAP1from *Arabidopsis* (de Pater et al., 1996; Ishiguro and Nakamura, 1994; Rushton, P.J. et al. 1995; Rushton, P.J. et al. 1996;). A common feature

among the WRKY transcription factors is the conserved amino acid sequence WRKY at the N-terminus and a putative zinc-finger motif, $Cx_4$–$5Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$, comprising a region of 60 amino acids in length at the C-terminus (Rushton et al., 1995).

The WRKY proteins play a role either as repressors or activators during transcriptional process (Xie et al., 2005). Robatzek and Somssich (2002) showed that a member of the WRKY family, *At*WRKY6, positively regulates genes involved in leaf senescence and pathogen defense. WRKY factors can also act as repressors, as observed for the *Os*WRKY71 protein, that represses the *Amy*32b promoter during GA signaling pathway (Zhang et al., 2004).

Many physiological and developmental processes are regulated by genes encoding WRKY proteins. These transcription factors are involved in disease response signaling through hormonal pathway by salicylic acid (SA) (Eulgem et al., 2000). It was also found that *At*WRKY70 mediates SA and jasmonic acid cross-talk during plant defense response (Li et al., 2004). This family members also participates in response to abiotic stresses like freezing (Huang and Duman, 2002), drought, salinity, cold, heat (Pnueli et al., 2002; Rizhsky et al., 2004; Seki et al., 2002), wounding (Hara et al., 2000) and oxidative stress (Rizhsky et al., 2004). They can also control seed germination and post-germination growth through ligation at box2/W-box of GA *α-Amy2* promoter site (Rushton et al., 1995).

Phylogenetic analysis allowed the division of these transcription factors into seven subfamilies: I, IIa + IIb, IIc, IId + IIe, and III (Rushton et al., 2010). Family III members are known to act as key regulators in processes like SA signalization during pathogen infection in a time dependent and host specific manner (Kalde et al., 2003).

In a previous work we compared the transcriptome of maize roots grown in a control soil without free Al with acidic soil containing toxic levels of Al (Mattiello et al.,

2010). In this work two different maize inbred lines were tested for aluminum tolerance Cat100-6 (aluminum tolerant) and S1587-17 (aluminum sensitive). After one day and three days of exposition to acid soil conditions and based on microarray analysis it was possible to group genes accordingly to their expression (Mattiello et al., 2010) and a candidate gene of the Cat100-6 inbred line treated during three days in acid soil was selected for posterior experiments and analysis. In this work we have characterized the *ZmWRKY69* gene. The overexpression of *ZmWRKY69* increased the tolerance of transgenic tobacco plants in a nutrient solution containing Al as well as in plants grown in acid soil. We also observed that ZmWRKY69 is located in the nucleus and found several proteins that interact with this transcription factor.

## 2. Materials and Methods

### 2.1 Sequence alignment

The complete coding region of *ZmWRKY69* (Genbank Accession number: NM_001153907.1) was obtained from the sequence Zm.10017.1.A1_at from the maize Affymetrix Gene Chip array (Affymetrix, USA).

The ZmWRKY69 protein sequence was aligned with sequences of other plants using the ClustalX2 program (Larkin et al., 2007). The phylogenetic tree was constructed using the MEGA 5.0 program via the neighbor-joining algorithm. The phylogenetic tree was visualized using the Tree View program (Page, 1996). Bootstrap values of 1000 replicates were used to confer the reliability of each node.

## 2.2 Subcellular localization of ZmWRKY69

The coding region of the *Zmwrky69* gene was cloned in frame upstream of the green fluorescent protein (GFP) gene sequence (Siemering et al., 1996) under the control of the constitutive CaMV35S promoter in the pRT104 expression vector (Topfer et al., 1987) for the construction of the 35S::*Zm*WRKY69-GFP. The oligonucleotides used to clone the fusion protein were: forward 5' CCATGGATGGCTGAAGCCGTGGAG 3' (NcoI site underlined), and reverse primer 5' GGTACCTGGATAGTAGAGGCTTGGCATTG 3' (KpnI site underlined). The PCR product was subcloned into pGEM®-T Easy Vector (Promega, USA). Then, the resulting vector was digested with NcoI and KpnI and the insert was ligated into the pRT104 vector containing the GFP gene to create recombinant plasmids for transient expression analysis. Constructs were confirmed by both restriction analysis and sequencing.

Onion epidermis was peeled out and the inner part was used for a transient expression assay. Tungsten M10 particles (Bio-Rad, USA) were carried with 5µg of plasmid DNA, 20 µl 0.1 M spermidine and 50 µl 2.5 M $CaCl_2$. Eight µl of the mixture were used to load the macrocarrier. Particle bombardment was performed using a biolistic helium particle accelerator system (Biomics, Brasília, Brazil) at a pressure of 1000 psi. Images were observed with a fluorescent microscope Leica DMI4000B (Leica, Germany).

## 2.3 Yeast two-hybrid analysis

Total RNA from maize root tips submitted to aluminum stress for three days was used to synthesize a cDNA library using the SMART technology (SMARTer™ PCR cDNA Synthesis Kit, Clontech, USA). This cDNA was able to recombine with the AD cloning vector (pGADT7-Rec) *in vivo* to yield a complete GAL4-AD expression vector. This construct expressed the cDNA insert as a GAL4-AD fusion protein. The complete coding sequence of *Zm*wrky69 gene was amplified with the oligonucleotides 5' CATATGATGGCTGAAGCCGTGGAG 3' (NdeI site underlined) and 5' CCATGGTCATGGATAGTAGAGGCTTGGC 3' (NcoI site underlined) and cloned into pGBKT7 in frame with the GAL4-BD domain (Clontech, USA). The screening was done by co-transformation in the yeast strain Y2HGold (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ,LYS2::GAL1UAS–Gal1TATA–His3, GAL2UAS–Gal2TATA–Ade2 URA3::MEL1UAS–Mel1TATA AUR1-C MEL1)* including the cDNA, the pGADT7-Rec and the construction pGBKT7::*Zmwrky69*, according to Clontech's Matchmaker™ System (Clontech, USA). Positive clones were screened using SD/–Ade/–His/–Leu/–Trp medium (QDO, quadruple drop-out medium), as described in Matchmaker™ Gold Yeast Two-Hybrid System user manual (Clontech, USA).The co-transformation efficiency and number of clones screened were calculated by plating appropriate dilutions in selective media. Positive colonies appeared within 5 days of the screen and reporter genes were subsequently tested for α-galactosidase activity. The interactions were confirmed by re-streaking the positive clones onto QDO medium containing x-α-gal to verify the

maintenance of the correct phenotype and to test the strength of the interaction. After that, the interactions were re-tested on QDO medium with aureobasidin A (500 µg/ml).

### 2.4 Isolation and identification of interacting proteins

After phenotype confirmation, plasmids of positive clones were isolated using a lyticase protocol, with minor modifications (Philippsen et al., 1991). *Escherichia coli* cells were chemically transformed and plated onto LB medium containing ampicillin to select for the AD plasmid only. The cDNA inserts were amplified using the BD Matchmaker AD LD-Insert Screening Amplimer Set (Clontech, USA) and the BD Advantage 2 PCR kit (Clontech, USA). The PCR product was sequenced and then compared with different databases.

### 2.5 Transformation of tobacco plants

The complete coding sequence of *Zmwrky69* was amplified by RT-PCR using High Capacity RNA-to-cDNA Kit (Life Technologies, USA) with gene specific primers: Zmwrky69 forward, 5' GGATCCCCATGGCTGAAGCCGTGGAGAG 3' (BamHI site underlined) and *Zm*wrky69 reverse 5'TCTAGATCATGGATAGTAGAGGCTTGGC 3' (XbaI site underlined). The insert was cloned into pGEM®-T Easy Vector (Promega, USA). Subsequently, a BamHI and XbaI insert comprising the *Zmwrky69* coding region was cloned into pRT104 linearized with the same enzymes. The cassette 35S::*Zm*wrky69::T35S was then recovered with HindIII and cloned into pCAMBIA 2301(Cambia, Australia) also

cleaved with HindIII, giving rise to the pCAMBIA2301_ *Zm*wrky69. Leaf discs of aprox. 1 $cm^2$ were excised from four months-old tobacco plants (*Nicotiana tabacum* var SR1) and inoculated with 200 µl of *Agrobacterium* (GV3101 strain) containing the pCAMBIA2301_ *Zm*wrky69 and left at 25°C for 48 hours in petri dishes with INFTAB medium (composed of 100 mg/L inosytol, 50 mg/L pantothenic acid, 50 mg/L nicotinic acid, 50 mg/L pyridoxine hydrochloride, 50 mg/L tiamin and 0.5 mg/L biotin, pH 5). Leaves were transferred to solid INFTAB medium containing BAP (6-benzylaminopurine) and timentin. Finally, plants were then transferred to a root induction medium containing timentin and kanamycin and once the root development was complete plants were transferred to a greenhouse (Brasileiro et al., 1998). A *β-glucuronidase* histochemical assay was performed in tobacco leaves overnight at 37°C to detect positive transformation events using X-Gluc as a substrate (Jefferson et al., 1987).

**2.6 Aluminum tolerance assay**

Tobacco seeds harboring the cassette 35S::*Zm*wrky69::T35S (T1 transgenic lines) were surface sterilized in 1.5% sodium hypochlorite for twenty minutes and then rinsed five times in distilled water. Approximately thirty seeds were germinated in MS medium (Murashige et al., 1962) containing kanamycin and maintained in 16/8 h light/dark photoperiod during twenty days. Primary tobacco transformants positive for GUS activity using X-Gluc and selected by kanamicyn medium were used in hydroponic experiment. Seedlings with root length of approximately 1.0 cm were transferred to erlenmeyer flasks containing hydroponic solution composed of MS medium (250 µM $NH_4NO_3$, 11 µM $H_3BO_3$, 2 µM $MgCl_2$ , 0.2 µM $CuSO_4.5H_2O$, 0.35 µM $ZnSO_4$, 250 µM $NH_4NO_3$ , 2 µM

$Na_2$-EDTA, 125 µM $MgSO_4$, 125 µM $MgSO_4.H_2O$, 0.2 µM $Na_2MoO_4$, 500 µM $KNO_3$, 500 µM $KNO_3$, 2 µM $KH_2PO_4$, 0.35 µM $ZnSO_4.7H_2O$, 500 µM $Ca(NO_3)_2.4H_2O$, 2 µM $Fe(NH_4)_2.6H_2O$ pH 5.8) kept at 90 rpm during seven days. Seedlings were then transferred to a new medium containing $CaCl_2$ medium [1 µM] pH 4.4 supplied with $AlCl_3$ 50 µM and 150 µM, kept under constant shaking at 90 rpm for 3 days. Concomitantly, seedlings were also germinated in acid soil with pH 4.1 containing 10 mmol/$dm^3$ of Al, and in control soil with pH 5.0. Relative root length was calculated as [(root elongation at a certain concentration of Al)/(root elongation at 0 Al)] × 100.

**2.7 Quantification of Al in root apices**

Approximately 10 root apices of tobacco maintained in acid soil pH 4.1 Al content of 10 mmol/$dm^3$ were cleaned with acidified water with HCl (pH 4. 1 – 4. 3). Roots were put in microtubes with 1ml nitric acid (pH 2.0) for 24 hours. The supernatant was maintained at -20°C for posterior analysis. The aluminum concentration in root apices was measured using a spectrofluorometer with 1 ml 8-hidroxiquinoline (1% (w/v) 8-hidroxoquiniline, 2.5% de acetic acid (v/v) in deionized water), 3 ml acetic acid/ammonium hydroxide pH 5.7 and 10 ml butilic acetate (Bloom et al., 1978). The reagents were mixed and the organic part (superior) was separated for analysis in a Cary Eclipse spectrofluorometer (Agilent, USA), with lamp intensity of 10 A, excitation wavelength 390 nm and emission wavelength was 515 nm. For each sample 4 measurements were done using a quartz cuvette (optical length of 1 cm). A standard curve was made with serial dilutions of $AlCl_3$.

## 3. Results

### 3.1 Molecular characterization of *ZmWRKY69*

The coding sequence of *ZmWRKY69* was obtained using the NCBI database (GenBank accession EU968283). The deduced protein contains 320 amino acids, molecular mass of 34 kD and a pI of 5.92 (ProtParam tool, Expazy). ZmWRKY69 has a core domain known as wrky domain composed by the amino acid sequence WRKYGKQ and a zinc-finger portion composed by a cysteine and a histidine in position 173 and 175, respectively (Rushton et al., 2010). WRKY proteins are divided in three well characterized WRKY family groups: I, II and III (Eulgem et al., 2004). The ZmWRKY69 protein was classified in the group III (Figure 1) and it is composed by only one wrky domain and the characteristic zinc-finger structure $Cx_7Cx_{23}HxC$ (Suttipanta et al., 2011).

As these factors appear throughout the plant kingdom, a sequence alignment was performed using ClustalW2 (EMBL-EBI European Bioinformatics Institute) to search for homologous sequences among other monocots and also dicot plants. The deduced amino acid sequence showed similarity to monocot sequences of *Hordeum vulgare* (*Hv*WRKY30) with 47% similarity, of wheat (*Ta*WRKY1B) with 50% similarity, of *Oriza sativa* (*Os*WRKY73) with 17% and of the dicot *At*WRKY30 with 19% similarity (Figure 1). These results are in accord to our phylogenetic tree made using the neighbor-joining method (Figure 2). This means that the WRKY domain is shared between monocot and dicotyledonous plants, showing an early origin during evolution. The high similarity observed between the proteins from monocots in the alignment is also supported by a subgroup containing all the monocots proteins inside the group III (Figure 2). We also

annotated in Figure 2 distinct types of regulation of genes encoding WRKY proteins, but no clear correlation with their phylogeny could be found.

### 3.2 Subcellular localization of ZmWRKY69

Transcription factors are elements localized in the nucleus and they are responsible for the regulation of gene expression by ligating to its own promoter or to another promoter sequences (Rushton et al., 1996). This transient assay using onion epidermis showed that *Zm*WRKY69 was found in the nucleus, as expected for a transcription factor (Figure 3). The control construct, the *Sc*TFIIA transcription factor from sugarcane (Gentile et al., 2010), was used as a positive control for nuclear localization (Figure 3).

### 3.3 Yeast two-hybrid assay

We used a construct containing the ZmWRKY69 transcription factor as bait (Figure 4A) to screen a cDNA library obtained from roots of Cat100-6, an Al-tolerant maize line that was exposed to Al stress. A total of 100 positive interacting clones were obtained. Positive clones were selected in QDO medium with x-α-gal and aureobasidin A (Figure 4B). Among the proteins that interact with the WRKY protein we found a gibberellin receptor, an auxin-repressed protein, a lipoxygenase, and an unknown protein (Table 1).

### 3.4 Effects of *ZmWRKY69* overexpression in transgenic tobacco

Tobacco plants were transformed with pCAMBIA2301_ *Zm*wrky69, containing the *ZmWRKY69* under the control of the 35S promoter. T1 tobacco transformants were accessed for GUS gene expression for confirmation of plasmid transformation. Leaves were placed in a buffer solution containing X-Gluc as substrate. A blue color was observed as a result of the *β*-glucuronidase enzyme activity and representative positive events containing the *Zmwrky69* gene are shown in Figure 5. Seeds from GUS positive T1 plants were germinated in MS medium containing kanamycin. Seedlings with pale yellow coloration were considered as not transgenic and were discarded, while seedlings with green leaves and normal development were used for further studies. Wild type seedlings were grown in media without kanamycin. Plants were accessed for aluminum tolerance using either hydroponic or soil conditions. In the hydroponic experiment, 30 seedlings of each of the three events previously selected on media with kanamycin and wild type plants were grown for three days in a $CaCl_2$ solution containing $AlCl_3$ in three different concentrations (0 µM, 50 µM and 150 µM), pH 4.4. Overexpression of *Zmwrky69* reduced root growth, because wild type plants had 1.65 common average, while *Zmwrky69* overexpressors had only 0.95 cm (Figure 6). In the presence of 50 µM $AlCl_3$, roots from wild type seedlings had a significant reduction in length (1,1 cm on average). This inhibitory effect of Al was more evident at 150 µM $AlCl_3$, (root length of 0.85 cm on average). The two events overexpressing the gene *Zmwrky69* had no changes in their root development after exposure to both aluminum concentrations (Figure 6).

## 4. Discussion

WRKY proteins participate in many biological processes like seed development, pathogen response, oxidative stress and hormone signalization. The maize ZmWRKY69 protein has the typical WRKY domain (Figure 1) and the proteins containing this domain are distributed among different types of plants (Figure 2). Analyzing the sequence of *Zm*WRKY69 it was possible to classify this transcription factor into group III (Figure 2), which possesses one WRKY core (WRKYGKQ) in the N-terminus and a characteristic zinc-finger motif $Cx_7Cx_{23}HxC$ in C-terminus (Figure 1). As a transcription factor, this protein was localized in the nucleus in accordance to our results of subcellular localization (Figure 3). Members of this subfamily participates in many developmental programs of plant immune system and knockout or overexpression changes this web of signaling (Qiu, 2009). It is a difficult task unraveling WRKY function in abiotic stresses, due to a crosstalk and redundancy that exist behind this process (Rushton et al., 2010). To our knowledge, this is the first work investigating the role of a gene encoding a WRKY factor in plant responses to acid soil containing toxic levels of Al.

The majority of works related to aluminum tolerance uses the ability of plants to grow in hydroponic medium containing Al and there was a clear correspondence between the concentration of the metal and a physiological change in root development, as observed in plants like maize (Cançado, 2008) and tobacco (Yin, 2010). A reduction in root length and root area decrease plant capacity to absorb nutrients in solution, and this is a primary symptom of aluminum toxicity (Yin et al., 2010). We observed that *Zm*wrky69 caused a reduction in the root length of seedlings grown in nutrient solution compared to wild type plants (Figure 6). Regarding tolerance to Al, the experiment in hydroponics clearly showed that the root length of the transgenic plants was not altered due to the presence of Al

(Figure 6). This indicates that ZmWRKY69 might activate genes that help to protect plants against Al toxicity.

A more accurate measurement was done using a spectrofluorometer to evaluate aluminum content in roots. Because we did not observed differences in the levels of Al present in the roots of both wild type and transgenic plants grown in acidic soil (data not shown), the protective role of ZmWRKY69 probably is not related to the ability to exclude Al from the rhizosphere, a common strategy to overcome Al toxicity (Kochian, 1995). Other works also have reported genes that protect plants but do not cause differences in aluminum accumulation between wild type plants and overexpressing lines. This was the case of *Arabidopsis thaliana* overexpressing a gene encoding a cytosolic dehydroascorbate reductase (DHAR) involved in antioxidative system in tobacco (Yin et al. 2010a). Tobacco plants overexpressing a gene encoding a 2-alkenal reductase and control plants also did not show differences in the Al content in the roots when grown in nutrient solution containing $AlCl_3$. Hence, there are strategies other than the avoidance of aluminum accumulation that are triggered by the presence of a transgene.

The yeast-two hybrid approach also was done to search for possible targets in *Zm*WRKY69 signaling pathway present in the roots of the Cat100-6 inbred line exposed to Al. Among the targets we identified a gibberellic acid (GA) receptor GID1L2 (Table 1). Upon binding to GA, GID1 is able to interact with DELLA proteins and trigger their degradation, allowing GA to perform its role in many aspects of plant development like growth, seed germination, leaf expansion, pollen maturation and induction of flowering (Ueguchi-Tanaka et al., 2007). DELLA proteins are key regulatory elements in GA action, being able to block the DNA binding activity of transcription factors (Gao et al., 2011). Recently He et al., (2012) found that the balance among the hormones GA, abscisic acid

(ABA) and indole acetic acid (IAA) is modulated by nitric oxide (NO). Together, these hormones influence Al tolerance in rye and wheat. Therefore, the maize GID1L2 homolog could interact with ZmWRKY69, modulating its action in a GA dependent way. Further work on the GID1L2 expression pattern in response to Al in maize would help to increase our knowledge on the role of hormone in Al tolerance.

Another interesting target found in our yeast two-hybrid essay was a lipoxygenase. Lipoxygenases (LOX) are widely distributed in animal and plant kingdom (Chateingner, 1999). These enzymes are involved in oxylipin biosynthesis pathway by adding oxygen to polyunsaturated fatty acids. These oxylipins are involved in plant defense responses to pathogens and pests (Rosahl and Feussner, 2005). Plant LOX are also involved in several physiological processes such as senescence (Paliyath and Droillard, 1992), wounding (Farmer and Ryan, 1992), growth and mobilization of storage lipids (Feussner and Kindl, 1992). The LOX gene expression is controlled by hormones like JA (jasmonic acid) (Creelman and Mullet, 1997), abscisic acid (Melan et. al., 1993) and also by biotic and abiotic stresses like pathogen attack (Melan et al., 1993) and water deficiency (Porta et al., 1999), respectively. In maize two lox genes *ZmLOX10* and *ZmLOX11* share 90% identity and control a range of biologically important processes (Nemchenko et al., 2006). *ZmLOX10* is expressed in leaves and induced by cold stress, defense hormone jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA). This indicates its role in plant defense against pathogens and pests. *Zm*LOX11 is expressed in silks and controls only ABA. This possibility indicates that is a gene involved in response to osmotic stress (Nemchenko et al., 2006). This work is also the first to characterize a plant LOX gene (*Zm*LOX10) regulated transcriptionally based on circadian clock. An *in silico* analysis using pSORT (Nakai and Keneshisa, 1992) and subnuclear (Lei and Dai, 2005) indicated

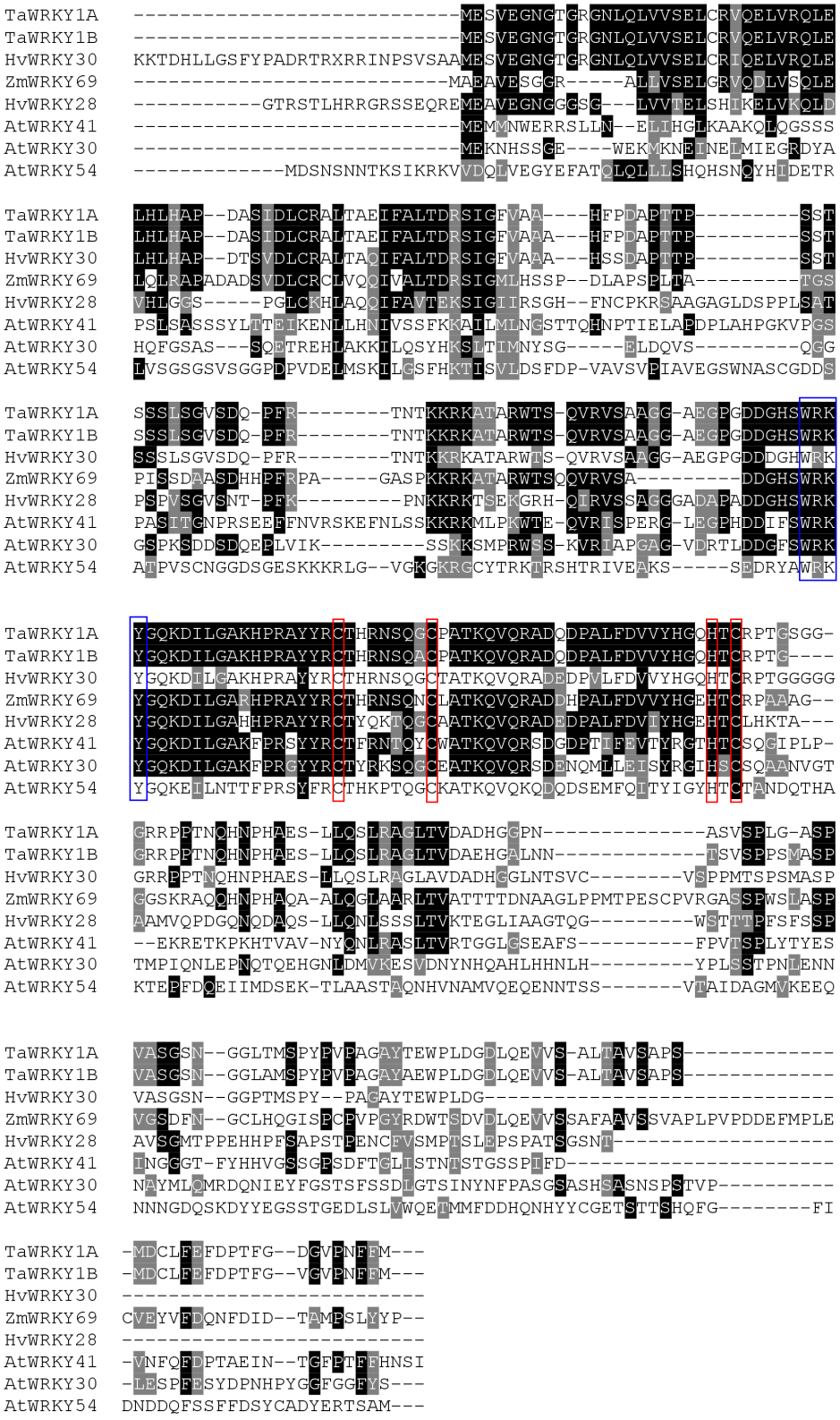
that *Zm*LOX11 protein is located in the nucleus. In humans, the arachidonate 5-lipoxygenase (5-LOX) is located in the nucleus and is able to alter the nuclear localization of the p53 protein, a key transcription factor that regulates apoptosis (Catalano et al., 2004). Interestingly, 5-LOX blocks p53 association with another nuclear protein, PML, but the mechanism remains unknown. Therefore, we can speculate that ZmLOX10 could interact with ZmWRKY69 regulating its action by an unknown mechanism, that could involve the localization in the nucleus.

A third target identified in the yeast two-hybrid encoded an auxin-repressed protein (ARP). This class of protein is not well understood yet and most studies relates the expression of the corresponding genes in response during plant development, such as bud dormancy in pea (Stafstrom, 1998), fruit maturation in strawberry (Reddy and Poovaiah, 1990) and pollen maturation in tobacco (Steiner et al., 2003). *ARP* genes also have been associated with plant responses to water stress in poplar roots (Kohler et al., 2003), but we are not aware of any report on plants exposed to Al stress. The putative interaction of the ARP protein with ZmWRKY69 suggests that a mechanism of action of this class of protein might be through the regulation of the activity of transcription factors. A protein with unknown function (NP_001144110.1) was also identified in our yeast two-hybrid essay. At this stage we are not able to provide any hypothesis on its role in Al tolerance nor on its effects on ZmWRKY69 and further studies will be needed in these directions.

**Acknowledgements**

This work was supported by grant 07/50993-5 from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to MM. DF received a fellowship from CNPq (National Council for Scientific and Technological Development)

## Figure legends




37

**Figure 1**. Alignment of *Zm*WRKY69 with other plant proteins. Identical amino acid residues are shown in black and grey background. Dashed lines indicate gaps. The WRKY domain is indicated by a blue square. Highly conserved residues within group III are shown in red square. In all cases, the ClustalW2 program was used. Triticum *aestivum* (Ta) ABC65846.1; *Triticum aestivum* (Ta) ABC65847.; *Hordeum vulgare* (Hv) ABI13396.1; *Zea mays* (Zm) NP_001147379; *Hordeum vulgare* (Hv) ABI13394.1; *Arabidopsis thaliana* (At) NP_192845.1; *Arabidopsis thaliana* (At) NP_568439.1; *Arabidopsis thaliana* (At) NP_181607.1

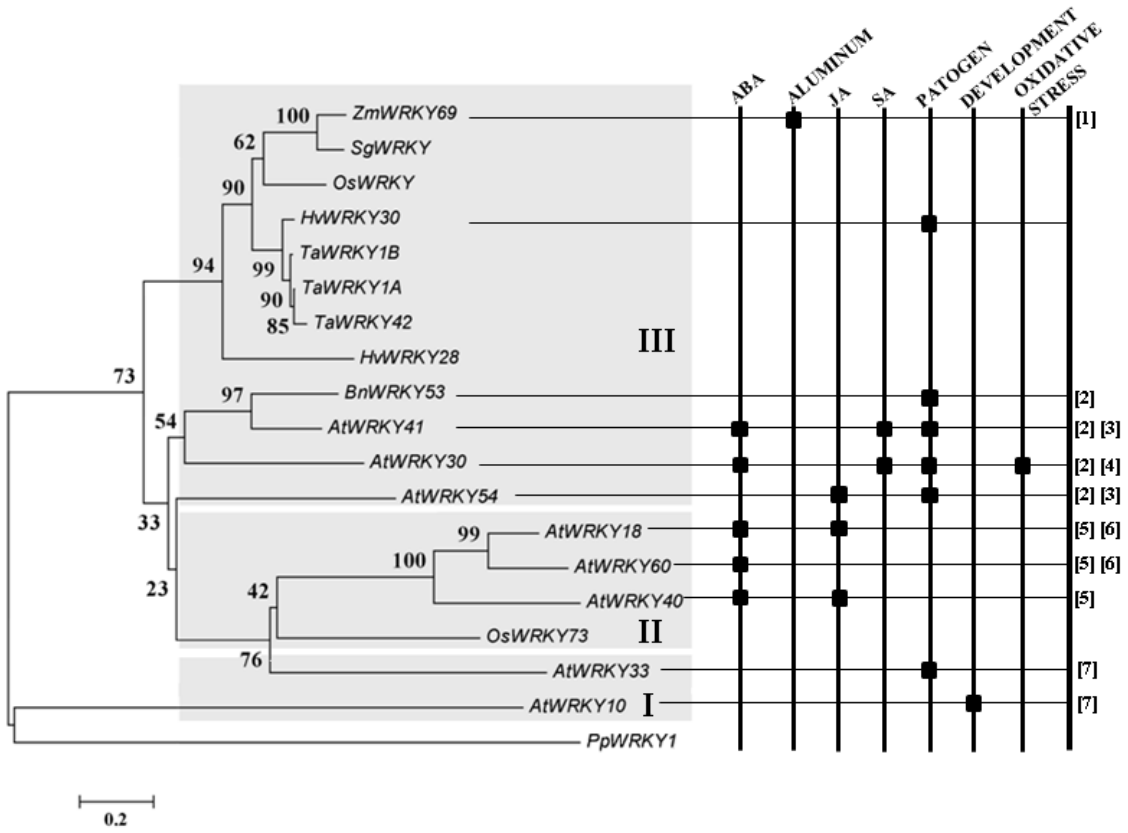



**Figure 2.** Phylogenetic tree of WRKY proteins. Sequences used for the alignment were from *Zea mays* (Zm) NP_001147379.1, *Sorghum* (Sg) XP_002444278.1, *Oryza sativa* (Oz) DAA05138.1; *Hordeum vulgare* (Hv) ABI13394.1; *Triticum aestivum* (Ta) ABC65846 and ABC65847.1; *Brassica napus* (Bn) ACQ76808.1; *Arabidopsis thaliana* (At) NP_192845.1 and NP_568439.1; and *Physcomitrella patens* (Pp) AAL78681.1. The phylogenetic tree was constructed using the MEGA 5.0 program with a consensus of 1000 bootstraps replicates using the neighbor-joining (NJ) method. [1] (Mattiello et al., 2010); [2] (Mangelsen et al., 2008); [3] (Kalde et al., 2003); [4] (Scarpeci et al., 2008); [5] (Yu et al., 2001); [6] (Pandey et al., 2010); [7] (Lippok et al., 2007).

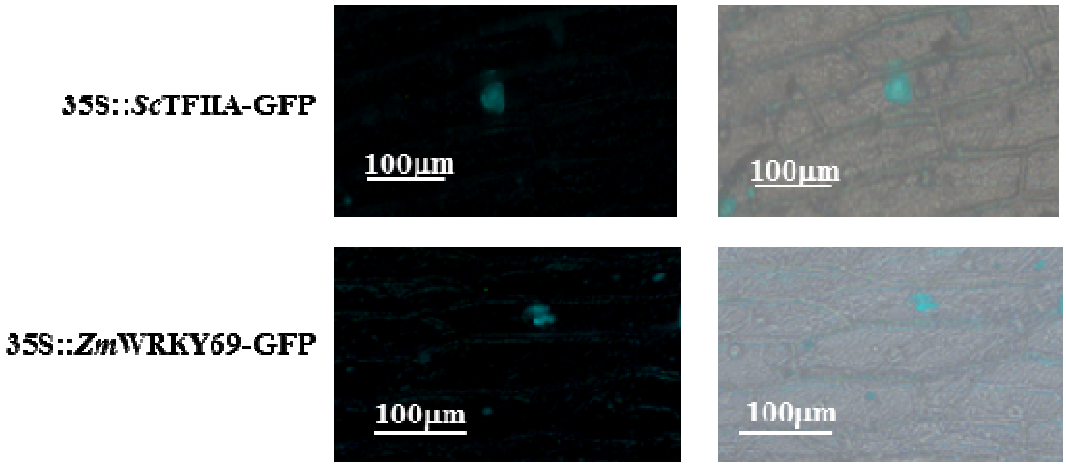



**Figure 3.** Subcellular localization of *Zm*WRKY69. A construct carrying the target gene *Zm*WRKY69 fused to the GFP protein under the control of the CaMV 35S was used in a transient assay using onion epidermal cells**.** A control construct carrying the *Sc*TFIIA transcription factor was used (Gentile et al., 2010). Epidermal cells were observed with a fluorescence microscope (Leica, Germany). Left: green fluorescence, right: merged image.

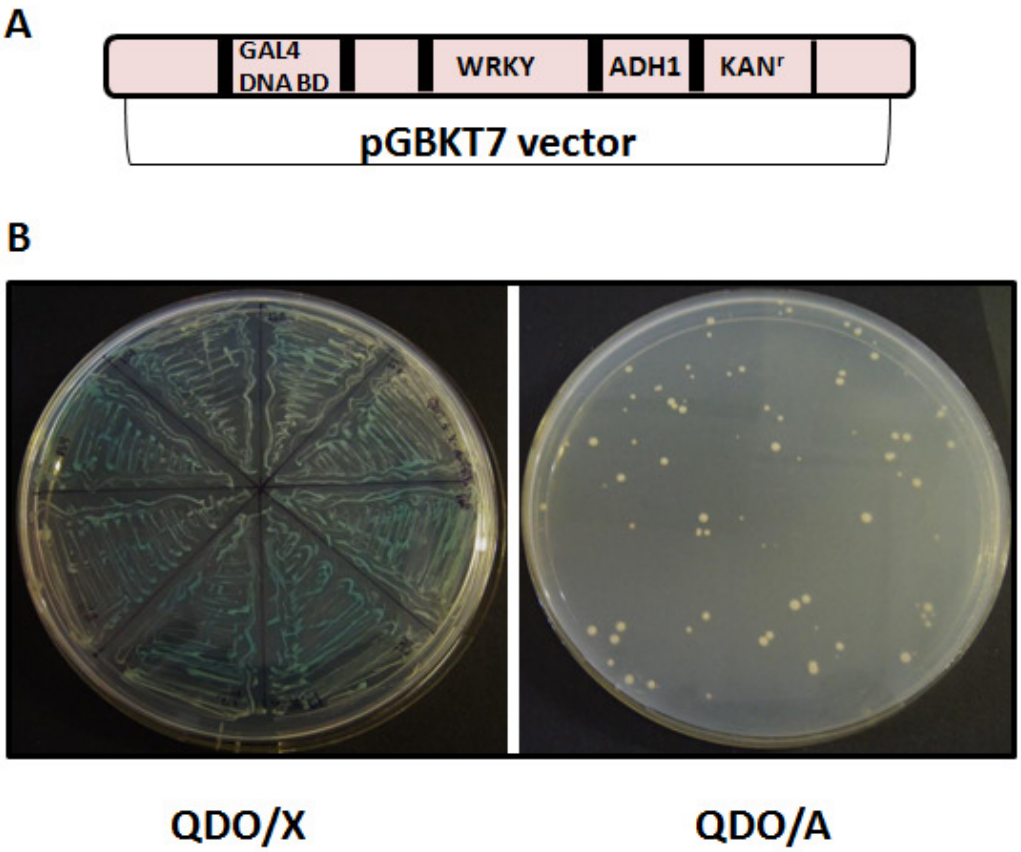



**Figure 4**. Yeast two hybrid assay using ZmWRKY69 as bait.. A: pGBKT7 vector carrying the coding sequence from *Zmwrky69* in frame after the GAL4 DNA-Binding Domain (DNA-BD). B: Positive clones indicating the interaction between the WRKY protein with other maize proteins. Left: QDO/X/ selective medium QDO containing X-α-Gal. Right: Selected clones in medium QDO with aureobasidin A.

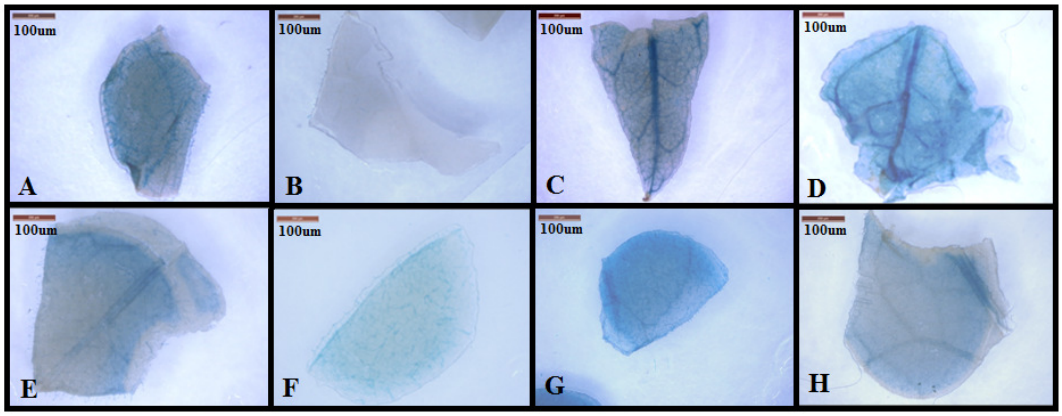



**Figure 5**. *β*-glucuronidase hystochemical assay on tobacco leaves. A) Positive control control containing a p35S::GUS::3´NOS (Modolo, 2009), B) Negative control (wild type *Nicotiana tabacum* SR1),. (C-H) Independent transgenic events (T1 generation) for *Zm*wrky69: 3, 18, 20, 22, 24 and 16, respectively. All the images were captured using the LEICA DMI400B (Leica, Germany).

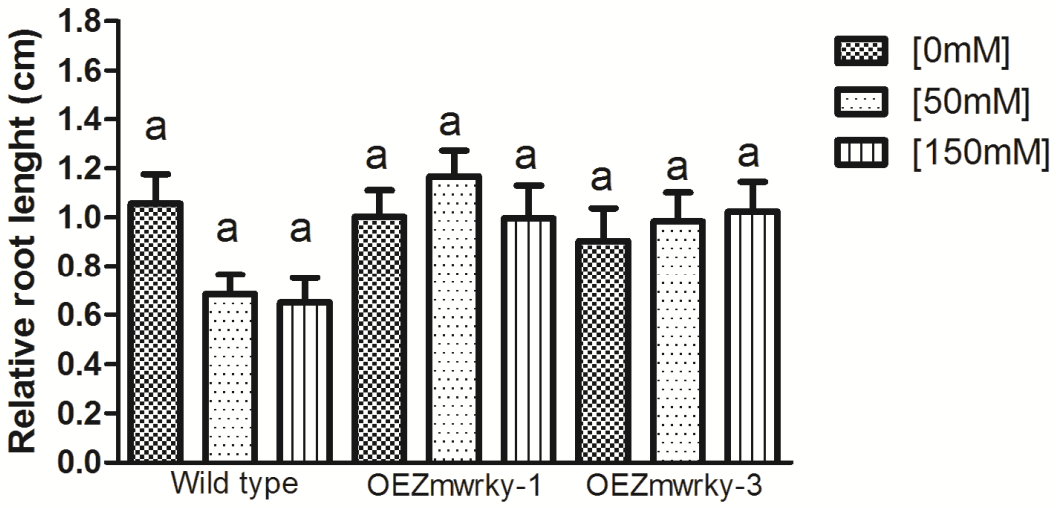



**Figure 6**. **Effect of aluminum in the root length under hydroponic conditions.** Wild type and T1 transgenic seedlings were grown during 10 days in $CaCl_2$ medium without aluminum and then transferred to a medium supplied with 0, 50 and 150 µM of aluminum and $CaCl_2$ [1 µM] pH 4.4. After 3 days under aluminum exposure, the main root was measured using a ruler (cm scale). Bars presented the average of 30 seedlings. Data were analyzed using the analysis of variance (ANOVA) and different letter indicates significant differences. Error bars indicate the SEM of three replications and each replication comprised 10 roots.

**Table 1**. Positive clones found in the yeast two- hybrid assay. The protein ID (NCBI) is indicated in the left column and annotation of the interacting protein in the right column.

| PROTEIN ID | INTERACTING PROTEIN |
|---|---|
| EU959343.1 | Zea mays clone 217231 gibberellin receptor GID1L2 mRNA |
| NP_001105981.1 | Zea mays lipoxygenase11 (lox11), mRNA |
| NP_001150581.1 | auxin-repressed protein |
| NP_001144110.1 | uncharacterized protein LOC100276950 |

## 5. References


Bloom PR, Weaver RM, Mcbride MC: The spectrophotometric and fluorometricdetermination with 8-hydroxyquinoline and butyl acetateextraction. Soil Sci Soc Am J 1978, 42:713-716.

Cançado, G.M.A., Nogueira, F.T.S., Camargo, S.R., Drummond, R.D., Jorge, R.A., Menossi, M. 2008. Gene expression profiling in maize roots under aluminum stress. Biologia Plantarum 52, 475-485.

Catalano, A., Rodilossi, S., Caprari, P., Coppola, V., Procopio, A. 2004. 5-Lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. EMBO J 24, 170-179.

Chateigner, A.L., Deunff, Y.L., Jalouzot, R. 1999. Germination-associated changes in the transcript content of pea seedling lipoxygenases. Lipoxygenase-g: a new marker of axis growth resumption. Planta 208, 606-613.

de Pater, S., Greco, V., Pham, K., Memelink, J., Kijne, J., 1996. Characterization of a zinc-dependent transcriptional activator from Arabidopsis. Nucleic Acids Res 24, 4624-4631.

Creelman, R.A., Mullet, J., 1997. Biosynthesis and action of jasmonates in plants.Annu. Rev. Plant Physiol. Plant Mol. Biol 48, 355-381.

Eulgem, T., Rushton, P.J., Robatzek, S., Somssich, I.E., 2000. The WRKY superfamily of plant transcription factors. Trends Plant Sci 5, 199-206.

Eulgem, T., Weigman, V.J., Chang, H.S., McDowell, J.M., Holub, E.B., Glazebrook, J., Zhu, T., Dangl, J.L., 2004. Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol 135, 1129-1144.

Stafstrom, J.P., B.D. Ripley, M.L. Devitt and B. Drake. 1998. Dormancy-associated gene expression in pea axillary buds. Planta 205:547–552

Farmer E, Ryan CA (1992) OctadecanoõÈd precursors of jasmonic acid activate the synthesis of wound inducible proteinase inhibitors. Plant Cell 4: 129±134

Feussner I, Kindl H (1992) A lipoxygenase is the main lipid body protein in cucumber and soybean cotyledons during the stage of triglyceride mobilization. FEBS Lett 298: 223-225

Gao XH, Xiao SL, Yao QF, Wang YJ, Fu XD. An updated GA signaling relief ofrepression regulatory model. Mol Plant 4, 601-606.

Gentile, A., Da Cruz, P., Tavares, R.G., Krug-Baldacin, M.G., Menossi, M., 2010. Molecular characterization of ScTFIIAgamma, encoding the putative TFIIA small subunit from sugarcane. Plant Cell Rep 29, 857-864.

Hara, K., Yagi, M., Kusano, T., Sano, H., 2000. Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding. Molecular and General Genetics 263, 30-37.

Huang, T., Duman, J.G., 2002. Cloning and characterization of a thermal hysteresis (antifreeze) protein with DNA-binding activity from winter bittersweet nightshade, Solanum dulcamara. Plant Mol Biol 48, 339-350.

Ishiguro, S., Nakamura, K., 1994. Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5' upstream regions of genes coding for sporamin and beta-amylase from sweet potato. Mol Gen Genet 244, 563-571.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6, 3901-3907.

Jofuku, K.D., den Boer, B.G., Van Montagu, M., Okamuro, J.K., 1994. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6, 1211-1225.

Kalde, M., Barth, M., Somssich, I.E., Lippok, B., 2003. Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. Mol Plant Microbe Interact 16, 295-305.

Kohler, A., Delaruelle, C., Martin, D., Encelot, N., Martin, F., 2003. The poplar root transcriptome: Analysis of 7000 expressed sequence tags. FEBS Lett 542, 37-41.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lei, Z., Dai, Y., 2005. An SVM-based system for predicting protein subnuclear localizations. BMC Bioinformatics 7, 291.

Li, J., Brader, G., Palva, E.T., 2004. The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16, 319-331.

Lippok, B., Birkenbihl, R.P., Rivory, G., Brummer, J., Schmelzer, E., Logemann, E., Somssich, I.E., 2007. Expression of AtWRKY33 encoding a pathogen- or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. Mol Plant Microbe Interact 20, 420-429.

Maeo, K., Hayashi, S., Kojima-Suzuki, H., Morikami, A., Nakamura, K., 2001. Role of conserved residues of the WRKY domain in the DNA-binding of tobacco WRKY family proteins. Biosci Biotechnol Biochem 65, 2428-2436.

Mangelsen, E., Kilian, J., Berendzen, K.W., Kolukisaoglu, U.H., Harter, K., Jansson, C., Wanke, D., 2008. Phylogenetic and comparative gene expression analysis of barley (Hordeum vulgare) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. BMC Genomics 9, 194.

Mattiello, L., Kirst, M., da Silva, F.R., Jorge, R.A., Menossi, M., 2010. Transcriptional profile of maize roots under acid soil growth. Bmc Plant Biol 10, 196.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., Vasil, I.K., 1991. The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. Cell 66, 895-905.

Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M., Peterman, T.K., 1993. An *Arabidopsis thaliana* Lipoxygenase Gene Can Be lnduced by Pathogens, Abscisic Acid, and Methyl Jasmonate. Plant Physiol 101, 441-450.

Murashige T, Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497.

Nakai K, Kanehisa M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics. 14, 897–911.

Nemchenko, A., Kunze, S., Feussner, I., Kolomiets, Michael., 2006. Duplicate maize 13-lipoxygenase genes are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection, and hormonal treatments. Journal of Experimental Botany 57, 3767-3779.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12, 357-358.

Paliyath G, Droillard MJ (1992) The mechanisms of membrane deterioration and disassembly during senescence. Plant Physiol Biochem 30: 789-812.

Pandey, S.P., Roccaro, M., Schon, M., Logemann, E., Somssich, I.E., 2010. Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. Plant J 64, 912-923.

Philippsen, P., Stotz, A., Scherf, C., 1991. DNA of Saccharomyces cerevisiae. Methods Enzymol 194, 169-182.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A., Mittler, R., 2002. Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume Retama raetam. Plant J 31, 319-330.

Porta, H., Rocha-Sosa, M., 2002. Plant Lipoxygenases. Physiological and Molecular Features. Plant Physiology 130, 15-21.

Qiu, Y., Yu, D., 2009. Over expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance. Environ. Exp. Bot. 65, 35-47.

Reddy, A.S.N. and B.W. Poovaiah. 1990. Molecular cloning and sequencing of a cDNA for an auxin-repressed messenger-RNA—correlation between fruit growth and repression of the auxin regulated gene. Plant Mol. Biol. 14:127–136.

Rizhsky, L., Davletova, S., Liang, H., Mittler, R., 2004. The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in Arabidopsis. J Biol Chem 279, 11736-11743.

Robatzek, S., Somssich, I.E., 2002. Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev 16, 1139-1149.

Rosahl S, Feussner I. 2005. Oxylipins. In: Murphy D (ed.) Plant lipids. Oxford: Blackwell Publishing, 329–354.

Rushton, P.J., Macdonald, H., Huttly, A.K., Lazarus, C.M., Hooley, R., 1995. Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of alpha-Amy2 genes. Plant Mol Biol 29, 691-702.

Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K., Somssich, I.E.. 1996. Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. The EMBO Journal 15, 5690 – 5700.

Rushton, P.J., Somssich, I.E., Ringler, P., Shen, Q.J., 2010. WRKY transcription factors. Trends Plant Sci 15, 247-258.

Sablowski, R.W.M., Meyerowitz, E.M., 1998. A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell 92, 93-103.

Scarpeci, T.E., Zanor, M.I., Carrillo, N., Mueller-Roeber, B., Valle, E.M., 2008. Generation of superoxide anion in chloroplasts of Arabidopsis thaliana during active photosynthesis: a focus on rapidly induced genes. Plant Mol Biol 66, 361-378.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., Shinozaki, K., 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant Journal 31, 279-292.

Siemering, K.R., Golbik, R., Sever, R., Haseloff, J., 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. Curr Biol 6, 1653-1663.

Steiner, C., J. Bauer, N. Amrhein and M. Bucher. 2003. Two novel genes are differentially expressed during early germination of the male gametophyte of *Nicotiana tabacum.* Biochim. Biophys. Acta 1625:123–133

Suttipanta, N., Pattanaik, S., Kulshrestha, M., Patra, B., Singh, S.K., Yuan, L., 2011. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in Catharanthus roseus. Plant Physiol 157, 2081-2093.

Topfer, R., Matzeit, V., Gronenborn, B., Schell, J., Steinbiss, H.H., 1987. A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res 15, 5890.Ueguchi-Tanaka, M., Nakajima, M., Ashikari, M., and Matsuoka, M.(2007). Gibberellin receptor and its role in gibberellin signaling in plants. Annu Rev Plant Biol 58: 183–198

Ulker, B., Somssich, I.E., 2004. WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol 7, 491-498.

Wyrick, J.J., Young, R.A., 2002. Deciphering gene expression regulatory networks. Curr Opin Genet Dev 12, 130-136.

Xie, Z., Zhang, Z.L., Zou, X., Huang, J., Ruas, P., Thompson, D., Shen, Q.J., 2005. Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol 137, 176-189.

Yin, L., Mano, J., Wang, S., Tsuji, W., Tanaka, K., 2010. The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots. Plant Physiol 152, 1406-1417.

Yin, L., Wang, S., Eltayeb, A.E.,Uddin, Md.I., Yamamoto, Y., Tsuji, W., Takeuchi, Y., Tanaka, K., 2010a. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta 231, 609-621.

Yu, D., Chen, C., Chen, Z., 2001. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 13, 1527-1540.

Zhang, Z.L., Xie, Z., Zou, X., Casaretto, J., Ho, T.H., Shen, Q.J., 2004. A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. Plant Physiol 134, 1500-1513.

## Conclusões

Este projeto teve como principal finalidade a caraterização funcional de um gene diferencialmente expresso sob tratamento em solos ácidos da linhagem de milho Cat100-6 (tolerante). As principais conclusões são:

- As análises filogenéticas indicam que o gene codifica um fator de transcrição da família WRKY, sendo denominado *ZmWRKY69*, sendo classificado na subfamília dos fatores WRKY tipo III.
- A análise de localização subcelular indica que ZmWRKY69 é uma proteína nuclear, como esperado para um fator de transcrição.
- O experimento de duplo híbrido em levedura indicou possíveis mecanismos de regulação da proteína ZmWRKY69, que envolvem um receptor de giberelina, uma proteína reprimida por auxina, uma lipoxigenase e uma proteína desconhecida.
- Os experimentos envolvendo plantas de tabaco que superexpressam o gene *ZmWRKY69* mostraram que esse gene confere tolerância ao alumínio;

## 8. Referências bibliográficas


Adámoli, J.; Macêdo, J.; Azevedo, L.G.; de.; Netto, J. (1985). Caracterização da região dos Cerrados. In: Goedert, W.J. (Ed). Solos dos Cerrados: tecnologias e estratégias de manejo. São Paulo: Nobel; Planaltina, DF: EMBRAPA CPAC. Cap 2.p. 33-74.

Akeson, M. A., D. N. Munns & G. Burau. (1989). Adsorption of $Al^{3+}$ to phosphatidylcholine vesicles.Biochim. Biophys. Acta 986: 33-40.

Boscolo, P.R.S.; Menossi, M. and Jorge, R.A. (2003). Aluminum-induced oxidative stress in maize. Phytochemistry (62): 181-189.

de la Fuente, J.M., Ramirez-Rodriguez, V., Cabrera-Ponce, J.L., Herrera-Estrella, L., (1997). Aluminum tolerance in transgenic plants by alteration of citrate synthesis. Science 276, 1566-1568.

Brasileiro, A. C. M..; de Campos Carneiro, V. T.Manual de transformação genética de plantas. 1. ed. Brasília, Embrapa SPI/Embrapa-Cenargen, 1998. 309p.

Delhaize, E. and Ryan, P.R. (1995). Aluminum toxicity and tolerance in plants. Plant Physiol. (107): 315-321.

Delhaize, E., Ryan, P.R., Hebb, D.M., Yamamoto, Y., Sasaki, T., Matsumoto, H.(2004). Engineering high-level aluminum tolerance in barley with the ALMT1 gene. Proc Natl Acad Sci U S A 101, 15249-15254.

Delhaize, E., Ma, J.F., Ryan, P.R. (2012). Transcriptional regulation of aluminium tolerance genes. Trends in Plant Science. 17: 341-348.

Doncheva, S., Amenós, M.; Poschenrieder, C and Barceló, J. (2004). Root cell patterning: a primary target for aluminum toxicity in maize. Journal of Experimental Botany (56) 414: 1213-1220.

Foy, C.D. (1988). Plant adaptation to acid, aluminum-toxic soils.Commun Soil Sci Plant Anal 19: 959-987.

Ganapathi, T.R., Suprasanna, P., Rao, P.S.,Bapat, V.A. (2004). Tobacco (Nicotiana tabacum L.) - A model system for tissue culture interventions and genetic engineering. Indian Journal of Biotechnology 3, 171-184.

Jones DL, Gilroy S, Larsen PB, Howell SH, Kochian LV. (1998). Effect of aluminum on cytoplasmic Ca2C homeostasis in root hairs of Arabidopsis thaliana (L.). Planta 206:378–87

Kochian, L.V. (1995). Cellular Mechanisms of Aluminum Toxicity and Resistance in Plants. Ann. Rev. Plant Physiol. Plant Mol. Biol.(46): 237-260.

Kochian, L.V., Hoekenga, O.A., Pineros, M.A. (2004). How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol 55, 459-493

Ma JF, Shen RF, Nagao S, Tanimoto E. (2004). Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. Plant Cell Physiol. 45:583–9.

Maron, L.G.; Kirst, M.; Mao, Chuanzao, M.; Milner, M.J.; Menossi, M. and Kochian, L. (2008). Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots. New Phytologist. 179: 116-128.

Maron, L.G.; Piñeros, M.A.; Guimarães, C.T.; Magalhães, J.V.; Pleiman, J.K.; Mao, C.; Shaff, J.; Belicuas, S.N.J. and Kochian, L.V. (2010). Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. The Plant Journal 61: 728-740.

Poschenrieder, C.; Gunsé, B.; Corrales, I.; Barceló, J. (2008). A glance into aluminum toxicity and resistance in plants. Science of total environment 400: 356-368.