Universidade de Brasília

Embrapa Recursos Genéticos e Biotecnologia
Plant-Pest Interaction Laboratory

INRA Sophia Antipolis

PhD thesis

Melhoramento da tolerância de plantas a estresses biótico e abiótico: Uso de regulação transcricional por CRISPR/dCas9 e Algodão Transgênico

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor in Biotechnology and Biodiversity

Programa de Biotecnologia e Biodiversidade da UnB

Brasília, the 8th of June 2018

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Biotechnological improvement of plants against Biotic and Abiotic stresses: Use of CRISPR/dCas9 transcriptional regulation and Transgenic cotton

Aprovado em 8 de Junho de 2018

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Agradecimientos

Agradezco en primer lugar a mi familia, mi base en esta aventura que emprendí hace años para estudiar fuera de mi país natal y formar parte de este mundo. A mis padres Mónica y Joaquin, por el amor, enseñanzas y un sinfín de aportes a lo que soy como persona. A mis hermanas Laura y Luisa por ser las compañeras y el pedal de confianza. A Mita, Sergio y Maia por ser parte del núcleo y por apoyarme en todas las decisiones. A ellos y a todo el resto por crear el cimiento de una vida plena, feliz y de sueños realizados.

Agradezco a los integrantes del «LIMPP» implicados en este trabajo que jamás habría podido desarrollar sin ellos.

A mi orientadora Fátima Grossi por permitirme hacer parte de su grupo y por liderar con pasión y energía.

A François Gillet por su enorme contribución, por incentivarme científicamente y ayudarme a sobrepasar las diferentes barreras.

A Thuanne Ribeiro por su incansable ayuda en los experimentos y sus incentivos en los momentos difíciles.

A Caroline Bournaud por su implicación desde el comienzo y sobre todo hasta el final.

A Deisy Amora por ayudarme en la corrección y concepción de este manuscrito.

A los últimos 4 por ser también mis grandes amigos.

A Isabela Tristán, Maria Eugenia Lisei y Regina Amorim por su contribución en los diferentes experimentos.

A Janice de Almeida Engler y su grupo en el INRA de Sophia Antipolis por permitirme realizar la última parte del trabajo en su laboratorio, por hacer parte de mi proyecto final y por dejarme hacer parte de la colaboración en el proyecto de las Fimbrinas.

Gracias a los amigos y amigas de siempre, así como a los nuevos y nuevas que conocí en este proceso. La vida académica no sería igual sin tener con quien compartir una vida personal increíble como la que he tenido. A los que viven en Colombia por las recepciones y los encuentros en las vacaciones. A los que viven en Europa, por ser mi segundo núcleo y hacer parte de mi formación como adulto. A los que encontré en Brasil, principalmente a Daniel Noriega por su parcería, consejos y confianza. A mis colegas y amigos del LIMPP que me ayudaron de alguna forma: Ludovic Saint-Felix, Leonardo Pepino, Danila Cabral, Daniela Amaral, Reneida Mendes, Dijair Souza, Fabricio Arraes, Dagna Maria, Rayssa Almeida, Arinaldo Silva.

Al «Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)» y a la «Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)», por el apoyo financiero.

A la «Embrapa Recursos Genéticos e Biotecnologia», por la estructura y recursos proporcionados.

A la Universidad de Brasília y al Programa de «Pós-Graduação em Biotecnologia y Biodiversidade».

Dans la vie, rien n'est à craindre, tout est à comprendre.

Marie Curie

Abstract

As sessile organisms, plants evolve towards a developmental and physiological flexibility to generate adaptation mechanisms against different abiotic and biotic stresses that often occur simultaneously. Water stress impacts different aspects of plant physiology, and can decrease their growth and productivity. *Meloidogyne incognita* is a root-knot nematode that infects and modifies the structure of plant roots. In an agronomical aspect, both stress episodes represent an elevated economic cost. Genetic manipulation of plant genomes can overcome some of these obstacles. We first implemented a CRISPR/dCas9 strategy in Arabidopsis to improve drought stress tolerance. Then, we introduced a cassette in cotton plants to induce drought stress tolerance and *M. incognita* resistance at the same time. The type II CRISPR/Cas system has been adapted in plants to control the genetic modification in a more targeted and precise procedure. In the light of controlling drought stress tolerance, we used the CRISPRi/CRISPRa mechanisms which make use of an engineered dead Cas9 fused to transcriptional modulators to regulate transcription. We evaluated the use the dCas9 fused to the tripartite activator VPR and two *Arabidopsis* epigenetic modification domains: The Acetyltransferase domain from AtHAC1 (HAT domain), and the methyltransferase domain from CURLY LEAF (CLF) gene (SET domain). During a transient assay, our results showed that the dCas9 fused to VPR and HAT increased the activity of promoters controlling a reporter gene, whereas the SET domain showed contrasted results. The strategy was also tested to control the endogene promoter of the transcription factor *AtAREB1*, known to control key genes in the response to drought stress. The *AREB1* expression was increased in plants carrying the $dCas9^{HAT}$ fusions, plants that showed a better tolerance to drought stress. Our data indicates that targeted epigenetic histone modifications can be used to modify promoter activity in plants. Here, we improved plant's response to drought stress, but the approach can be designed to control any promoter and include responses to other stresses. In a more classical approach, we engineered cotton genetically modified plants driving expression of AREB1ΔQT, a constitutive form of AREB1 transcription factor. We expressed within the same construct an RNAi targeting an essential gene in *M. incognita* that encodes for a splicing factor. We obtained three lines of GM cotton that had a better response to water withdrawal and nematode infection in the T1 generation. At term, both strategies represent new attractive opportunities in plant biotechnology.

Resumo

Como organismos sésseis, as plantas evoluem em direção a uma flexibilidade no desenvolvimento fisiológico para se adaptarem a estresses bióticos e abióticos e que freqüentemente ocorrem simultaneamente. O estresse hídrico afeta diferentes aspectos da fisiologia das plantas e pode diminuir seu crescimento e produtividade. *Meloidogyne incognita* é um nematoide de galhas que infecta e modifica a estrutura das raízes das plantas. Em um aspecto agronômico, ambos estresses representam um custo econômico elevado. A manipulação genética de genomas de plantas pode superar alguns destes obstáculos. Inicialmente, implementamos uma estratégia CRISPR / dCas9 em Arabidopsis para melhorar a tolerância à seca. Em seguida, introduzimos um cassete em plantas de algodão para induzir a tolerância ao estresse hídrico e a resistência ao *M. incognita* ao mesmo tempo. O sistema CRISPR/Cas tipo II foi adaptado em plantas para controlar a modificação genética em um procedimento mais direcionado e preciso. Com o intuito de controlar a tolerância ao estresse hídrico, utilizamos os mecanismos CRISPRi/CRISPRa, que empregam a «dead Cas9» fusionada à moduladores transcricionais. Avaliou-se o uso da dCas9 fusionada com ativador VPR e com dois domínios de

modificação epigenética de Arabidopsis: O domínio Acetiltransferase de AtHAC1 (domínio HAT) e o domínio metiltransferase do gene CURLY LEAF (CLF) (domínio SET). Nossos resultados mostraram que as dCas9 fusionadas a VPR e HAT aumentaram a atividade de promotores controlando um gene repórter. A estratégia também foi testada para controlar o promotor endógeno do fator de transcrição AtAREB1. A expressão de AREB1 foi aumentada em plantas portadoras das fusões dCas9HAT, plantas que apresentaram melhor tolerância ao estresse hídrico. Os dados indicam que modificações epigenéticas específicas de histonas podem ser usadas para modificar a atividade do promotor em plantas. Neste estudo melhoramos a resposta das plantas ao estresse hídrico, mas a abordagem pode ser projetada para controlar qualquer promotor e incluir respostas a outros estresses. Em uma abordagem mais clássica, nós projetamos plantas geneticamente modificadas (GM) de algodão, impulsionando a expressão de AREB1ΔQT. Dentro da mesma construção foram desenhados a expressão do gene AREB1ΔQT e o dsRNA para um gene essencial em *M. incognita,* que codifica para um fator de splicing. Foram obtidas linhagens de algodão GM que demonstraram melhor resposta ao estresse hídrico e à infecção por nematoides, na geração T1. A termo, ambas as estratégias representam novas oportunidades atraentes na biotecnologia vegetal.

Abbreviations

°C - Celsius degrees

3D - Three Dimensional

A, T, C, G - Adenine, Thymine, Cytosine et Guanine

AA - Aminoacids

ABA - Abscisic acid

acetylCoA - Acetyl coenzyme A

ADAR - Adenosine deaminases acting on RNA

AGROBEST - Agrobacterium-mediated enhanced seedling transformation

AREB - Abscisic Acid-responsive element binding protein 1

AREB1ΔQT - AREB1 lacking Q and T domains

AT - Acetyltransferase

avr - Avirulence

Bt - Bacillus thuringiensis

CAGE - Cap analysis of gene expression

Cas9 - CRISPR associated protein 9

cDNA - complementary DNA

CDS - Coding sequence

CLF - Curly Leaf

cm, mm - Centimeters, millimeters

CO_2 - Carbon dioxide

Cpf1 - CRISPR Prevotella and Francisella 1

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CRISPRa - CRISPR inhibition

CRISPRi - CRISPR inhibition

crRNA - CRISPR RNA

CS - Chromoshadow domain

DA - drought avoidance

dCas9 - dead Cas9

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DNMT3A - DNA methyltransferase 3A

dNTP - Nucleoside triphosphate

DSB - Double strand break

dsRNA - Double stranded RNA

DT - Drought tolerance

EDLL - Transcriptional activation *domain* from AP2/ERF transcription factors

EMS - Ethyl methanesulfonate

ERD - ETS2 repressor factor

ES - Embryogenic stem cells

EtOH - Ethanol

ExpB - Expansin B

FANTOM - Functional annotation of the mammalian genome

FAST - Fast *agrobacterium*-mediated seedling transformation

FIM - Fimbrins

g - Gram

GC - Giant cells

GFP - Green fluorescent protein

GM - Genetically modified

GMM - Genetically modified microorganism

GMO - Genetically modified organisms

GmUces - *Glycine max* Ubiquitin promoter

GmUcesMin - *Glycine max* Ubiquitin promoter minimal module

GUS - β-glucuronidase

h - Hour

H3K27me - Histone methylation occurring on Lysine 27 terminal tail of the core histone H3

H3K9me - Histone methylation occurring on Lysine 9 terminal tail of the core histone H3

HAT - Histone acetyltransferase

Hbf - Foetal haemoglobin

HEG - Homing endonuclease genes

HEPN - nucleotide binding endoRNase domain

HR - Homologous recombination

HSPC - Hematopoietic stem and progenitor cells

HT - Herbicide tolerance

ICSI - Intracytoplasmic sperm injection

indel - Insertion, deletion

INRA - Institut national de la recherche en Agronomie

ITN / ITP - Initiation of basal immunity

J2 - Second stage juveniles

JA - Jasmonate acid

JIRCAS - Japan International Research Center for Agricultural Sciences

KanR - Kanaycin resistance

kb - Kilobase

KI - Knock-in

KO - Knock-out

KRAB - Krüppe associated box

LB - Lysogeny broth

LEA - Late abundant protein

LIMPP - Laboratório de interação planta praga

LMO - Living modified Organisms

LSD1 - Lys-specific histone demethylase 1

MAMP, pAMP -M icrobial- or pathogen-associated molecular patterns

MCS - Multi-cloning site

mM - Milli Molar

mRNA - Messenger RNA

MS - Murashige and Skoog

MS2 - From MS2 bacteriophage

NC - Neighbouring cells

NeoR - Neomycine

NF-κB p65 - nuclear factor kappa-light-chain-enhancer of activated B cells

NGG - "Any nucleotide + two guanines

NHEJ - Non homologous end joining

NLS - Nuclear localization sequence

nm - Nanometer

NT - Non transformed plant

nt - Nucleotide

OE - Over-expression

OFP - Orange fluorescent protein

ORI - Origin of replication

PAGE - Polyacrilamide gel electrophoresis

PAM - Protospaced Adjacent Motif

pAREB1 - Promoter of AREB1

PBS - PUF binding site

PCR - Polymerase chain reaction

PD-1 - Programmed cell death 1

PEG - Polyethylene glycol

pGKII - Plasmid green K II

PhD - Philosophiae doctor

PP2C - Protein phosphatase 2C

PP7 - From PP7 bacteriophage

PPN - Plant parasitic nematode

qPCR - Quantitative PCR

QTL - Quantitative trait locus

Rda - The Epstein-Barr virus R transactivator

REPAIR - Programmable A to I Replacement

RFP - Red fluorescent protein

RKN - Root knot nematode

RNA - Ribonucleic acid

RNAi - RNA interference

RNAP - RNA polymerase

ROS - Reactive oxygen species

SA - Salicylic acid

SAM - Synergistic activation mediator

SCN - Cyst nematode

scRNA - Scaffold RNA

SET - (Su(var), E(z), and Trithorax) *domain*

SF - Splicing factor

sgRNA - Single guide RNA

SID - mSIN3 interaction domain

SID4x - Four motifs of mSin3

SnRK2 - Sucrose non-fermenting-1-related protein kinase 2

SRDX - EAR-motif repression domain

SRSR - Short regularly spaced resets

T-DNA - Transfer DNA

T1, T2…- Time 1, Time 2

TALE - Transcription activation-like effectors

TALEN - Transcription activation-like effectors nuclease

TCRα, TCRβ - T cell receptor

TCRα, TCRβ - T-cell recPDeptors

TF - Transcription factor

TGS - Transcriptional gene silencing

Ti - Tumor inducing plasmid

tracrRNA - trans-activating crRNA

TSS - Transcription start site

U. S. A - United States of America

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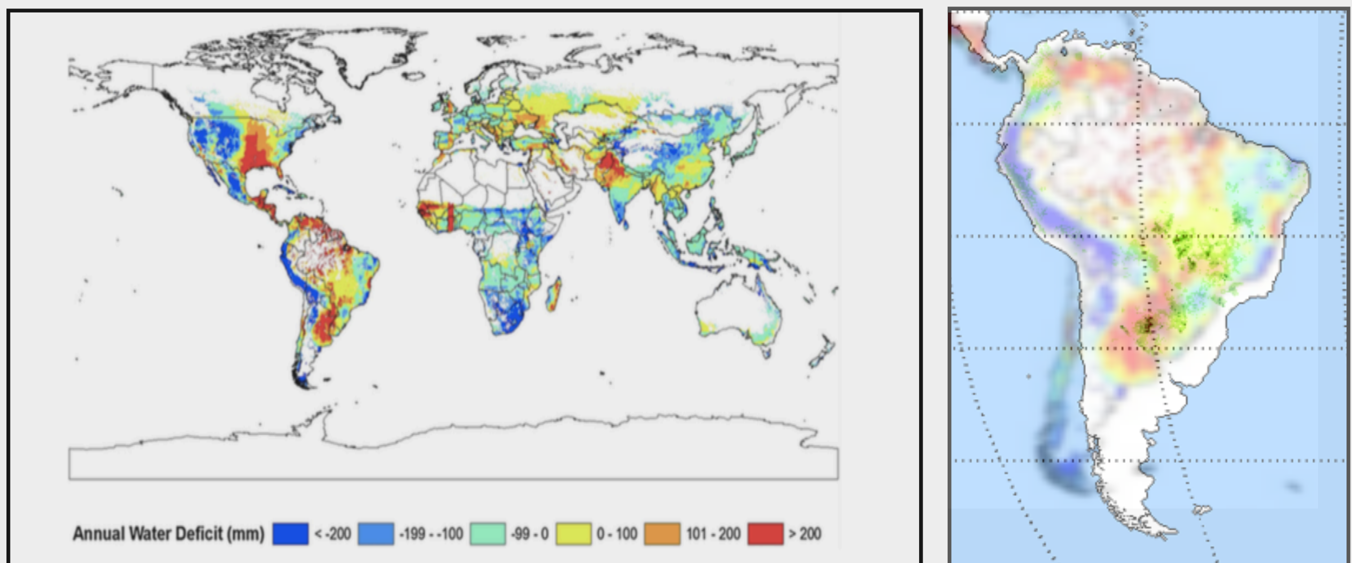
Chapter I - Improving plant responses to drought stress and nematode infection

1- General introduction

1.1 Climate change and drought stress response

Climate and natural variability at different spatial and temporal scales will result in important challenges for planted crops in the near future. The increasing global temperature predicted at short-term might also be associated with an intensification of drought (Lobell and Gourdji, 2012). In Europe, climate change will affect the spatial and time distribution of water resources with possible decreases in precipitations, particularly in the south and the center of the continent (Anderson et al., 2008). Brazil has already warmed by about 0,7 °C in the last 50 years (Marengo et al., 2011), and has experienced extreme droughts in 2005, 2010 and 2015. A water deficit prediction made of a forecast that might arise between 2077 and 2099, showed that in South America, Brazil could be one of the most impacted countries, with regions facing important deficits (0 to >200 mm) (**Figure 1**).

Meteorological drought is a climate event characterized by abnormal low precipitations and often accompanied by above normal temperatures, driving periods of dry soils and a decrease in the levels of water storages (rivers, lakes, reservoirs). Water resources are sensitive to climate change but also closely related to human water use behavior. The considerable growth in the population, industrial, agricultural



Adapted from Zhang and Cai, 2013 and Monfreda et al., 2008

Figure 1- Water deficit prediction for the years 2070 to 2099 in mm/year (A) Worldwide and (B) overlaid with the cotton planted regions in Brazil (green). The regions with the worst water deficit predictions overlay with the cotton planted regions in Brazil.

activities and living standards have intensified water demands, and future societies will face shortage on water availability (Mehran et al., 2017). Severe drought affects millions of people by impacting water resources, ecosystems and agriculture, as it alters different plant functions, principally disturbing plant growth and production, which in turn reduces carbon fixation (Dai, 2011; Alizadeh et al., 2014). Many plant traits have been reported to be affected by drought stress, such as plant height, stem dry weight, leaf weight, area and node number, leading to an important decrease in yield (Loka, Oosterhuis and Ritchie, 2011; Farooq et al., 2012; Sahito et al., 2015; Challinor et al., 2016; Fahad et el., 2017).

1.2 Plant strategies to face drought stress

Drought varies spatially, temporally, and in strength. Accordingly, plants have diversified their response and have evolved different strategies to endure drought conditions at different morphological, physiological, biochemical, and molecular layers. Drought resistance (DR) delineates the ability of certain plants to grow satisfactorily when exposed to water deficit, and in crops, it prescribes the ability to maintain a minimal yield loss in periods of water scarcity (Fang et al., 2014). DR in plants can be divided in drought avoidance (DA), drought tolerance (DT), and drought escape (DE) (Osakabe et al., 2014; Basu et al., 2016).

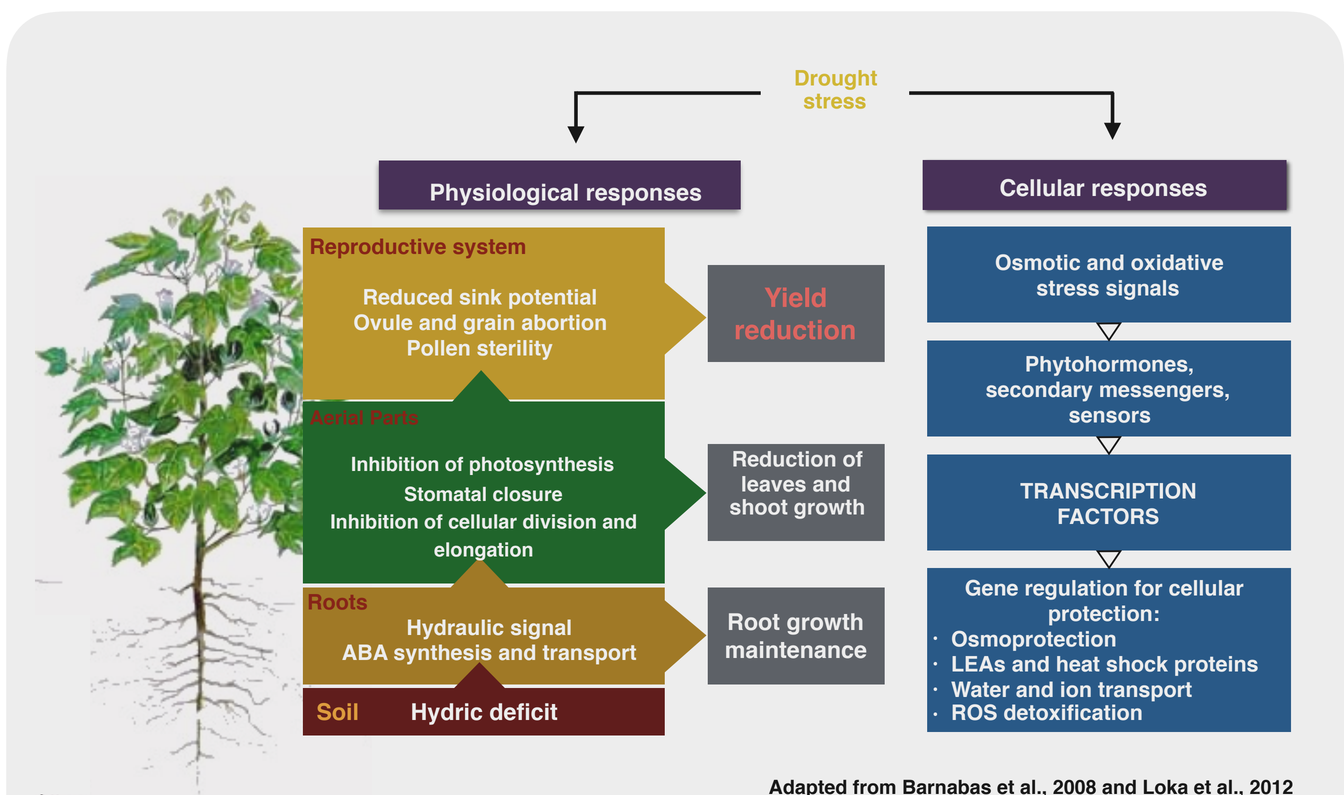


Figure 2- Drought stress physiological responses triggered by cellular and molecular responses in plants. Dry soils are sensed by roots that maintain their growth to retain water uptake. The physiological responses in aerial parts result in reduction of leaves and shoot growth. An arrest in the development of the reproductive system derives in yield reduction. The physiological responses are activated by a series of cellular and molecular responses that activate different transcription factors that control the expression of different protective and detoxifying proteins.

DA strategy relies on the capacity to avoid adverse effects by maintaining normal physiological processes through the adjustment of morphological structures and growth rates. DA plants maintain high water potential by maintaining the growth of the roots, having smaller and thicker cuticle, thicker leaves that can change their direction and have smaller stomata that close rapidly to prevent water loss. DA accomplishment also changes the time of the transition to reproductive stages to avoid abortion of grains and pollen (Fang et al., 2014).

DT strategies relate to the ability to maintain certain physiological states by regulating several response genes and pathways. DT plants keep an adequate cell turgor with osmotic adjustment by accumulating secondary metabolites and osmoprotectants to retain water and maintain membrane integrity while preventing disruptions in cellular metabolism and changing cellular and tissue elasticity. DE refers to the adjustment of plants of their growth period or life cycle to prevent encountering local or seasonal drought. The drought stress response varies in plants and can involve the combination at different degrees of drought escape, avoidance, and tolerance. The morphological and physiological changes, triggered by the molecular pathways to respond to water scarcity are summarized in **Figure 2** (Hare et al., 1998; Tezara et al., 1999; Silvente et al., 2012; Osakabe et al., 2014; Fang et al., 2015).

1.3 The Acid abscisic pathway

At a cellular level, Abscisic acid (ABA)-independent and ABA-dependent regulatory systems have been illustrated (Shinozaki and Shinozaki, 2006). Both pathways trigger different regulatory networks, by activating different transcription factors. ABA is a phytohormone that accumulates during drought stress conditions, triggering ROS-induced stomatal closure induced by ion-and water transport in guard cells to control loss of water by evapotranspiration and maintain turgor pressure (**Figure 3**). Consequently, the CO_2 absorption is reduced, impacting the electron transport in the thylakoids, and thus photosynthesis and plant growth (Touchette et al., 2007; Maes et al., 2009; Leach et al., 2011). The group of Shinozaki and Shinozaki at JIRCAS in Japan have been studying the different mechanisms, pointing how some key transcription factors are at the basis of drought stress response in plants (Uno et al., 2000; Shinozaki et al., 2003; Shinozaki and Shinozaki. 2006; Yoshida et al., 2010; Nakashima et al., 2014). In *A. thaliana*, there are 9 AREB TFs, and among them, A*REB1/ABF2, AREB2/ ABF4,* and *ABF3* are highly induced by ABA and osmotic treatments (Uno et al., 2000; Fujita et al., 2005; Yoshida et al., 2010). *AtAREB1* is a bZIP transcription factor (TF) and has shown good responses to drought stress when over-expressed in *A. thaliana*, and therefore, appears to be one of the best candidates to use in transgenic crops. AtAREB1/ABF2 is localized in the nucleus and expressed in roots and leaves under stress conditions. AREB1 is functionally regulated by phosphorylation in its four conserved regions (C1, C2, C3 in N-term and C4 in C-term). Upstream to AREB1, the *Receptor like kinases* have been identified in *A. thaliana* as sensors of the

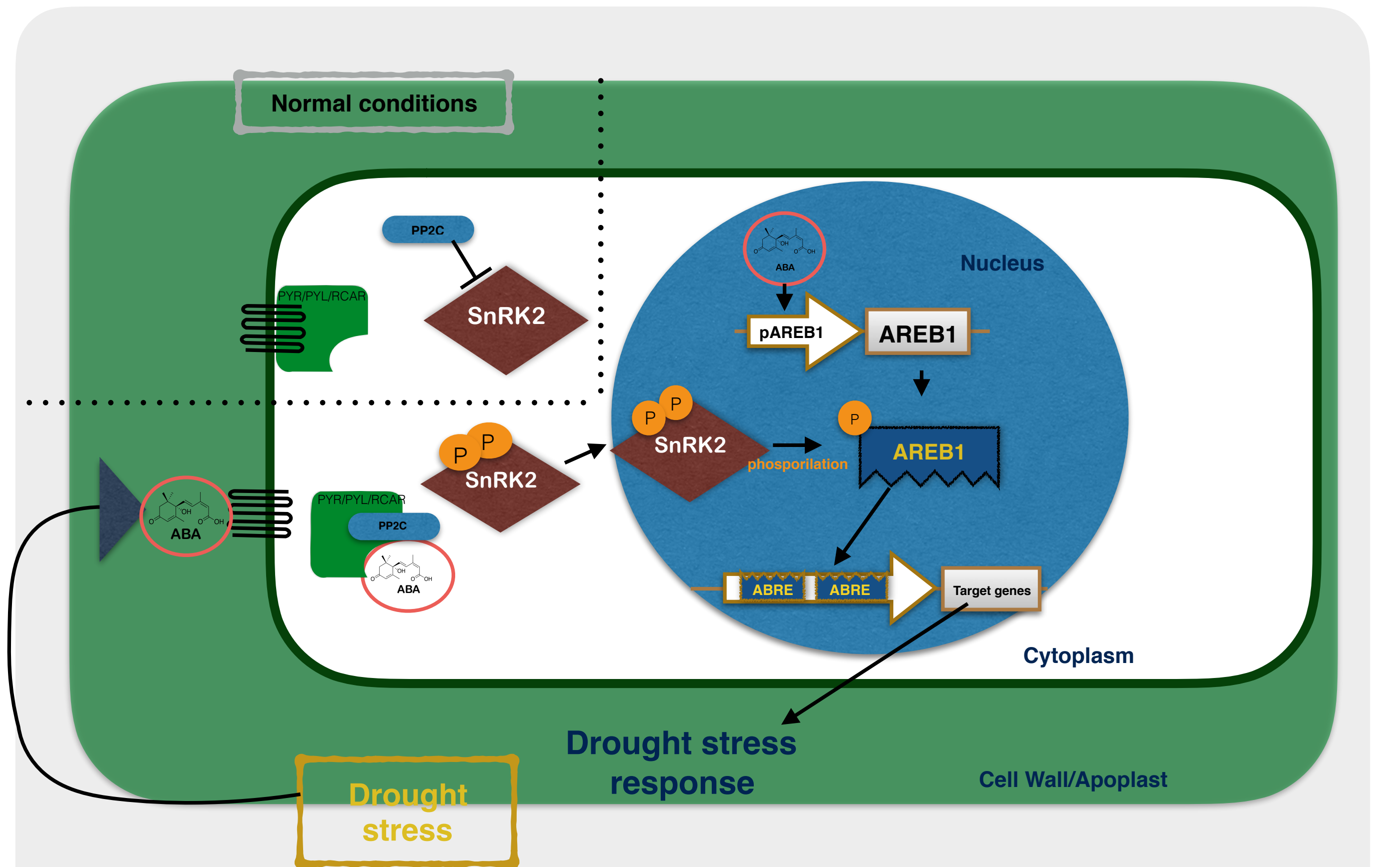


Figure 3- ABA pathway in normal and drought stress conditions. After sensing a water deficit, ABA is synthesized and interacts with its receptor PYR/PYL/RCAR. In the cytoplasm, the ABA receptor inactivates a PP2C phosphatase, leading to an autophosphorylation of an SnRK2 kinase. The SnRK2 is directed into the nucleus, where it phosphorylates the TF AREB1 to activate it. The activated AREB1 binds to ABRE sequences of target genes, that increase their expression and respond to drought stress. ABA is also able to increase AREB1 expression.

environment (Tardieu, 1996; Zhao et al., 2013; Osakabe et al., 2013). After sensing the water stress in the soil, ABA rapidly accumulates and targets the receptors PYR/PYL/RCAR (**Figure 3**). This generates within the protein a change of conformation permitting to suppress the PP2C phosphatases activity by direct interaction. PP2Cs inactivation is associated with the SnRK2 family protein kinases activation, capable to autophosphorylate and activate downstream effectors including AREB1 (Guzman et al., 2012; Finkelstein, 2013; Zhao et al., 2013). By the mean of its bzip DNA-binding domain, AREB1 will target multiple stress-responsive downstream genes such as LEA class genes, ABA-regulated genes and PP2Cs, osmotic stress protection genes, antioxidant signaling, and chromatin remodeling (**Figure 3**) (Fujita et al., 2005; Furihata et al., 2006; Yoshida et al., 2010; Li et al., 2013; Yoshida et al., 2015). AREB1 over-expression showed enhanced drought stress tolerance in *A. thaliana*, rice, and soybean (Oh et al., 2005; Fujita et al., 2005, 2013; Barbosa et al., 2013; Todaka et al., 2015). Therefore, *AtAREB1* results as an attractive target to improve plant drought stress tolerance.

2. M. incognita infection

It is also relevant to consider plants in the field being interconnected with different factors in their environment. Thus, understanding the interactions of multiple parameters of that environment with a plant might contribute both to have a complete view of the plant's biological system and to design an adapted biotechnological approach. For instance, if drought stress is considered as a serious problem at short term, plant crops are also exposed simultaneously to other stresses as pathogen attacks. Among plant pathogens, plant parasitic nematodes are responsible for crop production losses estimated from 8% to 14% depending on the season and country (Nicol et al., 2011). Thus, the loss of yield in crops attributed to plant-parasitic nematodes has been estimated in ~125 billion US dollars each year (in U.S.A) (Koenning et al, 1999; Chitwood et al, 2002). The most damaging nematodes are the cyst nematodes (SCN - *Heterodera spp.* and *Globodera spp*) and the root-knot nematodes (RKN - *Meloidogyne spp.*), both having a sedentary endoparasitic life style. The two laboratories where I worked focus on *M. incognita* species. This RKN is a microscopic plant obligatory parasite with a wide geographic distribution over the world (Souza Jr et al., 2013). After entering the root as a juvenile (J2), *M. incognita* migrates inter-cellularly until it reaches the elongation zone, and then goes up through the vascular system until the differentiation zone (**Figure 4 A**) (Goverse and Smant, 2014). It modulates host cells to form multinucleated giant cells to serve as feeding sites, by inducing karyokinesis without cell division. This results in the formation of galls that destroy the structure of the root (Bird et al., 2008). Its parasitism destabilizes the root system organization (**Figure 4 B**). As a result, the parasite reduces vegetative and reproductive growth, chlorophyll content, leaf expansion, plant weight, and photosynthetic rate in cotton plants, leading to a critical loss of yield (Lu, 2008). Managing nematodes associated pests require special attention. Currently, measures available to control nematodes are inefficient and/or harmful to the environment. Nematicides have toxic effect on health and environment and nematodes can be found deep enough in the soil to escape the action of pesticides. Controlling nematode populations by growing resistant cultivars can be transiently successful, but it has been observed that this strategy led to the replacement of one nematode species to another and must be complemented with crop rotation (Bird and Kaloshian, 2003). Biological control using nematodes parasites or predators lack of consistent results when considered in large-scale and at long term (Bird and Kaloshian, 2003). An economic and sustainable alternative strategy is to generate genetically modified plants bringing new resistance genes against nematodes. To date, transgenic plants expressing proteinase inhibitors, Cry toxins, peptide repellents, and silencing via RNAi have been engineered to control against nematodes in plants as banana, rice, and potato, but no strategy has been developed in cotton (Lilley et al., 2011; Ali et al., 2017; Dutta et al., 2018).

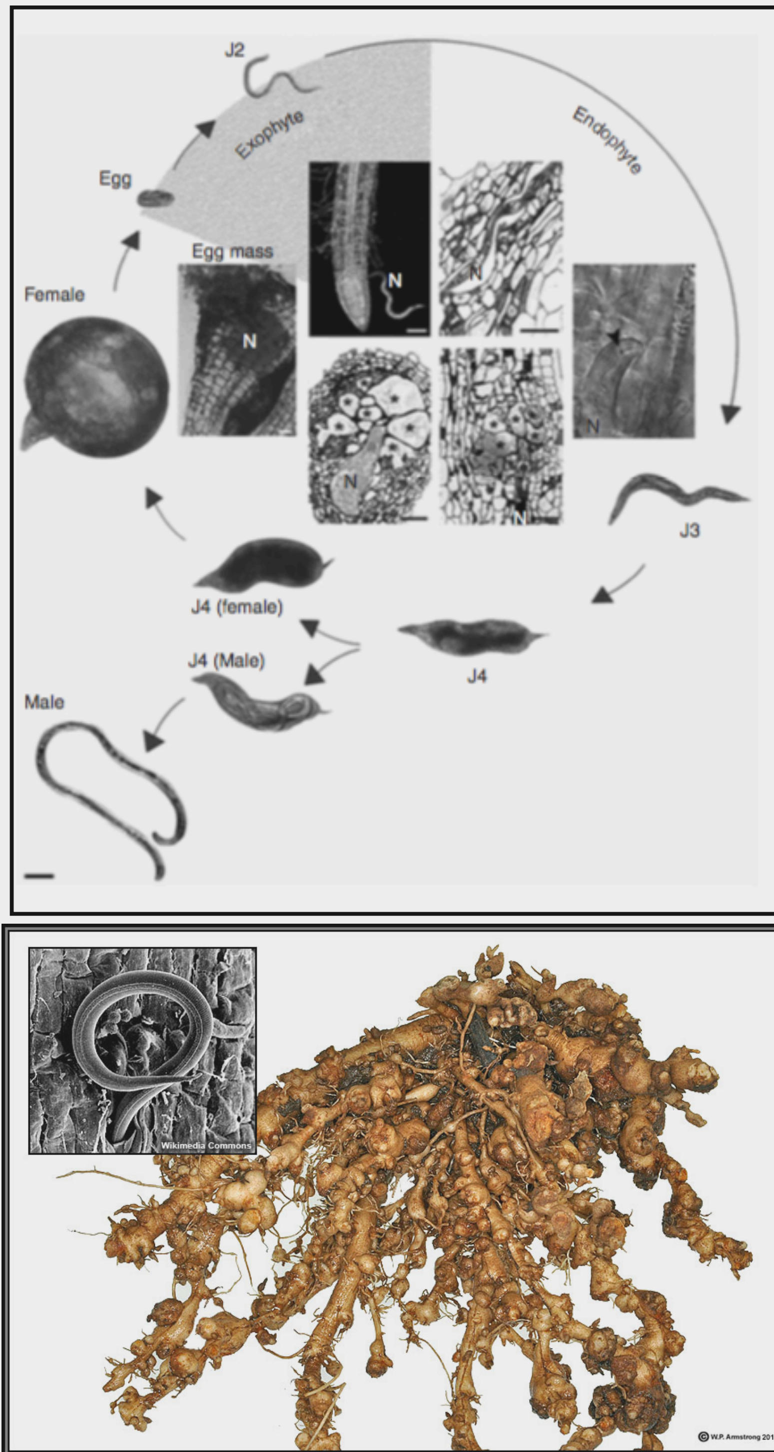


Figure 4. (A) *M. Incognita* life cycle. Infective second- stage juveniles (J2) penetrate the root and migrate between cells to reach the plant vascular cylinder. Each J2 induces the dedifferentiation of five to seven root cells into Giant cells (*). The nematode (N) becomes sedentary and goes through three molts (J3, J4, adult). Sporadically, males develop and migrate out of the roots. The pear-shaped female produces eggs that are released on the root surface. Embryogenesis within the egg is followed by the first molt, generating second-stage juveniles (J2). Scale bars, 50 mm. (B) Image of *M. Incognita* (J2) entering tomato roots (black and white) and root galls in celery resulting from the giant cells in the feeding site of the nematode. Photo by William Wergin & Richard Sayre.

The management of climate risks and pathogen attacks will need pertinent planning at different levels on how our society will adapt in the future. While different reconfigurations of distribution of the livelihoods, diets, or geography of farming will be essential, biotechnological scientists aim to bring parallel solutions to the problems related above. Some of these solutions rest on the tools that will be explained in the ongoing paragraphs.

2. Classical genetic engineering

2.1 Selective breeding

Before science even knew about DNA, humans were already artificially modifying genomes without understanding how. Selective breeding has existed since the birth of agriculture, and all crops, pets, or livestock have been breeding assets. In the *Origin of Species*, Charles Darwin states a first general conclusion that « species had not been independently created, but had descended, like varieties, from other species », which still seems to be a refuted assertion for some people in present days; and then opens with a first chapter entitled *Variation under domestication*. In this text, Darwin reviews that domesticated species undergo adaptations that do not correspond to their own good, but for man's use or fancy, arguing that the breeds have not been produced suddenly as useful as they are, but that the key is the power man applies to accumulate profitable selection. After WWII, Norman Borlaug, the first Nobel prize for a work related to plants, did an enormous work by selecting high yielding, disease resistant and stress tolerant wheat varieties. In modern breeding, traits that define a certain phenotype of a certain species are attributed to quantitative trait locus (QTL), regions in the genome that contain one or more genes responsible for that phenotype. QTL mapping studies aim to statistically link phenotypic data to genetic data with the aid of molecular markers (Miles and Wayne, 2008). Within crop plants as maize, studies have established that 2 to 4% of the genes are evidence of human selection and that there is an established number of loci associated directly to human preferences (Purugganan and Fuller, 2009; Hake and Ibarra, 2015). Several QTLs for drought stress resistance have been identified and some crops have been developed that retain a certain good yield at the same time with a better tolerance to drought (Witcombe et al., 2008., Fita et al., 2015; Mwadzingeni et al., 2016). However, while selective breeding is a powerful tool, it indirectly modifies entire genomes, is labor-intensive and time consuming.

2.2 Mutagenesis

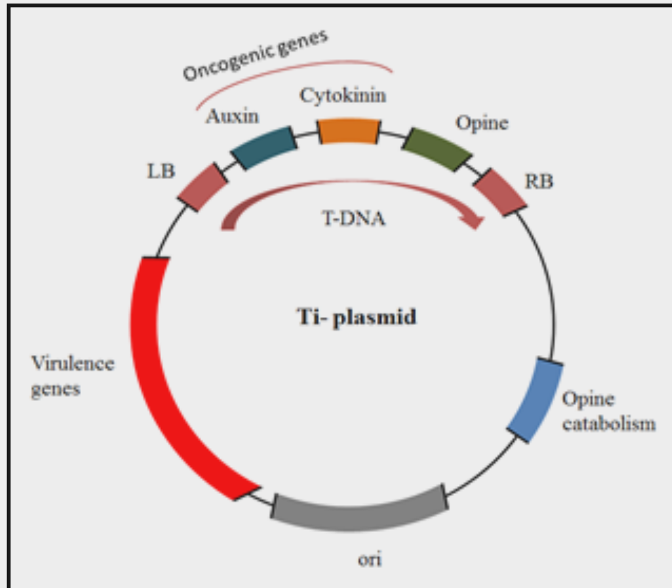
Once scientists knew how DNA structure existed in nature, its mechanisms of modification, its processes to change, adapt and evolve, and the machinery controlling these systems, the next step was to artificially control them. In the laboratory, one of the main intentions of modifying DNA is to change the sequences that code for proteins to study their role when they are mutated. Introducing mutations permits the creation non-functional proteins or new versions of proteins with new properties. One of the first procedures to create these mutated proteins was called mutagenesis and relied on the possible errors that can be introduced in a sequence while enzymes repair or synthesize it. First, classical mutagenesis based on the potentiality of physical or chemical agents to produce random mutations. Physical mutagens include γ rays, x rays, UV light or particle radiation (β and α particles) and induce large deletions or gross lesions. The list of chemical mutagens is extensive, but Ethyl methanesulfonate (EMS) is the most popular in plant mutagenesis. Briefly, the mutagen applied induces DNA breakage and removal of alkylated guanines, which will leave the DNA molecule depurinated and with a gap, causing deletions or insertions. Also, alkylated guanine can pair with thiamine, leading to base pair errors (Kodym and Afza, 2003). Biological consequences will depend on different factors such as target loci, size of the mutation, timing during the cell cycle and can have from no effect at all to profoundly modify gene expression, phenotype or even drive cell death (Schrader, 2003). Despite that random mutagenesis has been a method highly used in plants science, generating around 3000 crop cultivars with useful traits (Sikora et al., 2011; Oladosu et al., 2015).

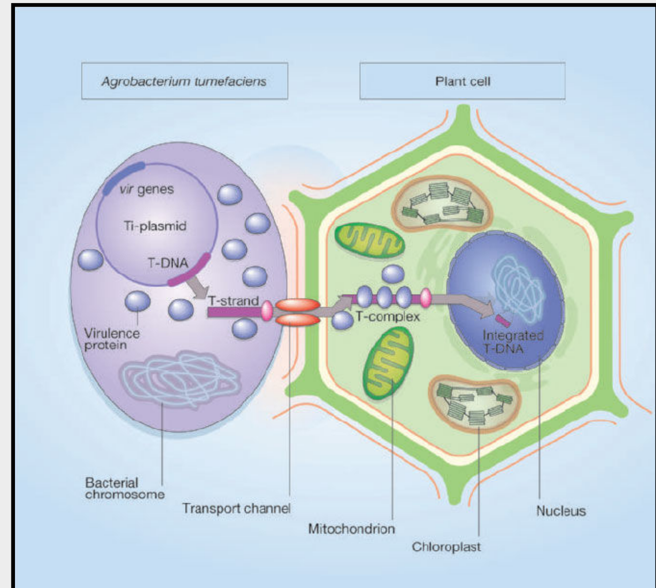
2.3 Gene transfer

Even if selecting, breeding or mutating living beings for the improvement of their characteristics has been a powerful tool, the introduction of genes into organisms opened a combination of new possibilities. As stated in the Cartagena protocol of biosafety, Living modified Organisms or Genetically modified organisms (LMOs/GMOs) are generated through a process whereby exogenous DNA is transfected directly into cells through the use of biotechnological tools (Secretariat of the Convention on Biological Diversity, 2000; Husby, 2007). In the lab, recombinant unicellular microorganisms, have become an excellent model for studying all cellular processes, but are also largely used different types of industries such as human health and agriculture. (Sherratt, 2001; American society for microbiology, 2007). Among GMOs, Genetically Modified (GM) plants and crops have attracted a large amount of attention both in the scientific community as for the general public.

2.3.1 Transgenic plants

The first assays to transfer genes into plant cells began in the late 1970's with the discovery of *Agrobacterium tumefaciens* and *A. rhizogenes* tumor inducing (Ti) and root inducing (Ri) plasmids,





Gelvin et al., 2005

Figure 5- *Agrobacterium Tumefasciens* mechanism of infection (A) Schematic representation of Ti plasmid and (B) schematic representation of T-DNA transfer from bacteria to the host nucleus. The sequences outside the T-DNA are conformed by a region of virulence (vir) genes, the origin of replication (ori), and an opine catabolism region. The opines are amino-acid like compounds that help into the induction of the tumors (crown galls). The products of the vir region and the T-DNA genes, induced by compounds present in exudate of wounded plants, generate the transfer of the region inside the border sequences into the host nucleus, where the T-strand will be integrated into a plant chromosome

respectively (**Figure 5 A**) (Kerr et al., 1976, Drummond and Chilton, 1978, Chilton et al., 1982). Though, the first stable transfer of an antibiotic bacterial resistance gene was made into protoplast petunia plant cells, and tobacco plants (Fraley et al., 1983, Bevan et al., 1983; Herrera-Estrella et al., 1983). These studies were followed rapidly by studies about transformation vectors, DNA delivery systems, and how to re-generate whole transgenic plants. For the next 30 years, the technologies to develop transgenic plants evolved depending on the interests that each geographical region had for research or agribusiness, expanding to other plant species and more methods of transformation (Vain, 2007). The principal responsible for the success in gene transfer into plants is the capacity of *Agrobacterium,* a soil bacteria with the ability to infect plant cells and induce different types of diseases by delivering DNA into plant cells. For instance, *A. tumefaciens* causes crown galls, *A. rhizogenes* causes hairy root disease, *A. vitas* causes galls on grape plants and *A. radiobacter* is avirulent. The *Agrobacterium* strains carry a Ti or rhizogenic (Ri) plasmid (200 to 800 kbp) with a transferable region (T-DNA) with border sequences flanking the T-region. The sequences outside the T-DNA are conformed by a region of virulence (vir) genes, the origin of replication (ori), and an opine catabolism region. The opines are amino-acid like compounds that help into the induction of the tumors (crown galls) (**Figure 5 A**). The products of the vir region and the T-DNA

genes, induced by compounds present in exudate of wounded plants, generate the transfer of the region inside the border sequences into the host nucleus, where the T-strand will be integrated into a plant chromosome (**Figure 5 B**). The biological processes of T-DNA insertion have been reviewed in several articles (Veluthambi et al., 1988; Zupan et al., 2000; Krishnamohan et al., 2001; Gelvin, 2003, 2012, 2017). By placing a gene of interest in the T-DNA and adapting the plasmids and the protocols to expand the host range of infection of the *Agrobacteria*, scientists have been able to transfer the genes to several different model plants, cereal, legumes, and industrial crops (Ziemienowicz, 2013; Christie and Gordon, 2014; *Agrobacterium* protocol Vol. 1 and 2). Likewise, other methods to transfer gene into plant genomes have been developed. A chemical procedure using PEG mediated transformation was established to transform protoplasts, which can be cultured in specific in-vitro media to develop cell walls and then regenerate whole plants through *calli* culture (Mathur and Koncz, 1997; Liu and Vidali, 2011). A series of physical methods to deliver directly a portion of DNA into the cell have also been conceived. Micro particle bombardment (biolistics) is now a commonly used method that delivers DNA-coated metal particles into the cells directly with a gene gun. Once inside the cell, the DNA detaches from the particle and can be integrated into the host's genome (Taylor and Fauquet, 2002). Other methods like electroporation, vacuum infiltration, micro and macro-injection, shock wave and ultrasound-mediated transformations have also been used to transfer DNA into plant cells (Rivera et al., 2012; Narusaka et al., 2012; Rashid and Lateef, 2016).

Transgenic plants can be divided into model plants for functional studies and crop plants for biofuels and human and animal consumption . In model plants the possibilities are infinite, as research questions concern all the fields of plants biology. Researchers have the possibility to choose among different model plant species, tissues, techniques of transformation and between a stable or a transient expression. The strategy will depend on the question and the time. For example, if working with *A. thaliana,* vast T-DNA insertion collections for most of the plant genes are available (Malley et al., 2015). Likewise, transient expression by agroinfiltration or biolistics, principally in *N. benthamiana*, remain one of the techniques more widely used in laboratories, because of its simplicity and effectiveness (Leckie et al., 2011; Leuzinger et al., 2013; Ueki et al., 2013).

2.3.2 GM crops

GM technology has also been used to produce o variety of crop plants, although few traits have been commercialized and adopted by agricultural markets. The first commercialized crop was the FlavrSavr™ tomato in 1994, that was engineered to delay rotting by inserting an anti-sense sequence of a polygalacturonase enzyme involved in the degradation of the cell wall. Since, two main GM crop types

engineered by immense companies have dominated GM agriculture: (1) Herbicide-tolerant (HT), GM crops that resist to a systemic herbicide (glyphosate) and (2) pest-resistance (Bt), GM crops with insertions of Cry toxins derived from *Bacillus thuringiensis* bacteria aiming the resistance to insect pests. The HT and Bt plants of GM soybean, cotton, maize, and canola represented in 2011 more than 16,7 million farmers around the world planting 160 million hectares in 29 countries. **Figure 6** shows the situation of GMOs around the world in 2016, according to an educational GMO internet site (Chassy, 2007; Lemaux, 2008; Kale and Ali, 2013; GMO answers, 2016). Despite the dominance of Bt and HT crops, scientists have tried to diversify GM crop research, giving rise to several different types of

Figure 6- Status of GM crops around the world in 2016, according to GM answers, an internet site aiming to answer non-scientific public's questions. (**https://gmoanswers.com/**, 2016)

engineered crops with other interesting characteristics including pathogen resistance, abiotic stress resistance or nutrition improvement. Numerous plant resistance genes (R genes) and corresponding avirulence genes (*avr* genes) from plant invaders, which make part of the evolutionary and fascinating equipment race between plant immunity and pathogen infection success have been the focus of research

at different labs around the world (Anderson et al., 2010; Dodds and Rathjen, 2010; Kushalappa et al., 2016; Eckardt, 2017). The study of these mechanisms has given the opportunity to develop different strategies of gain- and loss-of-function approaches in GM crops: (1) R-genes against one pathogen, pyramiding multiple R-genes for different pathogens or activating plant's immunity by over-expressing *avr* genes in the plants; (2) Over-expression of molecules that detoxify the effect of pathogen toxins such as protein toxin inhibitors, repressors; (3) antimicrobial proteins and metabolites (4) transcription factors that control entire defense pathways such as the salicylic acid (SA) or jasmonate acid (JA) pathways (Wally and Punja., 2010).

Besides pathogen attacks, crop plants must face a series of abiotic stresses that affect productivity. Therefore, several groups have focused their studies on abiotic tolerance mechanisms and their possible application for producing GM crops. One of the challenges is to improve DR in enhanced biotechnological plants while maintaining high yield. Drought resistant crops need to combine a better root system, stomatal regulation, water use efficiency whilst maintaining productive yield. The major emphasis has been to introduce genes encoding osmoprotectants, detoxifying genes, heat shock proteins, plant growth regulators, late embryogenesis abundant (LEA) proteins and transcription factors (Mathur et al., 2008; Lawlor, 2012; Liang et al., 2014; Bakhsh and Hussain, 2015). Hence, some candidate transgenes have been introduced in crops to enhance drought stress including tomato, rice, maize, soybean, wheat, and others (Wani et al., 2017).

2.3.3 RNAi strategy

The RNA interference (RNAi) gene silencing for targeting crop pests is widely investigated in our laboratory because of its effectiveness (Younis et al., 2014). To date, an important number of crops have been genetically modified with RNAi to improve resistance against hosts, tolerance against stresses, and yield traits (Kamthan et al., 2015). Since it was discovered in Petunias and decrypted in *C. elegans* (Napoli et al., 1990; Guo and Kemphues., 1995), the mechanism of silencing via RNAi has overcome with an entire field of study involving a new set of small none coding RNA's implicated in the mechanism of gene silencing and an aggregate of other type of cellular mechanisms (Sen and Blau, 2006; Wilson et al., 2013). Additionally, it has become an important tool for identifying gene functions, new cellular pathways and an important strategy for the new generation of biotechnological crops. Briefly, a sequence expressing a dsRNA is introduced in the plant genome, to be produced and delivered to the parasite either directly or via feeding of the cells. The dsRNA is then processed either by the plant's or the invader's RNAi machinery. The resulting siRNA silences critical genes and stops the infection by killing the host or restraining it to a given developmental step, as illustrated in the **Figure 7** (Lilley et al., 2011; Dutta et al., 2015; Tian et al., 2015).

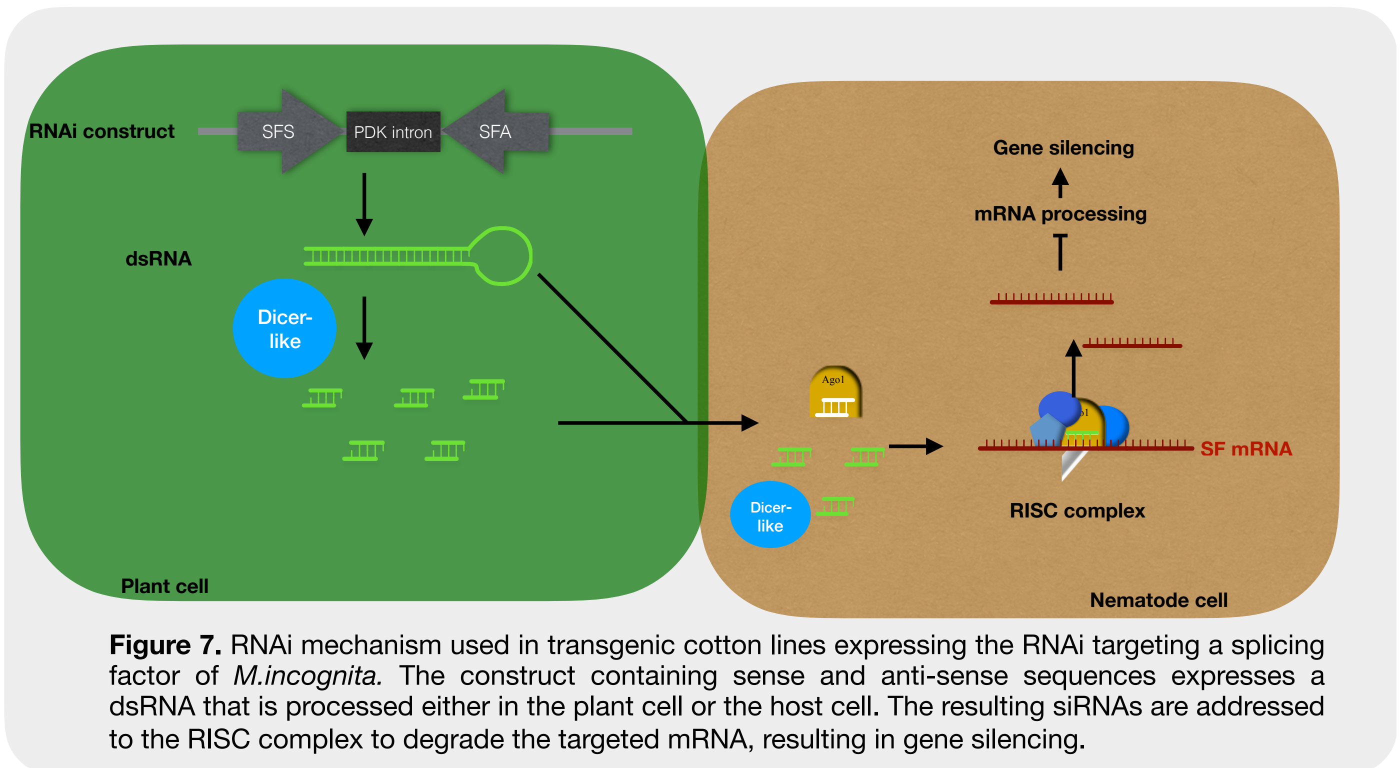


Figure 7. RNAi mechanism used in transgenic cotton lines expressing the RNAi targeting a splicing factor of *M.incognita.* The construct containing sense and anti-sense sequences expresses a dsRNA that is processed either in the plant cell or the host cell. The resulting siRNAs are addressed to the RISC complex to degrade the targeted mRNA, resulting in gene silencing.

Finally, micronutrient and macronutrient traits have also been enhanced in crop biotechnology, either by the increase or decrease of certain traits such as protein quality and levels, oils and fatty acids, carbohydrates, functional and secondary metabolites (McGloughlin, 2008). However, all these strategies in GM crops rely on the integration or one or very few traits. Governing entire metabolic pathways has become a new challenge and could be performed by targeting different genes at the same time. The new editing technologies have been for some time opening the door to multiple edition of genes in different organisms (Lopez-Obando et al., 2016; Liu et al., 2017; Yang et al., 2017).

3. Targeted genome editing era

The emerging methods of whole-genome sequencing and large-genome annotation resulted in a vast income of data about new coding and non-coding sequences. To determine how genotypes influenced phenotypes, there was a need for more directed and rapid methods to mutate genes to study their functions. The development of the new targeted gene inactivation approaches gave scientists powerful insights on how to evaluate gene function. This section introduces in a chronological manner how these technologies were implemented and introduced the technique that was most adopted: the CRISPR/Cas9 mechanism.

3.1 Repairing the DNA after double strand breaks

Genome editing relies on the mechanisms of DNA repair. DNA can be injured by a series of endogenous agents, some of them spontaneous as depurinations or induced by reactive oxygen species (ROS). Others are exogenous and induced by chemicals or radiations. The most damaging lesion is a double strand break (DSB), which must be repaired rapidly before DNA replication. The systems that repair lesions in the cells can be error-free, but some produce a series of repairs that prompt changes in the DNA sequence (Pagès and Fuchs, 2002). To repair a DSB, cells employ two pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (**Figure 8 A**). Classical NHEJ is mediated by recruiting a complex of proteins that contain a DNA ligase (LigIV) to rejoin the DNA ends, processes that may lead to deletions or insertions (indels) of aleatory sequences. These mutations can result in premature stop codons and are therefore helpful to create the lack of a functional protein. HDR uses a template to restore some lost information in the sequence. After a DSB, 3' overhangs are extended from the break site and a 5' end occupies the homologous strand forming a D-loop. Then, the gaps are filled by DNA synthesis with the aid of the DNA donor template, and the process finishes with the re-ligation of the 3' ends. Naturally, the DNA donor template is a homologous chromosome, but synthetic DNA can be introduced artificially that will be acquired in the site of the DSB, giving to opportunity to insert entire genes exactly in the site that the scientist intended (Kakarougkas and Jeggo, 2013; Malzahn et al., 2017). All the systems that will be explained below rely on this repair mechanisms to precisely edit the genome.

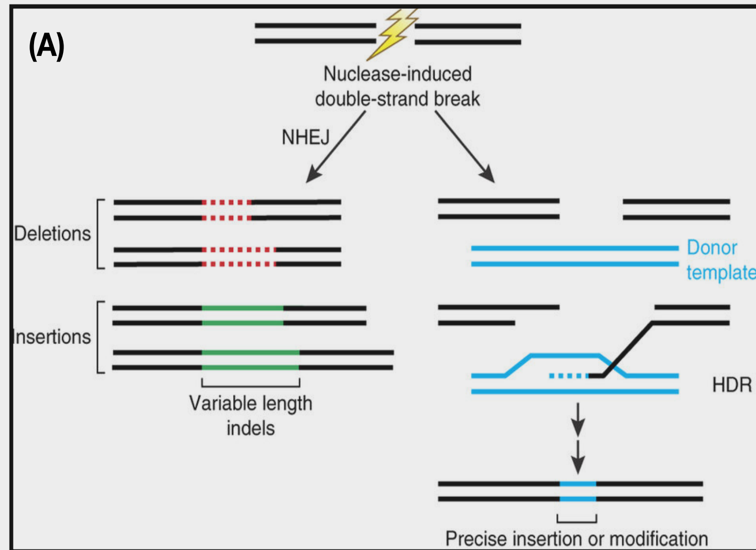
3.2 Homing endonucleases

In the 1970's, the first observation of a mechanism labeled as Homing was described for a genetic marker termed « omega », that was inherited from yeast strains (ω+) to strains lacking the marker (ω-) when crossed. This genetic marker was a group I intron, that was introduced into the ω- genome with the aid of a site-specific endonuclease that is encoded by the same intron and that generates a double-strand break in the target DNA, within an intron insertion site. The repair of the DNA lesion led to an integration of the sequence of the intron contained in the ω+ strains. The discovery of homing endonucleases drove the attention to the transfer, duplication and transmission of this Homing endonuclease genes (HEGs) and inteins (protein mobile segments) in bacteria, archaea, and eucaryotes, showing how this recycling of sequence segments can generate new functional proteins and drive important evolution processes (Chevalier and Stoddard, 2001). The particularity of homing endonucleases compared to class II restriction enzymes is that their cleavage site is larger (15-40bp), denoting an improvement in the specificity of the site reconnaissance. The idea of using them as a biotechnology tool

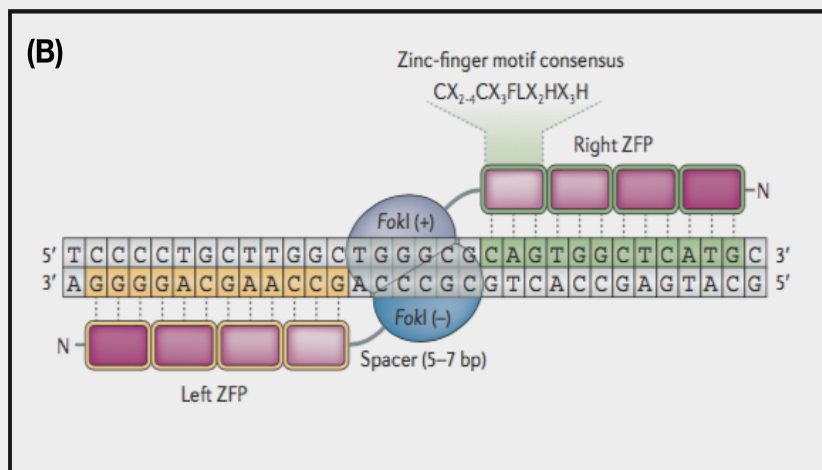
came rapidly as engineered enzymes to make malleable combinations of DNA-nuclease binding proteins. Hence, members of the LAGLIDADG homing endonuclease members were modified to create chimeric proteins as E-DreI, with altered residues in the protein-DNA interface to modify their specificity (Chevalier et al., 2002). Rapidly, other groups redesigned novel endonucleases derived from LAGLIDADG family with other site specificities (Silva and Belforth, 2004; Smith et al., 2006). Next, these enzymes were used for targeted genetic modifications by stimulating HR, still with very reduced efficiency (Stoddard, 2010). In plants, first assays were done to perform targeted mutations in the *liguleless1* locus in maize using I-*CreI* homing endonuclease, with an efficiency of 3% (Gao et al., 2010). At the same time, other new synthetic strategies were being implemented to target specific sites, which made homing endonucleases to be put on the side.

3.3 Zinc finger nucleases

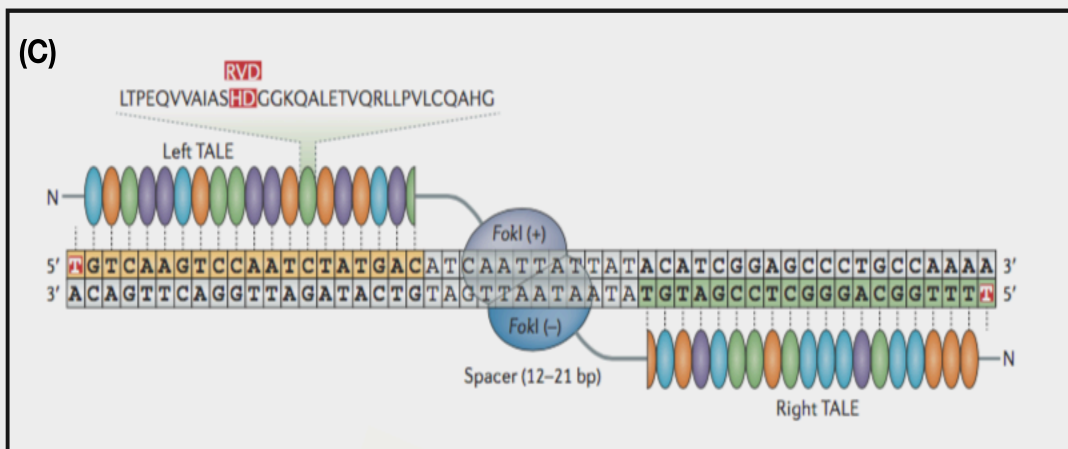
The findings in the 1990's of *Fok*I, a new nuclease that possessed a physically separated binding domain from a non-specific cleaving domain, permitted scientists to imagine that they would be able to fuse that cleaving C-terminal domain to another N-term binding domain that they could architecture. By chance, other studies had already described a DNA binding Cys2His2 zinc finger (ZFs) protein that contained tandem repetitions of (Tyr, Phe)-Xaa-Cys-Xaa_{2-4}-Cys-Xaa_3-Phe-Xaa_5-Leu- Xaa_2-His-Xaa_{3-5}-His sequences, forming a finger that binds to a zinc (II) ion and that interacts with a specific triplet of nucleotides in the DNA. The triplet sequence depended on the variation of each finger in amino acids (AA), giving rise to distinct possibilities of interactions with the triplets (Kim et al., 1996). Thus, by building different assemblies of three to five ZFs would permit to target DNA sequences from 9 to 15 bp, and by fusing this assembly to a nuclease, scientists were able to induce DSBs with these newly engineered ZF nucleases (ZFNs) (**Figure 8 B**) Yet, to cut DNA, ZFNs must form dimers, making that the best conformation to reach cleavage was to design two sets of inverted ZFN directed to neighboring sequences, with a spacer of 5 or 6bp between the inverted binding domains (**Figure 8 B**) (Händel et al., 2009; Shimizu et al., 2009). To virtually design the DNA binding domains before its delivery into the cells, different platforms were created with pre-selected ZFNs available to match the 64 possible nucleotide triplets and others (Maeder et al., 2008; Jabalameli et al., 2015). After a first success of targeted mutagenesis and gene replacement in *Drosophila* (Bibikova et al., 2002, 2003), ZFNs were used in organisms like nematodes, mice, frogs, tobacco, Maize, Zebrafish, *A. thaliana,* and human cells (Urnov et al., 2010; Carroll, 2011). Depending on the targeted host, scientists used DNA transfection, mRNA embryo injection, and all the plant transformation systems to deliver the ZFNs into the cells (Carroll, 2011). However, despite its promising capacity, ZFNs were proven to the source for several off targets, promoting aleatory chromosomal rearrangements that often led to cell death. The necessity to design complex



Sander and Young, 2014



KIm and Kim, 2014



KIm and Kim, 2014

Figure 8- Targeted genome editing before CRISPR/Cas9. (A) Double stranded breaks in DNA induces repair mechanisms using NHEJ or HFR pathways. (B) Engineered Zinc finger nucleases fused to nuclease FokI dimerizes to create targeted DSB and (C) Engineered TAL effector nucleases fused do FokI also dimerize to create DBSs in the targeted DNA sequence.

combinations of zinc fingers and the requirement for dimerization to cut the DNA rendered the experimental design difficult to carry out and unexpected results. Still, ZFNs was the first successful step in the editing genome era and gave important observations for the other technologies that came afterward.

3.4 Transcription activator-like effectors

First described as an avirulence gene (avrBs3) from Xanthomonas pathovars that induced hypersensitive response to pepper cultivars (Bonas et al., 1989), these effectors were characterized as type III transcription factors that entered plant's nucleus to induce developmental reprogramming of host cells (Römer et al., 2007; Kay et al., 2007). Therefore, they were labeled as transcription activation-like effectors (TALEs) that contributed to bacterial colonization, symptom development or pathogen dissemination (Bogdanov et al., 2010; Boch and Bonas; 2010). TALEs are composed by a central protein domain of 17,5 repeats of 34 amino acids, in which the residues 12 and 13 are hypervariable. Because of this variable residue (RVD), each TALE repeat recognizes a single specific base pair in a DNA sequence (**Figure 8 C**). The repeat domain being modular, rearranging the repeats would permit to generate specific binding domains for a given DNA sequence (Moscou and Bgodanov, 2009; Boch., 2011; Rusk, 2011). Similarly, as ZFNs, by fusing a TALEs to a nuclease like FokI (TALEN) and after their dimerization, TALEN would operate as specific nucleases to create site-specific DSBs, and consequently site-directed DNA editing after NHEJ or HR. NHEJ mutagenesis efficiency with TALENs attained up to 27%, similar as ZFN's highest efficiency (Cermak et al., 2011; Bogdanov and Voytas, 2011). Design of TALENs with custom repeat arrays can be achieved by different techniques, but Zhang et al. (2011) showed how Golden Gate cloning allowed to join multiple DNA fragments with unique 4-bp sticky-ends in a few PCR digestion steps. Several groups have then used TALENs to modify genomes in yeast, Drosophila, zebrafish, frog, rat, mice, human's cells, *arabidopsis*, maize, tomato, rice, wheat, tomato, among other organisms (Joung and Sander, 2013; Malzahn et al., 2017). Compared to ZFNs, TALEN has a greater specificity and less off-targets because of its ~30 pb requirement, but remains with the same efficiency rates and the protein must be engineered as well. Even so, TALENs have been widely used until today to modify different genomes, and has proved a powerful tool. TALENs real headache would be the forthcoming avenue of CRISPR/Cas9 that would bring important advantages over ZFNs and TALENs techniques.

3.5 CRISPR/Cas9 revolution

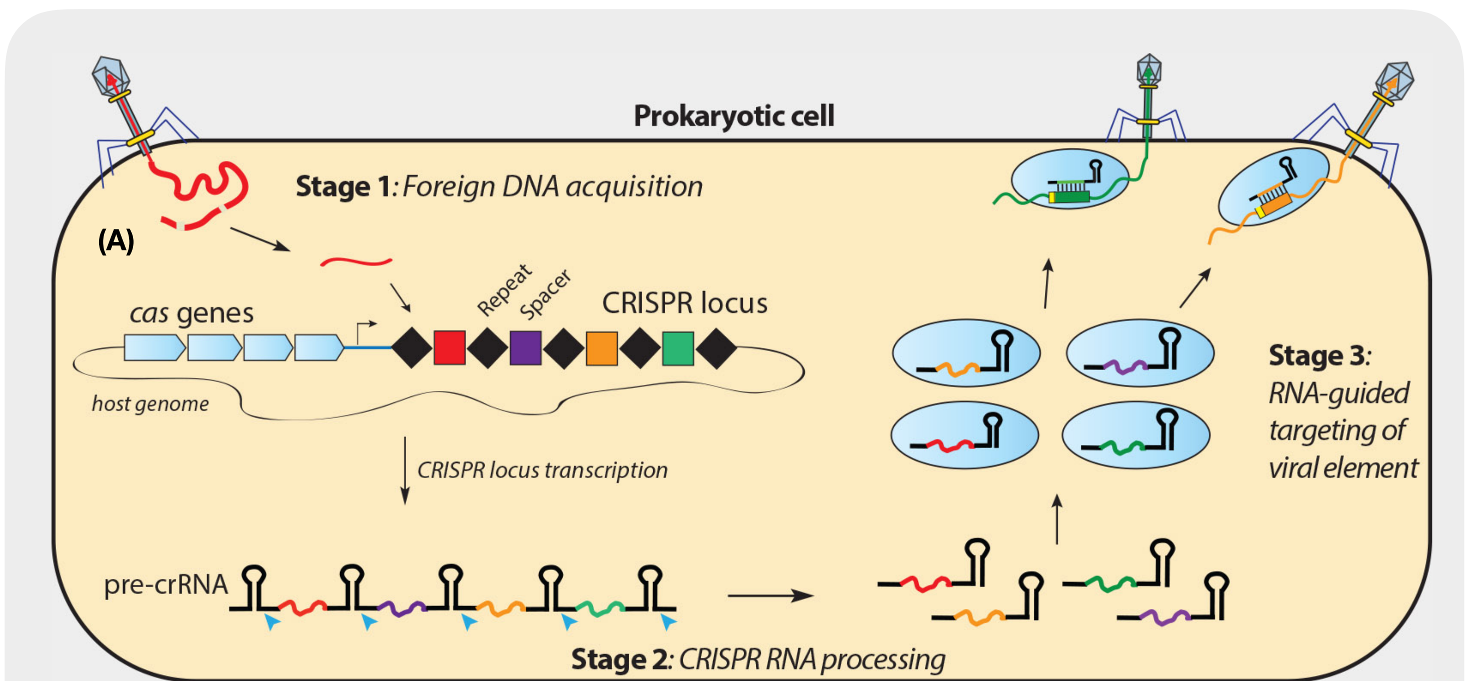
The political statesman Vladimir Lenin once said that "*You cannot make a revolution in white gloves.*" At that time, he meant no revolution could be done without nonviolent political action. Paradoxically, he would

never have thought that years later, a group of people would make a revolution only armed with some micropipets and various pairs of latex/nitrile and must probably white gloves.

3.5.1 CRISPR/Cas9: Beginnings, general mechanism and evolution

In 2012, the first reports of a new technique that would attain the same objectives as ZFNs and TALENs, editing DNA in precisely targeted sequences, came to light after a few years passed to decipher the bacterial mechanisms it comes from.

The story begins with a Spanish researcher who ought to answer why some different species of bacteria accumulated multiple copies of a repeated sequence he first called short regularly spaced resets (SRSRs) (Ishino et al., 1987; Mojica et al., 1993, 1995, 2000). In the same loci, that he would agree later with another research group to name as clustered regularly interspaced short palindromic repeats (CRISPR), were found to co-localize other crispr associated genes (cas) (Jansen et al., 2002). A few years later, a series of studies agreed that the CRISPR locus served as a defense mechanism, encoding information for a bacterial immune system against viruses' infection, and that probably some of the cas genes were nucleases that were involved in the resistance by cutting the viruse's DNA (Mojica et al., 2005; Pourcel et al., 2005; Bolotin et al., 2005; Barrangou et al., 2007). The next step went by different studies in which researchers found that the repeated sequences adjacent in the CRISPR locus corresponded to novel small RNA, called CRISPR-RNAs (crRNAs) that formed structures with another RNA encoded in the CRISPR locus: the *trans*-activating crRNA (tracrRNA). The complex crRNA-tracrRNA interacted with the Cas9 nuclease to cleave the viruse's DNA (Deveau et al., 2008; Horvath et al., 2008; Deltcheva et al., 2011; Jinek et al., 2012). The equation of the mechanism was practically resolved: following exposure to bacteriophage or an invading plasmid, bacteria can acquire and incorporate fragments of the alien DNA (protospacers) into a CRISPR locus array (spacers) that also contains a series of cas proteins, arranging a record of prior infections. After transcription of the repeated sequences into a pre-crRNA and the processing into mature crRNA with the help of an RNaseIII, the association of these crRNAs with a tracrRNA and a with a Cas nuclease or o complex of Cas proteins enables the recognition an invader DNA sequence, triggering its cleavage and destruction to stop the infection (**Figure 9 A**). The only condition is that the complex needs to identify a short-conserved sequence (2 to 5 nucleotides), named protospaced adjacent motif (PAM), in the targeted sequence of the invading DNA. Since its discovery, different phage resistance CRISPR-Cas systems have been found in different bacteria, arranged in two classes, five types and 16 subtypes according to the architecture of the CRISPR loci and the *cas* genes that the locus carries. The types I and III systems utilize a multi-Cas protein complex, whereas the type II CRISPR systems operates with a single endonuclease. The first described and more used is the Cas9 that



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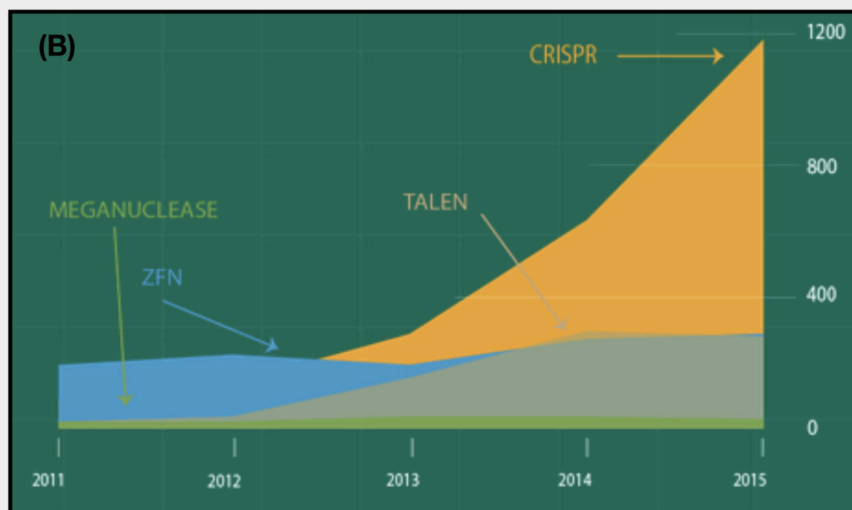

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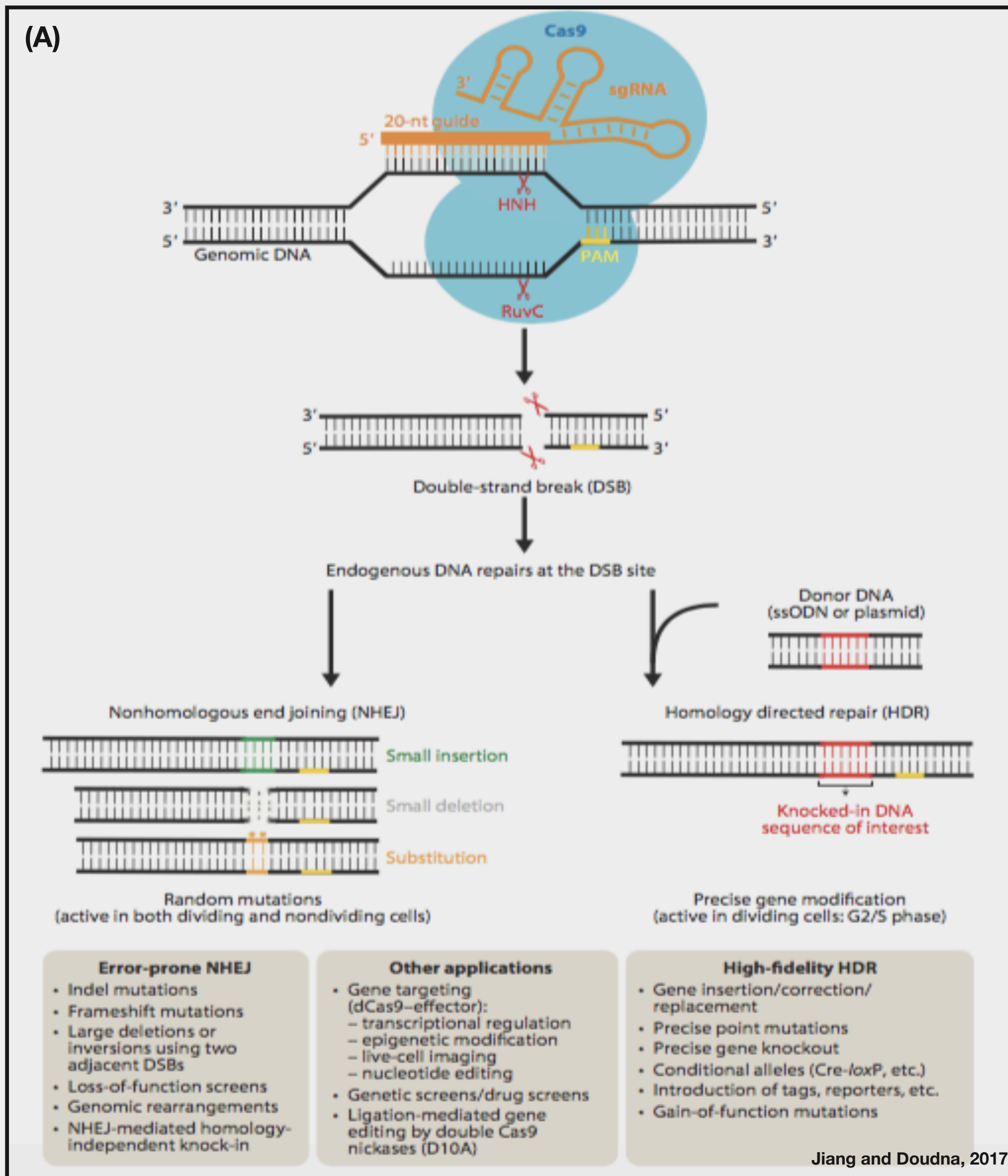
Figure 9- CRISPR-Cas systems come from adaptive immune system in prokaryotes. Protospacer sequences from phages are incorporated in a CRISPR array locus to form spacers, containing also genes encoding for Cas proteins to form spacers. Spacers are able to be transcribed into pre-crRNA and then crRNA. The complex crRNA-tracrRNA directs RNA guided targeting of new viral elements, and Cas nucleases degrade the targeted DNA to stop the infection. (B) Publication rate of CRISPR between 2012 and 2015 show the exponential grow of studies related to it.

possesses two nuclease domains (HNH or RuvC) that cleave DNA in a strand each (Makarova et al., 2015; Jiang and Doudna, 2017). Subsequently, the idea to use the mechanism to target other DNA sequences was already in different group's projects, but Emmanuelle Charpentier and Jennifer Doudna's publication appeared first in June 2012 (Jinek et al., 2012). They used a recombinant *S. pyogenes* Cas9 nuclease and a dual tracrRNA:crRNA artificially composed into a single guide RNA (sgRNA) to induce a targeted DSB in vitro, proposing an alternative to ZFNs and TALENs. However, at the same time, another

group that was already working to manipulate genes in mammals with TALEs, submitted a paper that appeared in January 2013, announcing in vivo mammalian genome editing with type II CRISPR/Cas9 (Cong et al., 2013; Hsu et al., 2013). After that, all was set for the birth of what is called today a revolutionary era in science, witnessed through the exponential way laboratories around the world began to publish after 2013, as shown in **Figure 9 B**. A worth reading review wrote by Lander (2016) narrates the story of this new technology from the point of view of the pioneers, which he entitles « The Heroes of CRISPR » showing at what point their discoveries are treated as revolutionary and somehow epical.

The system is very simple. The Cas9 endonuclease is guided by an engineered sgRNA that has two characteristics: a sequence at 3' that binds to the Cas9, and a sequence in 5' that interacts with DNA following the Watson-crick laws and determines the target sequence for the Cas9 to create a DSB. As shown in **Figure 10 A**, the DSB will be repaired by NHEJ or by HDR, allowing scientists to induce indels 3 pb from the PAM site, or resulting in precise genome insertions by using a homologous repair template. Thus, the system can be programmed to target new sites by changing the 5' sequence of the sgRNA (**Figure 10 A)**. The most used Cas (Cas9) enzyme has been the one from type II-A CRISPR from *Streptococcus pyogenes* that uses an NGG PAM site, where N is any nucleotide (Doudna and Charpentier, 2014). Biochemical and structural studies have been made to reveal the working model for RNA binding and target cleavage: Cas9 has a bilobed architecture composed of a nuclease lobe (contains the nuclease domains and a PAM-interacting (PI) domain) and an α-helical recognition lobe (contains regions that contribute to recognition of the guide RNA); Activation of Cas9 nuclease requires a conformational change in the HNH domain that depends on proper base pairing between sgRNA and DNA; The sgRNA-DNA heteroduplex is located in a region between the two lobes of the Cas9. Briefly, the model proposed states that binding of sgRNA to the Cas9 induces the conformational change of Cas9, that was in a autoinhibiteed conformation and now is in a recognition competent state. Then, Cas9-sgRNA surveys for PAMs, binds through the PI domain, induces strand separation for sgRNA-DNA interaction, forms an R loop that activates both nuclease domains and induces cleavage (**Figure 10 A**) (Wang et al., 2016).

To design a strategy, the first step is to choose a 20-nucleotide target region adjacent to the PAM site in a target sequence. Several computational platforms have been designed to select and scan the specificity of a certain sgRNA, being that effectivity of sgRNAs are one of the active research areas in CRISPR biology (Brazelton et al., 2015; Doench et al., 2016). Then, the system must be delivered into the cells as DNA, RNA or protein/sgRNA complex, with a template donor if the objective is to generate a sequence insertion (Glass et al., 2017). Once the delivery and the possible modification has been executed, scientists must identify the success of the editing. If the modification was intended to alter a phenotype or to insert a selection or reporter gene, the screening is more direct. If the intention was to



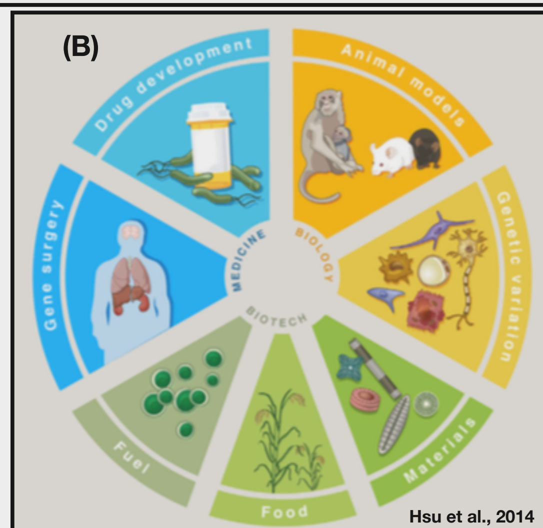


Figure 10- CRISPR-Cas9 mechanism of RNA directed DNA editing. (A) Cas9 system, repair mechanisms and applications. (B) Possible applications in different fields of the CRISPR/Cas9 systems.

provoke an indel, the resulting mutation has different possible outcomes, depending on the ploidy of the targeted cell: In diploid species, the possibilities are no mutation, a heterozygous mutation (one allele), biallelic mutation (different mutation in the two alleles) or homozygous mutation (same mutation in both alleles); In polyploid species, more possibilities can be generated. The methods of detection of on-targets are based either on mismatch based assays that consist summarily in amplifying the targeted region by PCR, denaturing the DNA to allow heteroduplex reannealing and detection of the heteroduplex. The detection of the heteroduplex can be made by restriction enzyme cleavage (T7 endonuclease 1, for example), High resolution melting analysis (based on qPCR technique) or Heteroduplex mobility assay by polyacrilamide gel electrophoresis (PAGE, will detect migration shifts). Alternatively, on-targets and off-targets can also be detected by sequencing the targeted amplicon or even sequencing entire regions (exome or whole genome). These and other techniques have been explained by different protocols and reviews (Clonetech tech note, 2015; Kim et al., 2017; Turtle-Schmidt et al., 2017; Zischewski et al., 2017). Although it is difficult to find a percentage of efficiency, likely because it depends on different factors such as sgRNA design, type of host, method of delivery, type of modification (NHEJ or HDR), CRISPR strategy has been recognized to be more efficient than ZFNs and TALENs, and with less off-target effects (Chira et al., 2017). When comparing with ZFNs and TALENs, CRISPR wins the debate because of the ensuing arguments: it does not have to be engineered and synthesized, it does not need to dimerize, its interaction relies on base RNA-DNA pairing instead of protein DNA pairing, targeting is only programmed by simply changing the sgRNA and CRISPR is more cost-effective (Boettcher and McManaus, 2015). However, some limitations have been described to the CRISPR/Cas9 technology, principally a certain appearance of off-targets, the large size of the Cas9 enzyme and the necessity to target a sequence before the PAM sequence (Crauciuc et al., 2017). These limitations would be one of the arguments to find new alternatives to spCas9.

In addition to the aspiration to overcome the few limitations of spCas9, the hype of CRISPR made possible to think about aiming at different targets at the same time and in the same cell, which was difficult with the only spCas9 being occupied with different sgRNAs. Hence, researchers from different labs became focused on finding new Cas9 alternatives and orthologs (**Table 1**). The first alternative came by mutating one or two of the nuclease domains of the Cas9. Mutating one of them created a nickase Cas9 (nCas9), that cleaves only one strand of the DNA at a time. A pair of nCas9 with the appropriate offset of sgRNAs was used to create DSBs, increasing the specificity (Ran et al., 2013). Mutating both nuclease domains generated a deadCas9 (dCas9), a matter that will be discussed later in the manuscript. In addition to Cas9 mutations, a collection of Cas9 orthologs is today available to use such as *Neisseria meningitides* Cas9 (*NmCas9*), *Staphylococcus aureus* Cas9 (*SaCas9*), *Campylobacter jejuni* (*CjCas9*) and others, as reviewed by different authors (Wang et al., 2016; Mitsunobu et a., 2017; Jiang and Doudna,

TABLE 1. Characterized PAMs for Cas9 orthologs

Cas9 system	PAM	References	Other notes
Streptococcus thermophilus CRISPR1	NNAGAAW	Horvath et al. 2008; Esvelt et al. 2013	NNAAAAW cleaved more efficiently (Fonfara et al. 2013)
Streptococcus thermophilus CRISPR3	NGGNG	Horvath et al. 2008	
Streptococcus pyogenes	NGG	Mojica et al. 2009	
Streptococcus agalactiae	NGG	Mojica et al. 2009	
Listeria monocytogenes	NGG	Mojica et al. 2009	
Streptococcus mutans	NGG	Van der Ploeg 2009	
Neisseria meningitidis	NNNNGATT	Zhang et al. 2013; Esvelt et al. 2013	
Campylobacter jejuni	NNNNACA	Fonfara et al. 2013	
Francisella novicida	NG	Fonfara et al. 2013	
Streptococcus thermophilus LMG18311	NNGYAAA	Chen et al. 2014	NNNGYAAA seems to also work
Treponema denticola	NAAAAN	Esvelt et al. 2013	

Braff et al, 2018

2017; Braff et al., 2018). The orthologs of Cas9 differ in size and require different PAM reconnaissance sequence, increasing the possibilities when choosing a target sequence, and permitting to use different Cas9 at the same time in the same cell (Murovec et al., 2017; Najm et al., 2017).

Along with Cas9 orthologs, other proteins have been found in different CRISPR classes. In the same as Cas9 CRISPR class 2, Zetsche et al. (2015, 2017) characterized the Cpf1 family. Cpf1 possesses an RuvC-like endonuclease domain distant from the one in Cas9, that cleaves both DNA strands but in a staggered way with an overhang in 5', and has no HNH domain. The PAM sequence in Cpf1 is not only different in the nucleotide motif, but is recognized in 5' of the protospacer, whereas Cas9 employs PAM sequences located on 3' of the protospacer. Additionally, Cpf1 mediates DNA interference with only crRNA and no need of tracrRNA, making the sgRNA for Cpf1 simpler than for the Cas9. In the same publication, the authors used Cpf1 to edit genes in human cells and found indels with comparable levels that with the Cas9. Along with Cpf1, other class 2 three distinct effectors were described: C2c1, C2c3 that contain RuvC-like endonuclease domains and C2c2 with two R(N)xxxH nucleotide binding (HEPN) RNase domains, endonuclease that also have new characterized PAMs (Shmakov et al., 2015; Abudayeh et al., 2016). Without no doubt, these characterized proteins and others that will assuredly come and the toolbox and the amount and quality of the publications will continue growing. The end is not near.

The possible applications of genome engineering with CRISPR/Cas9 are enormous. From basic biology to biotechnology and medicine, the **Figure 10 B** shows how all fields of science can benefit from such a tool (Hsu et al., 2014). In plants, the system has now today been extensively used. For instance, targeted inheritable mutations to knockout or edit genes have been generated in model plants *Arabidopsis Thaliana*, *N. benthamiana*, *N. Tabacum, Medicago trancatula* and in crops such as rice, tomato, maize, Sorghum, Soybean, poplar and wheat. Homologous recombination has also been achieved in *Arabidopsis*

th., *N. benthamiana*, soybean, maize and rice (Ding et al., 2016; Khatodia et al., 2016; Li et al., 2015; Schiml and Puchta, 2016; Song et al., 2016; Liu et al., 2017). An interesting and actual debate is whether CRISPR crops should be treated as classic GMOs and regulations for CRISPR-based foods are being relaxed in the U.S.A and Europe (https://www.nature.com/articles/d41586-018-01013-5; Waltz, 2016).

3.5.2 CRISPR/dCas9 or CRISPRi/CRISPRa

Synthetic biology can be defined as the « engineering, design or simulation of biology by the synthesis of complex, biologically based or inspired systems, which display functions that do not exist in nature » (Serrano, 2007). Thus, after the finding of the proteins that could interact with specific sequences of DNA, the idea of finding new properties and functions to these proteins by re-designing, modifying, or fusing to other factors came rapidly. In 1998, Sadowski et al. constructed a synthetic transcription factor consisting of GAL4 DNA binding domain from yeast and VP16, a portion of a herpex simplex virus protein described the same year (Triezenberg e al, 1988), to successfully activate transcription in mammalian cells. Ten years later, the fusion of ZFNs to the same VP16 and three repressor domains, the Krüppel associated box (KRAB), the ETS2 repressor factor (ERD) and mSIN3 interaction domain SID resulted in the ablation or activation of luciferase reporter gene in human epithelial cells (Ayer et al., 1992; Margolin et al., 1994; N. Sgouras et al, 1995; Beerli et al., 1998). Subsequently, TALEs, which were TFs already, were designed to address promoters from tomato or *A. thaliana,* resulting in the increase of the activity of these promoters with TALEN's own activation domain (Morbitzer et al., 2010), or fused to VP16 and VP16's tetramer VP64 in human cells (Geiβler et al., 2011; Miller et al., 2011; Zhan et al., 2011).

The CRISPR system has also been repurposed to accomplish what is labelled today as CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) (**Figure 11**). In 2013, Qi et al. used a catalytically inactive mutated (in both RuvC and HNH-domains) dead Cas9 (dCas9) to target promoter sequences controlling a red fluorescent protein (RFP) in *E. coli,* resulting in a repression of the transcription of RFP. Indeed, the dCas9 interferes with the association of RNAP and other TFs to the DNA in the promoter and achieved almost 99% of repression in prokaryote cells (Qi et al., 2013; Larson et al., 2013; Zuberi et al., 2017). Ergo, to improve CRISPRi, the dCas9 was fused to repressor domains as KRAB, chromoshadow domain (CS), Mxi1, WRPW or four repeats of mSin3 (SID4x) (Bikard et al., 2013; Konermann et al., 2013; Farzadfard et al., 2013; Gilbert et al., 2013, 2014), or even for more complex regulatory devices and circuits with on/off systems (Nielsen and Vought, 2014; Kiani et al., 2014). As an activator, the dCas9 has been used in different organisms by fusing it to simple activator domains such as the omega subunit of RNA polymerase, VP48, VP64, and VP160 (10 copies) and the NF-kB p65 subunit (p65AD) (Bikard et al., 2013; Maeder et al., 2013; Cheng et al., 2013; Mali et al., 2013; Gilbert et al.,

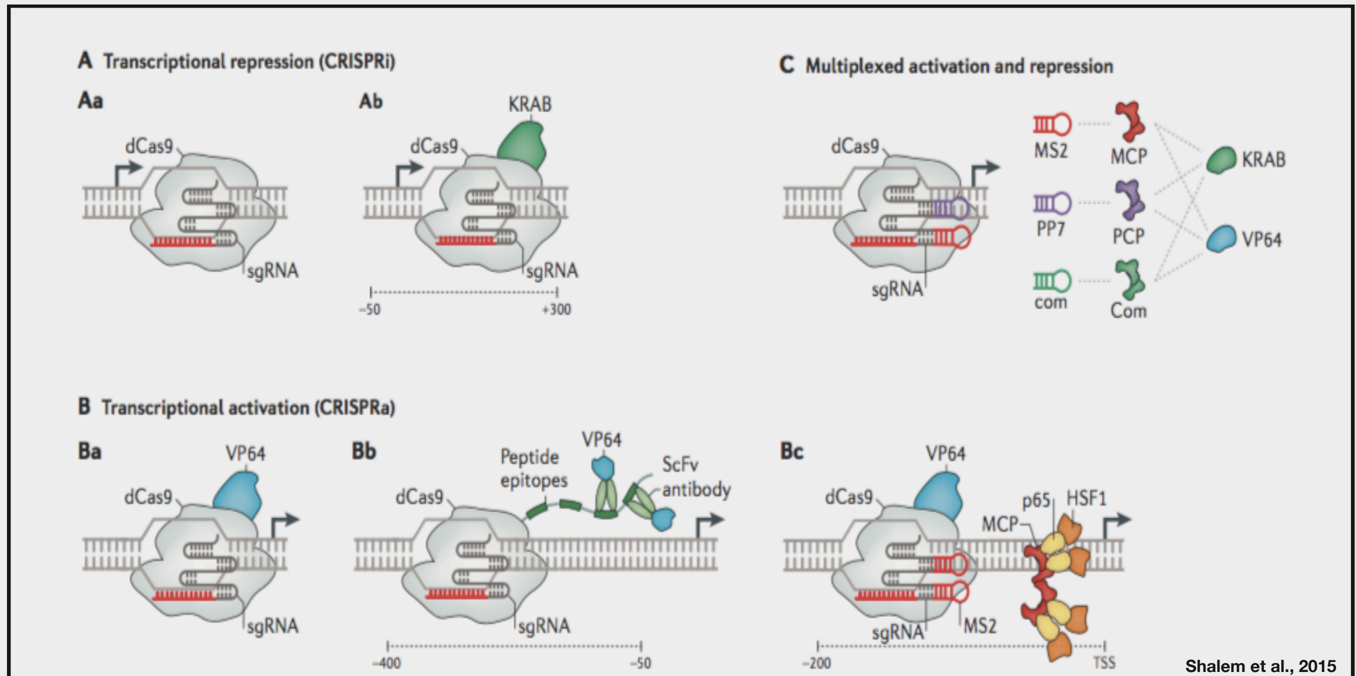


Figure 11- dCas9 mediated transcription modulation. (A) CRISPRi achieves repression with dCas9 alone by blocking other TFs or fused to inhibitor domains like KRAB. (B) dCas9 can be fused to transcriptional activators to accomplish CRISPRa, like VP64, SunTag array or RNA loops (CRISPR 2.0) to recruit activation domains. (C) Activation and repression can be obtained by using CRISPR 2.0 RNA loops recruiting activators and inhibitors in a same cell.

2014). The efficiency of CRISPRa was then improved by amplifying the signal of the activator. Chavez et al. (2015) fused the dCas9 to a tripartite activator VPR (VP64+p65AD+Rta), a system that proved to be more efficient than the VP64 alone, and that was used as an activator in animal cells. In another study, the SunTag peptide containing an array of epitope repeats that recruited several copies of VP64 was used to activate with a 50-fold transcription increase in K562 cells (**Figure 11 B**) (Tanenbaum et al., 2014). Besides engineering the Cas9, other groups used a synergestic activation mediator (SAM), a modification of the sgRNA to form a scaffold RNA (scRNA) containing an MS2, PP7, com or PUF binding site RNA hairpins, that each recruit other activator or repressor proteins. These mechanisms have been labelled as CRISPR2.0 and allow to target and tune up/down different promoters at the same in the same cell (**Figure 11 B, C**) (Konermann et al., 2013; Zalatan et al., 2015).

We know today that chromatin structure is affected by different histone post-translational modifications, and that these modifications apart from modeling chromatin states also influence transcription, DNA repair, replication, and modification. Histone modifications comprehend methylation, acetylation, phosphorylation, deamination, β-N-acetylglucosamine addition, Ubiquitylation and sumoylation among others. It has been shown that promoters with different acetylation or methylation

marks differ in their activity. For instance, silenced promoters are generally associated to histone H3 lysine 9 methylation (H3K9me), H3K27me or DNAme, and active promoters are related to histone acetylation or H3K4 methylations (Bannister and Kouzarides, 2011; ENCODE project consortium, 2012). Either way, the idea to fuse dCas9 to epigenetic factors to modify the epigenetic state in a targeted locus was rapidly applied. On the CRISPRa side, the dCas9 was fused to p300-core, a catalytic histone acetyltransferase (HAT) that augmented expression of the targeted genes while it was related to H3k27 acetylations (Hilton et al., 2015). In a system labelled as Casilio (CRISPR-Cas9-Pumilio), which consists in a scRNA containing multiple PUF binding sites (PBS) that recruit PUF proteins fused to other proteins, the fusions of PUF and a histone acetyltransferase CREB-binding protein increased expression, principally when labelled proximal to the transcription start site (TSS) (Cheng et al., 2016). The dCas9 was also fused to TET1 demethylase inducing targeted demethylation and driving transcriptional activation in different types of cells (Liu et al., 2016; Xu et al., 2016). On the CRISPRi edge, some examples have been used; for example, the fusion of dCas9 to LSD1 (Lys-specific histone demethylase 1) or KRAB were used to repress genes by removing dimethylations in H3K4 or inducing trimethylations in H3K9 respectively (Kearns et al., 2015). Methylation induced by a dCas9 engineered with a DNA methyltransferase 3A (DNMT3A) was used to reduce mRNA levels of different targeted genes in human and mice cells (Vojta et al., 2016; McDonald et al., 2016). The system of CRISPRi and CRISPRa has been proved very powerful, as shown by the constant evolution and different strategies adopted with the dCas9: use of mutated orthologs of the *sp*Cas9; conception DNase-dead Cpf1 mutant (ddCpf1); use of splitted or inactive dCas9 that assemblies after induction with chemicals, light, sgRNA presence or UV light exposure (Dominguez et al., 2016; Dydovik et al., 2016; Brocken et al., 2017; Lo and Qi, 2017; Baeumler et al., 2017; Zhang et al., 2017).

In plants, a few studies have successfully used dCas9-based transcription factors. In 2015, Piatek et al. fused the dCas9 with EDLL and TAD activation domains and SRDX repressor domain. To prove their system, they used the the GUS (uidA) reporter gene in a transient assay system in *N. benthamiana* leaves, showing that the fusions could induce or repress promoter activity depending on the sgRNA (Piatek et al., 2015). In the same year, a fusion of pco-dCas9 to VP64 and a combination of three sgRNAs could transiently activate a minimal synthetic promoter controlling the GUS gene. The researchers also stably transformed *A. thaliana* with the dCas9-VP64 and dCas9-SRDX and sets of three sgRNAs targeting *AtPAP1* and *miR319* showing successful activation of both genes although without the observed phenotypes of the classical over-expression of both genes. Additionally, they achieved repression of *AtCSTF64, miR159A* and *miR159B* by transforming *A. thaliana* with pco-dCas9-3X (SRDX) and the sgRNAs (Lowder et al., 2015). The same group used AsCpf1 and LbCpf1 to generate mutations but also repurposed the dAsCpf1 (D908A) and dLbCpf1 (D832A) fused to SRDX to repress *miR159B* in *A. thaliana*

*(*Lowder et al., 2017[1]). In a third publication of the same lab, Lowder et al. (2017) used the CRISPR 2.0 system by using a scRNA recruiting MS2-VP64 and MS2-EDLL to target genes in *A. thaliana* and rice with successful activation for scRNA-MS2-VP64 but not for scRNA-MS2-EDLL (Lowder et al., 2017). In an interesting approach, Dreissig et al (2017) used the dCas9 fused to eGFP/mRuby2 to visualize the dynamics of telomeres in leaf cells of *Nicotiana Benthamiana.* When we were preparing our study, another publication used dCas9 fused to VP64 and at the same time a scRNA to recruit MS2-p65-HSF activation domains to increase *AtPAP1* and *AtAVP1* genes. Their work resulted in an increase of the expression of both genes and they could mimic the phenotypes of a classical overexpression of both genes, hence leaf purple colors for AtPAP1 and more and larger leaves for AtAVP1 (Park et al., 2017). However, no studies have been achieved with dCas9 and epigenetic domain fusions in plants.

During my thesis work, I proposed two strategies to engineer plants to elevate drought stress tolerance. In the second and main chapter of this manuscript, I describe a strategy based in the new technology CRISPR/Cas9 to modify AREB1 expression by controlling its promoter activity in *A. thaliana*. Then, in the third chapter, I describe a more classical strategy in which *AtAREB1* constitutive form (AREB1ΔQT) was over-expressed in cotton plants, accompanied in the same cassette by an RNAi targeting a splicing factor (SF) of the phytoparasite *M. Incognita.*

Chapter II- CRISPR/dCas9 strategy

1. Introduction

This study aims the induction of drought stress tolerance by targeting the promoter of *AtAREB1*. As stated in the first chapter, AREB1 is a key transcription factor in the ABA-mediated response to drought stress in plants. Some evidences also point that AREB1 gene expression would be targeted by epigenetic mechanisms. The use of an inhibitor of histone deacetylation in peanut led to an increase in the expression of transcription factor of the ABA signaling, including AhAREB1 (Su et al., 2015). Moreover, several genes including genes from the ABA pathway are modulated by the mean of DNA methylation and/or histone modification during drought or osmotic stress, sometimes in a heritable way (Ding et al., 2011; Pandey et al., 2015; Kim et al., 201; Banerjee et al., 2017). Hence, a strategy would be to focus on the epigenetic mechanisms that modulate the ABA signaling pathway. More precisely, focusing on AREB1 expression remains an open question that could be addressed by using current tools in genome editing.

Hence, the possible regulation of ABA signaling by epigenetic modifications, focusing on AREB1 expression, remains an interesting and non-addressed approach in plants.

With the development of the targeted plant genome editing using nucleases, the possibilities to create new varieties to overcome the different challenges and demands have notably augmented (Kumar and Jain, 2014; Abdallah et al., 2016; Malzahn et al., 2017). More particularly, the CRISPR system has been repurposed to accomplish CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi). The dCas9 can be engineered in fusion with some activators/repressors domains to form an artificial transcriptional regulator (Dominguez et al., 2016; Didovyk et al., 2016; Brocken et al., 2017; Lo and Qi, 2017; Baeumler et al., 2017; Zhang et al., 2017). In plants, the dCas9 has been fused with the EDLL, TAD or VP64 activation domains in *N. benthamiana* (Piatek et al., 2015) and in *A. thaliana* (Lowder et al., 2015; Tang et al., 2017; Park et al., 2017). These fusion domains recruit the mediator complex for transcription initiation to activate gene expression (Poss et al., 2013). In animal cells, the dCas9 was fused with a tripartite transcriptional activator VP64-p65-Rta (VPR), increasing the efficiency over dCas9-VP64 (Chavez et al., 2015), but this activator has never been used in plants. The dCas9 can be also engineered in fusion with epigenetic domains allowing histone remodeling. The Histone acetyltransferase (HAT) catalyzes the acetylation of core histones through the addition of an acetyl group to the lysine residue on the ε-amino group on the terminal tail of histones (Schneider et al., 2013). Histone acetylation triggers DNA relaxation that is correlated with enhanced DNA-protein interactions, and thus with gene activation (Eberharter and Decker, 2002). In such a way, the activation of dCas9^{HAT}, combined with the directed targeting of sgRNAs appears promising for positively regulating gene expression (Bordoli et al., 2001; Deng et al., 2007). In the Polycomb-group (PcG) gene family, the conserved SET domain catalyzes methylation of lysine residues on the histone tail, which also leads to regulation of the chromatin structure (Thorstensen et al., 2011). Such post-translational modification induces chromatin compaction and gene expression repression (Shubert et al., 2006; Saleh et al., 2007). Exploring the possibility of CRISPR/ dCas9 epigenome editing represent a significant advance and open the door for more flexible approaches to tuning gene expression by directly targeting specific promoters of desired genes of interest.

2. Hypothesis and objectives

We focused on an engineered dCas9 allowing gene expression regulation either by transactivation or chromatin remodeling. We used the dCas9^{VPR} previously published to enhance gene transcription, and focused on two epigenetic mediators (**Figure 12**). The dCas9^{HAT} results from the fusion of an *A. thaliana* histone acetyltransferase (HAT) domain from AtHAC1. On the other hand, dCas9^{SET} carries a methyltransferase domain (SET) from the *A. thaliana* CURLY LEAF gene (AtCLF) (CURLY

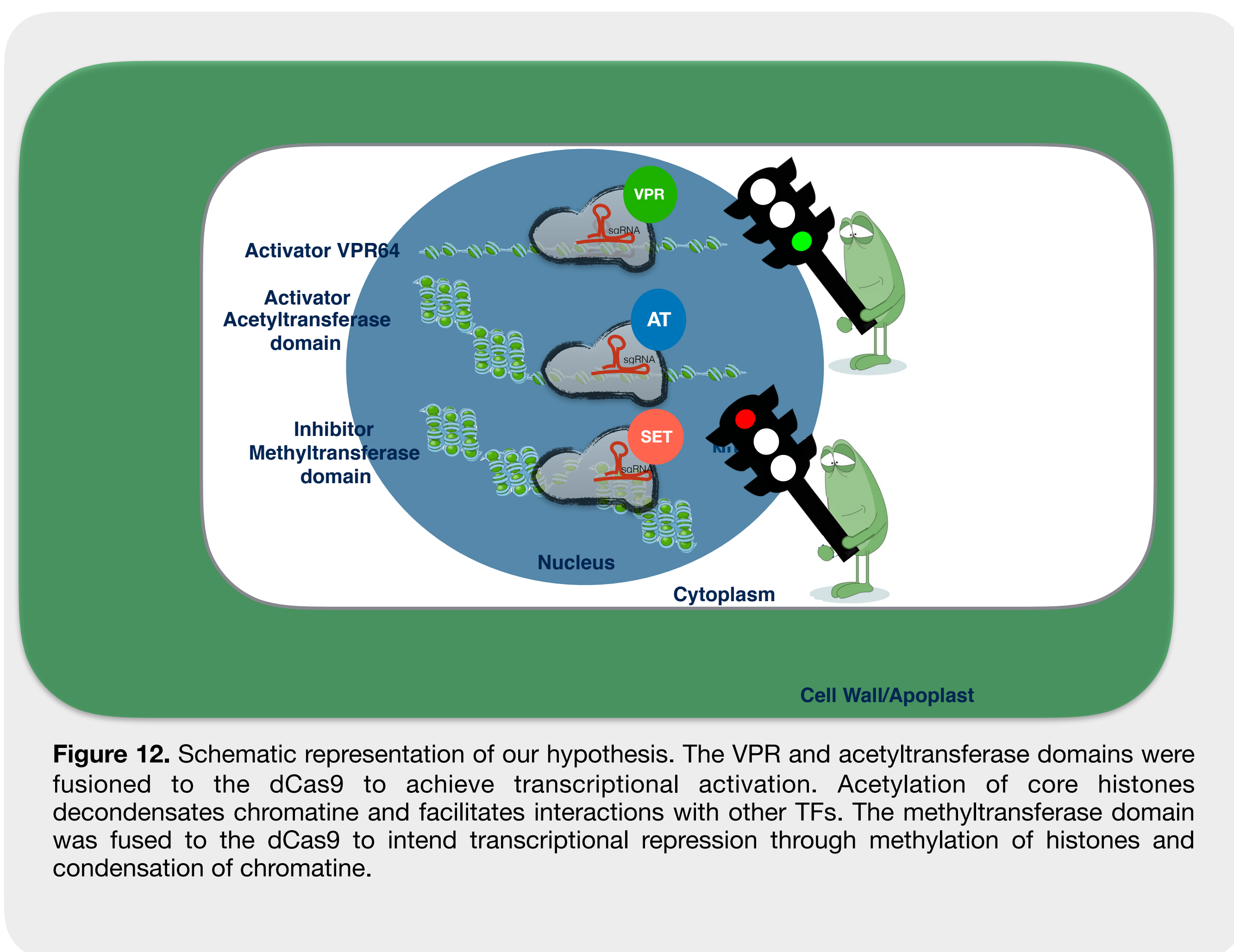


Figure 12. Schematic representation of our hypothesis. The VPR and acetyltransferase domains were fusioned to the dCas9 to achieve transcriptional activation. Acetylation of core histones decondensates chromatine and facilitates interactions with other TFs. The methyltransferase domain was fused to the dCas9 to intend transcriptional repression through methylation of histones and condensation of chromatine.

LEAF). In short, we hypotehzised that dCas9^{VPR} could positively regulate transciprtion in plants as it had worked for animal cells. Secondly, we thought that dCas9^{HAT} could enhance or activate transcription by acetylating and thus decondensing DNA to allow TF to interact with the sequence. FInally, dCas9^{SET} would act negatively on the expression of a target locus in the plant genome by methylating and condensing the DNA molecule (**Figure 12**). We first confronted our constructs with a reporter system of two soybean promoters (GmUceS) controlling the GUS gene. We then selected AREB1 to challenge the tuning of drought stress tolerance in Arabidopsis toward the expression of our different dCas9 constructs.

3. Results

3.1 Design of the dCas9 constructs

We first designed three dCas9 constructs to assay transcriptional regulation of the gene of interest. The gene encoding for dCas9^{VPR} was obtained from the Church's laboratory group (Addgene plasmid #63798, Chavez et al., 2015) and cloned in a modified version of the plant binary vector pGreenII

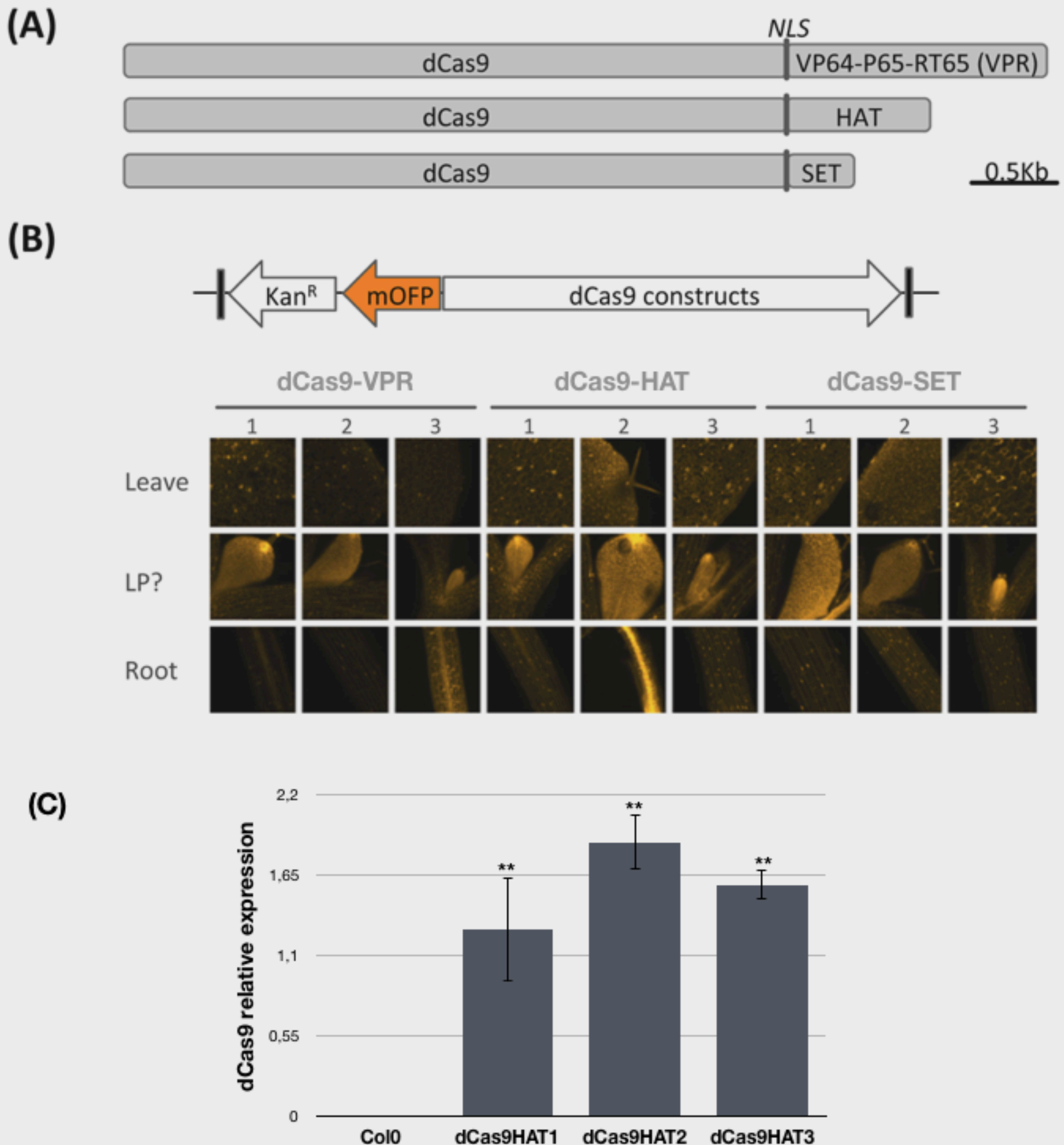


Figure 13. Characterization of dCas9 fusions and stable transformation in *A. Thaliana* plants. (A) Schematic representation of the three dCas9 fusions used in this study. VPR is a tripartite activator, HAT is an acetyltransferase domain and SET is a methyltrasnferae domain. (B) the dCas9 constructs were cloned in a pG plasmid containing two selection genes, Kan^R and mOrange fluorescent protein (mOFP). Images of leaves, Leaf primordia (LP) and roots show the mOFP of transformed lines assessed with the confocal microscope. (C) RT-qPCR was performed in Col0 and three lines of dCas9HAT transformed plants. For RT-qPCR, the gene expression levels were obtained by normalizing to the transcript levels of *GAPH* and *Actine*. Mean and sd were obtained from three biological replicates. The values were plotted relative to the lowest expression value (excluding the wild-type plants). Asterisks indicate significant differences between Col0 and the different lines (Student's T test P values, $^{**}P < 0.01$).

(**Figure 13 A, Figure S1 A**). The sequence encoding for VPR was replaced by the catalytic core from the Arabidopsis Histone Acetyl Transferase 1 gene (AtHAC1, AT1G79000) or the histone methyltransferase Curly Leaf gene (AtCLF, AT2G23380) to generate respectively dCas9^{HAT} and dCas9^{SET} (**Figure 13 A, Figure S1 A**). The T-DNA was designed to perform two rounds of transgenic plant selection based on antibiotic resistance (i.e kanamycine) and the level of a fluorescence reporter gene (i.e a nuclear mOrange fluorescent protein, mOFP) After positive transformants were selected upon kanamycine, fluorescence intensity was assessed by confocal microscopy (**Figure 13 B**). A considerable variation in transgene expression is often observed between different lines and even in different populations of the same line (Butaye et al., 2005). The fluorescence corresponding to mOFP could be seen all over the roots and leaves, at a higher intensity in the nucleus. Some lines presented stronger fluorescence, suggesting that the cassette was inserted in a region of the genome where gene expression is higher. Among these lines, the occurrence of the dCas9 constructs was checked by PCR (**Figure S1 B**). The three transgenic lines dCas9^{VPR}1, dCas9^{AT}2 and dCAS9^{SET}1 were retained in this study.

3.2 Testing the dCas9 constructs in a GUS reporter system

The study of promoter activity is performed in most cases with the use of an easily detectable reporter gene. The uidA/GUS gene that encodes for the enzyme β-glucuronidase has been widely used in functional studies in plants because it is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Karcher, 2002; Basu et al., 2004; Wang et al., 2005). Hence, we used this system to evaluate the dCas9 constructs. We focused on two versions of a promoter available at the laboratory that is the glycine max ubiquitin promoter (GmUces, 1133 bp length) and its truncated version GmUcesMin (GmUcesMin, 170 bp length) – (**Figure 14 A**). Previous studies suggested that distance of the specific sgRNA to transcription start site (TSS) might influence the transcription regulation of the gene of interest. Whilst some studies have reported that a specific sgRNA binding distance from TSS corroborates with higher target gene expression (Gilbert et al., 2014; Radzisheuskaya et al., 2016), others have pointed that dCas9 might generate steric hindrance, and thus interfere the transcriptional machinery activities (Larson et al., 2013; Hilton et al, 2015). We integrated these parameters for the selection of three sgRNAs. We also wished to assess the impact of the regulatory elements in a promoter on the efficiency of the CRISPR-based strategy. Hence, *in silico* analysis conferred the transcription regulatory sequences along both GmUces promoters. Based on the *in silico* analysis, we mapped the whole sgRNAs candidates in the GmUces and GmUcesMin and selected three sgRNAs. The sgRNA1 targets the region at -56 bp from TSS and located both GmUces and GmUcesMin. The sgRNA2 targets a region close to the TSS (+18 bp) in GmUcesMin. The sgRNA3 targets a region in the 5'UTR at +273 bp from the TSS (**Figure 14 A, Table S1**).

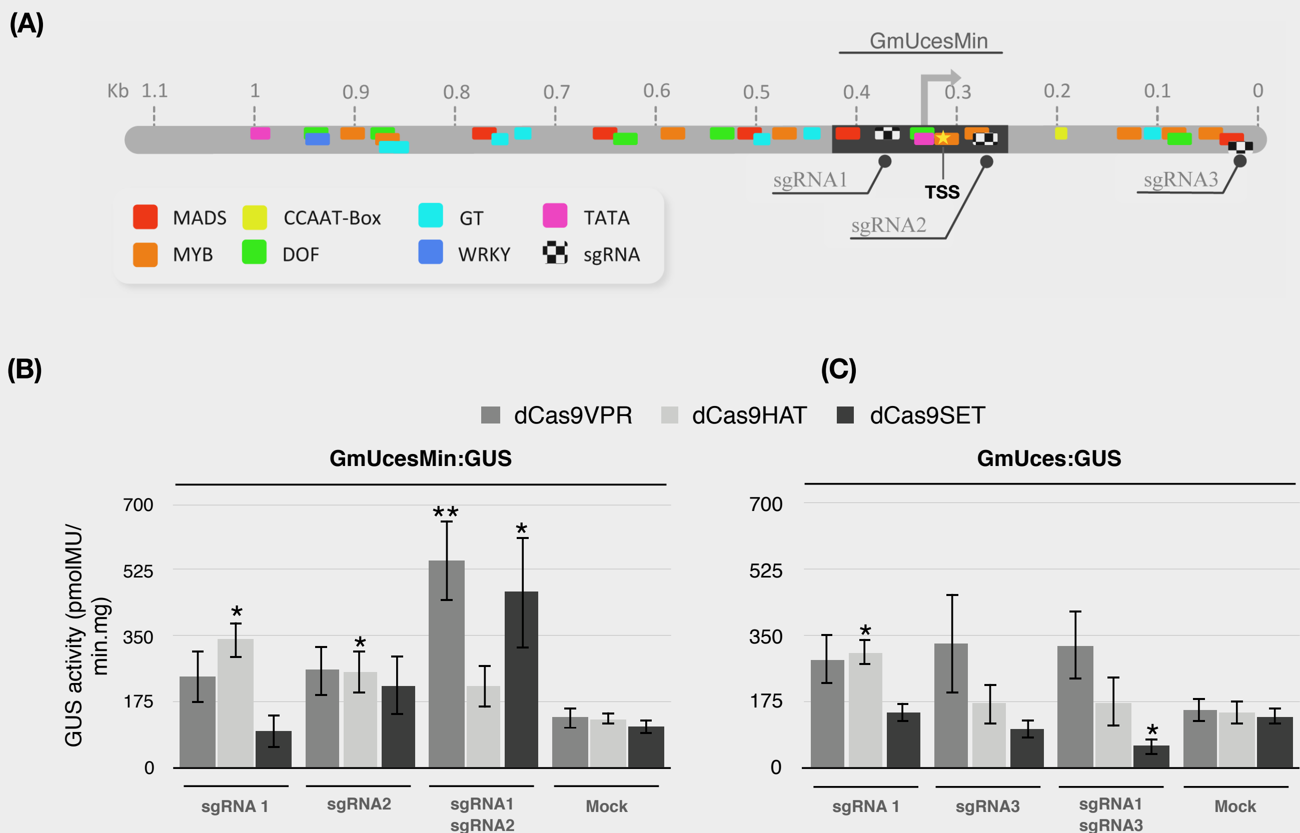


Figure 14. Testing das9 constructs in a GUS reporter system. (A) Schematic representation of GmUcesMin and GmUces promoters with regulatory boxes (colors) and sgRNA positions (black-white squares). Mock represents seedlings transiently expressing GmUces construction and GV3101 with an empty vector. (B-E) Seedlings from stably transformed dCas9 fusions were transiently transformed with GmUceSmin and GmUceS and combinations of sgRNAs. (B, C) GUS activity was assayed for GmUcesmin (B) and GmUces (C) Results represent means of 6 independent experiments in which were pooled n=20 seedlings for each. Statistical analysis were performed using Student's t-test to confirm significance of GUS activity for each dCas9 fusion guided by one sgRNA or a combination of two sgRNAs compared to Mock controls. Asterisks* represent values of p<0,05. Bars indicate standard error.

To test the functionality of the transcriptional regulators directed by the selected sgRNAs, we evaluated the efficiency of the dCas9 constructs in activating/inhibiting the uidA reporter system by quantifying GUS enzymatic activity in a β-glucuronidase assay (**Figure 14 B, C**). We used *agrobacterium* mediated transient expression in *Arabidopsis* as a simple and rapid assay (Li et al., 2010; Wu et al., 2014).). *Arabidopsis* seedlings (n=20) from lines dCas9^{VPR}, dCas9^{HAT} and dCas9^{SET} were incubated with *agrobacterium* carrying Ti plasmids to perform ectopic expression of GmUces-GUS or GmUcesMin-GUS in combination with the expression of a single or two sgRNAs. All test conditions were compared to the control (Mock) that corresponds to *A. thaliana* seedlings expressing transiently GUS under GmUcesmin (**Figure 14**

A) or GmUces (**Figure 14 B**) promoters. With GmUcesMin-GUS in the dCas9^{VPR} genetic background, GUS activity was ~2 fold higher using sgRNAs 1 or 2, and significantly higher (~4 fold) when using the two sgRNAs in combination. For the dCas9^{HAT} genetic background, the higher enzymatic activity was observed significantly for the sgRNA1 with a ~2,6-fold increase while it was ~1,9 fold for the sgRNA2. No difference was observed in dCas9^{SET} plants with sgRNAs 1 or 2. Nevertheless a surprising significant activation occurred with their combination. The maximum value measured corresponded to dCas9^{VPR} plants in association with the two sgRNAs (**Figure 14 B**). We next reproduced the experiment using GmUces-GUS to evaluate the impact of the full-length GmUces promoter on GUS expression (**Figure 14 C**). In this experiment, the sgRNA1 and sgRNA3 were tested. When we performed the same assay with the GmUces, we observed a similar activation of ~2 fold with the dCas9^{VPR} associated with sgRNAs 1 or 3 and their combination. With the dCas9^{HAT} plants, the results showed a significant activation with sgRNA 1 alone. In the dCas9^{SET} plants, we observed a significant inhibition (2,4-fold) with the combination of sgRNAs 1 and 3, which is the inverse from what we had observed with GmUceSmin. These results show that the sgRNAs, their combination and their association with the dCas9 fused to the regulatory domains led to highly variable GUS expression profiles. Under certain configurations, dCas9^{VPR} and dCas9^{HAT} can activate GUS expression significantly while dCas9^{SET} can be either an enhancer or an inhibitor.

We next were intrigued to investigate through a GUS staining assay whether the GUS expression profile was modified in the different dCas9 genetic backgrounds (**Figure 15**). As previously shown, we confirmed a variable intensity of GUS staining for all conditions compared with the control. GUS activity was broadly higher in the roots than in the leaves of all *Arabidopsis* seedlings and for all conditions. The roots had a more intense staining for dCas9^{VPR} and dCas9^{HAT} with almost all combinations of sgRNAs. More in depth, we also observed the GUS activity in the hypocotyl and cotyledons in some conditions. This tendency was particularly pronounced in the dCas9-^{VPR} and dCas9^{AT} plants with the combination of two sgRNAs. Concerning dCas9^{HAT}, the phenotype was clearer using GmUces rather than GmUcesMin. This result suggests that dCas9^{VPR} and dCas9^{HAT} can modify the expression profile of a target gene in plant tissues. We also discerned a slight decrease in the staining of the cotyledons when assessing the dCas9-SET background with different combinations of sgRNAs

We also transiently expressed the sgRNAs and dCas9 fusions in plants stably transformed with the promoters GmUcesMin and GmUces (**Figure 15 C, D**). In comparison with **Figure 15 (A, B)** results, the staining showed a staining essentially only in the cotyledons, suggesting that when the promoters are integrated in the plant's genome, they are less or not expressed in the roots. We observed an increase in the staining for dCas9^{VPR} and dCas9^{HAT} for all combinations, but once more, principally with the combination of two sgRNAs (**Figure 15 C, D**). Interestingly, with the dCas9^{SET}, we perceived that in the GmUcesMin plants, the GUS was present in the central nerve, but it was diminished on the other regions of the leave, suggesting

the methyltransferase domain could be inhibiting the promoter in certain tissues (**Figure 15 C, D**).

These results indicate that dCas9^{VPR} and dCas9^{HAT} could be efficient to enhance a target gene expression under certain conditions. The efficiency of the system depends on the association of the sgRNA

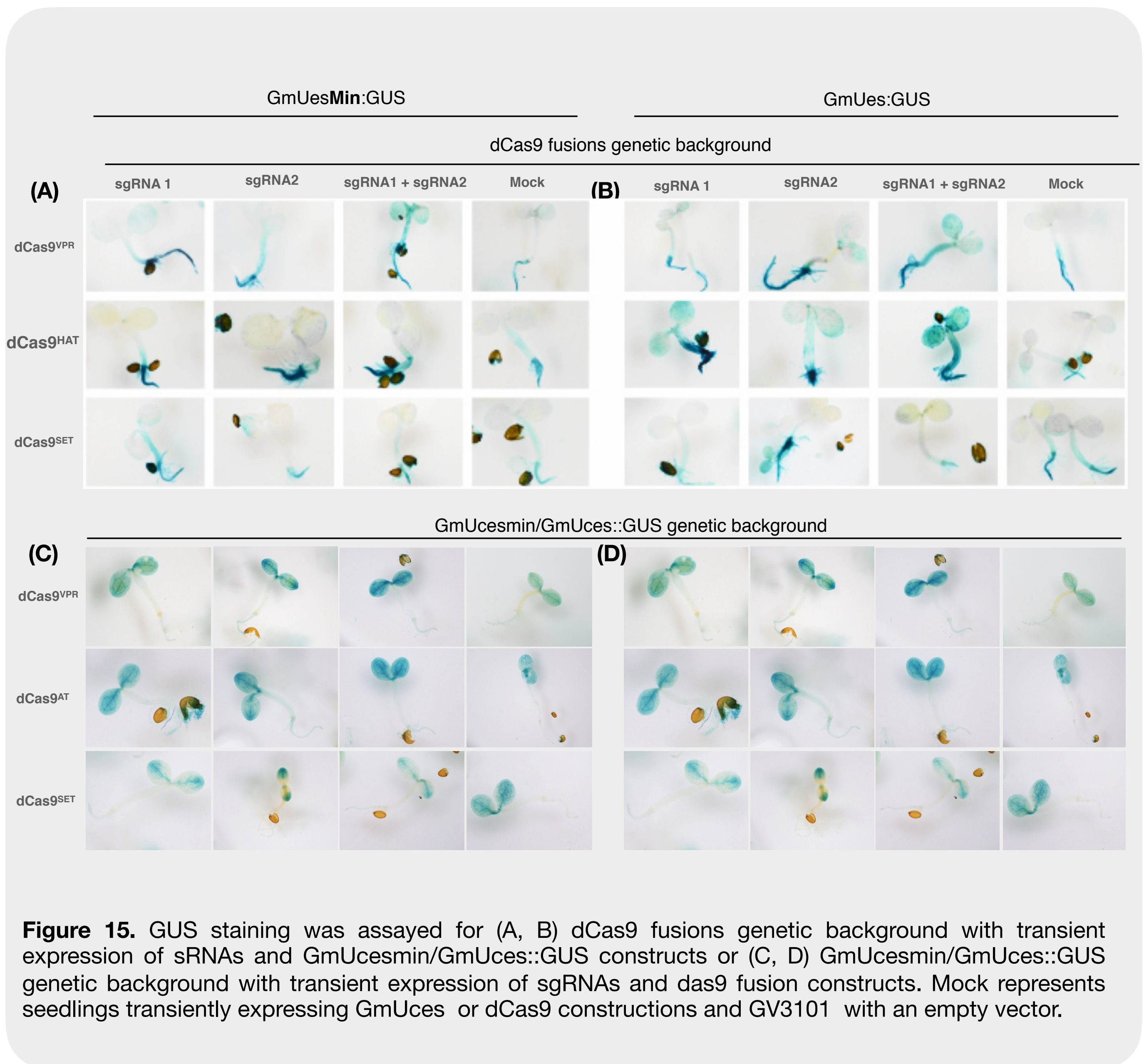


Figure 15. GUS staining was assayed for (A, B) dCas9 fusions genetic background with transient expression of sRNAs and GmUcesmin/GmUces::GUS constructs or (C, D) GmUcesmin/GmUces::GUS genetic background with transient expression of sgRNAs and das9 fusion constructs. Mock represents seedlings transiently expressing GmUces or dCas9 constructions and GV3101 with an empty vector.

and the genetic background (e.g. GmUces versus GmUcesMin). In a general manner, the use of the promoter GmUcesMin resulted in better transcriptional regulation to enhance GUS expression. Altogether these results encouraged us to use a similar approach for the regulation of one of the central gene implicated in drought stress response.

3.3 Challenging dCas9 constructs to regulate AREB1 promoter in stable Arabidopsis lines

As previously described with the design of sgRNA targeting GmUces, we also considered the position of the TSS and the occurrence of different regulatory elements in the AREB1 promoter (pAREB1). Our *in-silico* analysis revealed several regulatory elements in pAREB1 (**Figure 16 A**). Among the 105 existing PAM sites in pAREB1, we retained two sgRNAs: sgRNA AREB1_A is at -479 bp and the sgRNA

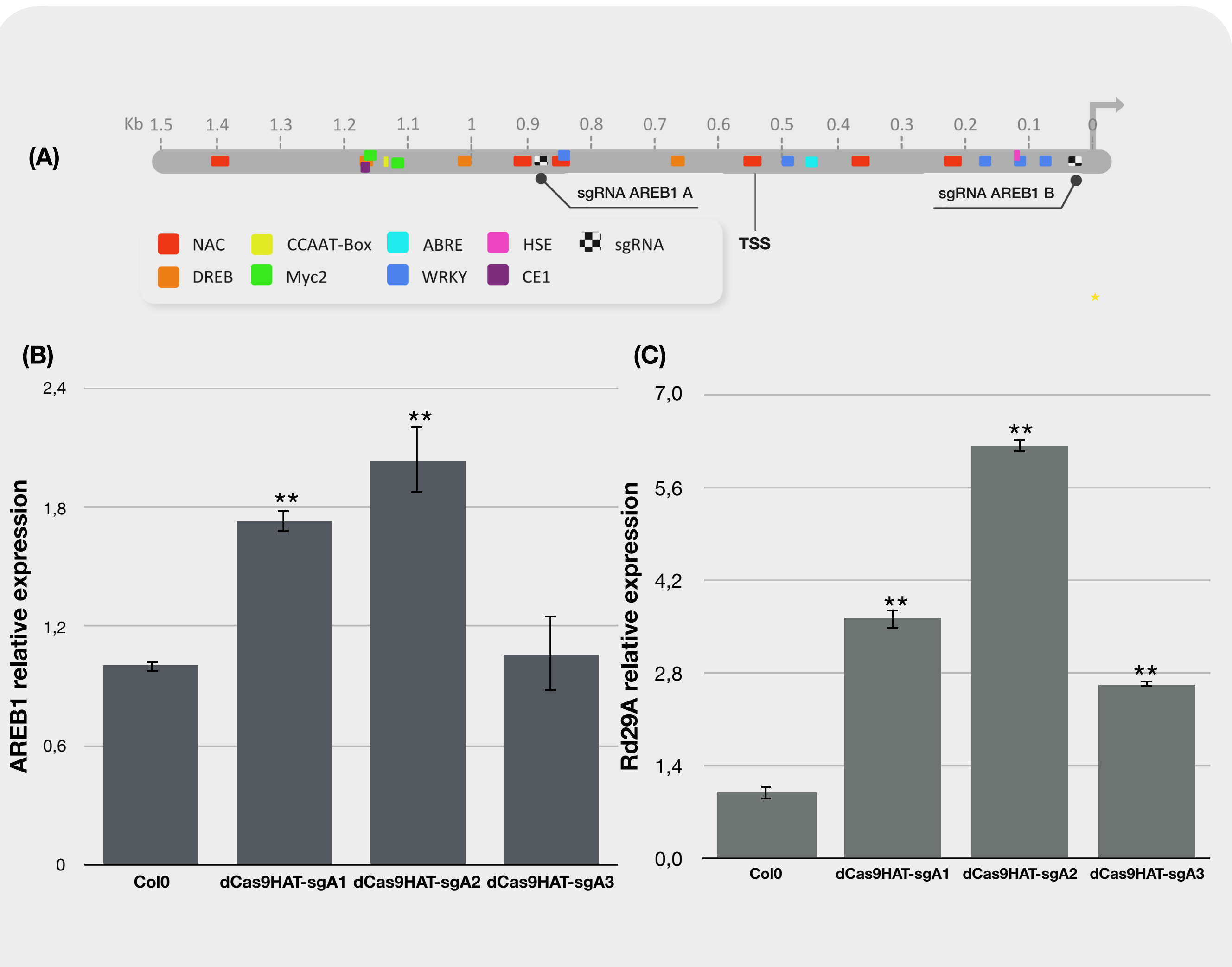


Figure 16. Challenging dCa9 constructs to regulation transcription of AREB1 by targeting pAREB1. (A) Schematic representation of pAREB1 with drought stress regulatory elements and position of the two sgRNAs designed. (B, C) Transcript levels of (B) *AREB1* and (C) *RD29A.* RT-qPCR was performed in dCas9HAT control and three lines of dCas9HAT-sgA transformed plants in normal conditions (no drought stress). For RT-qPCR, the gene expression levels were obtained by normalizing to the transcript levels of *GAPDH and Actine*. Mean and sd were obtained from three pooled biological replicates. For each gene, the expression level in the control plants under non-stressed conditions was defined as 1.0.

AREB1 B is at +356 bp from the TSS ; sgRNA AREB1 B) (**Figure 16 A; Figure S2**). The two sgRNAs were cloned in tandem in a single T-DNA and transformed in the *Arabidopsis* transgenic lines dCas9^{VPR,} dCas9^{HAT} and dCas9^{SET} to generate the dCas9^{VPR}-sgA dCas9^{HAT}-sgA and dCas9^{SET}-sgA lines respectively. However, gene expression results were only conclusive for dCas9^{HAT}-sgA lines and therefore they are the only ones shown. We verified whether the dCas9^{HAT}-sgA could activate or inhibit the AREB1

promoter (pAREB1) by real time qPCR in normal conditions (**Figure 16 B**). In each experiment, the control is referred to the parental line dCas9^{HAT}. We observed an increase in AREB1 expression of 1.7-fold for dCas9^{HAT}-sgA1 line and a significant increase 2-fold for dCas9HAT-sgA2 line, suggesting that dCas9^{HAT} could induce pAREB1 activity when directed by sgRNAs A and B. The gene Rd29A is very sensitive to various abiotic stresses and is a direct target of AREB1 TF (Fujita et al., 2005; Msanne et al., 2011). We assessed Rd29 expression in dCas9^{HAT}-sgA and found that the three independent lines had significant higher relative expression. However, dCas9HAT-sgA2 line had the highest expression with a 6-fold increase. Therefore, we chose **dCas9HAT-sgA2** for the rest of the study. Even if we did not obtain gene expression results for dCas9^{SET}-sgA lines, we also chose **dCas9SET-sgA3** line for the next experiments.

3.4 dCas9HAT-sgA plants have a dwarf phenotype

During the screening of the transgenic *A.thaliana* lines, we noticed that all dCas9^{HAT}-sgA lines grew slower than the parental dCas9^{HAT} control plants. We determined morphological measurements on the rosette and leaves in the dCas9^{HAT}-sgA2 and dCas9^{SET}-sgA3 plants. As previously described, the control is referred to the parental lines dCas9^{HAT} or dCas9^{SET} without the expression of the sgRNAs. Three weeks after germination, the rosette diameters in dCas9^{HAT}-sgA2 plants was ~3 fold significantly smaller than the controls (**Figure 17 A**). No differences were found between the different dCas9^{SET}-sgA3 plants and the control. The leaves length, width and area in dCas9^{HAT}-sgA 2 were respectively ~1.5, 1.3, and 1.9-fold shorter than the control (**Figure 17 B**). Once more, dCas9^{SET}-sgA leaves had comparable proportions as the dCas9^{SET} control. This data indicates that the biomass production is reduced in the dCas9^{HAT} plants expressing a sgRNA targeting the *AREB1* gene. However, as we would expect, an opposite phenotype (increased biomass) was not observed in dCas9^{SET}-sgA3.

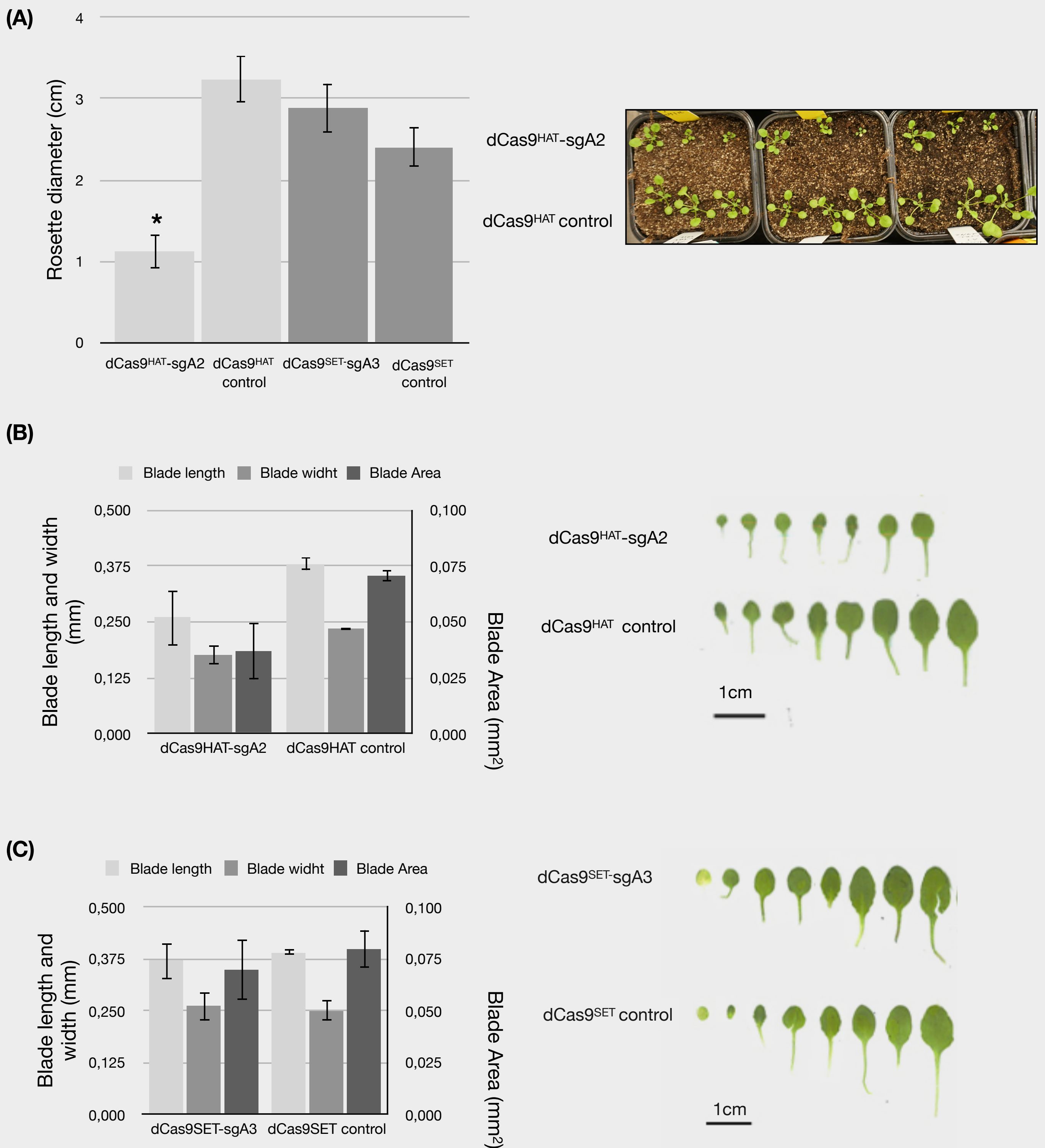


Figure 17. Phenotype dCas9^{HAT}-sgA2 and dCas9^{SET}-sgA3 lines. (A) Rosette diameter in three week-old plants. Results represent the mean of n=13 Statistical analysis were performed using Student's t-test to confirm significance of dCas9-sgA2 lines compared to dCas9HAT control lines Asterisk represent values of $p<0,05$. (B, C) Morphological measurements made with LeafJ plugin for IMageJ in of the scanned leaves (right) of dCas9^{HAT}sgA2 (B) and dCas9^{SET}sgA3 lines (C). Results represent data of leaf#7 of n=2 plants. Bars indicate standard error.

3.5 dCas9HAT-sgA2 plants have enhanced drought stress tolerance

To investigate the effect of dCas9^{HAT}-sgA2 and dCas9^{SET}-sgA3 expression on the drought stress response, we performed two sorts of drought stress. Severe drought stress consisted in removing plants from soil and kept on plates at 20% humidity on the bench (**Figure S3 A**). In another experiment, a mild-severe stress consisted of a water withdrawal for up to 20 days on the soil.

Inactivation of the photosystem II followed by a loss of the chlorophyll fluorescence are physiological indicators of drought stress. Thus, the measurement of the chlorophyll fluorescence is used as an indicator of the plant drought stress response (Chen et al., 2016). When plants are normally irrigated, the amounts of chlorophyll were comparable between the different dCas9 plants expressing or not the sgRNAs (**Figure 18 A, B**). In contrast, 4h after severe drought stress, the amount of total chlorophyll was 1.7-fold and 1.5-fold higher in dCas9^{HAT}-sgA2 and dCas9^{SET}-sgA3 respectively, compared to the control (**Figure 18 A, B**). Twenty days **after water withdrawal**, dCas9^{HAT}-sgA2 plants had a significantly higher content of chlorophyll (1.3 fold) than the control, inversely from dCas9^{SET}-sgA3 (**Figure 18 A, B**). Our data likely indicates that the activity of the photosystem is higher under drought stress in dCas9^{HAT}-sgA2. The observation of higher chlorophyll content in dCas9^{SET}-sgA3 also suggests that this construct maintains higher photosystem activity during drought stress, but was only observable during a 4h drought stress.

Another characteristic of the drought stress response is a decrease in the stomatal aperture to limit water loss (Daszkowska-Golec and Szarejko, 2013). We measured stomatal width and length of confocal images and calculated stomatal aperture (width/length) in non-stressed versus stressed plants after 1h, 2h and 20 days. We did not notice any difference between dCas9^{HAT}-sgA2 and the control without drought stress, and at any time with dCas9^{SET}-sgA3 plants (**Figure 18 C, D, E**). One hour after drought stress, the stomatal aperture was 1.6-fold lower in dCas9^{HAT}-sgA2 plants than the control **(Figure 18 C**). After 2h, the stomatal aperture of the control decreased almost to that of dCas9^{HAT}-sgA2, showing that stomatal closure is triggered more rapidly in dCas9^{HAT}-sgA2 plants. After 20 days of stress, the stomatal aperture was comparable with the control indicating that dCas9^{HAT}-sgA3 do not regulate differently the stomatal aperture during long-term drought stress. No differences were seen for stomatal aperture Cas9^{SET}-sgA3 plants (**Figure 18 D**).

To observe the phenotype during drought stress, we measured water loss rates (**Figure S4 A, B).** We did not observe differences between transformed plants (dCas9^{HAT}-sgA2 or dCas9^{SET}-sgA3) and their controls. However, the same experiment was performed in AREB1 over-expressing plants and areb1 mutant plants, and the same results were found between transformed and WT plants (**Figure S4 C, D**)

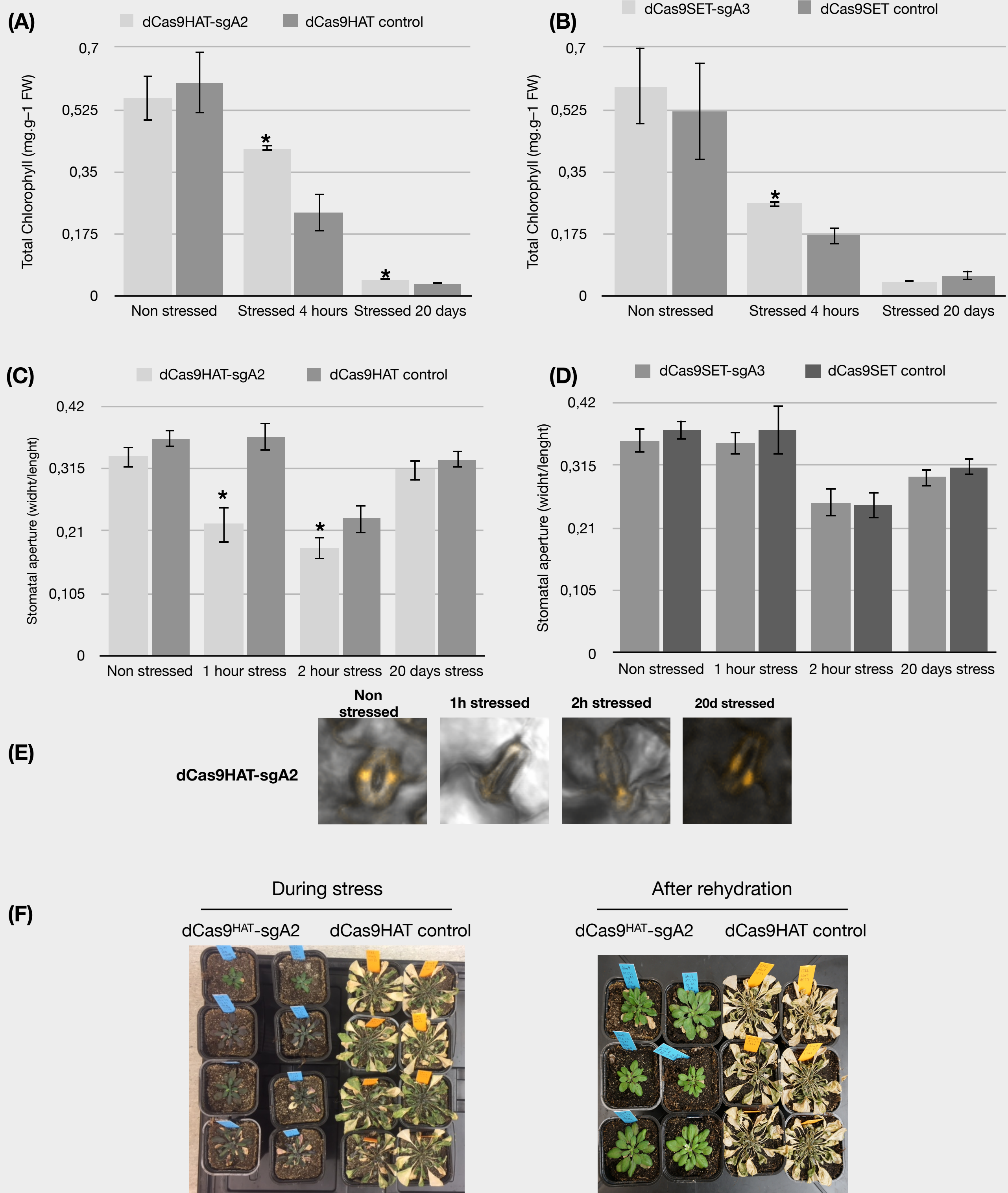


Figure 18. Physiological analysis of drought stress response of dCas9^{HAT}sgA2 and dCas9^{SET}sgA3 plants. (A, B) Total Chlorophyll contents in non stressed plants, 4 hours after severe drought stress and after 20 days in soil water stress. Results represent mean of n=6. (C, D) Stomatal aperture measurements after 2, 4 hour severe stress and 20 days of drought stress. Results represent means of n=30. Statistical analysis was performed using Student's t-test co-transfromed plants to control lines. Asterisks* represent values of p<0,05. Bars represent standard error. (E) Confocal images of stomates in dCas9^{HAT}sgA2 lines. (F) dCas9HATsgA2 pictures during stress and after rehydration

(Fujita et al., 2005; Yoshida et al., 2010).

Finally, we performed rehydration of the plants that had undergone either a severe 6h stress (**Figure S3 B**) or a mild stress on soil (**Figure 18 E**). We observed that after a severe stress, 85% of dCas9^{HAT}-sgA2 had survived compared to 50% for dCas9^{HAT} control plants (**Figure S3 B**). When we rehydrated dCas9^{HAT}-sgA2 plants that had suffered a mild drought stress on soil, all the transformed plants survived whereas all control plants died (**Figure 18 E**).

Altogether, these outcomes indicate that the dCas9^{HAT}-sgA2 plants trigger a faster stomatal closure in response to drought stress, conjugated with higher chlorophyll content suggesting that plants face lower physiological stress. These physiological responses could be part of the enhanced drought stress response shown in the recovery of dCas9^{HAT}-sgA2 after rehydration. On the other hand, dCas9^{SET}-sgA3 do not show any susceptibility to drought stress suggesting that the RNP dCas9^{SET}-sgA3 do not impact negatively on the drought stress response. However, the observation of altered chlorophyll content in these plants suggests the occurrence of a “side effect” of the construct on the plant physiology.

3.6 dCas9HAT-sgA2 phenotype is linked to targeted transcriptional regulation of pAREB1

To correlate the enhanced drought stress response in dCas9^{HAT}-sgA2 plants, we evaluated AREB1 and Rd29A expression during drought stress. We observed that AREB1 expression was increased

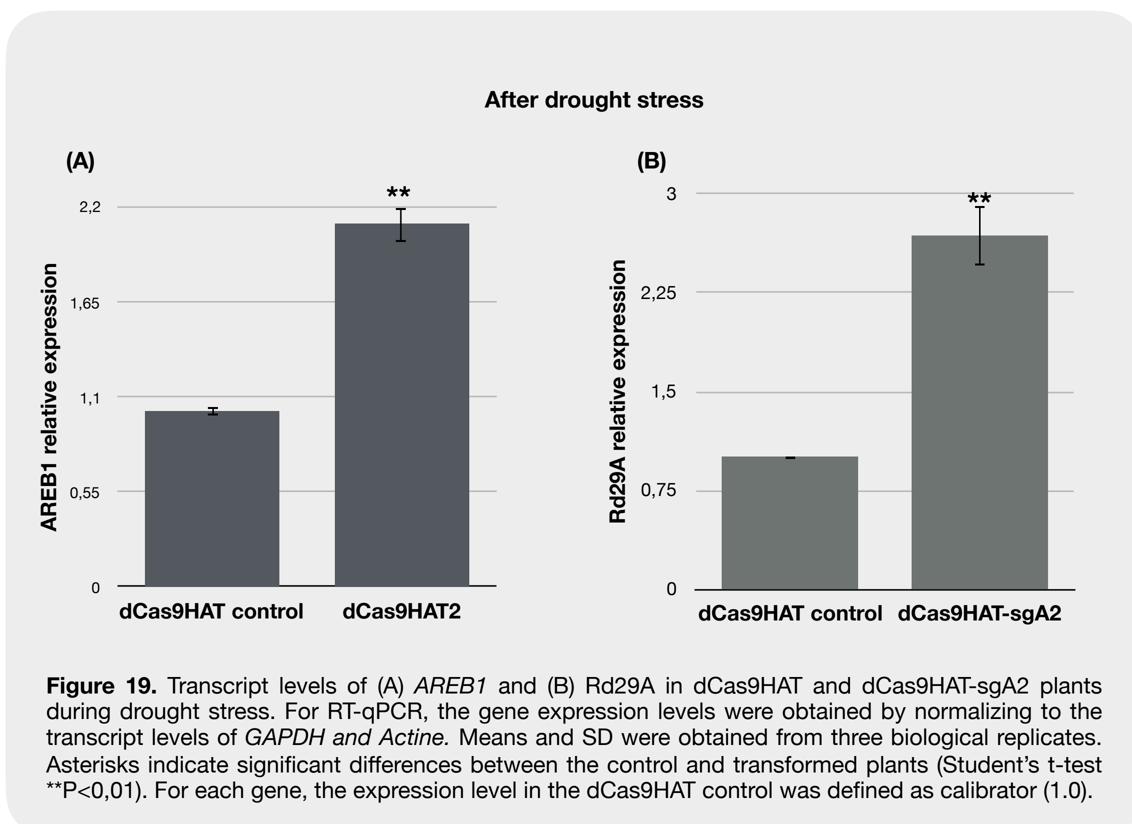


Figure 19. Transcript levels of (A) *AREB1* and (B) Rd29A in dCas9HAT and dCas9HAT-sgA2 plants during drought stress. For RT-qPCR, the gene expression levels were obtained by normalizing to the transcript levels of *GAPDH and Actine.* Means and SD were obtained from three biological replicates. Asterisks indicate significant differences between the control and transformed plants (Student’s t-test **P<0,01). For each gene, the expression level in the dCas9HAT control was defined as calibrator (1.0).

by 2-fold and Rd29 expression was increased by 2,6-fold (**Figure 19 A, B**). These results suggest that during drought stress, dCas9^{HAT} could enhance AREB1 expression and Rd29 expression and advance that the drought response of dCas9^{HAT}-sgA2 plants could be linked to the increase in AREB1 expression.

4. Discussion

Our present study expands the use of dCas9 as a transcriptional regulator of targeted promoters in plants (Piatek et al., 2015; Lowder et al., 2015, 2017; Park et al., 2017). Rather than using modulators of the transcription to aid pol II recruitement, we found that the use of domains that modify the epigenetic state also modify transcription when guided by sgRNAs to promoters in plants, and were capable of increasing drought stress response in planta. We observed that the cassette containing the dCas9 fusions was expressed in all the tissues with variations in abundance depending on the line as shown by mOFP expression.

Choosing the right sgRNAs

Design of functional and specific sgRNAs with minimal off-target effects has been an essential issue since the discovery of the CRISPR-Cas mechanisms (O'Geen et al., 2015; Wong et al., 2015; Zheng et al., 2016). Once the TSS location predicted, we designed sets of two sgRNAs for each targeted promoter in both the transient transcriptional regulation and the in-planta regulation of the endogenous promoter of pAREB1. For each promoter, we decided to target two regions located in 3' and 5' from the TSS (**Figures 12 A, 14 A**). However, the publications relating the existence of several tools for prediction of cis-regulatory sequences seem to agree that prediction of the TSS stays a difficult task and that CRISPRi efficiency relies on recruiting the effector complex to the TSS (Radzisheuskaya et al. 2016; Shahmuradov et al., 2017). As a matter of fact, Gilbert et al. (2014) found that the target area should be of -50 to +300 bps around the TSS, while other studies using the dCas9 fused to an acetyltransferase domain showed that targeting distal regions (>1 kb) from the promoter resulted in successful transcriptional activation (Hilton et al. 2014). Besides predicting the TSS, our in-silico analysis predicted several regulatory sequences and we confronted this data to the different putative positions of sgRNAs in the sequence of our three promoters. Indeed, several studies reported that a possible steric hindrance could interfere with endogenous regulatory elements because of the binding of the dCas9 (La Russa and

Qi, 2015; O'Geen et al., 2017). Therefore, based on TSS location, the position of the different regulatory boxes and the efficiency of the sgRNA (implemented by CHOPCHOP web-tool), we chose our sets of sgRNAs for the study. After we already had chosen our sgRNAs, some studies reported that choosing a sgRNA for CRISPRi/a studies, the parameters differed from CRISPR-cas editing approaches. First, while for genome editing the sequence in 3' of the sgRNA seems to be significant for the specificity, optimal parameters for CRISPRi/a sgRNAs depend on all nucleotides throughout the spacer region. Also, it seems that a sequence of 19 nucleotides is more efficient than less or more nucleotides. The differences in specificity are accountable to several differences between the mechanisms: The structure and function of the dCas9 fusions is different from Cas9; CRISPRi/a targets mainly promoters, where chromatin context is different from the coding regions, from the state of condensation to the different protein complexes interacting in promoters; mismatches at certain positions of sgRNA leads to association/dissociation changes, introducing a dimension of kinetic and thermodynamic dCas9 behaviors (Xu et al., 2015; Boyle et al., 2017).

Is VPR more robust than other activators in plants?

We used the same dCas9 fused to the VPR tripartite activator that was used by Chavez et al. (2015) in animal cells. We found that the VPR could activate expression in plant systems in the transient assay. The increase was robust as it augmented transcription for both synthetic promoters and all combinations of sgRNAs, and attained equal or higher GUS activities that the ones found with EDLL and TAD activators by Piatek et al., (2015). Furthermore, with $dCas9^{VPR}$, we obtained either a better activation with a combination of two sgRNAs (**Figure 12**). This result corroborates previous studies in which the authors also found that CRISPRi/a was more performant when using two or more sgRNAs (Piatek et al., 2015; Park et al., 2017). Moreover, we observed that the best combination resulted when using a small synthetic promoter with two targeted regions (**Figure 14**). Interestingly this tripartite artificial domain containing two viral activators (VP64 and Rta) and a vertebrate activator (p65) is able to induce the mediator complex during transcriptional regulation in plants cells as well. Chavez et al. (2015) also tested it in yeast, drosophila cells, and *Mus musculus* cells, confirming the versatility/universality of this type of activators. Unfortunately, we did not obtain $dCas9^{VPR}$ plants expressing the sgRNAs AREB1 A and B, and thus we could not test its capacity of inducing drought stress response.

Did acetylation of histones work to increase transcription?

The activation of promoters through H3K27 acetylation of histones employing $dCas9^{p300}$ Core domains has been evaluated in animal cells, showing to be more potent than activation domains (de Groote et al., 2012; Cheng et al., 2013; Hilton et al., 2015). To our knowledge, our study is the first to

adopt HAT domains for targeted transcriptional activation in plants. Our data showed that the use of a plant's HAT domain could increase the activity of a reporter gene controlled by synthetic promoters (**Figure 14**). We found that the activation mediated with acetylation was more important when targeted with the single sgRNA1. Our data showed that acetylation was sufficient to increase *AREB1* expression by targeting two loci in the p*AREB1* and to induce an increased drought stress tolerance in the transformed plants. Contrastingly, while Okada et al. (2017) obtained their best activation with one single and close from TSS sgRNA as we did with sgRNA1, Hilton et al. (2015) found that he could activate genes with dCas9^{p300} and various sgRNAs at distant enhancers. This observations combined to ours suggest that targeted transcription acetylation to activate promoters depend than only the position of sgRNAs, but in diverse factors concerning structure of the DNA. In relation with plants, previous studies reported that the over-expression of histone deacetylases had a role in ABA response, and that the use of an inhibitor of Histone deacetylases induced hyperacetylation and increased in the expression of AREB1 in peanut (Sridah et al., 2006; Chen Su et al., 2015). These results endorse our hypothesis in which *AREB1* is highly inducible by epigenetic changes in p*AREB1*.

dCas9^{HAT} combined to sgA caused an effect on size

The stable integration of the dCas9^{HAT} accompanied by sgRNAs targeting pAREB1 also showed a strong phenotype in which the plants were significantly smaller at the vegetative state (**Figure 17**). Although previous studies showed that the over-expression of AREB1 and its constitutive form AREB1ΔQT had smaller phenotypes and the *areb1* mutants had larger rosettes (Fujita et al., 2005), the dCas9^{HAT}-sgA2 co-expression presented even smaller rosette sizes. These differences could be explained because of the possible side effects of the expression of dCas9^{HAT}- and sgRNAs. Some publications have assessed the off-target activity of dCas9 (Xu et al., 2015; Boyle et al., 2017) that could result on affecting phenotypes. Moreover, HAT domains have been found to catalyze acetylation of other histones and proteins, being able to regulate different biological processes (Enríquez et al., 2016). Likewise, one hypothesis that has not been tested is if targeting promoters could influence the expression of neighboring genes. For instance, AREB1 is situated in a region in the chromosome 1 surrounded by several CDS for other genes, where a hAT-like transposase family (AT1G45832.1) is concatenated and could be probably sharing regions of the same promoter.

Was the methylation mediated by dCas9^{SET} specific?

Anterior studies showed that the fusion of dCas9 with KRAB repressor domain induced gene repression through H3K9me3 in human cells (de Groote et al., 2012; Thakore et al., 2015). Additionally, H3K27me3 marks have been highly associated with repressive marks in animals and plants (Tie et al.,

2009; Thorstensen et al., 2011; Hosogane et al., 2016; Moody et al. 2017). We hypothesized that the fusion of a dCas9 with a plant SET methyltransferase domain from AtCLF (Saleh et al., 2007) could inhibit transcription by introducing H3K27me3 in targeted promoters. However, our results were contrasted in both transient and stable assays. We observed that the dCas9^{SET} could either inhibit or activate GUS activity in independent experiments of the transient assay (**Figures 14, 15**). A similar result was observed when addressing the drought stress response of lines co-expressing dCas9^{SET} and sgRNAs targeting the AREB1 promoter. Indeed, the phenotypes were also contrasted, with some lines exhibiting a normal drought stress response, and other lines displaying a better tolerance to drought stress. During the time in which we were performing our assays, O'Geen et al. (2017) published an approach in which they included a dCas9 fused to full length Ezh2 and another fusion with only the SET domain of Ezh2, a writer of H3K27me3. They found that only the full length of Ezh2 could deposit H3K27me3 at the targeted promoter and that the SET domain alone could not deposit other H3K9me2 or H3K9me3 marks, suggesting that the rest of the Ezh2 residues besides the SET domain are necessary to produce the exact H3K27me3. Our fusion consists of the dCas9 with the SET catalytic domain alone. However, our results showed that our fusion could either inhibit or activate depending on the scenery or the expression lines. Therefore, we advance different conjectures: (1) our dCas9^{SET} is inhibiting in some cases because of the steric interference with other regulatory elements; (2) because of the lack of the entire AtCLF protein that directs the deposition of methylation marks, our dCas9^{SET} domain could be depositing other marks; for example, H3K4me, H3K36me and H3K79 me are associated with active transcription.

Was dCas9 able to induce Drought Stress response?

Previous studies by Park et al. (2017) showed that CRISPRa with VP64 activators of *AVP1* resulted in similar phenotypes as AVP1 over-expressors and enhancement of drought stress response. However, no physiological measurements were accomplished. Our study revealed that CRISPRa of AREB1 with dCas9 fusion to HAT domain resulted in a better drought stress tolerance during water withdrawal and after rehydration (**Figure 18, Figure S3**). Chlorophyll degradation can be used as an indicator of drought stress response as it leads to ROS production and cell death (Hörtensteiner et al., 2011; Misyura et al., 2013). Chlorophyll contents were higher in dCas9^{HAT} line after 4h severe stress and 20 days in soil stress, suggesting that dCas9^{HAT}-sgA could inhibit chlorophyll degradation during drought stresses. Yoshida et a. (2010) stated that AREB1 may be partially associated with stomatal closure under conditions of drought stress. Our data showed that all plants (transformed and control) close their stomates after severe drought stress very rapidly, but interestingly dCas9^{HAT}-sgA2 line closed the stomates faster than the rest of the plants, reinforcing the drought stress response of these line. Finally, dCas9^{HAT}-sgA2 plants were the only ones to remain alive after dehydration and rehydration. All these

results confirm that the increase in *AREB1* expression mediated by a dCas9^{HAT} guided by sgRNAs to p*AREB1* could mediate drought stress response.

CRISPRi/a results are difficult to anticipate

Our study presented unexpected results at different layers. In the transient assay for GmUcesMin, while for dCas9^{VPR} we observed a synergistic effect for the combination of sgRNAs, we only observed an increase for dCas9^{HAT} for individual sgRNAs. We also expected less that we would obtain an activation with the dCas9^{SET} in GmUcesMin and inhibition in GmUces when using a combination of two sgRNAs (**Figure 14**). In the same line of thought, dCas9^{SET}-sgA lines presented total chlorophyll than the control after 4 hours of stress, while they did not show this behavior after 20 days of stress. As stated before, the unexpected results with dCas9^{SET} could be explained because of the non-specific deposition of methylation marks, but other more complex factors should be considered. CRISPRi/a is a synthetic mechanism relying on the interaction of an engineered protein that does not exist in the host organism with chromatin structure and other protein complexes. Chromatin on its side has a complex structure with different states regulated by diverse biochemical and biophysical mechanisms. In conjunction with promoter regulation, the genome carries enhancer regions that also contribute to transcription regulation. The genome has a three-dimensional structure that has roles in the organization and function of the nucleus (Pombo et al., 2015; Keung et al., 2015; Bonev et al., 2016). This complexity makes that synthetically modifying this structure can produce unexpected results. Future studies should focus on understanding the relation between the fusions of dCas9 with the 3D structure and the proteins around chromatin if biotechnological products are to be created. For example, in an interesting approach, Dreissig et al., (2017) utilized the dCas9 fused to eGFP/mRuby2 to visualize telomeric movements in live leaf cells of *N. benthamiana.*

Concluding remarks

To date, the common strategy is based in the introduction of one only gene preceded by strong constitutive promoters, strategy that already showed positive results with the amelioration of many drought-resistant crops with enhanced parameters such as survivability, relative yield, biomass and others (Hu and Xiong, 2014). However, this strategy is limited because it relies on modifying only one pathway in a non-controlled manner. The possibility to use dCas9 fusions with different sgRNAs targeting diverse promoters opens the opportunity to expand the enhanced traits or tune at different points in a pathway. Besides engineering the Cas9, other groups used a synergestic activation mediator (SAM), a modification of the sgRNA to form a scaffold RNA (scRNA) containing an MS2, PP7, com or PUF binding site RNA

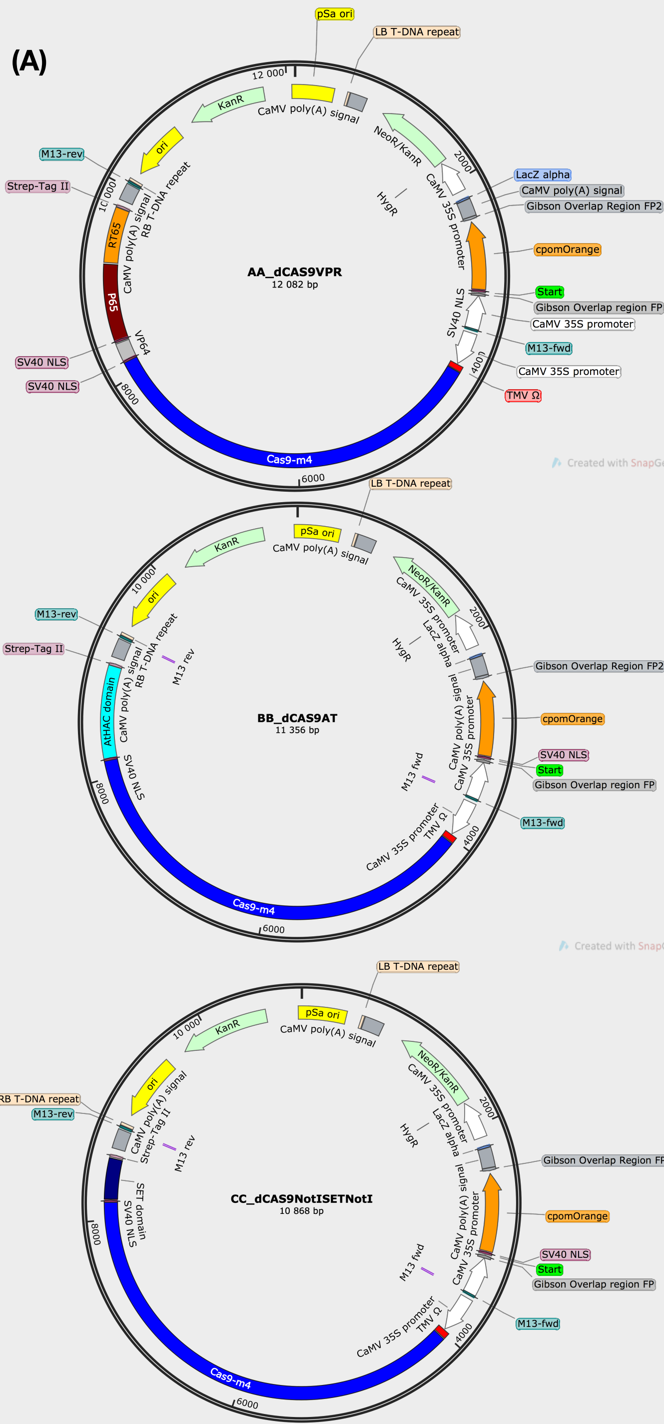
hairpins, that each recruit other activator or repressor proteins. These mechanisms have been labelled as CRISPR2.0 and allow to target and tune up/down different promoters at the same in the same cell (Konermann et al., 2013; Zalatan et al., 2015) or even for more complex regulatory devices and circuits with on/off systems (Nielsen and Voigt, 2014; Kiani et al., 2014). Our approach is among the first ones used in plants to propose solutions for the obstacles that agriculture will face in the future.

5. Supplemental Information

Table S1. sgRNAs used during this study

sgRNA	Target name	Target sequence	PAM	Location (from TSS)
sgRNA 1	GmUcesMin/GmUces	GATTGATTTAAATCAATTTT	AGG	-107 bp
sgRNA 2	GmUcesMin	GCAAAATGTCCCTTTTTGGT	TGG	-9 bp
sgRNA 3	GmUces	GATACGCGTTCAGATCTGAA	AGG	+226 bp
sgRNA-AREB1_1	AREB1 promoter	GTTCAGATCCAGTTATTAGG	TGG	-25 bp
sgRNA-AREB1_2	AREB1 promoter	GGATTTGTCCAAGCAACATT	TGG	-883bp

(A)



(B)

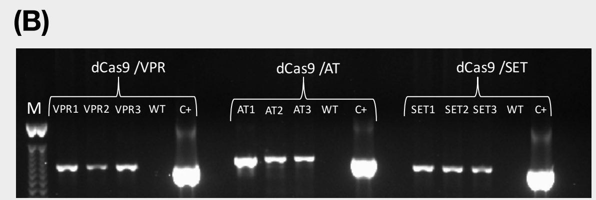
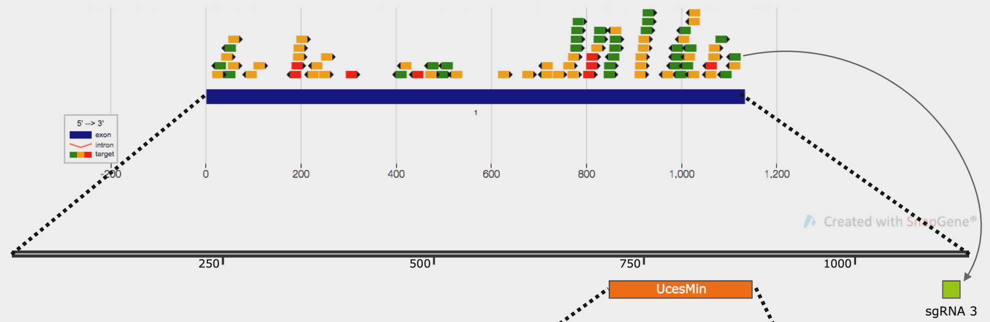
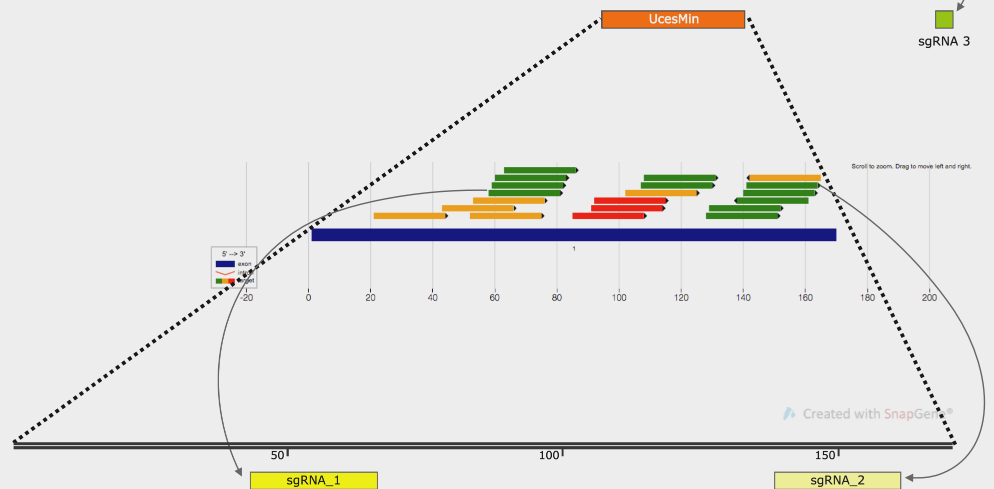


Figure S1. (A) Plasmid maps of the three dCas9 fusion constructs (B) PCR amplification of dCas9 and the three fusions.

UceSmax promoter



UceSmin promoter



AREB1 promoter

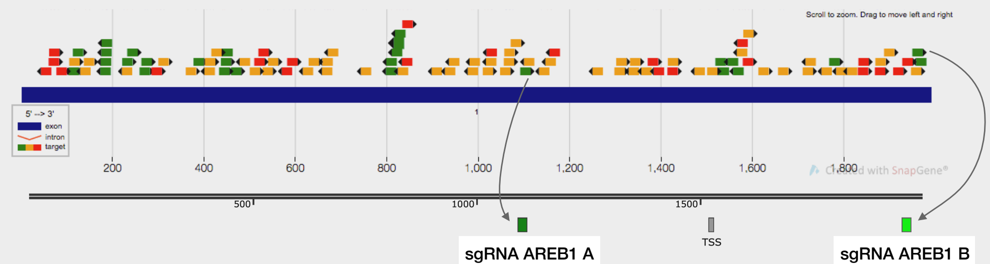
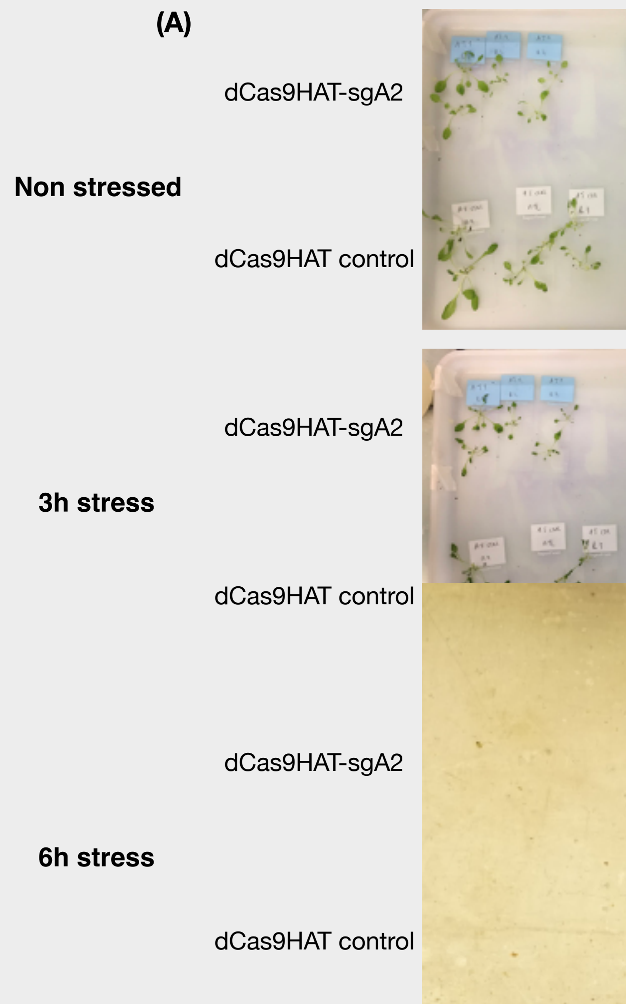


Figure S2. sgRNA design and position. (A) for UcesMax promoter where sgRNA3 is close to TSS, (B) for UcesMin promoter where sgRNA 1 is distant to TSS and shared with UcesMax and sgRNA 2 which is close to UcesMin TSS. (C) sgRNA_AREB1_1 and sgRNA_AREB1_2 design at close and distant regions from the TSS in the AREB1 promoter. Images show all possible sgRNAs as presented by **web tool inhttp://chopchop.cbu.uib.no/.** Color code represent rankings of sgRNAs scored by different ranking factors green (best), amber (okay), and red (bad).



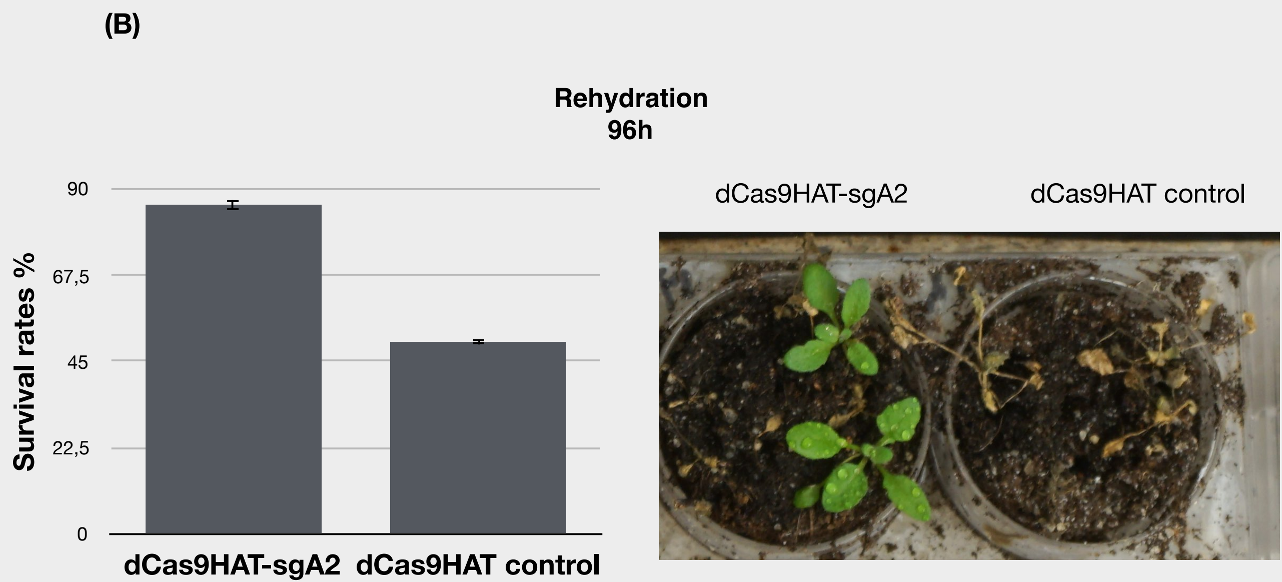
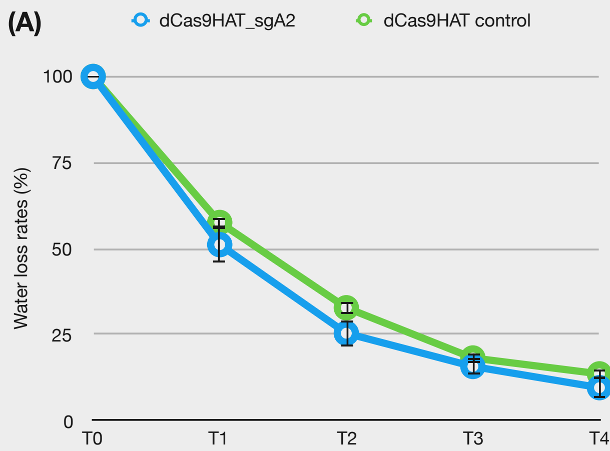
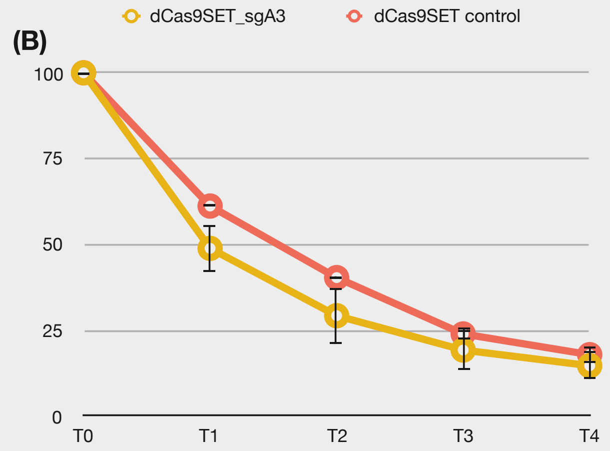
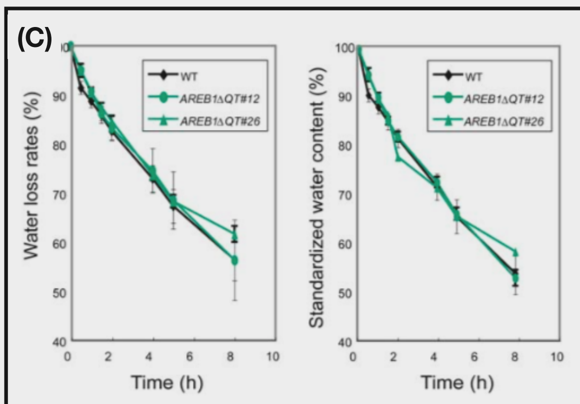


Figure S3. Severe drought stress applied in dCas9HAT-sgA2 plants. (A) Pictures of experiment. (B) Percentages and pictures of Survival rates after 96 h rehydration







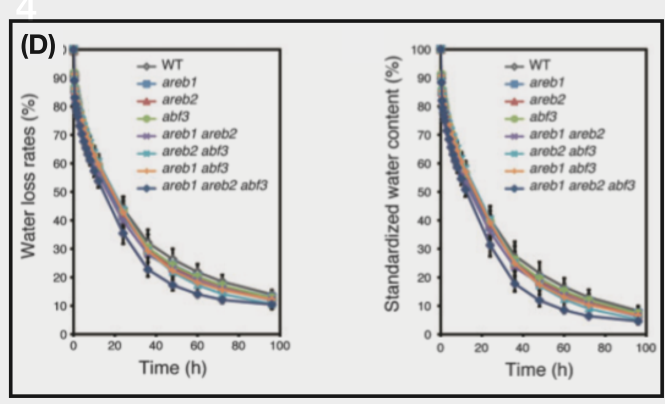


Figure S4. (A, B) (C, D) Water loss assays performed by Fujita et al. (2005) and Yoshida et al. (2010) in 35S-AREB1DQT and *areb1* mutants. The results are similar to the ones from dCas9-AT-sgA and dCas9-SET lines in Fig. 19. (C) stomatal closure after ABA treatment in *A. Thaliana* plants.

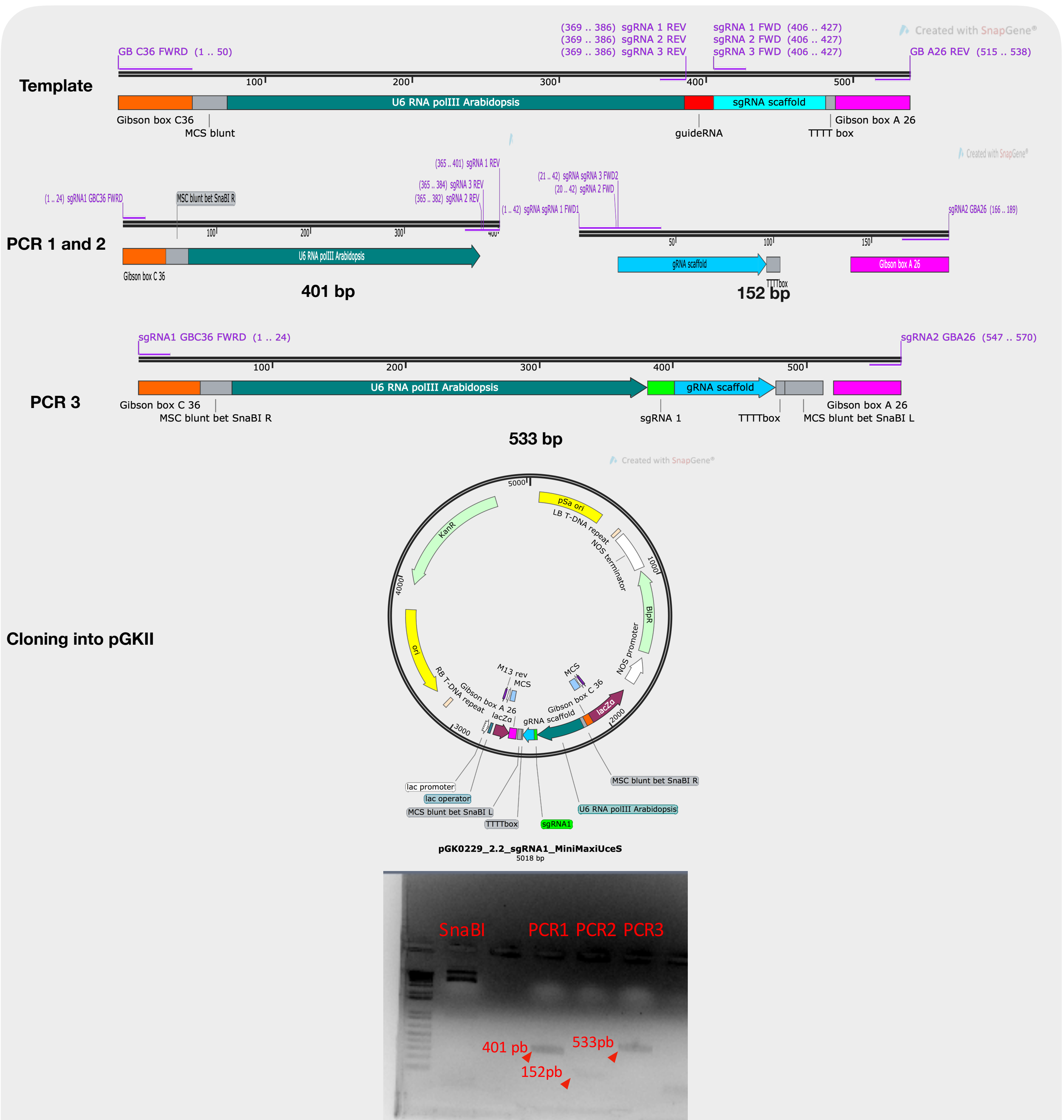


Figure S5. sgRNA synthesis ad cloning. Using a pre-existing sgRNA (Epoch) as template, PCR 1 (GBC36 FWRD-sRNAx REV) and PCR 2 (sgRNAx FWRD-GBA26) amplify two fragments containing the new synthesized sgRNA. PCR 3 (GBC36 FWRD-GBA26 REV) is performed to generate one fragment from the PCR 1 and 2 templates PCR 3 fragment was cloned in a *SnabI* linear pGK((vector.

6. Publication

Considering that we observed good results for the lines containing dCas9^{HAT}, we decided to submit a first publication concerning only the activation with the HAT domain.

Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with an Histone Acetyl Transferase

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Abstract

Drought episodes decrease plant growth and productivity that in turn represent an elevated economic cost. Plants naturally sense and respond to water stress by activating specific signaling pathways leading to physiological and developmental adaptations. Genetic manipulations of the genes belonging to these pathways could improve the tolerance of plants against drought. The ABA-responsive element binding protein 1 (AREB1)/ABF2 (ABRE binding factor) is a key positive regulator of the drought stress response. We investigated whether a CRISPR activation (CRISPRa) system that targets AREB1 might contribute to improve drought stress tolerance in Arabidopsis. The Arabidopsis histone acetyltransferase 1 (AtHAT1) promotes gene expression activation by switching the chromatin into a relaxed state. We generated stable transgenic plants expressing the chimeric dCas9-HAT. We first showed that the CRISPRa dCas9-HAT mechanism increased the activity of the promoter controlling the beta-glucuronidase reporter gene. We next set up the CRISPRa dCas9-HAT system to activate the endogenous promoter of *AREB1*. Plants showed a dwarf phenotype. Our qRT-PCR experiments indicated that both *AREB1* and *RD29A*, a gene positively regulated by AREB1, exhibited higher gene expression compared to the control. Plants maintained higher chlorophyll content and stomatal aperture under water deficit in addition to a better

survival rate after a drought episode. Altogether we report that CRISPRa dCas9-HAT is a valuable biotechnological tool to improve drought stress tolerance through the positive regulation of AREB1.

Keywords: Drought stress, CRISPRa, dCas9, Epigenetics, Histone Acetyltransferase

Introduction

Improving agronomical traits in plants against biotic and abiotic stresses to increase their economic value is a perennial concern. The consciousness about climate change and global warning embodies the need to carry out some efficient and sustainable solutions. In several countries, a current important issue is to maintain plant performance while facing a drought episode (Lobell et al., 2007, 2012; Zhang et al., 2013; Alizadeh et al., 2014). Drought varies spatially, temporally and in strength. Accordingly, plants have diversified their response and evolved toward the emergence of multiple morphological and physiological behaviors (Fahad et al., 2017, Tiwari et al., 2017). These behaviors comprise the combination at different degrees of drought escape, avoidance and tolerance. One driving motivation in drought resistance crop management is to harness genetic traits that improve these behaviors whilst ensuring high agronomical value. Several efforts have been made in this field of research. Conventional breeding and transgenic approaches showed improved drought stress tolerance in plants such as maize, soybean, rice and wheat (Ashraf, 2009; Wani et al., 2017).

Transcriptional studies have begun deciphering the cellular pathways that orchestrate the drought stress response (Nakashima et al., 2014; Singh and Laxmi, 2015). Physiological traits of drought resistance are mainly driven by hormonal control in plants. Abscissic acid (ABA) is a key regulator of drought stress response in plants by controlling stomatal closure (to prevent water losses by transpiration) and regulation of gene expression (Osakabe et al., 2014). The bZIP transcription factors, termed ABA-responsive element binding proteins (AREB)/ABF (ABRE binding factor), are important determinant in ABA signaling (Nakashima et al., 2014). The over-expression of *AREB1* (also named *ABF2*) showed enhanced drought stress tolerance in Arabidopsis, rice and soybean, while *areb1* loss of function displayed drought stress sensitivity (Oh et al., 2005; Fujita et al., 2005, 2013; Yoshida et al., 2010; Barbosa et al., 2013; Todaka et al., 2015). Indeed, AREB1 regulates a large set of genes downstream of the ABA signaling in response to drought stress (Yoshida et al., 2010), which participates in osmotic stress protection, ABA biosynthesis or antioxidant signaling (Fuijta et al., 2005; Li et al., 2013). Thus, *AREB1* represents one attractive candidate gene to improve the drought stress response.

With the promising avenues of research in targeted plant genome editing using nucleases, the possibilities to create new plant varieties to overcome the different challenges and demands have notably

augmented (Kumar and Jain, 2014; Abdallah et al., 2016; Malzahn et al., 2017). Beyond genome editing, CRISPR is currently repurposed for genome to accomplish CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) (Brocken et al., 2017). The catalytically inactive form of Cas9 (dead Cas9, dCas9 for short) could be engineered in fusion with some chromatin modulator domains allowing a change in the chromatin conformation (Hilton et al., 2015; Liu et al., 2016; Dominguez et al., 2016). Histone acetyltransferase (HAT) catalyzes the acetylation of core histones through the addition of an acetyl group to the lysine residue on the terminal tail of histones (Schneider et al., 2013). Histone acetylation triggers DNA relaxation and leads to the exposition of the DNA to the transcriptional machinery (Eberharter and Decker, 2002). Thus, the HAT activity is correlated to gene expression activation. In such a way, the use of dCas9 in fusion with HAT (dCas9^{HAT}) and combined with the directed targeting of sgRNA's appears promising for positively regulating a targeted gene promoter activity (Bordoli et al., 2001; Deng et al., 2007). Exploring the possibility of CRISPR/dCas9 epigenome editing represents an important advance and opens the door for more flexible approaches to tune gene expression by directly targeting specific promoters of desired genes of interest.

In our study, we asked whether an engineered dCas9^{HAT} could efficiently enhance *AREB1* gene expression in *Arabidopsis thaliana* in response to drought stress. We first generated stable Arabidopsis transgenic lines expressing dCas9 in fusion with the core catalytic domain of an Arabidopsis HAT. We next validated our CRISPR system using sgRNA targeting a GUS reporter system. Finally, we transformed our dCas9^{HAT} with a construct containing sgRNAs that allowed targeting of the *AtAREB1* promoter and observed by a molecular and physiological approach an enhanced response to drought stress in these transgenic plants.

Results and Discussion

Generation of Arabidopsis transgenic lines expressing dCas9^{HAT}

We first designed the dCas9^{HAT} construct to assay transcriptional regulation of the gene of interest. The catalytic core from the Arabidopsis *Histone Acetyl Transferase 1* gene (*AtHAC1*, AT1G79000) was fused in the n-terminal part of the dCas9 and cloned in a modified version of the plant binary vector pGreenII (Figure 1A, table S1). The T-DNA was designed to perform two rounds of plant transgenic selection based on antibiotic resistance (i.e kanamycine) and the level of a fluorescence reporter gene (i.e a nuclear mOrange fluorescent protein, mOFP). Once positive transformants were selected, fluorescence intensity was assessed by confocal microscopy (Figure 1B). The fluorescence corresponding to mOFP could be seen all over the roots and leaves, at higher intensity in the nucleus. Some lines displayed stronger fluorescence, suggesting that the cassette was inserted in an active region of the genome for

gene expression. Among these lines, the occurrence of the dCas9 construct was checked by PCR (Figure S1), and their expression by qPCR (Figure 1C). The transgenic lines dCas9^{HAT} number 2 had the highest mOFP and dCas9 expression and was retained for this study (named thereafter dCas9^{HAT}).

Challenging the dCas9 constructs in a GUS reporter system

To evaluate the dCas9 constructs, we set up a surrogate reporter system based on the regulation of the uidA (GUS encoding for the β-glucuronidase) reporter gene expression. We used a 170-bp minimal truncated version of a *Glycine max* ubiquitin promoter (accession number) herein designated GmUcesMin (Fragoso et al., non-published; Zhang et al., 2015). We selected two sgRNAs nearby to the transcription start site (TSS) of GmUcesMin (Figure 2A, table S2). The efficiency of the dCas9^{HAT} in activating the uidA reporter system was quantified by GUS enzymatic activity. Seedlings of stably transformed Arabidopsis lines expressing dCas9^{HAT} were incubated with *Agrobacterium* carrying Ti plasmids to perform a transient ectopic expression of GmUcesMin-uidA in combination with the expression of a single or two sgRNAs. The higher enzymatic activity was significantly observed for sgRNA1 with a ~2,4-fold increase and ~2-fold significant increase for sgRNA2, while it was of ~1,4 fold higher for the combination of the two sgRNAs. This result indicates that the expression of dCas9^{HAT}-sgA enhanced in *trans* the GUS gene expression. Remarkably some substantial differences were noticed depending on the location of the sgRNA and/or their combination. Previous studies suggested that the distance of the specific sgRNA from TSS might influence the transcription regulation of the gene of interest. Whether some studies have reported that a specific sgRNA binding distance from TSS corroborates with higher target gene expression (Gilbert et al., 2014; Radzisheuskaya et al., 2016), others have pointed that dCas9 might generate steric hindrance, and thus interfere transcriptional machinery activities (Larson et al., 2013; Hilton et al, 2015).

We performed the expression profile of GmUcesMin-iudA by GUS staining (Figure 2C). Since dCas9^{HAT} and the sgRNA are supposed to be broadly expressed in the whole *Arabidopsis* plant, we would expect to observe GUS activity in any tissue. Conversely, GUS activity was restricted in roots and was higher in transgenic plants compared to the control. Hence the transgenic plants differ from the control only by their intensity. The mapping of different regulatory boxes in GmUcesMin led us to hypothesize that some root specific transcription activators might trigger GUS expression specifically in roots (Figure S2). These results suggest that dCas9^{HAT} in combination with sgRNA enhances gene expression but do not activate their expression in other tissues. Notably Zhang et al. (2015) previously reported that the full length of GmUcesMin (called GmScreamM2, lenght 1391 bp) triggers gene expression principally in soybean seeds. It is tempting to postulate that the truncated form of GmUcesMin or our expression conditions might favor the expression of a target gene in roots.

Molecular and phenotypic characterization of dCas9^{HAT}-sgA

We next inquired whether AREB1 gene expression could be regulated by dCas9^{HAT}. We designed two sgRNAs to target the endogenous promoter of AtAREB1 (Figure 3A). One is located at 3' from the TSS (-479 bp) and the second is in the 5'UTR (+356 bp). The two sgRNAs (sg-pAREB1.1 and sg-pAREB1.2) were cloned in tandem within a single T-DNA and transformed in the *Arabidopsis* transgenic lines dCas9^{HAT} to generate the dCas9^{HAT}-sgA. We verified the *AREB1* gene expression by real time qPCR in three transgenic lines (Figure 3B). In each experiment, the control is referred to the parental line dCas9^{HAT}. We observed a slight but significant increase in *AREB1* expression of 1.7-fold for dCas9^{HAT}-sgA1 line and 2-fold for dCas9^{HAT}-sgA2 line compared to the control, suggesting that targeting dCas9^{HAT} to the *AREB1* gene could trigger its reading. *RD29A* gene is positively regulated by AREB1 and is a reference reporter to monitor AREB1 activity (Fujita et al., 2005; Msanne et al., 2011). We found that *RD29A* gene expression is significantly higher in the three dCas9^{HAT}-sgA2 lines. The dCas9^{HAT}-sgA2 line 2 had the highest expression (6-fold increase) and was selected for further morphological and physiological analyses. Three weeks after germination, the rosette diameter in dCas9^{HAT}-sgA2 plants was ~3 fold smaller than the control (Figure 4A, Figure S3A). The leaves length, width and area in dCas9^{HAT}-sgA were respectively ~1.5, ~1.3, and ~1.9-fold shorter than the control (Figure S3 B, C), suggesting the mutant caused a dwarf phenotype under normal plant growth conditions. Altogether these results revealed some similarities to plant phenotype responding to drought stress. Without water deficit, *AREB1* is slightly positively regulated indicating that dCas9^{HAT} activates *AREB1* beyond a drought context. The activation of promoters through H3K27 acetylation of histones employing dCas9$^{p300\ Core}$ domains has been evaluated in animal cells, showing to be more potent than activation domains (de Groote et al., 2012; Cheng et al., 2013; Hilton et al., 2015). Interestingly, previous studies reported that the over-expression of histone deacetylases (HDACs) had a role in ABA response, and that the use of an HDAC inhibitor induced hyperacetylation and increased *AREB1* gene expression in peanut (Sridah et al., 2006; Chen Su et al., 2015). Our findings suggest that histone acetylation by dCas9^{HAT} in *AREB1* loci is a determinant parameter in its expression and allows local chromatin rearrangement.

dCas9^{HAT}-sgA plants have enhanced drought stress tolerance

We next analyzed the efficiency of dCas9^{HAT} activator at a molecular level by measuring the gene expression of *AREB1* and *RD29A* under drought stress. AREB1 and *RD29A* gene expression was significantly increased by ~2-fold and ~2,6-fold in dCas9^{HAT}-sgA2 plants (Figure 4 A). This data suggest that the drought stress response is enhanced in dCas9^{HAT}-sgA2 line. We next investigated the physiological traits of transgenic Arabidopsis seedlings during drought. We performed two drought

conditions. A severe drought stress (SDS) consisted in removing plants from soil and kept on plates at 20% humidity on the bench. In another experiment, a mild-severe stress (MSDS) consisted to a water withdrawal up to 20 days. One physiological indicator of the drought stress is the inactivation of the photosystem II followed by a loss of the chlorophyll fluorescence (Chen et al., 2016). The measurement of the chlorophyll fluorescence was used to monitor the plant drought stress response. When plants were normally irrigated, the amounts of chlorophyll were comparable between dCas9^{HAT}-sgA2 and the control (Figure 4B). In contrast, 4h after SDS, the amount of total chlorophyll was 1.7-fold higher in dCas9^{HAT}-sgA2. After twenty days of MSDS, dCas9^{HAT}-sgA2 plants had a significant higher content of chlorophyll (1.3 fold) than the control. Our findings likely indicate that the activity of the photosystem is higher under drought stress in dCas9^{HAT}-sgA2.

Another characteristic of the drought stress response is a decrease in the stomatal aperture to limit water loss (Daszkowska-Golec and Szarejko, 2013). We measured stomatal width and length by confocal images, and calculated stomatal aperture (width/length) in non-stressed versus stressed plants after SDS (1h, 2h) and MSDS. In dCas9^{HAT}-sgA2 without drought stress, the stomatal aperture was comparable to the control. However, 1h after drought stress, the value was 1.6-fold lower in dCas9^{HAT}-sgA2 plants than in the control (Figure 4 C). After 2h, the stomatal aperture was still significantly lower in dCas9^{HAT}-sgA2, even though control plants closed the stomata between 1h and 2h of stress. Yet, our data revealed that stomatal closure is triggered more rapidly in dCas9^{HAT}-sgA plants. After 20 days of stress the stomatal aperture was comparable with the control, suggesting that dCas9^{HAT}-sgA2 does not regulate differently the stomatal aperture during a long-term drought stress. Our results indicate that the expression of dCas9^{HAT}-sgA2 led to a faster stomatal closure after a severe drought stress and corroborates with a previous study stating that AREB1 may partially be associated with stomatal closure (Yoshida et al., 2010).

Finally, we performed a survival assay consisting to analyze the ability of transformed plants to recover after SDS and MSDS (Fig 4 E; Figure S5). When SDS was applied and after 48h of rehydration, we observed that 85% of dCas9^{HAT}-sgA2 had a significant total recovery whereas only 50% of control plants were still alive. However, after 96h of re-hydration, only the transgenic plants showed a total recovery (Fig. 4 E). When we submitted the plants to MSDS and then rehydrated for 48h, we observed that all the transgenic plants survived, whereas all the controls died (Figure S5). Altogether, our data indicate that dCas9^{HAT} directed to AREB1 loci improve Arabidopsis drought response.

Conclusion

Further than using modulators to aid the recruitment of RNA polymerase (Pol II) transcription machinery, the use of domains that modify the chromatin folding is also an interesting mean to fine-tune gene expression. The expression of the dCas9^{HAT} could unwind the chromatin and allow the formation of the transcriptional machinery. This approach has been demonstrated in animals and we herein report its feasibility in plants. Our main result is that dCas9^{HAT} positively regulates AREB1 and provides an enhanced drought stress response. It is noteworthy that dCas9^{HAT} activity depends on the cellular context. The enhancer effect of dCas9^{HAT} is stronger when the drought stress response is activated. The finding suggests that the chromatin folding in AREB1 locus constitutes a regulatory mechanism for AREB1 gene expression. We also notify that GUS expression varies according to the position and the number of sgRNAs used. Having a better understanding of the chromatin context for a specific locus will help for the rational design of CRISPRi/a strategies.

Experimental procedures

Plasmid construction and sgRNA design

The sequence coding for *A. thaliana* acetyltransferase domain were gathered from TAIR, from the protein HAC1 (AT1G79000- Amino acids (AA) between 1119 to 1408), synthesized (EPOCH) and cloned in a vector containing the sequence coding for the dCas9 ((plasmid #63802, George Church lab). The cassette containing dCAS9-HAT was cloned into pGREEN KII (pGKII_dCas9-VPR) vector containing Neo/KanR selection and a sequence coding for the orange fluorescent protein (OFP). The sgRNAs were designed and screened using chopchop (http://chopchop.cbu.uib.no). The promoter sequence of AREB1 (AT1G45249.3) was subtracted from SeqViewer tool in TAIR site and the sgRNAs targeting AREB1 promoter (sg-pAREB1.1 and sg-pAREB1.2) were designed at -479 bp bp and +356 bp from the predicted TSS (approximately 25 bp downstream TATA box). A second vector was designed containing two cassettes for sgRNA expression, with sg-pAREB1.1 and sg-pAREB1.2 controlled by the U6 promoter, and containing a sgRNA scaffold. Box sequences were designed at the edges and the middle of each sgRNA template, called Gibson boxes (Gibson, 2009) to facilitate amplification and cloning. The sgRNAs targeting GmUcesMin promoter were chosen to have two different locations around the TSS. The sgRNA templates were amplified in a three step PCR with a pair of primers in the Gibson boxes (extremities), and a second

pair spanning the new 20-nt sgRNA and 20-nt in the template (Table S3). Each sgRNA template was sub-cloned into *SmaI* linearized pGKII0229.

Plant material, growth conditions and stable transformation

Col-0 *A. thaliana* seeds were surface-sterilized and germinated *in vitro* in Murashige and Skoog (MS) medium including vitamins (with or without selection agents). After stratification (2 days at 4 °C), they were germinated and grown under a 12-hour photoperiod growth chamber at 21 °C. Alternatively, plants were sown on soil/sand mixture, stratified and grown under 16h photoperiod in a growth chamber at 21 °C. The constructs were transferred into *Agrobacterium tumefaciens* GV3101 via heat-shock (30 min at 0°C, 5 min at 37°C and 2 h at 28° C in Luria-Bertani (LB) medium) (Holsters et al., 1978). The floral dip method was used to transform the *A. thaliana* plants (Clough and Bent, 1998).

Transient expression in *A. thaliana* seedlings

Transient expression in Arabidopsis seedlings was adapted from the FAST and AGROBEST protocols (Li et al., 2017; Wu et al., 2014). Briefly, 20 seedlings were stratified and germinated in ½ MS in 96 well plates. After 4 days in growth chamber (12h photoperiod, 21 °C), seedlings were co-incubated with *Agrobacterium tumefasciens* strain GV3101 (OD600 = 0,2) carrying different constructions allowing the expression of GmUcesMin-GUS, sg-UcesMin.1 and/or sg-UcesMin.2. The co-culture medium consisted to a combination of 50% ½ MS, 0,25% sucrose and 50% ABMES salts medium (17,2 mM K_2HPO_4, 8,3 mM NaH_2PO_4, 18,7 mM NH_4Cl, 2 mM KCl, 1,25 mM $MgSO_4$, 100 uM $CaCl_2$, 10 µM $FeSO_4$, 50mM MES, 2% Glucose, 200 µM acetosyringone (extemporaneously) at pH 5,5). The ratio of *A. tumefaciens* for each construct was identical corresponding to a third of the bacterial population in the inoculum. In the experimental conditions testing the expression of one single RNA or the mock control (without sgRNA), the corresponding *A. tumefaciens* strain was replaced by that expressing the empty vector. After two days of co-cultivation, a part of the seedlings (5 from 20) were washed in distilled water for GUS staining. The rest of the seedlings were put on MS with Carbenicillin (100 µg/mL) for 1 day and then collected for GUS activity assay.

GUS staining and GUS activity assays

β-glucoronidase (GUS) histochemical staining was performed by adding X-Gluc solution (2mM X-Gluc ((5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid) in 50 mM Na_2HPO_4, pH 7.0 and 0,1% Triton X-100) to *A. thaliana* seedlings for 10 hours at 37 °C. Seedlings were discolored in ethanol 70% overnight and observed using bright field optics under a digital camera (Olympus MVX10). GUS activity was monitored

by a fluorometric GUS enzymatic assay adapted from Jefferon et al., 1989. The total soluble protein was extracted with a GUS extraction buffer (50mM sodium phosphate buffer, 10mM EDTA, 0,1% Sarcosil, and 0,1% Triton X-100; 10mM β-mercaptoethanol) and quantified using the Bradford Method (Bradford, 1976). The assay was carried out by adding 20 µg of total soluble protein with 1mM 4-MUG (4-methyl-umbelliferyl-glucuronide) fluorogenic substrate (excitation: 356 nm; emission: 455nm) and sampling 200 µL into a stop buffer (Sodium carbonate 0,2M) at different times. A standard dilution curve was accomplished with different concentrations of 4MU (4-methyl umbelliferone). The fluorescence was measured using the fluorometer Spectramax M3 (Molecular devices).

***In silico* analysis of GmUcesMin promoter**

The presence of cis-acting elements GmUcesMin promoter was examined using the bioinformatics tool MatInspector version 8.0 (Genomatix®) using "plants" as matrix group, "0.85" as the value for the similarity of the main bases that constitute each cis-acting element (core similarity), and "Optimized +1" as the value for the similarity matrix (similarity matrix) (Cartharius et al., 2005).

Gene expression

RNA extractions from *Arabidopsis thaliana* leaves were performed with Concert ™ Plant RNA Reagent kit protocol (Invitrogen) following the manufacturer's protocol.

For cDNA synthesis, the following reagents were added in a 0,2-mL tube: 1 µg total RNA, 1 µL of 10µM NV-dT30 (2µM), 1µl of 10mM dNTP (2mM), Milli-Q water q.s.p 12µL. The reaction was then incubated at a temperature of 65°C for 5 minutes. Then 4 µl of Buffer First Stand Buffer 5X, 0.1M DTT and 1 µl of RNAse Out were added and the reaction was incubated at a 37°C for 2 minutes. Finally, 1 µL of the MMLV enzyme was added to the reaction followed by incubation at 37°C for 50 minutes and inactivation of the reaction for 15 minutes at 70°C. The cDNAs obtained in the previous step were diluted 1:20 and used for analysis of the differential expression of *AREB1*, *RD29A* and *dCAS9* genes by real-time PCR in biological and experimental triplicates. The qPCR reaction followed the following proportions: 2 µL of cDNA (1:20), 5 µL of 2X GoTaq qPCR Master Mix Sybr Green and 0.5 µL of each pimer and 2 µL of water to a final volume of 10 µL in CFX96 Biorad equipment. GAPDH (AT1G16300) and Actine2 (AT3G18780) were used as reference genes for relative quantification with the $2^{(\Delta\Delta Ct)}$ method (Pfaffl, 2001).

Drought stress, morphologic and physiologic measurements

Transgenic *A. thaliana* seeds were sown in pots completed with the same amount of soil/sand, and watered always with the same volume of water. After one week, plants were transferred to pots containing

three transformed plants and three control plants. For mild-severe drought stress (MSDS), approximately after 3-4 weeks, soil was water-saturated and plants were kept without watering. After 25 days, plants were rehydrated for survival rate measurements. Rosette radius measurements and scanning of the leaves for leaf morphology were performed in three-weeks-old plants. Leaf morphology was performed as described by Maloof et al. (2013) with the aid of LeafJ plugin for ImageJ (Schneider et al., 2012). A more severe water withdrawal (SDS) was performed by removing whole plants or cutting the rosettes and placing them on the bench for different amount of times depending on the experiment. Whole rosette dehydration during SDS was achieved by cutting rosettes of three-weeks-old plants and weighing hourly. For survival rates, whole plants were removed from soil without harming the roots and SDS for was applied for 6h in the bench. Plants that survived were counted after rehydration for 48h. Stomatal aperture was assessed from stressed-leaves for a period of 2h, 4h or 20 days. Confocal images of stomata were acquired with equal settings and processed with Zeiss confocal fluorescence microscope 100M using the software package LSM 510 version 3.2. The width and the length of the stomatal aperture were captured by confocal and measured by the ratio width to length. For chlorophyll content, total chlorophyll was extracted from leaves after treatment of 24h in 80% acetone. After centrifugation at 12,000g for 5 min, absorbance was measured at wavelengths 645 and 663 nm with a spectrophotometer. Chlorophyll concentration was estimated following Arnon's equations (Chlorophyll a (μg/mL) = 12.7 (A663) − 2.69 (A645); Chlorophyll b (μg/mL) = 22.9 (A645) − 4.68 (A663); Total chlorophyll (μg/mL) = 20.2 (A645) + 8.02 (A663)).

Legends of the figures

Figure 1. Molecular characterization of transgenic *A. thaliana* dCas9^{HAT} lines. (A) Schematic representation of the construct allowing the selection of *A. thaliana* dCas9^{HAT} lines. Kan^{R}: kanamycine resistant gene; mOFP: monomeric Orange fluorescent protein; NLS: nuclear localization signal. (B) Fluorescence microscopy imaging of *A. thaliana* leaves, primordia and roots expressing the mOFP in nucleus in three dCas9^{HAT} lines and compared with Col0 plants. All confocal images were acquired under identical parameters (excitation. 549 nm /emission 565 nm). (C) Assessment of the dCas9^{HAT} expression. RT-qPCR was performed in Col0 and three lines of dCas9^{HAT} transformed plants. Transcript expression levels were normalized against the geometric mean of the housekeeping genes (GADPH and Actin2). Mean and standard deviation (SD) were calculated from three independent biological replicates. The values were plotted relative to the lowest expression value (excluding Col0 plants). Asterisks indicate significant differences between Col0 and the different lines (Student's T-test P values, $^{**}P < 0.01$).

Figure 2. Challenging dCas9^{HAT} in a GUS reporter system. (A) Schematic representation of the GmUcesMin promoter with the sgRNA positions (black- white squares). The TATA-box is represented by a black square. Black and white curved arrows represent TSS and ATG codon respectively. (B) Arabidopsis seedlings from stably transformed dCas9^{HAT} fusions were transiently transformed with GmUcesMin in combinations of different sgRNAs. The results represent means and SD of 6 independent experiments in which were pooled n=20 seedlings for each. Statistical analysis was performed using Student's t-test to confirm significance of GUS activity for each dCas9 fusion guided by one sgRNA or a combination of two sgRNAs compared to Mock controls. Asterisks* represent values of p<0,05. Bars indicate standard error. (C) GUS staining was assayed for the same seedlings in GmUcesMin promoter.

Figure 3. Challenging dCa9^{HAT} constructs towards the regulation transcription of AREB1 by targeting pAREB1. (A) Schematic representation of pAREB1 with the two sgRNAs designed. The TATA-box is represented by a black square. (B) Relative expression of *AREB1* and (C) *RD29A* genes in non-stressed plants. Transcript expression levels were normalized against the geometric mean of the housekeeping genes (GADPH and Actin2). Means and SD were obtained from three biological replicates. Asterisks indicate significant differences between Col-0 and transgenic plants (Student's t-test **P<0,01). For each gene, the expression level in the dCas9^{HAT} control was defined as calibrator (1.0). (D) Phenotypic analysis of dCas9^{HAT}-sgA. Rosette diameter of three-week-old plants. Results represent the mean of n=13. dCas9HAT control plants are represented by a – sign and dCas9^{HAT}sgA2 plants by + sign. Statistical analysis was performed using Student's t-test to confirm significance of dCas9-sgA lines compared to dCas9 control lines. Asterisk represent values of p<0,05. Bars indicate standard error.

Figure 4. Molecular and physiological analyses of drought stress response in dCas9^{HAT}sgA. Transcript levels of (A) *AREB1* and (B) *RD29A* in dCas9^{HAT} and dCas9^{HAT}-sgA2 plants during drought stress. Transcript expression levels were normalized against the geometric mean of the housekeeping genes (GADPH and Actin2). Means and SD were obtained from three biological replicates. Asterisks indicate significant differences between the control and transformed plants (Student's t-test **P<0,01). For each gene, the expression level in the dCas9^{HAT} control was defined as calibrator (1.0). (C) Total chlorophyll contents in non-stressed plants, 4 hours after SDS and after MSDS. Results represent mean of n=6. (D) Stomatal aperture measurements after 2, 4-hour severe stress and 20 days of drought stress. Results

represent means of n=30. Statistical analysis was performed using Student's t-test co-transformed plants to control lines. Asterisks* represent values of p<0,05. Bars indicate standard error. (E) Survival rates after 6h SDS and rehydration for 48h and 96h. Results represent percentage of survived plants from n=20. Asterisk indicates significant difference between the control dCas9^{HAT} and dCas9^{HAT}-sgA2 plants (chi-square test *P<0,05). dCas9^{HAT} control plants are represented by a – sign and dCas9^{HAT}sgA2 plants by + sign.

Figure S1. Detection of positive dCas9^{HAT} Arabidopsis plants via PCR. Genomic DNA was extracted from the leaves of selected plants to amplify the region between the dCas9 sequence and the HAT domain sequence (1214 bp amplicon. C-, negative control; C+, positive control (PCR template is the original dCas9^{HAT} expression cassette used for plant transformation; M, 1.0-kb ladder (INVITROGEN" Cat. # 10787018);

Figure S2. *In silico* analysis of GmcUcesMin promoter. The sequence of GmUcesmin was analyzed with MatInspector version 8.0, Genomatix® tool and regulatory boxes were schematized.

Figure S3. Dwarf phenotype of dCas9^{HAT}-sgA2 plants. (A) Image of 3-week-old dCas9^{HAT}-sgA2 plants. (B) Morphological measurements made with LeafJ plugin for IMageJ in of the scanned leaves (right) of dCas9^{HAT}sgA2 plants. Results represent data of leaf#7 of n=2 plants. Bars indicate standard error.

Figure S4. Images of dCas9^{HAT}-sgA2 and dCas9^{HAT} control plants submitted to MSDS and re-hydrated for 48h.

References

Abdallah, N.A., Prakash, C.S., McHughen, A.G., 2015. Genome editing for crop improvement: Challenges and opportunities. GM Crops & Food 6, 183–205. https://doi.org/10.1080/21645698.2015.1129937

Alizadeh, V., Shokri, V., Soltani, A., Yousefi, M.A., n.d. Effects of Climate Change and Drought-Stress on Plant Physiology 5.

Ashraf, M., 2010. Inducing drought tolerance in plants: Recent advances. Biotechnology Advances 28, 169–183. https://doi.org/10.1016/j.biotechadv.2009.11.005

Barbosa, E.G.G., Leite, J.P., Marin, S.R.R., Marinho, J.P., de Fátima Corrêa Carvalho, J., Fuganti-Pagliarini, R., Farias, J.R.B., Neumaier, N., Marcelino-Guimarães, F.C., de Oliveira, M.C.N., Yamaguchi-Shinozaki, K., Nakashima, K., Maruyama, K., Kanamori, N., Fujita, Y., Yoshida, T., Nepomuceno, A.L., 2013. Overexpression of the ABA-Dependent AREB1 Transcription Factor from Arabidopsis thaliana Improves Soybean Tolerance to Water Deficit. Plant Molecular Biology Reporter 31, 719–730. https://doi.org/10.1007/s11105-012-0541-4

Bordoli, L., 2001. Plant orthologs of p300/CBP: conservation of a core domain in metazoan p300/CBP acetyltransferase-related proteins. Nucleic Acids Research 29, 589–597. https://doi.org/10.1093/nar/29.3.589

Brocken, D.J.W., Tark-Dame, M., Dame, R.T., 2018. dCas9: A Versatile Tool for Epigenome Editing. Current Issues in Molecular Biology 15–32. https://doi.org/10.21775/cimb.026.015

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., Werner, T., 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942. https://doi.org/10.1093/bioinformatics/bti473
Cheng, A.W., Jillette, N., Lee, P., Plaskon, D., Fujiwara, Y., Wang, W., Taghbalout, A., Wang, H., 2016. Casilio: a versatile CRISPR-Cas9-Pumilio hybrid for gene regulation and genomic labeling. Cell Research 26, 254–257. https://doi.org/10.1038/cr.2016.3
Cheng, A.W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T.W., Rangarajan, S., Shivalila, C.S., Dadon, D.B., Jaenisch, R., 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Research 23, 1163–1171. https://doi.org/10.1038/cr.2013.122
Clough, S.J., Bent, A.F., 1998. Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana: Floral dip transformation of Arabidopsis. The Plant Journal 16, 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x
Daszkowska-Golec, A., Szarejko, I., 2013. Open or Close the Gate – Stomata Action Under the Control of Phytohormones in Drought Stress Conditions. Frontiers in Plant Science 4. https://doi.org/10.3389/fpls.2013.00138
de Groote, M.L., Verschure, P.J., Rots, M.G., 2012. Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. Nucleic Acids Research 40, 10596–10613. https://doi.org/10.1093/nar/gks863
Deng, W., Liu, C., Pei, Y., Deng, X., Niu, L., Cao, X., 2007. Involvement of the Histone Acetyltransferase AtHAC1 in the Regulation of Flowering Time via Repression of FLOWERING LOCUS C in Arabidopsis. PLANT PHYSIOLOGY 143, 1660–1668. https://doi.org/10.1104/pp.106.095521
Dominguez, A. A., Lim, W. A., & Qi, L. S. (2016). Beyond editing: Repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nature Reviews Molecular Cell Biology*, *17*(1), 5–15. http://doi.org/10.1038/nrm.2015.2
Eberharter, A., 2002. Histone acetylation: a switch between repressive and permissive chromatin: Second in review series on chromatin dynamics. EMBO Reports 3, 224–229. https://doi.org/10.1093/embo-reports/kvf053
Fahad, S., Bajwa, A.A., Nazir, U., Anjum, S.A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M.Z., Alharby, H., Wu, C., Wang, D., Huang, J., 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. Frontiers in Plant Science 8. https://doi.org/10.3389/fpls.2017.01147
Fujita, Y., 2005. AREB1 Is a Transcription Activator of Novel ABRE-Dependent ABA Signaling That Enhances Drought Stress Tolerance in Arabidopsis. THE PLANT CELL ONLINE 17, 3470–3488. https://doi.org/10.1105/tpc.105.035659
Gibson, D.G., 2009. Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Research 37, 6984–6990. https://doi.org/10.1093/nar/gkp687
Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H., Guimaraes, C., Panning, B., Ploegh, H.L., Bassik, M.C., Qi, L.S., Kampmann, M., Weissman, J.S., 2014. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159, 647–661. https://doi.org/10.1016/j.cell.2014.09.029
Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E., Reddy, T.E., Gersbach, C.A., 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nature Biotechnology 33, 510–517. https://doi.org/10.1038/nbt.3199
Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M., Schell, J., 1978. Transfection and transformation of Agrobacterium tumefaciens. Molecular and General Genetics MGG 163, 181–187. https://doi.org/10.1007/BF00267408
Jefferson, R. A. (1989). The GUS reporter gene system. Nature, 342(6251), 837–838. http://doi.org/10.1038/342837a0
Kumar, V., Jain, M., 2015. The CRISPR–Cas system for plant genome editing: advances and opportunities. Journal of Experimental Botany 66, 47–57. https://doi.org/10.1093/jxb/eru429
Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nature Protocols 8, 2180–2196. https://doi.org/10.1038/nprot.2013.132
Li, J.F., Nebenfuhr, A., 2010. FAST Technique for Agrobacterium-Mediated Transient Gene Expression in Seedlings of Arabidopsis and Other Plant Species. Cold Spring Harbor Protocols 2010, pdb.prot5428-pdb.prot5428. https://doi.org/10.1101/pdb.prot5428
Li, X.-Y., Liu, X., Yao, Y., Li, Y.-H., Liu, S., He, C.-Y., Li, J.-M., Lin, Y.-Y., Li, L., 2013. Overexpression of Arachis hypogaea AREB1 Gene Enhances Drought Tolerance by Modulating ROS Scavenging and Maintaining Endogenous ABA Content. International Journal of Molecular Sciences 14, 12827–12842. https://doi.org/10.3390/ijms140612827
Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., … Jaenisch, R. (2016). Editing DNA Methylation in the Mammalian Genome. *Cell*, *167*(1), 233–247.e17. http://doi.org/10.1016/j.cell.2016.08.056
Lobell, D.B., Gourdji, S.M., 2012. The Influence of Climate Change on Global Crop Productivity. PLANT PHYSIOLOGY 160, 1686–1697. https://doi.org/10.1104/pp.112.208298

Malzahn, A., Lowder, L., Qi, Y., 2017. Plant genome editing with TALEN and CRISPR. Cell & Bioscience 7. https://doi.org/10.1186/s13578-017-0148-4
Msanne, J., Lin, J., Stone, J.M., Awada, T., 2011. Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. Planta 234, 97–107. https://doi.org/10.1007/s00425-011-1387-y
Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. Frontiers in Plant Science 5. https://doi.org/10.3389/fpls.2014.00170
Oh, S.-J., 2005. Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth. PLANT PHYSIOLOGY 138, 341–351. https://doi.org/10.1104/pp.104.059147
Osakabe, Y., Osakabe, K., Shinozaki, K., & Tran, L.-S. P. (2014). Response of plants to water stress. *Frontiers in Plant Science*, *5*(March), 86. http://doi.org/10.3389/fpls.2014.00086
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, 45e – 45. https://doi.org/10.1093/nar/29.9.e45
Radzisheuskaya, A., Shlyueva, D., Müller, I., Helin, K., 2016. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucleic Acids Research 44, e141–e141. https://doi.org/10.1093/nar/gkw583
Schneider, A., Chatterjee, S., Bousiges, O., Selvi, B.R., Swaminathan, A., Cassel, R., Blanc, F., Kundu, T.K., Boutillier, A.-L., 2013. Acetyltransferases (HATs) as Targets for Neurological Therapeutics. Neurotherapeutics 10, 568–588. https://doi.org/10.1007/s13311-013-0204-7
Singh, D., Laxmi, A., 2015. Transcriptional regulation of drought response: a tortuous network of transcriptional factors. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00895
Sridha, S., Wu, K., 2006. Identification of *AtHD2C* as a novel regulator of abscisic acid responses in Arabidopsis. The Plant Journal 46, 124–133. https://doi.org/10.1111/j.1365-313X.2006.02678.x
Su, L.-C., Deng, B., Liu, S., Li, L.-M., Hu, B., Zhong, Y.-T., Li, L., 2015. Isolation and characterization of an osmotic stress and ABA induced histone deacetylase in Arachis hygogaea. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00512
Tiwari, S., Lata, C., Chauhan, P.S., Prasad, V., Prasad, M., 2017. A Functional Genomic Perspective on Drought Signalling and its Crosstalk with Phytohormone-mediated Signalling Pathways in Plants. Current Genomics 18. https://doi.org/10.2174/1389202918666170605083319
Todaka, D., Shinozaki, K., Yamaguchi-Shinozaki, K., 2015. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00084
Wu, H.-Y., Liu, K.-H., Wang, Y.-C., Wu, J.-F., Chiu, W.-L., Chen, C.-Y., Wu, S.-H., Sheen, J., Lai, E.-M., 2014. AGROBEST: an efficient Agrobacterium-mediated transient expression method for versatile gene function analyses in Arabidopsis seedlings. Plant Methods 10, 19. https://doi.org/10.1186/1746-4811-10-19
Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K., 2010. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. The Plant Journal 61, 672–685. https://doi.org/10.1111/j.1365-313X.2009.04092.x
Zhang, N., McHale, L.K., Finer, J.J., 2015. Isolation and characterization of “GmScream” promoters that regulate highly expressing soybean (Glycine max Merr.) genes. Plant Science 241, 189–198. https://doi.org/10.1016/j.plantsci.2015.10.010
Zhang, X., Cai, X., 2013. Climate change impacts on global agricultural water deficit: MORE WATER NEEDED FOR AGRICULTURE? Geophysical Research Letters 40, 1111–1117. https://doi.org/10.1002/grl.50279

Figure 1

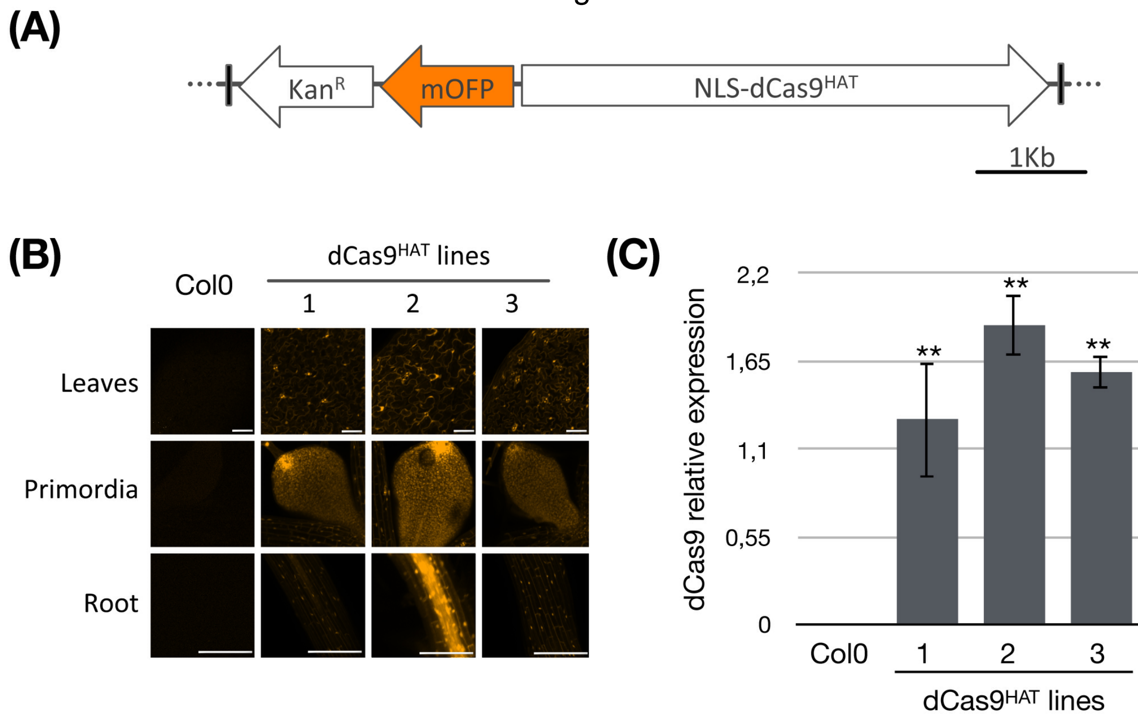


Figure 2

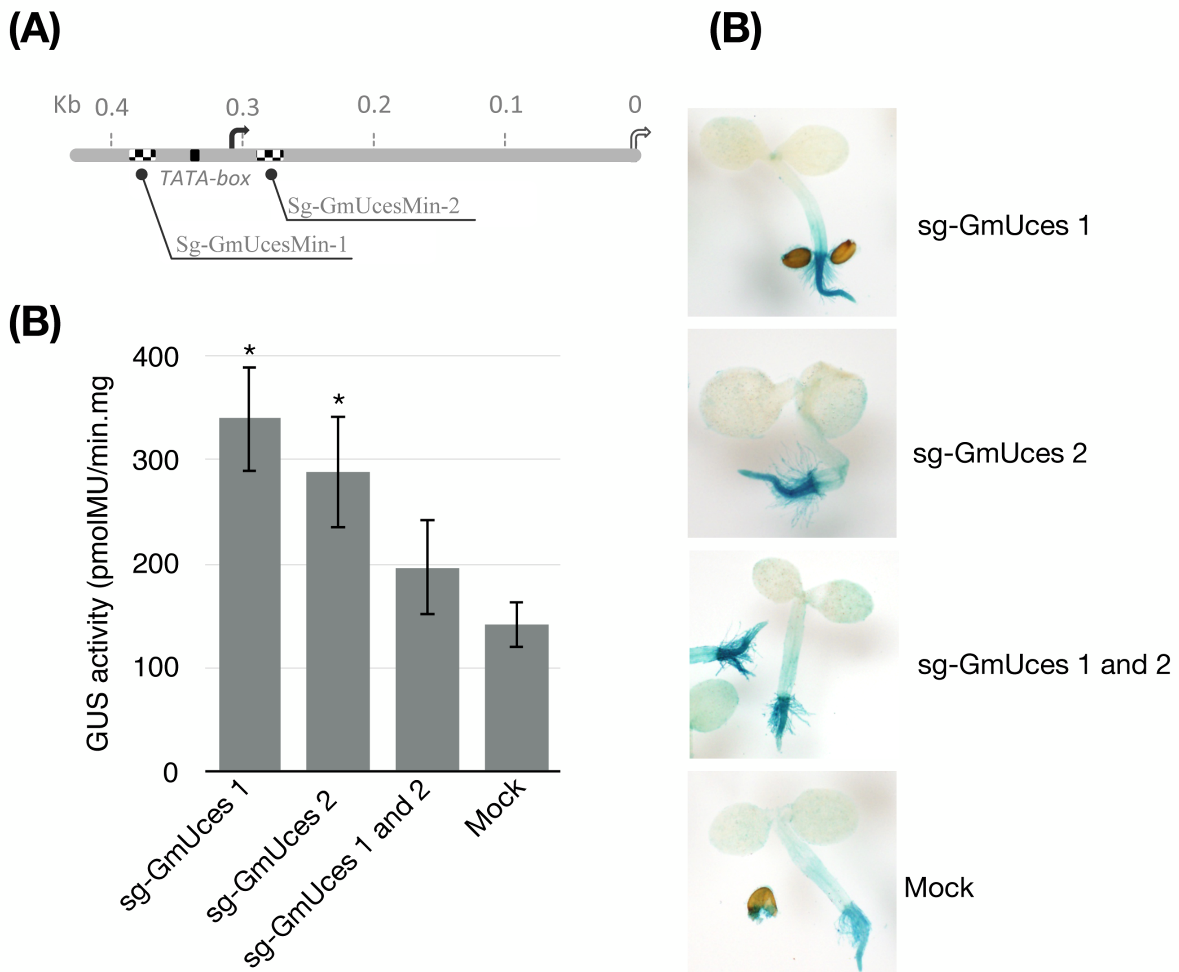


Figure 3

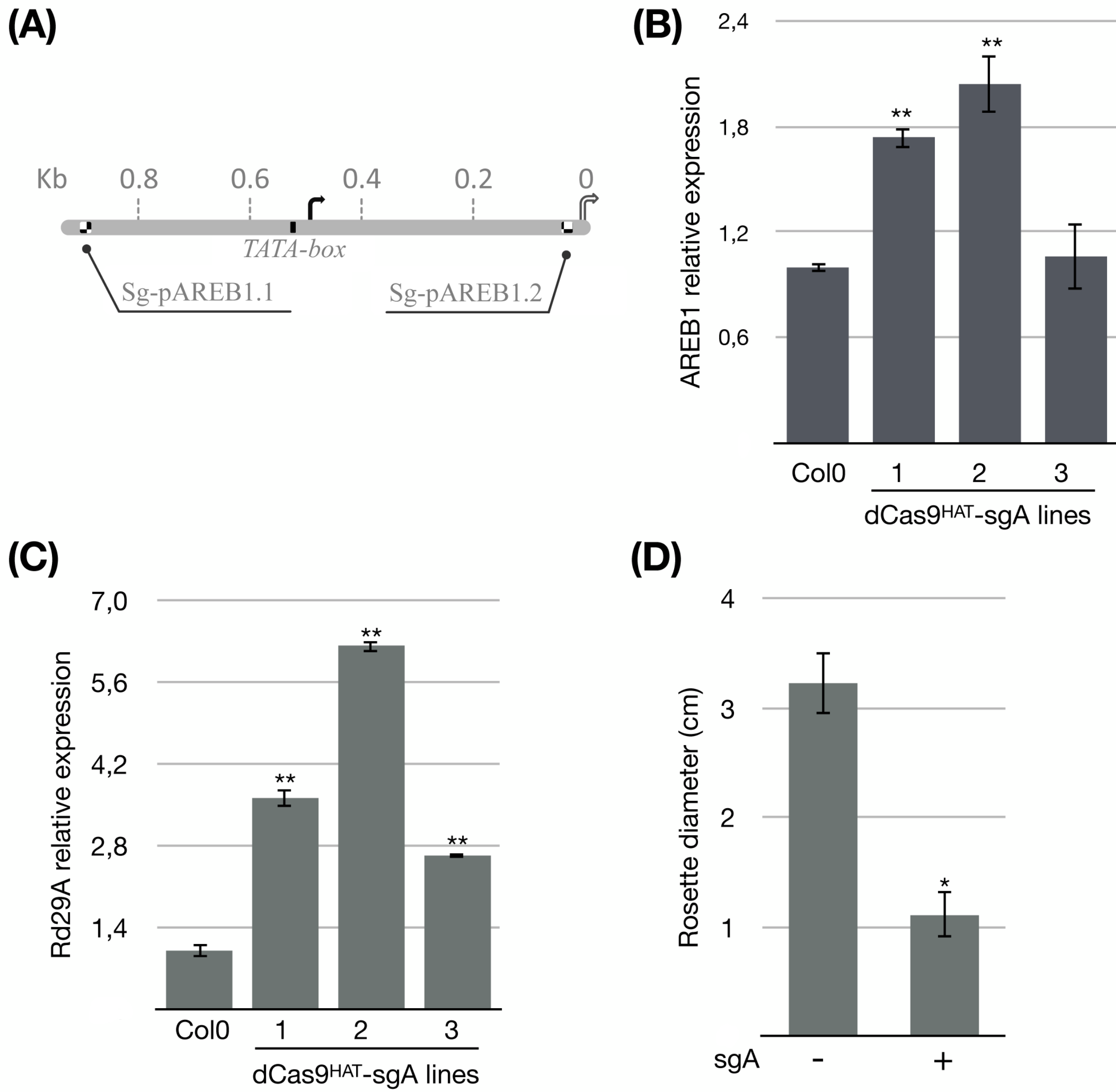
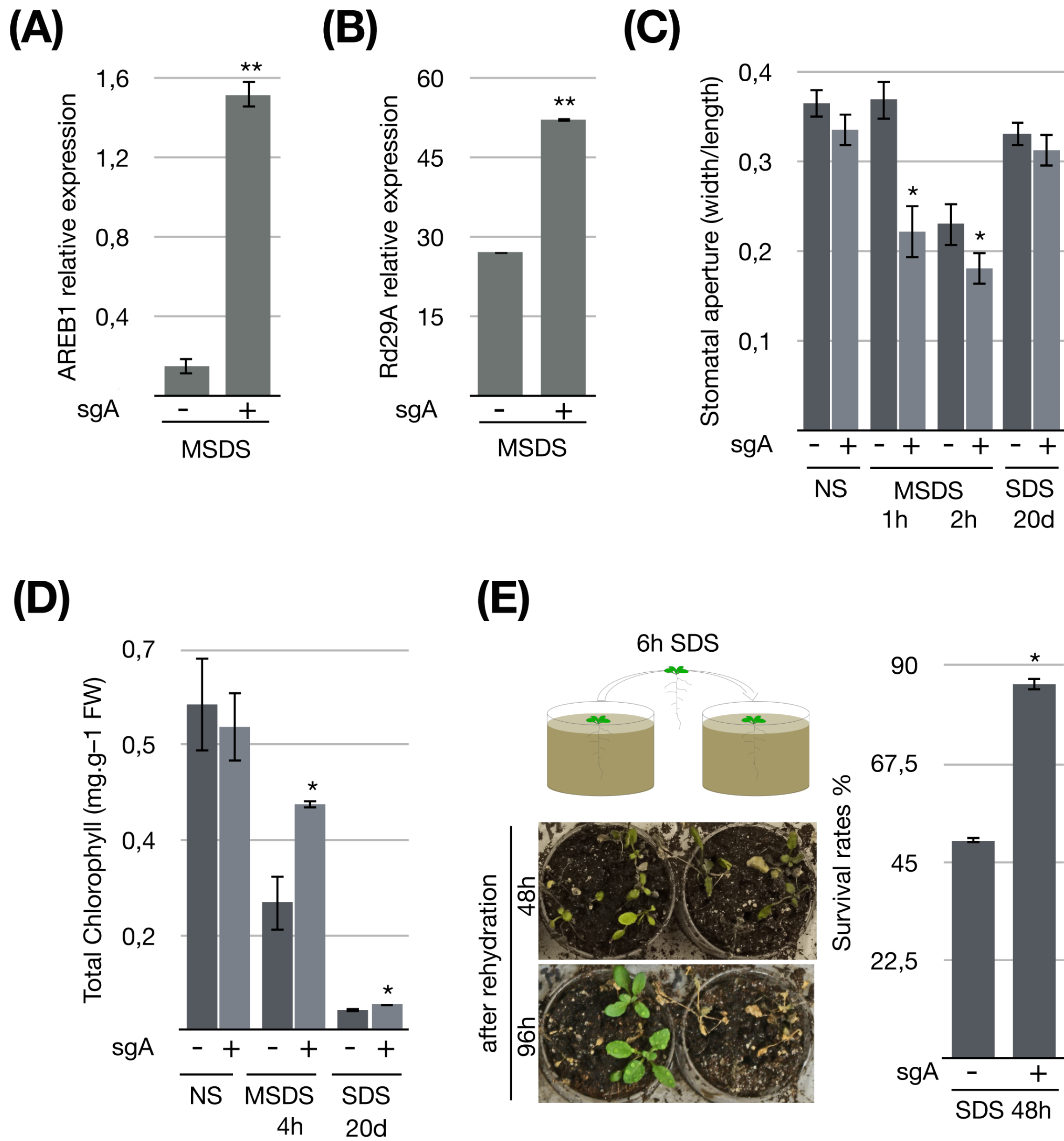


Figure 4



7. Experimental procedures

Plasmid construction and sgRNA design

The vector pAWG-dCas9-VPR was obtained at Addgene (plasmid #63802, George Church lab) containing the full-length sequence coding for the dCAS9 and the tripartite activator VPR (VP64-p65-RT65). The cassette containing dCAS9^{VPR} was cloned into a pGREEN KII (pGKII_dCas9-VPR) vector containing Neo/KanR selection and a sequence coding for an orange fluorescent protein (OFP). The sequences coding for *A. thaliana* acetyltransferase and methyltransferase domains were gathered from TAIR, from the proteins HAC1 (AT1G79000- Aminoacids (AA) between 1119 to 1408) and Curly leaf (AT2G23380.1 -AA between 751 to 871) respectively, synthesized by EPOCH and sub cloned in the position of the VPR domain in the pGKII_dCas9-VPR (**Figure S1**). A fourth vector was designed containing two cassettes for sgRNA expression, with sgRNA_AREB_A and sgRNA_AREB1_B controlled by U6 promoter, and containing a sgRNA scaffold. The sgRNAs were designed with the aid of the web tool in http://chopchop.cbu.uib.no (**Figure S2**). We predicted the TSS in the GmUcesmin GmUces by following the Y Patch and YR Rules, where the TSS is in GmUcesmin where the 5'UTR region was excluded (Yamamoto et al., 2007[a, b]). Promoter sequence of AREB1 (AT1G45249.3) was subtracted from SeqViewer tool in TAIR site and the sgRNAs targeting AREB1 promoter were designed at -25 bp and -883 from the TSS (transcription starting site). Box Sequences were designed at the edges and the middle of each sgRNA template, called Gibson boxes (Gibson, 2009) to facilitate amplification and cloning. The sgRNA templates were amplified in a three step PCR with a pair of primers in the Gibson boxes (extremities), and a second pair spanning the new 20 nt sgRNA and 20 nt in the template (**Figure S3, Table S1**). Each sgRNA template was sub-cloned into *SmaI* linearized pGKII0229.

Plant material, Growth conditions and stable transformation

Col-0 *A. thaliana* seeds were surface-sterilized and germinated in vitro in MS medium including vitamins (with or without selection agents). After stratification (2 days at 4 °C), they were germinated and grown under a 12-hour photoperiod growth chamber at 21 °C. Alternatively, plants were sown on soil/sand mixture, stratified and grown under 16h photoperiod in a growth chamber at 21 °C. The constructs were transferred into *Agrobacterium Tumefasciens* GV3101 via heat-shock (30 min at 0°C, 5 min at 37°C and 2 h at 28° C in LB medium) (Holsters et al., 1978). The Floral dip method was used to transform the *A. thaliana* plants (Clough and Bent, 2008).

Transient Expression in *Arabidopsis thaliana* seedlings

Transient expression in Arabidopsis seedlings was adapted from the FAST and AGROBEST protocols (Li et al., 2017; Wu et al., 2014). Briefly, 20 seedlings were stratified and germinated in 1/2 MS in 96 well plates. After 4 days in a growth chamber (12h photoperiod, 21 °C), seedlings were put in contact with the different *A. tumefasciens* carrying the different constructions, in a combination of 50% 1/2 Ms and 50% ABMES salts medium (17,2 mM K2HPO4, 8,3 mM NaH2PO4, 18,7 mM NH4Cl, 2 mM KCl, 1,25 mM MgSO4, 100 uM CaCl2, 10 μM FeSO4, 50mM MES, 2% Glucose, AS 200 uM (only before co-cultivation); pH 5,5) and 0,25% sucrose. After two days of co-cultivation, a part of the seedlings (5 from 20) were washed in distilled water for GUS staining. The rest of the seedlings were put on MS with Carbenicillin (100 μg/mL) for 1 day and then collected for GUS activity assay.

GUS staining and GUS activity assays

β-glucoronidase (GUS) histochemical staining was performed by adding 2mM X-Gluc solution (Galagher et al., 1992) to *A. thaliana* seedlings for 10 hours at 37 °C. The seedlings were discolored in EtOH 70% overnight and observations were carried out using bright field optics and images were acquired with a digital camera (Olympus MVX10). Fluorometric GUS enzymatic assay to measure GUS activity was adapted from Jefferon et al., 1989. The protein was extracted with GUS extraction buffer (50mM sodium phosphate buffer, 10mM EDTA, 0,1% Sarcosil, and 0,1% Triton X-100; β-mercaptoethanol was added to final concentration of 10mM) and quantified with the Bradford Method (Bradford, 1976). The assay was carried out by adding 20 μg of protein in 4-MUG fluorogenic substrate (1mM in GUS extraction buffer) and sampling 200 μL into Stop buffer (Sodium carbonate 0,2M) at different times. A standard dilution curve was accomplished with different concentrations of 4MU. The fluorescence was measured in a Spectramax M3 (Molecular devices).

RNA extraction cDNA synthesis and Gene expression

RNA extractions from Arabidopsis thaliana plants were performed according to the Concert ™ Plant RNA Reagent kit protocol (Invitrogen). 100 mg of fresh leaf tissue in liquid nitrogen were macerated in liquid nitrogen and transferred to 500 μL of the ice-cold Concert reagent (4 ° C), which were then homogenized in vortex. Subsequently the tubes were left at room temperature for five minutes. After this time, the material was centrifuged for two minutes at room temperature at a speed of 12,000 g, and the supernatant was transferred to a new tube. Then 100 μL of 5 M NaCl was added and in the sequence 300 μL of chloroform was added and the tubes were inverted for 5 X. To separate the phases, the samples were subjected to centrifugation of 12,000 g for ten minutes, at 4 ° C, and the upper aqueous phase was transferred to a new tube. Thereafter, a volume equivalent to the ice-cold isopropanol aqueous phase was added and homogenized by inversion for five seconds. Samples were kept at room temperature for ten minutes and then further centrifuged for ten minutes at 4 ° C and 12,000 g. The supernatant was discarded, the pellet was

washed with 1 mL of 75% ethanol and the tubes were centrifuged for 1 minute at room temperature (12,000 g). The residual liquid was removed from the tube with the pipette and the RNA was resuspended in 20 μl of autoclaved Milli-Q water. Samples were stored at -20 ° C. Integrity of the RNA was verified by 1% agarose gel electrophoresis and the RNA samples were quantified in nanodrop.

For cDNA synthesis, the following reagents were added in a 0,2 mL tube: 1μg of total RNA, 1μL of 10μM NV-dT30 (2μM), 1μl of 10mM dNTP (2mM), Milli-Q water q.s.p 12μL. The reaction was then incubated at a temperature of 65 ° C for 5 minutes. Then 4 μl of Buffer First Stand Buffer 5X, 0.1M DTT and 1 μl of RNAse Out were added and the reaction was incubated at a 37 ° C for 2 minutes. Finally, 1 μL of the MMLV enzyme was added to the reaction followed by incubation at 37 ° C for 50 minutes and inactivation of the reaction for 15 minutes at 70 ° C. The cDNAs obtained in the previous step were diluted 1:20 and used for analysis of the differential expression of areb, rd29 and dcas9 genes by real-time PCR in biological and experimental triplicates. The qPCR reaction followed the following proportions: 2 μL of cDNA (1:20), 5 μL of 2X GoTaq qPCR Master Mix Sybr Green and 0.5 μL of each pimer and 2 μL of water to a final volume of 10 μL in CFX96 Biorad equipment.

Drought stress, morphologic and physiologic measurements

Seeds from transformed *A. thaliana* were sown in pots containing the same amount of soil/sand, and watered always with the same volume of water. After one week, plants were transferred to pots containing three transformed plants and three control plants as shown in Figure S3. Approximately after 3-4 weeks, soil was saturated with water and plants were kept without watering. After 25 days, plants were rehydrated for survival rate measurements. Rosette radius measurements and scanning of the leaves for leaf morphology were performed in three-week-old plants. Leaf morphology was performed as described by Maloof et al. (2013) with the aid of LeafJ plugin for ImageJ. A more severe water withdrawal was performed by removing whole plants or cutting the rosettes and placing them on the bench for different amount of times depending on the experiment. Whole rosette dehydration during severe water withdrawal was achieved by cutting rosettes of three-week-old plants and weighing hourly. Survival rates were measured after severe dehydration for 6 hours in the bench and plants that survived were counted after rehydration for 48h. Stomatal aperture was assayed in leafs of plants stressed for 2h, 4h or 20 days, where leafs were collected and stomatal images were acquired with Confocal microscope. Width and length of Stomates were assessed with Zeiss software tools and stomatal aperture expressed by widht/lenght. For chlorophyll content, total chlorophyll was extracted from leaves after treatment of 24h in 80% acetone. After centrifugation at 12,000g for 5 min, absorbance at wavelengths 645 and 663 nm with a spectrophotometer. Chlorophyll concentration was estimated following Arnon's equations (Chlorophyll a (μg/mL) = 12.7 (A663) − 2.69 (A645); Chlorophyll b (μg/mL) = 22.9 (A645) − 4.68 (A663); Total chlorophyll (μg/mL) = 20.2 (A645) + 8.02 (A663)).

8. CRISPR Imagination

The rate of publications in CRISPR/Cas9 grew so vastly, making very difficult of a complete overview of all the publications. The hype of CRISPR/Cas9 proof of concepts is now beginning to cease, and laboratories around the world are using CRISPR/Cas9 as a tool, which traduces its puissance for both fundamental and applied research. Yet, I assumed interesting to finish to main chapter of this thesis by picturing some interesting and recent publications that used the technology in original or innovative approaches. The objective of this sub-part is not to make an extensive review of what has been published, but more to tinkle the reader's imagination by describing a few authentic research papers.

Inspired by the basal mechanism of bacterial immunity which acquires virus DNA information (protospacers) with the aid of CRISPR-cas adaptation proteins (Cas1 and Cas2) into a CRISPR array of spacers (**Figure 20)**, Shipman et al. (2016, 2017) described an experimental approach to record and recreate an image of a hand and then a digital movie into living Bacteria. In a first publication, they integrated chosen synthetic DNA sequences into *E. Coli* populations containing a plasmid containing the CRISPR array to allow the cells to record chronologically the fragments inserted. Interestingly, they saw that if including a PAM (AAG) in 5' of the integrated sequence, the protospacers were included in an oriented manner if making serial electroporation of the sequences (Shipman et al., 2016). This was the first step to create the first biological recorder, that they reused to record the information of a digitalized image of a human hand and a sequence of five frames of a galloping horse adapted from British photographer Eadweard Muybridge's *Human and Animal Locomotion* series (**Figure 20 A**). Succinctly, they designed two strategies to define the information: (1) a rigid system in which each one of the 4 bases (A, T, C, G) was assigned to 4 pixel color each or (2) a more flexible system in which a triplet of nucleotides encoded for one pixel color, giving the possibility to encode 21 possible colors. Then, they designed 28-base protospacers that defined a set of pixels, that they denoted pixet, with a PAM after each protospacer. The protospacers were then introduced as oligonucleotides in hairpin formats and electroplated in pools into *E. Coli* populations. The images were reconstructed by decoding the acquired protospacers by high-throughout sequencing. The galloping horse movie used the same strategy, yet by encoding five different images over the course of five days by electroplating frame after frame (Shipman et al., 2017). A few months later, a third publication displayed a similar approach to record temporal signals into a DNA substrate, allegorizing a tape recorder that records analog audio into recordable data in a tape. To achieve it, they inserted an inducible plasmid (pTrig) that replicated after an external signal into the same population of *E. Coli* containing a plasmid with Cas1-Cas2 and a CRISPR array (pRec). In the absence of an induction signal, the acquisition of spacers into pRec derived from pRec itself and other plasmids from the genome. In the presence of the induction signal, pTrig numbers were multiplied, and subsequently induced the inclusion of fragments of pTrig as spacers into pRec. They proved that they could record systematic time-course recordings over 4 days, giving the opportunity to record fluctuations of states in the cells, somehow record into DNA the story of a given cell (**Figure 20 B**) (Sheth et al., 2017).

Most part of the time we hear about the CRISPR advances in mammalian cells because of its repercussion in human health. However, CRISPR has attained labs that study all types of organisms. In

my opinion, the ants are the most fascinating beings, and they could not escape being modified with CRISPR technology. Ant genetics aim to reveal the molecular foundations of the collective behavior and advanced sociality of different ant species. Being that ant communication is based on pheromone communication, Yan et al. (2017) mutated the orco gene that encodes a co-receptor of an odorant receptor with the aid of CRISPR/Cas9 in *Harpegnathos saltator* species. They exploited the ability of *H. Saltator* female workers to become reproductive gamergates when a queen dies, by injecting CRISPR-sgRNA into embryos and converting the developed workers into mutated gamergate queens in the F0 generation. Thus, the next mutated (F1) haploid male generation were out- and back-crossed to obtain F4 heterozygous or homozygous queens. The mutation of orco gene displayed altered social behavior consistent with the loss of pheromone sensing like not perceiving the difference between the inside and outside of the nest among others, and *orco* mutants were unable to transition into gamergates (Friedman et al., 2017; Yan et al., 2017). This is only one of the examples of how this editing technology has revolutionized all fields of science, here representing an advance in the study of eusocial insects.

As mentioned before, the most renown publications in the CRISPR revolution are in the field of human health research, with the objective of curing genetic diseases. CRISPR has been used as genetic

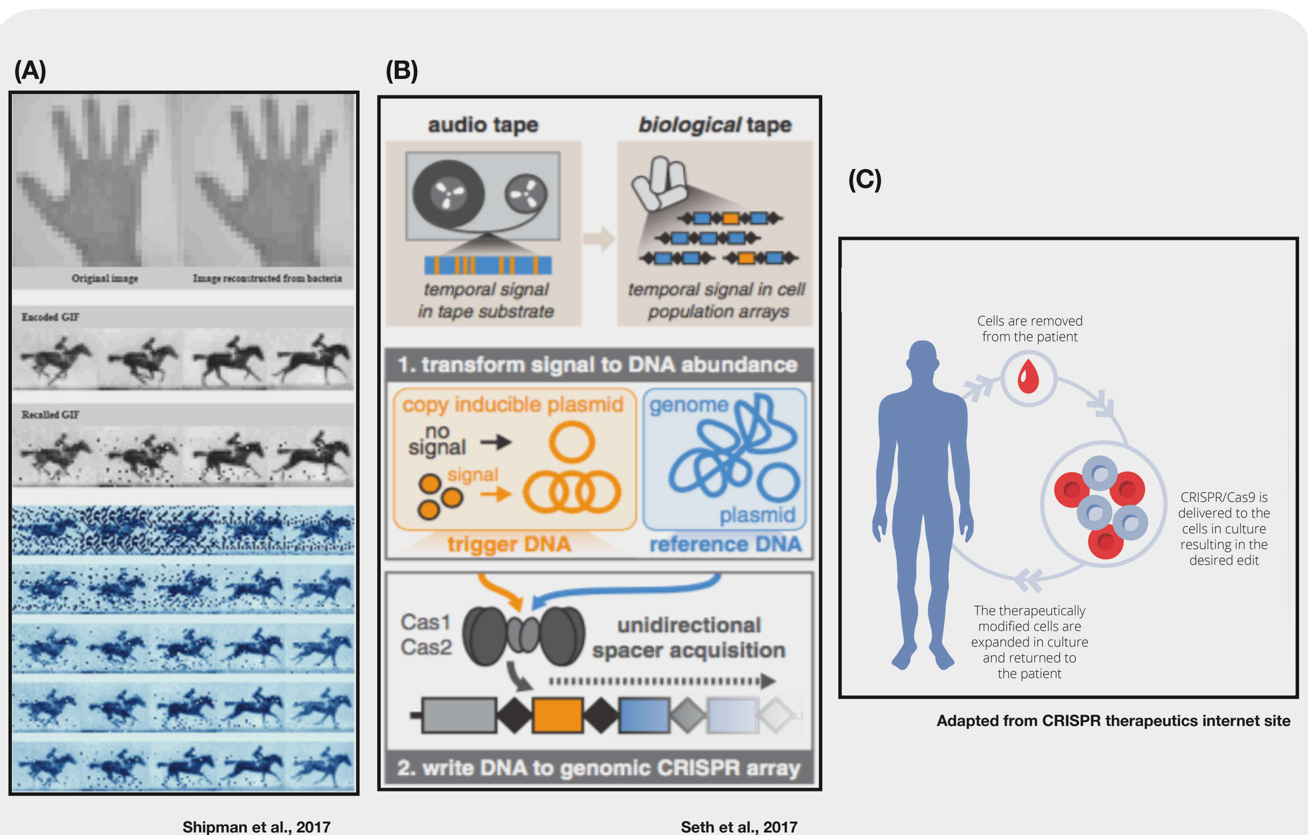


Figure 20. CRISPR novel publications and mechanisms. (A) Image of a hand and digital movie performed by Shipman et al. (2017) in Bacteria. (B) Biological tape implemented bu Sheth et al. (2017). (C) Strategy implemented in possible future therapy trials to mutate with CRISPR/Cas9 genome editing immune system cells from patients with cancer, Sickle Cell Disease and β-thalassaemia.

screening replacing RNAi and cDNA screens for CRISPR loss of function and gain of function libraries to identify essential genes that play roles, principally for cancer lethal genes. Discovering these genes has permitted to construct cell lines and animal models that carry disease genetic backgrounds in order to study them. Also, this model lines have been used to study gene therapy to cure them (Luo, 2016; Patel et al., 2017). Thus, delivery of CRISPR to mutate genes that cause the disease to restore a healthy form of the protein has been performed, giving positive results for diseases like Duchenne muscular distrophy, Cystic fibrosis, haemoglobinopathy, β-thalassaemia, Leber con- genital amaurosis, haemophilia and others (Baigioni et al., 2017). Recently, press news have been announcing the first human trials with CRISPR therapy in cancer patients in 2018. Though in china several therapies seem to be under way, no publications have been released at this day, as showed in clinicaltrial.gov . In occident, two major proposals were submitted to begin in 2018. The first one, from a group in the University of Pennsylvania, aims to treat autologous T cells from different cancer patients with CRISPR/Cas9 to mutate the genes TCRα, TCRβ (T-cell recPDeptors) and PD-1, proteins known to regulate anti-tumor activity of T-cells (Burr et al., 2017; https://clinicaltrials.gov/show/NCT03399448). The other company, « CRISPR therapeutics » in which Emmanuelle Charpentier is member of the scientific advisory team, intends to treat Sickle Cell Disease and β-thalassaemia disease by editing human primary CD34+ hematopoietic stem and progenitor cells (HSPCs) from sick patients. The point mutations or deletions will be made in genes that lead to the up-regulation of fetal hemoglobin (Hbf) to control this protein in patients with the pathology (Lin et al., 2017). The strategy will be similar in both trials, as showed in **Figure 20 C**, by removing the T-cells or CD34+ HSPCs, treating them with the CRISPR agents, and reinserting them into the patients.

To finish this sub-part about the CRISPR imagination, two very recent papers showed how the system evolves rapidly, making CRISPR possibilities to seem endless and infinite. Besides all the Cas and Cpf1 orthologs already described to edit DNA, the discovery of type VI CRISPR systems Cas13 proteins that have nucleotide-binding (HEPN) endoRNase domains that mediate precise RNA cleavage permitted the editing of RNA. In their paper, Cox et al. (2017) describe how they interfered with the mRNA levels in mammalian cells by using two Cas13 (PspCas13b and LwaCas13a). Moreover, in the same publication they used two engineered dCas13b fused to a adenosine deaminases acting on RNA (ADAR) that convert adenine into inosine (a nucleotide equivalent to guanosine). With these dCas13b-ADAR, that they referred as RNA editing pro Programmable A to I Replacement (REPAIR), they could edit transcripts containing pathogenic mutations. In comparison with DNA editing, their REPAIR system does not need a PAM preference, and thus can target any adenosine in the transcriptome. Also, they argue it will be useful in more temporary and reversible cases in which therapies require short term changes in gene expression, such as inflammations or organ transplants, where the rejection of the organ could be prevented over a temporary time.

The aim of this sub-part was to show how the constant publications in CRISPR can trigger a scientist's imagination by showing onPly some of the examples in recent advances with CRISPR technology. Reading this type of publications was the spark that convinced me that I wanted to enter into the CRISPR world, and even if not with such an important impact as the publications above, I will try to explain from now on what we did with the tools we had in the lab.

Chapter III - Transgenic cotton to induce drought stress tolerance and *M. Incognita* resistance

1.Cotton and drought stress

Cotton is one of the most important fibers producing crops but is also an important source of plant proteins and oil. China, U.S.A, India, Pakistan, and Brazil are the leading producing countries, and the world production in 2015 was of 25,9 million metric tons (Cotton Incorporated, 2015). In Brazil, the export of cotton represented more than US$ 482,117 million in 2015 (ABRAPA, 2015). Considering the social and economic importance of cotton, it will be worthwhile to improve its agronomical value using genetic engineering.

The first transgenic lines appeared in the 90's, and until today, the three major methods to transform cotton are A*grobacterium*-mediated, microparticle bombardment and the pollen tube pathway-mediated transformation (Zhang et al., 2016). The genotype is still the major constraint because of the limited number of cultivars that can be cultured in vitro (Zhang et al., 2016). Several transgenes have already

been developed to improve yield quality (Jian et al., 2011; Bajwa et al., 2015) or to reduce the toxicity of its seed relying on the gossypol, a cotton metabolite that plays important role in the cotton-environment interaction (Palle et al., 2012; Wagner et al., 2015; Tian et al., 2016).

Cotton is a glycophyte, has a C3 metabolism, and is considered a drought-tolerant crop, with a wide sensitivity variation among genotypes (Ullah et al., 2017). Still, cotton is often planted in dry regions from Egypt, China (Xinjiang), Brasil or India (**Figure 1, for regions in Brazil),** meaning it must be permanently irrigated and its culture faces high evaporation and salt accumulation in the soils (Soth et al., 1999). Many traits have been reported to be affected in cotton by drought stress, such as plant height, dry stem weight, leaf weight, area and node number, leading to an important decrease in yield (Loka, et al., 2011; Sahito et al., 2015; Yu et al., 2016; Ullah et al., 2017). Some cotton lines have been created to induce drought stress tolerance with stress proteins such as GHSP26 (Heat Shock Protein Gene), GUSP1 (Universal Stress Protein Gene), Phyto-B (Phytochrome-B Gene), AtRAV1 and AtRAV2, AtLOS5 (Yue et al., 2012; Shamim et al., 2012; Mittal et al., 2015), and to resist to other abiotic stresses (Luo et al., 2013; Juturu et al., 2015). Despite many efforts in the past, there is still a need to genetically improve cotton to adapt this plant to our agronomical constraints but also to climate changes.

2. Hypothesis and objectives

Considering the general introduction of this manuscript and the introduction of this chapter, in which is stated the need to answer to different stresses that cotton plants can endure, we ought to design a strategy to improve drought stress tolerance and *M. Incognita* infection at the same time.

The Q and T regions in N-terminal of AREB1 TF contain the regulatory domain of the transcription factor. The truncation of these domains leads to shape a constitutively active form of AREB1, called AREB1ΔQT. AREB1ΔQT mutants showed in improved tolerance to desiccation and constitutively activated the same genes as the AREB1-responsive pathway (Fujita et al., 2005). With that basis, it would be interesting to observe if the over-expression of this *Arabidopsis* TF's can activate the ABA-pathways in other species, such as the one in cotton. As a matter of fact, it has already been described that the over-expression of *SlAREB1*(tomato) in tomato, *AhAREB1* (*Arachis hypogaea*) in *Arabidopsis*, *AtAREB1* in soybean and *OsbZIP46/Abi5* in rice regulate and improve drought stress responses pathways, suggesting a high conservation of the pathway among different plants, and that it would be conceivable in cotton (Oh et al., 2005; Barbosa et al., 2013; Tang et al., 2012; Li et al., 2013; Bastías et al., 2014). The central role of AREB1, and more particularly its constitutive mutant *AREB1ΔQT*, in the ABA-mediated pathway in responses to drought stress, makes it a powerful candidate to manipulate it into cotton plants (**Figure 3**).

In 2006, Yadav et al. chose to target an *M. Incognita* splicing factor (SF) following three criteria: (a) Its *C. elegans* orthologs are essential genes and RNAi silencing could would result lethal; (b) Its functions are being conserved in diverse organisms, so the chance that their functions have been conserved in the parasitic nematodes is very high. (c) Its sequence is dissimilar enough that the RNAi is nematode specific. Importantly, they introduced the dsRNA targeting the SF into tobacco plants, observing an important decrease in the formation of root-knots, an interference with the normal development of the nematode, and a decrease of the SF's cDNA. Thus, targeting with an RNAi the *M. incognita*'s splicing factor appears to be an attractive hypothesis for an efficient and sustainable cotton resistance strategy.

Thus, to obtain cotton plants with an enhanced response to drought stress and *M. Incognita* infection, the gene encoding for the transcription factor *AREB1ΔQT* and an RNAi that targets a mRNA sequence of the *M. incognita* splicing factor were used, with both genes in the same vector construct. By over-expressing *AREB1ΔQT*, we expected to increase in intensity the ABA-mediated pathway to improve drought stress responses. Working with the *AREB1ΔQT* constitutive version might lead to anticipate drought stress all along the plant life. We also prospected to reinforce cotton resistance to *M. incognita* the growth within the feeding site relying on the RNAi strategy **(Figure 21 A**).

3 Results and discussion

3.1 Design of vectors and Transformation of cotton plants

To improve cotton plants with a better response to both drought stress and *M. incognita* infection, vector constructs were designed bearing both *AREB1ΔQT* and an RNAi targeting a Splicing factor (SF) mRNA from the nematode (**Figure. 21 B**). The sequence of *AREB1ΔQT* was put under the control of CaMV35S promoter. To design the RNAi, a region of 200 bp was selected from *M. incognita*'s splicing factor sequence with the aid of BLOCK-iTTM RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress). The 200 bp region was positioned in sense and antisense surrounding a PDK intron as in Yan et al. (2012). The RNAi sequence was put in control of *UceA1.7*, a cotton ubiquituitination-related promoter characterized for having important constitutive expression in roots and flower tissues (Viana et al., 2011). The sequences were synthesized and sub-cloned into vector pGS52123-1. To positively select the transformed plants, we used the barley acetohydroxy acid synthase gene (*AHAS*) that confers resistance to imidazolinone herbicides (**Figure 21**) (Lee et al., 2011).

The vector was multiplied and digested with *Bam*HI to release the cassette containing the construct (*AREB1ΔQT-FS*), and was inserted into BRS cedro cotton cultivar embryos by microparticle bombardment (Rech et al., 2008). We bombarded 2190 embryos that were put in a media with Imazapyr herbicide from which we could positively select 16 (0,75%) plants from the T0 generation. The major

Figure 21. Schematic representation of the strategy used in the transgenic cotton. (A) Representation of roots and aerial parts of non transformed (left) and transformed plants (right) and the expected effect on drought stress and nematode infection. (B) cassette inserted by micro particle bombardment; The AREB1ΔQT gene is in control of the CMV35S and the RNAi in control of UceA promoter. The AHAS gene confers resistance to herbicide Imazapyr. *BamHI* restriction site was used to isolate the cassette before bombardment.

problem encountered in this part of the work was the difficulty to control a fungus contamination, which wasted 40,7% of the embryos bombarded with *AREB1ΔQT-SF*. The contamination with this fungus started growing in the apical meristem of some embryos at the start of the culture, and spread all over the medium in one or two days. A sample of this organism was analyzed by the Biological control laboratory at Embrapa Cenargen, and the species Fusarium oxysporum f. sp. vasinfectum was identified. This fungus is one of the principal pathogen of cotton agriculture, and transmits mostly via the seeds (DV Araújo, 2008; Hall et al., 2013), suggesting the BRS cedro seeds were already infected from the field where they were multiplied. To restrain the fungus spreading, two fungicides (Nistatine, Viyavax-Thiram 200SC) were used

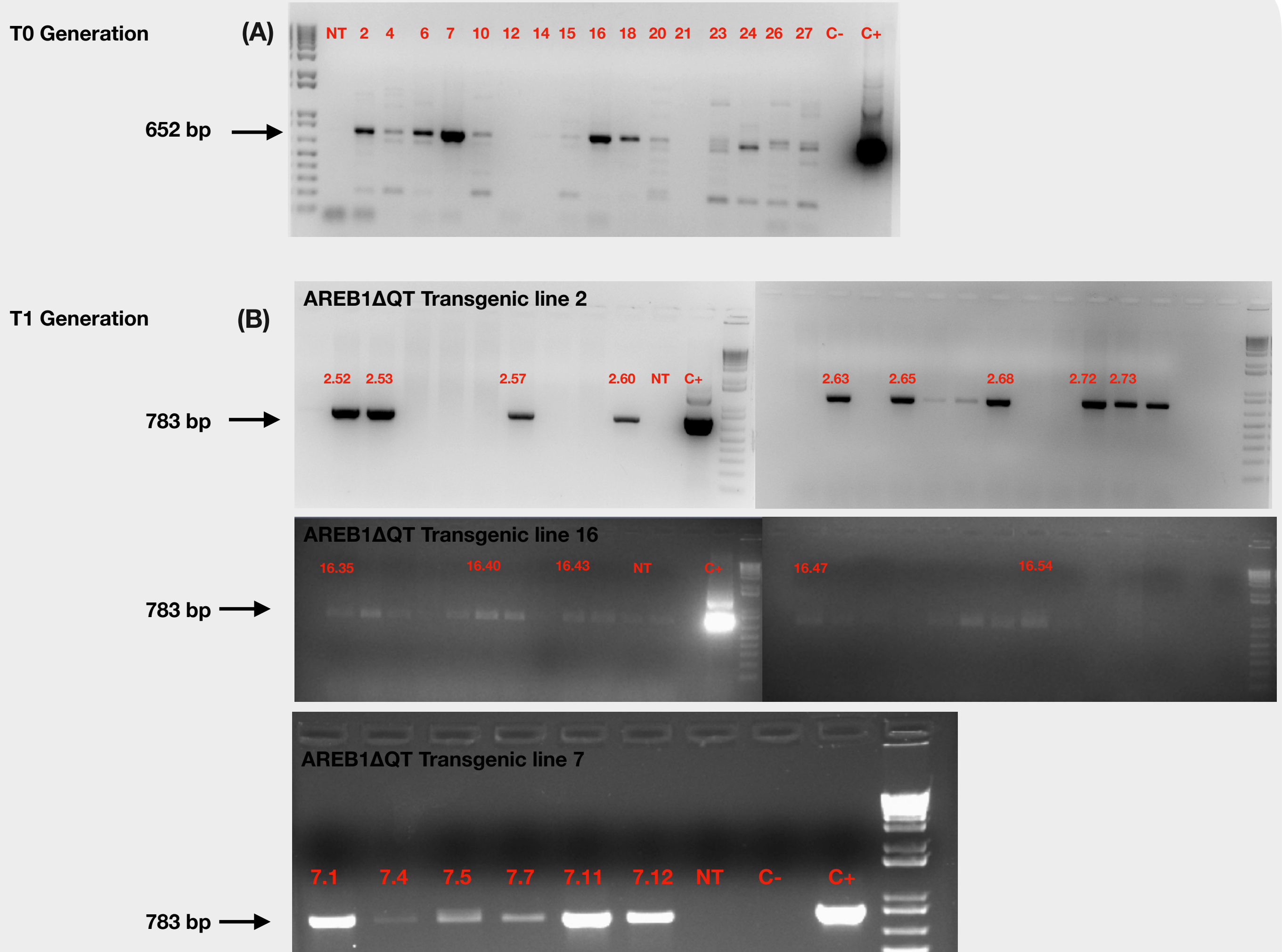


Figure 22. PCR amplification of transformed cotton plants at T0 and T1 generation. (A) amplification of DNA extracted from leaves of T0 generation with primers AHAS FWRD and REV (Table S). (B) Amplification at T1 generation of DNA extracted from seeds, with primers 35S FWRD and T-NOS REV (Table S). Numbers represent the putative transformed lines. NT represent DNA from non transformed lines. C- represents water negative control. C+ represents positive control of amplification with the original plasmid.

when sterilizing the seeds and included in the culture media, without good results, principally because the spores are inside the cells of the embryos, unable to reach by the fungicides.

To confirm if the T0 plants were positively transformed, total DNA was extracted PCR

Table 2 . Cotton transformation and provisional efficiency

Construction	Nbr Embryos bombarded	Embryos contaminated	Plants selected by Imazapir	Putative PCR positive plants	Efficiency
AREB1ΔQT-SF	2190	40,7 %	0,75 %	3	0,04 %

amplifications were made with primers amplifying a region in the *AHAS* gene (652 bp) (**Table S2**). We obtained clear bands for plants 2, 6, 7, 16, 18, and less visible bands for plants 4, 10, 20 and 24 (**Figure 22 A)** As expected, there were no amplifications for NT (non-transformed) plants. We considered plants 2,

7 and 16 to generate the T1 generation. Considering these three positive plants, the partial efficiency for both constructs is low, being of 0,04% for AREB1ΔQT-SF (**Table 2**). Nonetheless, the efficiency attained is comprised between 0,027% and 0,2%, which is the efficiency described in Rech et al. in 2007 for cotton transformation. the herbicide in the leaves.

We performed DNA extraction of individual seeds of the T1 generation to confirm the presence of the insert with PCR amplification (primers p35S FWRD, T-NOS REV; **Table S2**). We could amplify several plants with a positive insertion in each line (**Figure 22 B**). According to Mendelian inheritance pattern for gene integration, the ratio of the transgenic lines over not transgenic should be of 3:1. However, we did not obtain mendelian ratios in our study, as shown in **table 3.** Our ratios could be explained because of the transformation method we utilized. After particle bombardment of meristem tissues at the T0 generation, the resulting plants are chimeras with transformed and non-transformed aerial parts. Chimera plants after shoot biolistics transformation were also reported in anterior studies (Homrich et al., 2012). Hence, the number of transformed seeds was less than the expected by inheritance laws.

Table 3 Analysis of T1 putative transgenic seeds of GM cotton lines

Line	Number of analyzed seeds	PCR positives
2	73	22
7	12	6
16	55	24

3.2 Drought stress and nematode infection assays at T1 generation

The T1 seeds that were positive were germinated and planted in pots in a greenhouse. To assess if plants had a better tolerance to drought stress, we performed a series of water withdrawal assays with plants from lines 2 and 16. First, we performed a drought assay in which, after water saturation, plants were left for 9 days without re-hydration. Images of the plants at day 9 show that transformed plants from lines 2 and 16 resist better to water stress than NT plants (**Figure 23 A, B**) All the transformed plants have extended leaves and a better performance when compared to NT plants that show wilted leaves. Next, we measured water loss in whole plants or disc leaves from the same lines 2 and 16 (**Figure 24**). For whole plants that were left without watering and weighed every day, we observed that plants from line 2 lost less water from day 1, with a better performance than NT plants until day 9 (**Figure 24 A**). However, plants

(A)

First drought assay

Second drought assay

(B)

Figure 23. Images of transformed cotton lines 2 and 16 (AREB1ΔQT and RNAi-FS) of T1 generation after a 9 days of water withdrawal. NT plants correspond to non transformed plants. (A) and (B) refer to two independent experiments realized at different times and with different plants. Transformed plants showed healthy expanded leaves, whereas NT plants showed wilted leaves after 9 days of stress.

from line 16 had a very similar response compared to NT plants. We also cut leaf discs and left them in plastic cups while weighing them hourly. Plants from line 2 had a slightly better response than NT plants. This time, the disc from plant 16 lost water more rapidly and more importantly (**Figure 24 B**).

The preliminary results in the T1 generation of the plants show that the expression of the transgene *AREB1ΔQT-SF* could be inducing drought stress tolerance, principally for line 2. Still, the

assays were realized at T1 generation, in which homozygosity has yet been attained, thus a possible explanation for the results for plant 16. The expression of *AtAREB1ΔQT* has also been reported in soybean and presented higher survival after drought stress (Leite et al., 2014). Interestingly, AtAREB1ΔQT over-expression showed drought tolerance during drought stress and after rehydration.

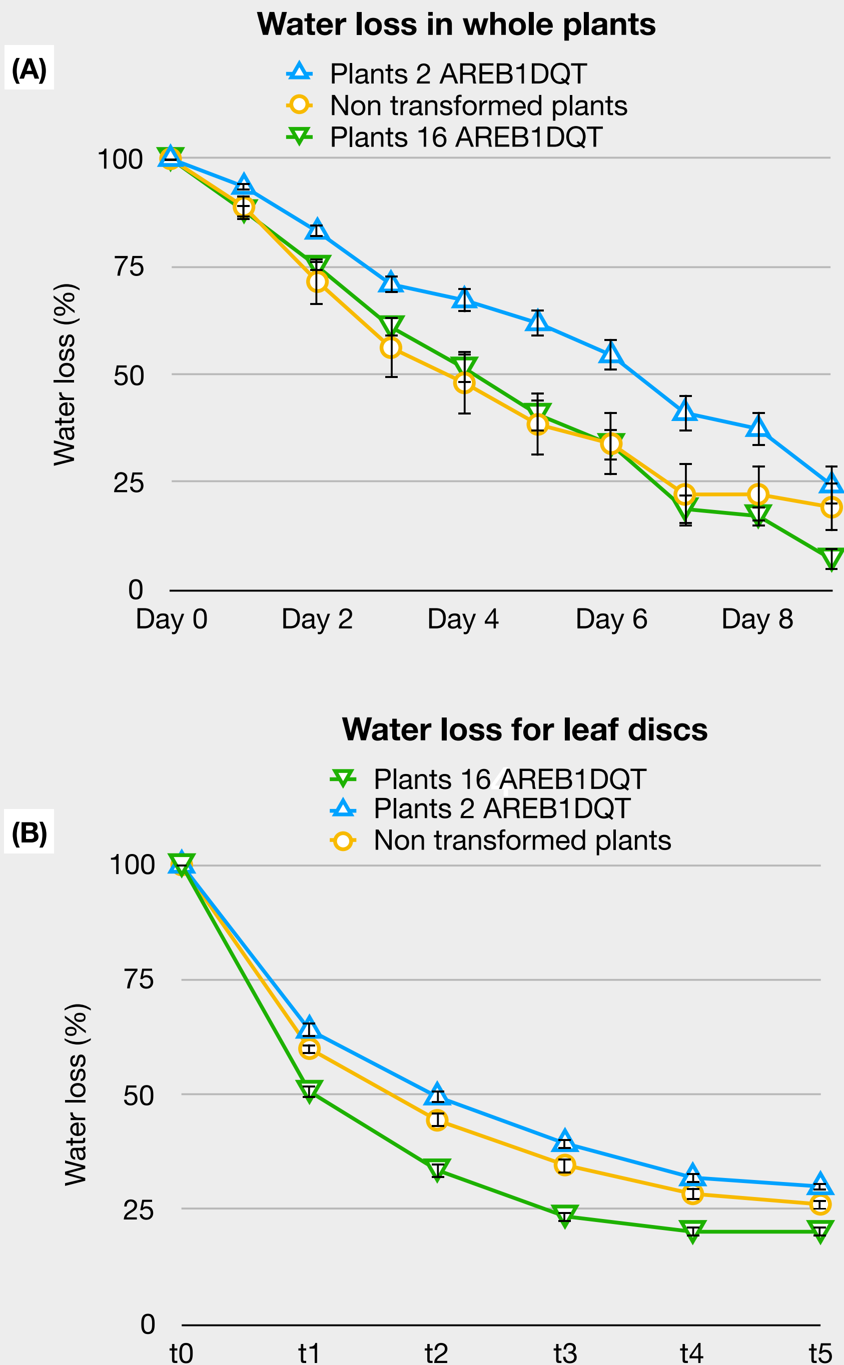


Figure 24. Water loss experiments realized on lines 2 and 16 (AREB1ΔQT and RNAi-FS). (A) Water loss was measured by weighing whole plants each day during 9 days of water withdrawal. Results represent mean and SD of n=4 to, n=7. (B) Water loss was measured for independent leaf discs weighed every hour after they were excised. Results represent means from 4 independent repetitions of n=3.

These results indicate that AREB1 from *A. thaliana* can interact with the ABA pathway of other plant hosts by activating endogene downstream ABA response genes. The data also suggests that the structure of protein is well conserved among different plant species. Comparatively, a recent study showed that *GhABF2*, the ortholog of *AtAREB1* in cotton conferred drought and salt stress both in Arabidopsis and in cotton (Liang et al., 2016)

To test the ability of the transformed plants to resist to a nematode infection, we performed a test in which independent leaves of the transformed plants were cultivated to grow roots. The roots were incubated with *M. Incognita* and galls and egg masses were counted after 30 days of infection (**Figure 25**). As for drought stress plants from line 16 showed a slight increase of galls and egg masses compared to NT. However, the results show that plant 7 had a significant low number of galls and egg masses compared to NT plants. These results suggest that the RNAi expressed in the cotton plants has a putative effect on M. Incognita infection.

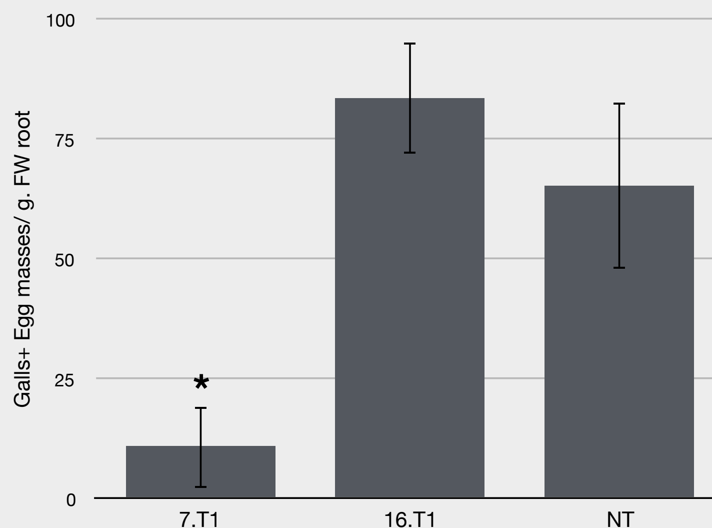


Figure 25. Nematode infection test realized on lines 7 and 16 (AREB1ΔQT and RNAi-FS). Leaves from both lines were excised and cultivated to induce root growth. The roots were infected with *M. Incognita.* and after 30 days galls and egg masses were counted. Results represent mean and SD from n=5. Statistical analysis was performed using Student's t-test between transformed and NT plants. Asterisks* represent values of $p<0,05$. Bars represent standard error.

3.3 Partial conclusion

The preliminary results show that at the T1 generation, some of the lines have a better response to drought stress and nematode infection. The T2 generation was being collected when I left for France to work at INRA. Among the perspectives, the gene expression and the number of copies will be measured by q-PCR. Also, plants will be assayed in a new drought stress assay and will be challenged to nematode

infection on soil. The combination of the presented results and the forthcoming ones will generate the second scientific paper of my thesis work.

4. Experimental procedures

AREB1ΔQT-RNAi-FS construct

The cassette containing AREB1ΔQT and dsRNA SFMi (Splicing factor *M. Incognita*) were designed as presented below. The sequence of AREB1ΔQT was disponibilized by JIRCAS (Japan). To design the RNAi-FS, a region of 200 bp was selected from *M. incognita*'s splicing factor sequence (Genbank acc. AW828516) with the aid of BLOCK-iTTM RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress). The 200 bp region was positioned in sense and antisense surrounding a PDK intron from plasmid pKannibal as in Yan et al. (2012). The gene coding for *AREB1ΔQT* was placed under the control of doubled cAMV 35S promoter and the SFMi under the control of *uceA1.7* (Viana et al., 2011). The fragments were synthesized by EPOCH, and sub-cloned into a vector pGS52123-1 containing the AHAS selection gene. The construct was transformed into Omnimax *E. Coli* with the Heat shock method.

Plant material, transformation and culture conditions

Plants of cotton (*G. hirsutum*) cultivar BRS372 Cedro harvested in «EMBRAPA Algodão» were used for all the experiments. The transformation with AREB1ΔQT-FS was performed by Microparticle bombardment as described before (Rech et al., 2008). Succinctly, the seeds were sterilized 3 days before (Washed in water, 3 minutes in 70% EtOH, 20 minutes in 6% bleach, and washed 3 times in sterilized water) and left in water for 24 hours with 250ug/mL Cefotaxime, 1000ppt of Nistatin. After 48h in the dark, the embryos were separated and organized in 6cm Petri plates. 5 µg of the DNA plasmid was coated in tungsten and the embryos were bombarded with 1200 psi with PDS-1000 / He™ (Biorad). The embryos were placed in MSI Imazapyr selection medium (MSI), and the plants that developed shoots after 30 days, were transferred to a greenhouse. The T0 plants in the greenhouse (average temperature 26 ± 1°C; average humidity 70 ± 10%) were maintained in pots containing soil as substrate. Every 15 days, they were supplemented with fertilizers for leaves and roots (Biofert). When cotton buds appeared, they were sealed with nail polish to prevent crosses, and seeds were harvested to obtain T1 generation.

Molecular analysis

Genomic DNA from cotton was extracted with a protocol adapted from Zhang and Stewart, (2000). Three foliar discs were collected in a micro-centrifuge tube with 3 steel beads and frozen with liquid nitrogen.

The tissue was disrupted with the TissueLyser (Qiagen) for 30 seconds at 30 Hz. After separation, precipitation and desalting of DNA, the pellets were left for 2 hours at Room T in a cleaning solution. Finally, the pellets were washed in 70% and 100% EtoH, resuspended in water and treated with RNAse A. For PCR detection primers used are shown in **Table S2**.

For seed genomic DNA extraction, a 50-mg fragment of the seed was dissected carefully and macerated with 300 µL extraction buffer (200 mM Tris-HCl pH 7,5; 300 mM NaCl; 500 mM EDTA; 1% SDS). Then, 700 µL of extraction buffer were added and microtubes were incubated at 55°C for 20 minutes. The tubes were then centrifuged at 14.000 rpm and supernatant was transferred to a new tube. After separation, precipitation and desalting, the DNA was incubated with RNAse A. A new round of precipitation was performed and pellets were resuspended in water.

Physiological measurements, nematode infection

Plants from T1 transgenic positive seeds were germinated and transferred to pots containing the same amount of soil. The same volume of water was given to the plants each time they were hydrated. Whole plant dehydration was performed in 3-month-old plants: plants were saturated with water and weighed after 1 day for 10 days at the same hour of the day. For disc dehydration measurements, three discs of 1,5 cm discs were isolated from different leaves and put on tubes with water for 2 hours. Excess water was removed and discs were weighed and put on plastic plates in the bench. Next, discs were weighed hourly.

Because nematode infection and analysis destroys the plant, we performed an assay in which roots were induced from independent leaves. Completely expanded young leaves were detached from cotton plants leaving approximately 5 cm of petiole. The leaves were then sterilized with 0.5% sodium hypochlorite for 20 minutes and washed three times with distilled water. After drying on paper towels, the leaves were individually packed in Petri dishes (150 x 20 mm) containing sterile sand and gel substrate until half the plate, where the petiole was inserted. A glass slide was placed under the abaxial face of the sheet so that it did not encounter the moisture. The substrate was moistened regularly with nutrient solution containing Murashige and Skoog salts and the plates were maintained in a 16 h photoperiod growing chamber at 26 ° C. After root emergence, about 20 days, two holes were made in the surface of the substrate and inoculated 500 juveniles of second stage (J2) of M. incognita by root. At the end of 30 days the number of galls and egg masses / g root were counted.

Table S2. Primers sequences used in this study

Name	Sequence	Use	Study
CAS9 FWRD	CATCAGGGAGCAGGCAGAAAAC	Insertion confirmation	CRISPR/dCas9
VPR REV	CTGAGCCAGTGCAGACACCATG	Insertion confirmation	
HAC REV	CCTTAAGAGGAGGACAAGCCC	Insertion confirmation	
SET REV	CAACTCTATGATCTCCAGCAACC	Insertion confirmation	
VPR FWRD	ACGCATTGGACGATTTTGAT	Insertion confirmation	
HAC FWRD	TTCCTACTGCTGAATCTCTTGTT	Insertion confirmation	
SET FWRD	TGGATGGGGAGCTTTTCTTA	Insertion confirmation	
sgRNA1 GBC36 FWRD	TTT CAC ACC GCA GGG TAA TAA CTG	sgRNA synthesis and ampification	
sgRNA1 GBD23 REV	TGCAAATAGTCCTCTTCCAACAA	sgRNA synthesis and ampification	
sgRNA2 GBE24 FWRD	tttgtcgctcttcgcaatgtc	sgRNA synthesis and ampification	
sgRNA2 GBA26 REV	tgctcaaagagacatgggtggaag	sgRNA synthesis and ampification	
sgRNA 1 FWD	GATTGATTTAAATCAATTTTGTTTTAGAGCTAGAAATAGCAA	sgRNA synthesis	
sgRNA1 REV	AAAATTGATTTAAATCAATCCAATCACTACTTCGTCT	sgRNA synthesis	
sgRNA 2 FWD	GCAAAATGTCCCTTTTTGGTGTTTTAGAGCTAGAAATAGCAA	sgRNA synthesis	
sgRNA 2 REV	ACCAAAAAGGGACATTTTGCCAATCACTACTTCGTCT	sgRNA synthesis	
sgRNA 3 FWD	GATACGCGTTCAGATCTGAAGTTTTAGAGCTAGAAATAGCAA	sgRNA synthesis	
sgRNA 3 REV	TTCAGATCTGAACGCGTATCCAATCACTACTTCGTCT	sgRNA synthesis	
AREB1_qPCR_2017_F	a a c a g g c t t a c a c c g t g g a g	qPCR	
AREB1_qPCR_2017_R	c t t t g g a c c t c c t t g c a g a a	qPCR	
dCas9_qPCR_F	AAAGCTCAAAGGGTCTCCCG	qPCR	
dCas9_qPCR_R	TTATCGAGGTTAGCGTCGGC	qPCR	
Rd29A_qPCR_FWRD	TGGATCTGAAGAACGAATCTGATATC	qPCR	
Rd29A_qPCR_REV	GGTCTTCCCTTCGCCAGAA	qPCR	
GAPDH A.thaliana_Fow_qPCR	TTGGTGACAACAGGTCAAGCA	qPCR	
GAPDH A.thaliana_Rev_qPCR	AAACTTGTCGCTCAATGCAATC	qPCR	
ACT2 A.thaliana_Fow_qPCR	CTTGCACCAAGCAGCATGAA	qPCR	
ACT2 A.thaliana_Rev_qPCR	CCGATCCAGACACTGTACTTCCTT	qPCR	
p35S FWRD	CTATCCTTCGCAAGACCCTTCC	Insertion confirmation	Transgenic cotton
T-NOS REV	GCAAGACCGGCAACAGGATTC	Insertion confirmation	
AHAS FWRD	ACTAGAGATTCCAGCGTCAC	Insertion confirmation	
AHAS REV	GTGGCTATACAGATACCTGG	Insertion confirmation	

References

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.-C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Ségurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.-N., Schiex, T., Smant, G., Weissenbach, J., Wincker, P., 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26, 909–915. https://doi.org/10.1038/nbt.1482

Abdallah, N.A., Prakash, C.S., McHughen, A.G., 2015. Genome editing for crop improvement: Challenges and opportunities. GM Crops & Food 6, 183–205. https://doi.org/10.1080/21645698.2015.1129937

Abdeeva, I., Abdeev, R., Bruskin, S., Piruzian, E., n.d. Transgenic Plants as a Tool for Plant Functional Genomics. Transgenic Plants 28.

Abe, K., Ichikawa, H., 2016. Gene Overexpression Resources in Cereals for Functional Genomics and Discovery of Useful Genes. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.01359

Abudayyeh, O.O., Gootenberg, J.S., Konermann, S., Joung, J., Slaymaker, I.M., Cox, D.B.T., Shmakov, S., Makarova, K.S., Semenova, E., Minakhin, L., Severinov, K., Regev, A., Lander, E.S., Koonin, E.V., Zhang, F., 2016. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 353, aaf5573. https://doi.org/10.1126/science.aaf5573

Adams, D., Baldock, R., Bhattacharya, S., Copp, A.J., Dickinson, M., Greene, N.D.E., Henkelman, M., Justice, M., Mohun, T., Murray, S.A., Pauws, E., Raess, M., Rossant, J., Weaver, T., West, D., 2013. Bloomsbury report on mouse embryo phenotyping: recommendations from the IMPC workshop on embryonic lethal screening. Disease Models & Mechanisms 6, 571–579. https://doi.org/10.1242/dmm.011833

Adrio, J.-L., Demain, A.L., 2010. Recombinant organisms for production of industrial products. Bioengineered Bugs 1, 116–131. https://doi.org/10.4161/bbug.1.2.10484

Agrobacterium protocols, 2014. Springer Science+Business Media, New York, NY.

Ahuja, I., de Vos, R.C.H., Bones, A.M., Hall, R.D., 2010. Plant molecular stress responses face climate change. Trends in Plant Science 15, 664–674. https://doi.org/10.1016/j.tplants.2010.08.002

Ali, M. A., Azeem, F., Abbas, A., Joyia, F. A., Li, H., & Dababat, A. A. (2017). Transgenic Strategies for Enhancement of Nematode Resistance in Plants. Frontiers in Plant Science, 8(May), 1–13. http://doi.org/10.3389/fpls.2017.00750

Alizadeh, V., Shokri, V., Soltani, A., Yousefi, M.A., n.d. Effects of Climate Change and Drought-Stress on Plant Physiology 5.

Allison, L.A., 2007. Fundamental molecular biology. Blackwell Pub, Malden, MA.

Alqudah, A.M., Samarah, N.H., Mullen, R.E., 2011. Drought Stress Effect on Crop Pollination, Seed Set, Yield and Quality, in: Lichtfouse, E. (Ed.), Alternative Farming Systems, Biotechnology, Drought Stress and Ecological Fertilisation. Springer Netherlands, Dordrecht, pp. 193–213. https://doi.org/10.1007/978-94-007-0186-1_6

Ameen Rashid, A.H., Dhahir Lateef, D., 2016. Novel Techniques for Gene Delivery into Plants and Its Applications for Disease Resistance in Crops. American Journal of Plant Sciences 07, 181–193. https://doi.org/10.4236/ajps.2016.71019

Anderson, J., Arblaster, K., Bartley, J., & Cooper, T. (2008). Climate Change–Induced Water Stress and its Impact on Natural and Managed Ecosystems. European

Anderson, J.P., Gleason, C.A., Foley, R.C., Thrall, P.H., Burdon, J.B., Singh, K.B., 2010. Plants versus pathogens: an evolutionary arms race. Functional Plant Biology 37, 499. https://doi.org/10.1071/FP09304

Antonino de Souza Júnior, J.D., Ramos Coelho, R., Tristan Lourenço, I., da Rocha Fragoso, R., Barbosa Viana, A.A., Lima Pepino de Macedo, L., Mattar da Silva, M.C., Gomes Carneiro, R.M., Engler, G., de Almeida-Engler, J., Grossi-de-Sa, M.F., 2013. Knocking-Down Meloidogyne incognita Proteases by Plant-Delivered dsRNA Has Negative Pleiotropic Effect on Nematode Vigor. PLoS ONE 8, e85364. https://doi.org/10.1371/journal.pone.0085364

Araújo, D.V.D., n.d. Caracterização molecular, patogenicidade e transmissão pela 113.

Ashraf, M., 2010. Inducing drought tolerance in plants: Recent advances. Biotechnology Advances 28, 169–183. https://doi.org/10.1016/j.biotechadv.2009.11.005

Ayer, D.E., Laherty, C.D., Lawrence, Q.A., Armstrong, A.P., Eisenman, R.N., 1996. Mad proteins contain a dominant transcription repression domain. Molecular and Cellular Biology 16, 5772–5781. https://doi.org/10.1128/MCB.16.10.5772

Baeumler, T.A., Ahmed, A.A., Fulga, T.A., 2017. Engineering Synthetic Signaling Pathways with Programmable dCas9-Based Chimeric Receptors. Cell Reports 20, 2639–2653. https://doi.org/10.1016/j.celrep.2017.08.044

Bajwa, K.S., Shahid, A.A., Rao, A.Q., Bashir, A., Aftab, A., Husnain, T., 2015. Stable transformation and expression of GhEXPA8 fiber expansin gene to improve fiber length and micronaire value in cotton. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00838

Bakhsh, A., Hussain, T., 2015. Engineering crop plants against abiotic stress: Current achievements and prospects. Emirates Journal of Food and Agriculture 27, 24. https://doi.org/10.9755/ejfa.v27i1.17980

Ball, P., 2005. Quantum optics: Cheat detection. Nature 433, 584–584. https://doi.org/10.1038/433584a

Banerjee, A., Roychoudhury, A., 2017. Epigenetic regulation during salinity and drought stress in plants: Histone modifications and DNA methylation. Plant Gene 11, 199–204. https://doi.org/10.1016/j.plgene.2017.05.011

Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell Research 21, 381–395. https://doi.org/10.1038/cr.2011.22

Barbosa, E.G.G., Leite, J.P., Marin, S.R.R., Marinho, J.P., de Fátima Corrêa Carvalho, J., Fuganti-Pagliarini, R., Farias, J.R.B., Neumaier, N., Marcelino-Guimarães, F.C., de Oliveira, M.C.N., Yamaguchi-Shinozaki, K., Nakashima, K., Maruyama, K., Kanamori, N., Fujita, Y., Yoshida, T., Nepomuceno, A.L., 2013. Overexpression of the ABA-Dependent AREB1 Transcription Factor from *Arabidopsis thaliana* Improves Soybean Tolerance to Water Deficit. Plant Molecular Biology Reporter 31, 719–730. https://doi.org/10.1007/s11105-012-0541-4

Bärlocher, C., Holland, R., Box, P.O., Zeist, A., n.d. FOR MORE INFORMATION: 48.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science 315, 1709–1712. https://doi.org/10.1126/science.1138140

Bastías, A., Yañez, M., Osorio, S., Arbona, V., Gómez-Cadenas, A., Fernie, A.R., Casaretto, J.A., 2014. The transcription factor AREB1 regulates primary metabolic pathways in tomato fruits. Journal of Experimental Botany 65, 2351–2363. https://doi.org/10.1093/jxb/eru114

Basu, C., Kausch, A.P., Chandlee, J.M., 2004. Use of β-glucuronidase reporter gene for gene expression analysis in turfgrasses. Biochemical and Biophysical Research Communications 320, 7–10. https://doi.org/10.1016/j.bbrc.2004.05.128

Basu, S., Ramegowda, V., Kumar, A., Pereira, A., 2016. Plant adaptation to drought stress. F1000Research 5, 1554. https://doi.org/10.12688/f1000research.7678.1

Beerli, R.R., Segal, D.J., Dreier, B., Barbas, C.F., 1998. Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. Proceedings of the National Academy of Sciences 95, 14628–14633. https://doi.org/10.1073/pnas.95.25.14628

Bertioli, D.J., Seijo, G., Freitas, F.O., Valls, J.F.M., Leal-Bertioli, S.C.M., Moretzsohn, M.C., 2011. An overview of peanut and its wild relatives. Plant Genetic Resources 9, 134–149. https://doi.org/10.1017/S1479262110000444

Bevan MW, Flavell RB, Chilton MD (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304 184–187

Bhatnagar-Mathur, P., Vadez, V., Sharma, K.K., 2008. Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Reports 27, 411–424. https://doi.org/10.1007/s00299-007-0474-9

Biagioni, A., Chillà, A., Andreucci, E., Laurenzana, A., Margheri, F., Peppicelli, S., Del Rosso, M., Fibbi, G., 2017. Type II CRISPR/Cas9 approach in the oncological therapy. Journal of Experimental & Clinical Cancer Research 36. https://doi.org/10.1186/s13046-017-0550-0

Bibikova, M., 2003. Enhancing Gene Targeting with Designed Zinc Finger Nucleases. Science 300, 764–764. https://doi.org/10.1126/science.1079512

Bibikova, M., Golic, M., Golic, K.G., Carroll, D., n.d. Targeted Chromosomal Cleavage and Mutagenesis in Drosophila Using Zinc-Finger Nucleases 7.

Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., Marraffini, L.A., 2013. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Research 41, 7429–7437. https://doi.org/10.1093/nar/gkt520

Bird, D.M., Kaloshian, I., 2003. Are roots special? Nematodes have their say. Physiological and Molecular Plant Pathology 62, 115–123. https://doi.org/10.1016/S0885-5765(03)00045-6

Bird, D.M., Opperman, C.H., Williamson, V.M., 2008. Plant Infection by Root-Knot Nematode. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/7089_2008_31

Block, M., Herrera-Estrella, L., 1983. Chimeric Genes as Dominant Selectable Markers in Plant Cells, in: Potrykus, I., Harms, C.T., Hinnen, A., Hütter, R., King, P.J., Shillito, R.D. (Eds.), Protoplasts 1983. Birkhäuser Basel, Basel, pp. 130–131. https://doi.org/10.1007/978-3-0348-6556-2_60

Boch, J., 2011. TALEs of genome targeting. Nature Biotechnology 29, 135–136. https://doi.org/10.1038/nbt.1767

Boch, J., Bonas, U., 2010. *Xanthomonas* AvrBs3 Family-Type III Effectors: Discovery and Function. Annual Review of Phytopathology 48, 419–436. https://doi.org/10.1146/annurev-phyto-080508-081936

Boettcher, M., McManus, M.T., 2015. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Molecular Cell 58, 575–585. https://doi.org/10.1016/j.molcel.2015.04.028

Bogdanove, A. J., & Voytas, D. F. (2013). TAL Effectors : Customizable Proteins, 1843(2011), 1843–1847. http://doi.org/10.1126/science.1204094
Bogdanove, A. J., Schornack, S., & Lahaye, T. (2010). TAL effectors: Finding plant genes for disease and defense. Current Opinion in Plant Biology, 13(4), 394–401. http://doi.org/10.1016/j.pbi.2010.04.010
Bonas, U., Stall, R.E., Staskawicz, B., 1989. Genetic and structural characterization of the avirulence gene avrBs3 from *Xanthomonas campestris* pv. vesicatoria. MGG Molecular & General Genetics 218, 127–136. https://doi.org/10.1007/BF00330575
Bondy-Denomy, J., Davidson, A.R., 2014. To acquire or resist: the complex biological effects of CRISPR–Cas systems. Trends in Microbiology 22, 218–225. https://doi.org/10.1016/j.tim.2014.01.007
Bonev, B., Cavalli, G., 2016. Organization and function of the 3D genome. Nature Reviews Genetics 17, 661–678. https://doi.org/10.1038/nrg.2016.112
Bordoli, L., 2001a. Plant orthologs of p300/CBP: conservation of a core domain in metazoan p300/CBP acetyltransferase-related proteins. Nucleic Acids Research 29, 589–597. https://doi.org/10.1093/nar/29.3.589
Bordoli, L., 2001b. Plant orthologs of p300/CBP: conservation of a core domain in metazoan p300/CBP acetyltransferase-related proteins. Nucleic Acids Research 29, 589–597. https://doi.org/10.1093/nar/29.3.589
Bortesi, L., Fischer, R., 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnology Advances 33, 41–52. https://doi.org/10.1016/j.biotechadv.2014.12.006
Bouabe, H., Okkenhaug, K., 2013. Gene Targeting in Mice: A Review, in: Bailer, S.M., Lieber, D. (Eds.), Virus-Host Interactions. Humana Press, Totowa, NJ, pp. 315–336. https://doi.org/10.1007/978-1-62703-601-6_23
Boyle, E.A., Andreasson, J.O.L., Chircus, L.M., Sternberg, S.H., Wu, M.J., Guegler, C.K., Doudna, J.A., Greenleaf, W.J., 2017. High-throughput biochemical profiling reveals sequence determinants of dCas9 off-target binding and unbinding. Proceedings of the National Academy of Sciences 114, 5461–5466. https://doi.org/10.1073/pnas.1700557114
Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1–2), 248–254. http://doi.org/10.1016/0003-2697(76)90527-3
Braff, J.L., Yaung, S.J., Esvelt, K.M., Church, G.M., 2016. Characterization of Cas9–Guide RNA Orthologs: TABLE 1. Cold Spring Harbor Protocols 2016, pdb.top086793. https://doi.org/10.1101/pdb.top086793
Brasileiro, A.C.M., Araujo, A.C.G., Leal-Bertioli, S.C., Guimarães, P.M., 2014. Genomics and Genetic Transformation in Arachis 11.
Brasileiro, A.C.M., Morgante, C.V., Araujo, A.C.G., Leal-Bertioli, S.C.M., Silva, A.K., Martins, A.C.Q., Vinson, C.C., Santos, C.M.R., Bonfim, O., Togawa, R.C., Saraiva, M.A.P., Bertioli, D.J., Guimaraes, P.M., 2015. Transcriptome Profiling of Wild Arachis from Water-Limited Environments Uncovers Drought Tolerance Candidate Genes. Plant Molecular Biology Reporter 33, 1876–1892. https://doi.org/10.1007/s11105-015-0882-x
Brazelton, V.A., Zarecor, S., Wright, D.A., Wang, Y., Liu, J., Chen, K., Yang, B., Lawrence-Dill, C.J., 2015. A quick guide to CRISPR sgRNA design tools. GM Crops & Food 6, 266–276. https://doi.org/10.1080/21645698.2015.1137690
Brocken, D.J.W., Tark-Dame, M., Dame, R.T., 2018. dCas9: A Versatile Tool for Epigenome Editing. Current Issues in Molecular Biology 15–32. https://doi.org/10.21775/cimb.026.015
Burr, M.L., Sparbier, C.E., Chan, Y.-C., Williamson, J.C., Woods, K., Beavis, P.A., Lam, E.Y.N., Henderson, M.A., Bell, C.C., Stolzenburg, S., Gilan, O., Bloor, S., Noori, T., Morgens, D.W., Bassik, M.C., Neeson, P.J., Behren, A., Darcy, P.K., Dawson, S.-J., Voskoboinik, I., Trapani, J.A., Cebon, J., Lehner, P.J., Dawson, M.A., 2017. CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity. Nature 549, 101–105. https://doi.org/10.1038/nature23643
Butaye, K.M.J., Cammue, B.P.A., Delauré, S.L., De Bolle, M.F.C., 2005. Approaches to Minimize Variation of Transgene Expression in Plants. Molecular Breeding 16, 79–91. https://doi.org/10.1007/s11032-005-4929-9
Carroll, D., 2011. Genome Engineering With Zinc-Finger Nucleases. Genetics 188, 773–782. https://doi.org/10.1534/genetics.111.131433
Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., … Werner, T. (2005). MatInspector and beyond: Promoter analysis based on transcription factor binding sites. Bioinformatics, 21(13), 2933–2942. http://doi.org/10.1093/bioinformatics/bti473
Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., Voytas, D.F., 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Research 39, e82–e82. https://doi.org/10.1093/nar/gkr218
Challinor, A.J., Koehler, A.-K., Ramirez-Villegas, J., Whitfield, S., Das, B., 2016. Current warming will reduce yields unless maize breeding and seed systems adapt immediately. Nature Climate Change 6, 954–958. https://doi.org/10.1038/nclimate3061

Chambers, P.J., Pretorius, I.S., 2010. Fermenting knowledge: the history of winemaking, science and yeast research. EMBO reports 11, 914–920. https://doi.org/10.1038/embor.2010.179
Chassy, 2007. The History and Future of GMOs in Food and Agriculture. Cereal Foods World. https://doi.org/10.1094/CFW-52-4-0169
Chavez, A., Scheiman, J., Vora, S., Pruitt, B.W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C.D., Wiegand, D.J., Ter-Ovanesyan, D., Braff, J.L., Davidsohn, N., Housden, B.E., Perrimon, N., Weiss, R., Aach, J., Collins, J.J., Church, G.M., 2015. Highly efficient Cas9-mediated transcriptional programming. Nature Methods 12, 326–328. https://doi.org/10.1038/nmeth.3312
Chen, N., Zhou, W.-B., Wang, Y.-X., Dong, A.-W., Yu, Y., 2014. Polycomb-group histone methyltransferase CLF is required for proper somatic recombination in *Arabidopsis*: Role of *CLF* in homologous recombination. Journal of Integrative Plant Biology 56, 550–558. https://doi.org/10.1111/jipb.12157
Chen, T., Gao, D., Zhang, R., Zeng, G., Yan, H., Lim, E., Liang, F.-S., 2017. Chemically Controlled Epigenome Editing through an Inducible dCas9 System. Journal of the American Chemical Society 139, 11337–11340. https://doi.org/10.1021/jacs.7b06555
Chen, Y.-E., Liu, W.-J., Su, Y.-Q., Cui, J.-M., Zhang, Z.-W., Yuan, M., Zhang, H.-Y., Yuan, S., 2016. Different response of photosystem II to short and long-term drought stress in *Arabidopsis thaliana*. Physiologia Plantarum 158, 225–235. https://doi.org/10.1111/ppl.12438
Cheng, A.W., Jillette, N., Lee, P., Plaskon, D., Fujiwara, Y., Wang, W., Taghbalout, A., Wang, H., 2016. Casilio: a versatile CRISPR-Cas9-Pumilio hybrid for gene regulation and genomic labeling. Cell Research 26, 254–257. https://doi.org/10.1038/cr.2016.3
Cheng, A.W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T.W., Rangarajan, S., Shivalila, C.S., Dadon, D.B., Jaenisch, R., 2013a. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Research 23, 1163–1171. https://doi.org/10.1038/cr.2013.122
Chesneau, A., Sachs, L.M., Chai, N., Chen, Y., Pasquier, L., Loeber, J., Pollet, N., Reilly, M., Weeks, D.L., Bronchain, O.J., 2008. Transgenesis procedures in Xenopus. Biology of the Cell 100, 503–529. https://doi.org/10.1042/BC20070148
Chevalier, B.S., 2001. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. Nucleic Acids Research 29, 3757–3774. https://doi.org/10.1093/nar/29.18.3757
Chevalier, B.S., Kortemme, T., Chadsey, M.S., Baker, D., Monnat, R.J., Stoddard, B.L., 2002. Design, Activity, and Structure of a Highly Specific Artificial Endonuclease. Molecular Cell 10, 895–905. https://doi.org/10.1016/S1097-2765(02)00690-1
Chira, S., Gulei, D., Hajitou, A., Zimta, A.-A., Cordelier, P., Berindan-Neagoe, I., 2017. CRISPR/Cas9: Transcending the Reality of Genome Editing. Molecular Therapy - Nucleic Acids 7, 211–222. https://doi.org/10.1016/j.omtn.2017.04.001
Chitwood, D.J., 2002. P HYTOCHEMICAL B ASED S TRATEGIES FOR N EMATODE C ONTROL. Annual Review of Phytopathology 40, 221–249. https://doi.org/10.1146/annurev.phyto.40.032602.130045
Clinical trials https://clinicaltrials.gov/ct2/results/map/click?map.x=633&map.y=534&term=CRISPR&map=ES&mapw=1243
Clonetech note, 2015.Note, T. (n.d.). Characterization of CRISPR / Cas9 introduced Mutations using the Guide it Indel Identification Kit, .
Clough, S. J., & Bent, A. F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant Journal, 16(6), 735–743. http://doi.org/10.1046/j.1365-313X.1998.00343.x
Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin, 2005. . Microbiology 151, 2551–2561. https://doi.org/10.1099/mic.0.28048-0
Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F., 2013. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 339, 819–823. https://doi.org/10.1126/science.1231143
Cox, D.B.T., Gootenberg, J.S., Abudayyeh, O.O., Franklin, B., Kellner, M.J., Joung, J., Zhang, F., 2017. RNA editing with CRISPR-Cas13. Science 358, 1019–1027. https://doi.org/10.1126/science.aaq0180
Crauciuc, A., Tripon, F., Gheorghiu, A., Nemes, G., Boglis, A., Banescu, C., 2017. Review. Development, Applications, Benefits, Challenges and Limitations of the New Genome Engineering Technique. An Update Study. Acta Medica Marisiensis 63. https://doi.org/10.1515/amma-2017-0007 CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies, 2005. . Microbiology 151, 653–663. https://doi.org/10.1099/mic.0.27437-0
Dai, A., 2011. Drought under global warming: a review: Drought under global warming. Wiley Interdisciplinary Reviews: Climate Change 2, 45–65. https://doi.org/10.1002/wcc.81
Darwin, C. 1861 The Origin of Species.
Daszkowska-Golec, A., Szarejko, I., 2013. Open or Close the Gate – Stomata Action Under the Control of Phytohormones in Drought Stress Conditions. Frontiers in Plant Science 4. https://doi.org/10.3389/fpls.2013.00138

de Groote, M.L., Verschure, P.J., Rots, M.G., 2012. Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. Nucleic Acids Research 40, 10596–10613. https://doi.org/10.1093/nar/gks863
Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J., Charpentier, E., 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471, 602–607. https://doi.org/10.1038/nature09886
Deng, W., Liu, C., Pei, Y., Deng, X., Niu, L., Cao, X., 2007. Involvement of the Histone Acetyltransferase AtHAC1 in the Regulation of Flowering Time via Repression of FLOWERING LOCUS C in Arabidopsis. PLANT PHYSIOLOGY 143, 1660–1668. https://doi.org/10.1104/pp.106.095521
Deveau, H., Barrangou, R., Garneau, J.E., Labonte, J., Fremaux, C., Boyaval, P., Romero, D.A., Horvath, P., Moineau, S., 2008. Phage Response to CRISPR-Encoded Resistance in *Streptococcus thermophilus*. Journal of Bacteriology 190, 1390–1400. https://doi.org/10.1128/JB.01412-07
Didovyk, A., Borek, B., Tsimring, L., Hasty, J., 2016. Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications. Current Opinion in Biotechnology 40, 177–184. https://doi.org/10.1016/j.copbio.2016.06.003
Ding, Y., Fromm, M., Avramova, Z., 2012. Multiple exposures to drought “train” transcriptional responses in Arabidopsis. Nature Communications 3. https://doi.org/10.1038/ncomms1732
Ding, Y., Li, H., Chen, L.-L., Xie, K., 2016. Recent Advances in Genome Editing Using CRISPR/Cas9. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.00703
Dodds, P.N., Rathjen, J.P., 2010a. Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics 11, 539–548. https://doi.org/10.1038/nrg2812
Dodds, P.N., Rathjen, J.P., 2010b. Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics 11, 539–548. https://doi.org/10.1038/nrg2812
Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H.W., Listgarten, J., Root, D.E., 2016. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nature Biotechnology 34, 184–191. https://doi.org/10.1038/nbt.3437
Domingo, J.L., 2016. Safety assessment of GM plants: An updated review of the scientific literature. Food and Chemical Toxicology 95, 12–18. https://doi.org/10.1016/j.fct.2016.06.013
Dominguez, A.A., Lim, W.A., Qi, L.S., 2016a. Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. Nature Reviews Molecular Cell Biology 17, 5–15. https://doi.org/10.1038/nrm.2015.2
Dominguez, A.A., Lim, W.A., Qi, L.S., 2016b. Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. Nature Reviews Molecular Cell Biology 17, 5–15. https://doi.org/10.1038/nrm.2015.2
Doudna, J.A., Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. Science 346, 1258096–1258096. https://doi.org/10.1126/science.1258096
Dreissig, S., Schiml, S., Schindele, P., Weiss, O., Rutten, T., Schubert, V., Gladilin, E., Mette, M.F., Puchta, H., Houben, A., 2017. Live-cell CRISPR imaging in plants reveals dynamic telomere movements. The Plant Journal 91, 565–573. https://doi.org/10.1111/tpj.13601
Drummond, M.H., CHILTON, M.-D., 1978. Tumor-Inducing (Ti) Plasmids of Agrobacterium Share Extensive Regions of DNA Homology. J. BACTERIOL. 136, 6.
Dutta, T.K., Banakar, P., Rao, U., 2015. The status of RNAi-based transgenic research in plant nematology. Frontiers in Microbiology 5. https://doi.org/10.3389/fmicb.2014.00760
Eberharter, A., 2002. Histone acetylation: a switch between repressive and permissive chromatin: Second in review series on chromatin dynamics. EMBO Reports 3, 224–229. https://doi.org/10.1093/embo-reports/kvf053
Eckardt, N.A., 2017. *The Plant Cell* Reviews Plant Immunity: Receptor-Like Kinases, ROS-RLK Crosstalk, Quantitative Resistance, and the Growth/Defense Trade-Off. The Plant Cell 29, 601–602. https://doi.org/10.1105/tpc.17.00289
Eibl, D., Eibl, R., Pörtner, R., 2009. Mammalian Cell Culture Technology: An Emerging Field, in: Cell and Tissue Reaction Engineering. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 3–11. https://doi.org/10.1007/978-3-540-68182-3_1
Enríquez P., et al., .D., LL.M., Ph.D. Candidate CRISPR-Mediated Epigenome Editing
Ericsson, A.C., 2014. A Brief History of Animal Modeling 8.
Fahad, S., Bajwa, A.A., Nazir, U., Anjum, S.A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M.Z., Alharby, H., Wu, C., Wang, D., Huang, J., 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. Frontiers in Plant Science 8. https://doi.org/10.3389/fpls.2017.01147
Fang, Y., Xiong, L., 2015a. General mechanisms of drought response and their application in drought resistance improvement in plants. Cellular and Molecular Life Sciences 72, 673–689. https://doi.org/10.1007/s00018-014-1767-0
Fang, Y., Xiong, L., 2015b. General mechanisms of drought response and their application in drought resistance improvement in plants. Cellular and Molecular Life Sciences 72, 673–689. https://doi.org/10.1007/s00018-014-1767-0

Farooq, M., Hussain, M., Wahid, A., Siddique, K.H.M., 2012. Drought Stress in Plants: An Overview, in: Aroca, R. (Ed.), Plant Responses to Drought Stress. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–33. https://doi.org/10.1007/978-3-642-32653-0_1
Farzadfard, F., Perli, S.D., Lu, T.K., 2013. Tunable and Multifunctional Eukaryotic Transcription Factors Based on CRISPR/Cas. ACS Synthetic Biology 2, 604–613. https://doi.org/10.1021/sb400081r
Ferrer-Miralles, N., Domingo-Espín, J., Corchero, J., Vázquez, E., Villaverde, A., 2009. Microbial factories for recombinant pharmaceuticals. Microbial Cell Factories 8, 17. https://doi.org/10.1186/1475-2859-8-17
Finkelstein, R., 2013. Abscisic Acid Synthesis and Response. The Arabidopsis Book 11, e0166. https://doi.org/10.1199/tab.0166
Fita, A., Rodríguez-Burruezo, A., Boscaiu, M., Prohens, J., Vicente, O., 2015. Breeding and Domesticating Crops Adapted to Drought and Salinity: A New Paradigm for Increasing Food Production. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00978
Forsburg, S.L., Rhind, N., 2006. Basic methods for fission yeast. Yeast 23, 173–183. https://doi.org/10.1002/yea.1347
Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L., Woo, S.C., 1983. Expression of bacterial genes in plant cells. Proceedings of the National Academy of Sciences 80, 4803–4807. https://doi.org/10.1073/pnas.80.15.4803
Friedman, D.A., Gordon, D.M., Luo, L., 2017. The MutAnts Are Here. Cell 170, 601–602. https://doi.org/10.1016/j.cell.2017.07.046
Fujita, Y., 2005a. AREB1 Is a Transcription Activator of Novel ABRE-Dependent ABA Signaling That Enhances Drought Stress Tolerance in Arabidopsis. THE PLANT CELL ONLINE 17, 3470–3488. https://doi.org/10.1105/tpc.105.035659
Fujita, Y., 2005b. AREB1 Is a Transcription Activator of Novel ABRE-Dependent ABA Signaling That Enhances Drought Stress Tolerance in Arabidopsis. THE PLANT CELL ONLINE 17, 3470–3488. https://doi.org/10.1105/tpc.105.035659
Fujita, Y., Yoshida, T., Yamaguchi-Shinozaki, K., 2013. Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. Physiologia Plantarum 147, 15–27. https://doi.org/10.1111/j.1399-3054.2012.01635.x
Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K., Yamaguchi-Shinozaki, K., 2006. Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proceedings of the National Academy of Sciences 103, 1988–1993. https://doi.org/10.1073/pnas.0505667103
Gaj, T., Gersbach, C.A., Barbas, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology 31, 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004
Gallagher, S.R. (Ed.), 1992. GUS protocols: using the GUS gene as a reporter of gene expression. Academic Press, San Diego.
Gao, H., Smith, J., Yang, M., Jones, S., Djukanovic, V., Nicholson, M.G., West, A., Bidney, D., Falco, S.C., Jantz, D., Lyznik, L.A., 2010. Heritable targeted mutagenesis in maize using a designed endonuclease. The Plant Journal 61, 176–187. https://doi.org/10.1111/j.1365-313X.2009.04041.x
Garneau, J.E., Dupuis, M.-È., Villion, M., Romero, D.A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A.H., Moineau, S., 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468, 67–71. https://doi.org/10.1038/nature09523
Geiβler, R., Scholze, H., Hahn, S., Streubel, J., Bonas, U., Behrens, S.-E., Boch, J., 2011. Transcriptional Activators of Human Genes with Programmable DNA-Specificity. PLoS ONE 6, e19509. https://doi.org/10.1371/journal.pone.0019509
Gelvin, S.B., 2017. Integration of *Agrobacterium* T-DNA into the Plant Genome. Annual Review of Genetics 51, 195–217. https://doi.org/10.1146/annurev-genet-120215-035320
Gelvin, S.B., 2012. Traversing the Cell: Agrobacterium T-DNA's Journey to the Host Genome. Frontiers in Plant Science 3. https://doi.org/10.3389/fpls.2012.00052
Gelvin, S.B., 2003. Agrobacterium-Mediated Plant Transformation: The Biology behind the "Gene-Jockeying" Tool. Microbiology and Molecular Biology Reviews 67, 16–37. https://doi.org/10.1128/MMBR.67.1.16-37.2003
Gibson, D.G., 2009. Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Research 37, 6984–6990. https://doi.org/10.1093/nar/gkp687
Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H., Guimaraes, C., Panning, B., Ploegh, H.L., Bassik, M.C., Qi, L.S., Kampmann, M., Weissman, J.S., 2014. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159, 647–661. https://doi.org/10.1016/j.cell.2014.09.029
Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. Cell 154, 442–451. https://doi.org/10.1016/j.cell.2013.06.044
Glass, Z., Lee, M., Li, Y., Xu, Q., 2018. Engineering the Delivery System for CRISPR-Based Genome Editing. Trends in Biotechnology 36, 173–185. https://doi.org/10.1016/j.tibtech.2017.11.006

GMO handbook.
Gonzalez-Guzman, M., Pizzio, G.A., Antoni, R., Vera-Sirera, F., Merilo, E., Bassel, G.W., Fernández, M.A., Holdsworth, M.J., Perez-Amador, M.A., Kollist, H., Rodriguez, P.L., 2012. *Arabidopsis* PYR/PYL/RCAR Receptors Play a Major Role in Quantitative Regulation of Stomatal Aperture and Transcriptional Response to Abscisic Acid. The Plant Cell 24, 2483–2496. https://doi.org/10.1105/tpc.112.098574
Goverse, A., Smant, G., 2014a. The Activation and Suppression of Plant Innate Immunity by Parasitic Nematodes. Annual Review of Phytopathology 52, 243–265. https://doi.org/10.1146/annurev-phyto-102313-050118
Goverse, A., Smant, G., 2014b. The Activation and Suppression of Plant Innate Immunity by Parasitic Nematodes. Annual Review of Phytopathology 52, 243–265. https://doi.org/10.1146/annurev-phyto-102313-050118
Greco, M., Chiappetta, A., Bruno, L., Bitonti, M.B., 2018.
Guimaraes, L.A., Mota, A.P.Z., Araujo, A.C.G., de Alencar Figueiredo, L.F., Pereira, B.M., de Passos Saraiva, M.A., Silva, R.B., Danchin, E.G.J., Guimaraes, P.M., Brasileiro, A.C.M., 2017a. Genome-wide analysis of expansin superfamily in wild Arachis discloses a stress-responsive expansin-like B gene. Plant Molecular Biology 94, 79–96. https://doi.org/10.1007/s11103-017-0594-8
Guimaraes, L.A., Pereira, B.M., Araujo, A.C.G., Guimaraes, P.M., Brasileiro, A.C.M., 2017b. Ex vitro hairy root induction in detached peanut leaves for plant–nematode interaction studies. Plant Methods 13. https://doi.org/10.1186/s13007-017-0176-4
Guimarães, P.M., Brasileiro, A.C.M., Proite, K., de Araújo, A.C.G., Leal-Bertioli, S.C.M., Pic-Taylor, A., da Silva, F.R., Morgante, C.V., Ribeiro, S. da G., Bertioli, D.J., 2010. A Study of Gene Expression in the Nematode Resistant Wild Peanut Relative, *Arachis stenosperma*, in Response to Challenge with Meloidogyne arenaria. Tropical Plant Biology 3, 183–192. https://doi.org/10.1007/s12042-010-9056-z
Guimaraes, P.M., Guimaraes, L.A., Morgante, C.V., Silva, O.B., Araujo, A.C.G., Martins, A.C.Q., Saraiva, M.A.P., Oliveira, T.N., Togawa, R.C., Leal-Bertioli, S.C.M., Bertioli, D.J., Brasileiro, A.C.M., 2015. Root Transcriptome Analysis of Wild Peanut Reveals Candidate Genes for Nematode Resistance. PLOS ONE 10, e0140937. https://doi.org/10.1371/journal.pone.0140937
Guo, S., Kemphues, K.J., 1995. par-1, a gene required for establishing polarity *in C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81, 611–620. https://doi.org/10.1016/0092-8674(95)90082-9
Hake, S., Ross-Ibarra, J., 2015. Genetic, evolutionary and plant breeding insights from the domestication of maize. eLife 4. https://doi.org/10.7554/eLife.05861
Hall, C., Heath, R., Guest, D., 2013. The infection process of Fusarium oxysporum f.sp. vasinfectum in Australian cotton. Australasian Plant Pathology 42, 1–8. https://doi.org/10.1007/s13313-012-0169-8
Han, L., n.d. Genetically Modified Microorganisms 25.
Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. Journal of Molecular Biology 166, 557–580. https://doi.org/10.1016/S0022-2836(83)80284-8
Händel, E.-M., Alwin, S., Cathomen, T., 2009. Expanding or Restricting the Target Site Repertoire of Zinc-finger Nucleases: The Inter-domain Linker as a Major Determinant of Target Site Selectivity. Molecular Therapy 17, 104–111. https://doi.org/10.1038/mt.2008.233
Hare, P.D., Cress, W.A., Van Staden, J., 1998. Dissecting the roles of osmolyte accumulation during stress. Plant, Cell and Environment 21, 535–553. https://doi.org/10.1046/j.1365-3040.1998.00309.x
Hilbeck, A., Meier, M., Römbke, J., Jänsch, S., Teichmann, H., Tappeser, B., 2011. Environmental risk assessment of genetically modified plants - concepts and controversies. Environmental Sciences Europe 23, 13. https://doi.org/10.1186/2190-4715-23-13
Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E., Reddy, T.E., Gersbach, C.A., 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nature Biotechnology 33, 510–517. https://doi.org/10.1038/nbt.3199
Hinnen et al., 1978.pdf, n.d.
Holbein, J., Grundler, F.M.W., Siddique, S., 2016. Plant basal resistance to nematodes: an update. Journal of Experimental Botany 67, 2049–2061. https://doi.org/10.1093/jxb/erw005
Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M., Schell, J., 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Molecular and General Genetics MGG 163, 181–187. https://doi.org/10.1007/BF00267408
Homrich, M.S., Wiebke-Strohm, B., Weber, R.L.M., Bodanese-Zanettini, M.H., 2012. Soybean genetic transformation: a valuable tool for the functional study of genes and the production of agronomically improved plants. Genetics and Molecular Biology 35, 998–1010. https://doi.org/10.1590/S1415-47572012000600015
Hörtensteiner, S., Kräutler, B., 2011. Chlorophyll breakdown in higher plants. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1807, 977–988. https://doi.org/10.1016/j.bbabio.2010.12.007
Horvath, P., Romero, D.A., Coute-Monvoisin, A.-C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C., Barrangou, R., 2008. Diversity, Activity, and Evolution of CRISPR Loci in

Streptococcus thermophilus. Journal of Bacteriology 190, 1401–1412. https://doi.org/10.1128/JB.01415-07
Hosogane, M., Funayama, R., Shirota, M., Nakayama, K., 2016. Lack of Transcription Triggers H3K27me3 Accumulation in the Gene Body. Cell Reports 16, 696–706. https://doi.org/10.1016/j.celrep.2016.06.034
Houdebine, L.-M., 2014. Impacts of genetically modified animals on the ecosystem and human activities. Global Bioethics 25, 3–18. https://doi.org/10.1080/11287462.2014.894709
Houdebine, L.-M., 2009. Methods to Generate Transgenic Animals, in: Engelhard, M., Hagen, K., Boysen, M. (Eds.), Genetic Engineering in Livestock. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 31–48. https://doi.org/10.1007/978-3-540-85843-0_2
Hsu, P.D., Lander, E.S., Zhang, F., 2014. Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell 157, 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010
Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G., Zhang, F., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nature Biotechnology 31, 827–832. https://doi.org/10.1038/nbt.2647
Hu, H., Xiong, L., 2014. Genetic Engineering and Breeding of Drought-Resistant Crops. Annual Review of Plant Biology 65, 715–741. https://doi.org/10.1146/annurev-arplant-050213-040000
HUSBY, J., 2007. Chapter 23 Definitions of GMO/LMO and modern biotechnology 8.
Ikonomou, L., Schneider, Y.-J., Agathos, S.N., 2003. Insect cell culture for industrial production of recombinant proteins. Applied Microbiology and Biotechnology 62, 1–20. https://doi.org/10.1007/s00253-003-1223-9
Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., Nakata, A., 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. Journal of Bacteriology 169, 5429–5433. https://doi.org/10.1128/jb.169.12.5429-5433.1987
Jabalameli, H.R., Zahednasab, H., Karimi-Moghaddam, A., Jabalameli, M.R., 2015. Zinc finger nuclease technology: Advances and obstacles in modelling and treating genetic disorders. Gene 558, 1–5. https://doi.org/10.1016/j.gene.2014.12.044
Jaenisch, R., Mintz, B., 1974. Simian Virus 40 DNA Sequences in DNA of Healthy Adult Mice Derived from Preimplantation Blastocysts Injected with Viral DNA. Proceedings of the National Academy of Sciences 71, 1250–1254. https://doi.org/10.1073/pnas.71.4.1250
Jansen, R., Embden, J.D.A. van, Gaastra, W., Schouls, L.M., 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology 43, 1565–1575. https://doi.org/10.1046/j.1365-2958.2002.02839.x
Jefferson, R. A. (1989). The GUS reporter gene system. Nature, 342(6251), 837–838. http://doi.org/10.1038/342837a0
Jennings, B.H., 2011. Drosophila – a versatile model in biology & medicine. Materials Today 14, 190–195. https://doi.org/10.1016/S1369-7021(11)70113-4
Jewell, M.C., Campbell, B.C., Godwin, I.D., 2010. Transgenic Plants for Abiotic Stress Resistance, in: Kole, C., Michler, C.H., Abbott, A.G., Hall, T.C. (Eds.), Transgenic Crop Plants. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 67–132. https://doi.org/10.1007/978-3-642-04812-8_2
Jiang, F., Doudna, J.A., 2017. CRISPR–Cas9 Structures and Mechanisms. Annual Review of Biophysics 46, 505–529. https://doi.org/10.1146/annurev-biophys-062215-010822
Jiang, Y., Guo, W., Zhu, H., Ruan, Y.-L., Zhang, T., 2012. Overexpression of GhSusA1 increases plant biomass and improves cotton fiber yield and quality: Role of a novel sucrose synthase gene in cotton. Plant Biotechnology Journal 10, 301–312. https://doi.org/10.1111/j.1467-7652.2011.00662.x
Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816–821. https://doi.org/10.1126/science.1225829
Joung, J.K., Sander, J.D., 2013. TALENs: a widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology 14, 49–55. https://doi.org/10.1038/nrm3486
Juturu, V.N., Mekala, G.K., Kirti, P.B., 2015. Current status of tissue culture and genetic transformation research in cotton (Gossypium spp.). Plant Cell, Tissue and Organ Culture (PCTOC) 120, 813–839. https://doi.org/10.1007/s11240-014-0640-z
Kakarougkas, A., Jeggo, P.A., 2014. DNA DSB repair pathway choice: an orchestrated handover mechanism. The British Journal of Radiology 87, 20130685. https://doi.org/10.1259/bjr.20130685
Kamle, S., Ali, S., 2013. Genetically modified crops: Detection strategies and biosafety issues. Gene 522, 123–132. https://doi.org/10.1016/j.gene.2013.03.107
Kamthan, A., Chaudhuri, A., Kamthan, M., Datta, A., 2015. Small RNAs in plants: recent development and application for crop improvement. Frontiers in Plant Science 06. https://doi.org/10.3389/fpls.2015.00208
Karcher, S.J., n.d. Blue Plants: Transgenic Plants Whith The Gus Reporter Gene 14.

Kawai, S., Hashimoto, W., Murata, K., 2010. Transformation of *Saccharomyces cerevisiae* and other fungi: Methods and possible underlying mechanism. Bioengineered Bugs 1, 395–403. https://doi.org/10.4161/bbug.1.6.13257

Kay, S., Hahn, S., Marois, E., Hause, G., Bonas, U., 2007. A Bacterial Effector Acts as a Plant Transcription Factor and Induces a Cell Size Regulator. Science 318, 648–651. https://doi.org/10.1126/science.1144956

Kearns, N.A., Pham, H., Tabak, B., Genga, R.M., Silverstein, N.J., Garber, M., Maehr, R., 2015. Functional annotation of native enhancers with a Cas9–histone demethylase fusion. Nature Methods 12, 401–403. https://doi.org/10.1038/nmeth.3325

Kerr, A., Panagopoulos, C.G., 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their Biological Control. Journal of Phytopathology 90, 172–179. https://doi.org/10.1111/j.1439-0434.1977.tb03233.x

Keung, A.J., Joung, J.K., Khalil, A.S., Collins, J.J., 2015. Chromatin regulation at the frontier of synthetic biology. Nature Reviews Genetics 16, 159–171. https://doi.org/10.1038/nrg3900

Khatodia, S., Bhatotia, K., Passricha, N., Khurana, S.M.P., Tuteja, N., 2016. The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.00506

Kiani, S., Beal, J., Ebrahimkhani, M.R., Huh, J., Hall, R.N., Xie, Z., Li, Y., Weiss, R., 2014. CRISPR transcriptional repression devices and layered circuits in mammalian cells. Nature Methods 11, 723–726. https://doi.org/10.1038/nmeth.2969

Kim, H., Kim, J.-S., 2014. A guide to genome engineering with programmable nucleases. Nature Reviews Genetics 15, 321–334. https://doi.org/10.1038/nrg3686

Kim, J.-M., Sasaki, T., Ueda, M., Sako, K., Seki, M., 2015. Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00114

Kim, Y.G., Cha, J., Chandrasegaran, S., 1996. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proceedings of the National Academy of Sciences 93, 1156–1160. https://doi.org/10.1073/pnas.93.3.1156

Klebe, R.J., Harriss, J.V., Sharp, Z.D., Douglas, M.G., 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene 25, 333–341. https://doi.org/10.1016/0378-1119(83)90238-X

Koch, M.S., Ward, J.M., Levine, S.L., Baum, J.A., Vicini, J.L., Hammond, B.G., 2015. The food and environmental safety of Bt crops. Frontiers in Plant Science 06. https://doi.org/10.3389/fpls.2015.00283

Kodym, A., Afza, R., n.d. Physical and Chemical Mutagenesis 15.

Koenning K, S. R., O, C., N, J. W., D, P. A., & B, J. O. (1999). Survey of Crop Losses in Response to Phytoparasitic Nematodes in the United States for 1994 1, 31(1987), 587–618..

Kollist, H., Nuhkat, M., Roelfsema, M.R.G., 2014. Closing gaps: linking elements that control stomatal movement. New Phytologist 203, 44–62. https://doi.org/10.1111/nph.12832

Konermann, S., Brigham, M.D., Trevino, A.E., Hsu, P.D., Heidenreich, M., Le Cong, Platt, R.J., Scott, D.A., Church, G.M., Zhang, F., 2013. Optical control of mammalian endogenous transcription and epigenetic states. Nature 500, 472–476. https://doi.org/10.1038/nature12466

Krenek, P., Samajova, O., Luptovciak, I., Doskocilova, A., Komis, G., Samaj, J., 2015. Transient plant transformation mediated by *Agrobacterium tumefaciens:* Principles, methods and applications. Biotechnology Advances 33, 1024–1042. https://doi.org/10.1016/j.biotechadv.2015.03.012

Krishnamohan, A., Balaji, V., Veluthambi, K., 2001. Efficient vir Gene Induction in *Agrobacterium tumefaciens* Requires virA, virG, and vir Box from the Same Ti Plasmid. Journal of Bacteriology 183, 4079–4089. https://doi.org/10.1128/JB.183.13.4079-4089.2001

Kumar, V., Jain, M., 2015. The CRISPR–Cas system for plant genome editing: advances and opportunities. Journal of Experimental Botany 66, 47–57. https://doi.org/10.1093/jxb/eru429

Kushalappa, A.C., Yogendra, K.N., Karre, S., 2016. Plant Innate Immune Response: Qualitative and Quantitative Resistance. Critical Reviews in Plant Sciences 35, 38–55. https://doi.org/10.1080/07352689.2016.1148980

La Russa, M.F., Qi, L.S., 2015. The New State of the Art: Cas9 for Gene Activation and Repression. Molecular and Cellular Biology 35, 3800–3809. https://doi.org/10.1128/MCB.00512-15

Lander, E.S., 2016. The Heroes of CRISPR. Cell 164, 18–28. https://doi.org/10.1016/j.cell.2015.12.041

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S., Qi, L.S., 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nature Protocols 8, 2180–2196. https://doi.org/10.1038/nprot.2013.132

Leach, K.A., Hejlek, L.G., Hearne, L.B., Nguyen, H.T., Sharp, R.E., Davis, G.L., 2011. Primary Root Elongation Rate and Abscisic Acid Levels of Maize in Response to Water Stress. Crop Science 51, 157. https://doi.org/10.2135/cropsci2009.12.0708

Leckie, B.M., Neal Stewart, C., 2011. Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. Plant Cell Reports 30, 325–334. https://doi.org/10.1007/s00299-010-0961-2

Lee, Y. W., Jin, S., Sim, W. S., & Nester, E. W. (1996). The sensing of plant signal molecules by Agrobacterium: Genetic evidence for direct recognition of phenolic inducers by the VirA protein. Gene, 179(1), 83–88. http://doi.org/10.1016/S0378-1119(96)00328-9

Lee, H., Rustgi, S., Kumar, N., Burke, I., Yenish, J.P., Gill, K.S., von Wettstein, D., Ullrich, S.E., 2011. Single nucleotide mutation in the barley acetohydroxy acid synthase (AHAS) gene confers resistance to imidazolinone herbicides. Proceedings of the National Academy of Sciences 108, 8909–8913. https://doi.org/10.1073/pnas.1105612108

Leite, J.P., Barbosa, E.G.G., Marin, S.R.R., Marinho, J.P., Carvalho, J.F.C., Pagliarini, R.F., Cruz, A.S., Oliveira, M.C.N., Farias, J.R.B., Neumaier, N., Guimarães, F.C.M., Yoshida, T., Kanamori, N., Fujita, Y., Nakashima, K., Shinozaki, K.Y., Desidério, J.A., Nepomuceno, A.L., 2014. Overexpression of the activated form of the AtAREB1 gene (AtAREB1ΔQT) improves soybean responses to water deficit. Genetics and Molecular Research 13, 6272–6286. https://doi.org/10.4238/2014.August.15.10

Lemaux, P.G., 2008. Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part I). Annual Review of Plant Biology 59, 771–812. https://doi.org/10.1146/annurev.arplant.58.032806.103840

Lenin, V. Vladimir Ilyich Lenin. (n.d.). LibraryofQuotes.com. Accessed Tue 12 Jun 18, from LibraryofQuotes.com Web site: http://libraryofquotes.com/quote/872248 Read more at http://libraryofquotes.com/quote/872248

Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X., Chen, Q., 2013. Efficient Agroinfiltration of Plants for High-level Transient Expression of Recombinant Proteins. Journal of Visualized Experiments. https://doi.org/10.3791/50521

Li, X. (2011). Tissue Staining protocol.

Li, J.F., Nebenfuhr, A., 2010. FAST Technique for Agrobacterium-Mediated Transient Gene Expression in Seedlings of Arabidopsis and Other Plant Species. Cold Spring Harbor Protocols 2010, pdb.prot5428-pdb.prot5428. https://doi.org/10.1101/pdb.prot5428

Li, X., Jun, Y., Erickstad, M.J., Brown, S.D., Parks, A., Court, D.L., Jun, S., 2016. tCRISPRi: tunable and reversible, one-step control of gene expression. Scientific Reports 6. https://doi.org/10.1038/srep39076

Li, X.-Y., Liu, X., Yao, Y., Li, Y.-H., Liu, S., He, C.-Y., Li, J.-M., Lin, Y.-Y., Li, L., 2013. Overexpression of Arachis hypogaea AREB1 Gene Enhances Drought Tolerance by Modulating ROS Scavenging and Maintaining Endogenous ABA Content. International Journal of Molecular Sciences 14, 12827–12842. https://doi.org/10.3390/ijms140612827

Li, Z., Liu, Z.-B., Xing, A., Moon, B.P., Koellhoffer, J.P., Huang, L., Ward, R.T., Clifton, E., Falco, S.C., Cigan, A.M., 2015. Cas9-Guide RNA Directed Genome Editing in Soybean. Plant Physiology 169, 960–970. https://doi.org/10.1104/pp.15.00783

Liang, C., Prins, T.W., van de Wiel, C.C.M., Kok, E.J., 2014. Safety aspects of genetically modified crops with abiotic stress tolerance. Trends in Food Science & Technology 40, 115–122. https://doi.org/10.1016/j.tifs.2014.08.005

Liang, C. *et al.* GhABF2, a bZIP transcription factor, confers drought and salinity tolerance in cotton (*Gossypium hirsutum* L.). *Sci. Rep.* **6**, 35040; doi: 10.1038/srep35040 (2016) Lievens, A., Petrillo, M., Querci, M., Patak, A., 2015. Genetically modified animals: Options and issues for traceability and enforcement. Trends in Food Science & Technology 44, 159–176. https://doi.org/10.1016/j.tifs.2015.05.001

Lilley, C.J., Kyndt, T., Gheysen, G., 2011. Nematode Resistant GM Crops in Industrialised and Developing Countries, in: Jones, J., Gheysen, G., Fenoll, C. (Eds.), Genomics and Molecular Genetics of Plant-Nematode Interactions. Springer Netherlands, Dordrecht, pp. 517–541. https://doi.org/10.1007/978-94-007-0434-3_24

Ling, M.M., Robinson, B.H., 1997. Approaches to DNA Mutagenesis: An Overview. Analytical Biochemistry 254, 157–178. https://doi.org/10.1006/abio.1997.2428

Liu, H., Naismith, J.H., 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnology 8, 91. https://doi.org/10.1186/1472-6750-8-91

Liu, X., Wu, S., Xu, J., Sui, C., Wei, J., 2017. Application of CRISPR/Cas9 in plant biology. Acta Pharmaceutica Sinica B 7, 292–302. https://doi.org/10.1016/j.apsb.2017.01.002

Liu, X.S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R.A., Jaenisch, R., 2016. Editing DNA Methylation in the Mammalian Genome. Cell 167, 233-247.e17. https://doi.org/10.1016/j.cell.2016.08.056

Liu, Y.-C., Vidali, L., 2011. Efficient Polyethylene Glycol (PEG) Mediated Transformation of the Moss *Physcomitrella patens*. Journal of Visualized Experiments. https://doi.org/10.3791/2560

Lo, A., Qi, L., 2017. Genetic and epigenetic control of gene expression by CRISPR–Cas systems. F1000Research 6, 747. https://doi.org/10.12688/f1000research.11113.1

Lobell, D.B., Gourdji, S.M., 2012. The Influence of Climate Change on Global Crop Productivity. PLANT PHYSIOLOGY 160, 1686–1697. https://doi.org/10.1104/pp.112.208298

Lopez-Obando, M., Hoffmann, B., Gery, C., Guyon-Debast, A., Teoule, E., Rameau, C., Bonhomme, S., Nogue, F., 2016. Simple and Efficient Targeting of Multiple Genes Through CRISPR-Cas9 in *Physcomitrella patens*. G3: Genes|Genomes|Genetics. https://doi.org/10.1534/g3.116.033266

Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W., Tang, X., Zheng, X., Voytas, D.F., Hsieh, T.-F., Zhang, Y., Qi, Y., 2015a. A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation. Plant Physiology 169, 971–985. https://doi.org/10.1104/pp.15.00636

Lowder, L.G., Zhang, D., Baltes, N.J., Paul, J.W., Tang, X., Zheng, X., Voytas, D.F., Hsieh, T.-F., Zhang, Y., Qi, Y., 2015b. A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation. Plant Physiology 169, 971–985. https://doi.org/10.1104/pp.15.00636

Lowder, L.G., Zhou, J., Zhang, Yingxiao, Malzahn, A., Zhong, Z., Hsieh, T.-F., Voytas, D.F., Zhang, Yong, Qi, Y., 2018. Robust Transcriptional Activation in Plants Using Multiplexed CRISPR-Act2.0 and mTALE-Act Systems. Molecular Plant 11, 245–256. https://doi.org/10.1016/j.molp.2017.11.010

Loyola-Vargas V.M., Vázquez-Flota F. (2006) An Introduction to Plant Cell Culture. In: Loyola-Vargas V.M., Vázquez-Flota F. (eds) Plant Cell Culture Protocols. Methods in Molecular Biology™, vol 318. Humana Press

Lu, P., n.d. EFFECT OF SOUTHERN ROOT-KNOT NEMATODE (MELOIDOGYNE INCOGNITA) ON COTTON GROWTH, YIELD, AND FIBER QUALITY 149.

Luo, J., 2016. CRISPR/Cas9: From Genome Engineering to Cancer Drug Discovery. Trends in Cancer 2, 313–324. https://doi.org/10.1016/j.trecan.2016.05.001

Luo, X., Wu, J., Li, Y., Nan, Z., Guo, X., Wang, Y., Zhang, A., Wang, Z., Xia, G., Tian, Y., 2013. Synergistic Effects of GhSOD1 and GhCAT1 Overexpression in Cotton Chloroplasts on Enhancing Tolerance to Methyl Viologen and Salt Stresses. PLoS ONE 8, e54002. https://doi.org/10.1371/journal.pone.0054002

Ma, Y., Zhang, J., Yin, W., Zhang, Z., Song, Y., Chang, X., 2016. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. Nature Methods 13, 1029–1035. https://doi.org/10.1038/nmeth.4027

Maeder, M.L., Linder, S.J., Cascio, V.M., Fu, Y., Ho, Q.H., Joung, J.K., 2013. CRISPR RNA–guided activation of endogenous human genes. Nature Methods 10, 977–979. https://doi.org/10.1038/nmeth.2598

Maeder, M.L., Thibodeau-Beganny, S., Osiak, A., Wright, D.A., Anthony, R.M., Eichtinger, M., Jiang, T., Foley, J.E., Winfrey, R.J., Townsend, J.A., Unger-Wallace, E., Sander, J.D., Müller-Lerch, F., Fu, F., Pearlberg, J., Göbel, C., Dassie, J.P., Pruett-Miller, S.M., Porteus, M.H., Sgroi, D.C., Iafrate, A.J., Dobbs, D., McCray, P.B., Cathomen, T., Voytas, D.F., Joung, J.K., 2008. Rapid "Open-Source" Engineering of Customized Zinc-Finger Nucleases for Highly Efficient Gene Modification. Molecular Cell 31, 294–301. https://doi.org/10.1016/j.molcel.2008.06.016

Maes, W.H., Achten, W.M.J., Reubens, B., Raes, D., Samson, R., Muys, B., 2009. Plant–water relationships and growth strategies of *Jatropha curcas* L. seedlings under different levels of drought stress. Journal of Arid Environments 73, 877–884. https://doi.org/10.1016/j.jaridenv.2009.04.013

Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J.J., Charpentier, E., Haft, D.H., Horvath, P., Moineau, S., Mojica, F.J.M., Terns, R.M., Terns, M.P., White, M.F., Yakunin, A.F., Garrett, R.A., van der Oost, J., Backofen, R., Koonin, E.V., 2015. An updated evolutionary classification of CRISPR–Cas systems. Nature Reviews Microbiology 13, 722–736. https://doi.org/10.1038/nrmicro3569

Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L., Church, G.M., 2013. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nature Biotechnology 31, 833–838. https://doi.org/10.1038/nbt.2675

Malik, H.J., Raza, A., Amin, I., Scheffler, J.A., Scheffler, B.E., Brown, J.K., Mansoor, S., 2016. RNAi-mediated mortality of the whitefly through transgenic expression of double-stranded RNA homologous to acetylcholinesterase and ecdysone receptor in tobacco plants. Scientific Reports 6. https://doi.org/10.1038/srep38469

Maloof, J. N., Nozue, K., Mumbach, M. R., & Palmer, C. M. (2013). LeafJ: An ImageJ Plugin for Semi-automated Leaf Shape Measurement. Journal of Visualized Experiments, (71), 2–7. http://doi.org/10.3791/50028

Malzahn, A., Lowder, L., Qi, Y., 2017. Plant genome editing with TALEN and CRISPR. Cell & Bioscience 7. https://doi.org/10.1186/s13578-017-0148-4

Mandel, M., & Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. Journal of Molecular Biology, 53(1), 159–162. http://doi.org/10.1016/0022-2836(70)90051-3

Marengo, J. A., Nobre, C. A., Chou, S. C., Tomasella, J., Sampaio, G., Alves, L. M., … Kay, G. (2011). Dangerous Climate Change in Brazil, 56. Retrieved from http://mudancasclimaticas.cptec.inpe.br/~rmclima/pdfs/destaques/relatorio_ingl.pdf

Margolin, J.F., Friedman, J.R., Meyer, W.K., Vissing, H., Thiesen, H.J., Rauscher, F.J., 1994. Kruppel-associated boxes are potent transcriptional repression domains. Proceedings of the National Academy of Sciences 91, 4509–4513. https://doi.org/10.1073/pnas.91.10.4509

Marowa, P., Ding, A., Kong, Y., 2016. Expansins: roles in plant growth and potential applications in crop improvement. Plant Cell Reports 35, 949–965. https://doi.org/10.1007/s00299-016-1948-4
Master, Z., Resnik, D.B., 2013. Hype and Public Trust in Science. Science and Engineering Ethics 19, 321–335. https://doi.org/10.1007/s11948-011-9327-6
Mathur, J., Koncz, C., 1998. PEG-Mediated Protoplast Transformation with Naked DNA, in: Martinez-Zapater, J.M., Salinas, J. (Eds.), Arabidopsis Protocols. Humana Press, Totowa, NJ, pp. 267–276. https://doi.org/10.1385/0-89603-391-0:267
McDonald, J.I., Celik, H., Rois, L.E., Fishberger, G., Fowler, T., Rees, R., Kramer, A., Martens, A., Edwards, J.R., Challen, G.A., 2016. Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. Biology Open 5, 866–874. https://doi.org/10.1242/bio.019067
Mehran, A., AghaKouchak, A., Nakhjiri, N., Stewardson, M.J., Peel, M.C., Phillips, T.J., Wada, Y., Ravalico, J.K., 2017. Compounding Impacts of Human-Induced Water Stress and Climate Change on Water Availability. Scientific Reports 7. https://doi.org/10.1038/s41598-017-06765-0
Meyerowitz, E.M., 2001. Prehistory and History of Arabidopsis Research. PLANT PHYSIOLOGY 125, 15–19. https://doi.org/10.1104/pp.125.1.15
Miles, C. & Wayne, M. (2008) Quantitative trait locus (QTL) analysis. Nature
Education 1(1):208____https://www.nature.com/scitable/topicpage/quantitative-trait-
locus-qtl-analysis-53904
Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., Dulay, G.P., Hua, K.L., Ankoudinova, I., Cost, G.J., Urnov, F.D., Zhang, H.S., Holmes, M.C., Zhang, L., Gregory, P.D., Rebar, E.J., 2011. A TALE nuclease architecture for efficient genome editing. Nature Biotechnology 29, 143–148. https://doi.org/10.1038/nbt.1755
Mitsunobu, H., Teramoto, J., Nishida, K., Kondo, A., 2017. Beyond Native Cas9: Manipulating Genomic Information and Function. Trends in Biotechnology 35, 983–996. https://doi.org/10.1016/j.tibtech.2017.06.004
Mittal, A., Jiang, Y., Ritchie, G.L., Burke, J.J., Rock, C.D., 2015. At RAV1 and At RAV2 overexpression in cotton increases fiber length differentially under drought stress and delays flowering. Plant Science 241, 78–95. https://doi.org/10.1016/j.plantsci.2015.09.013
Modrich, P., 1979. Structures and mechanisms of DNA restriction and modification enzymes. Quarterly Reviews of Biophysics 12, 315. https://doi.org/10.1017/S0033583500005461
Mojica, F.J.M., Diez-Villasenor, C., Soria, E., Juez, G., 2000. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Molecular Microbiology 36, 244–246. https://doi.org/10.1046/j.1365-2958.2000.01838.x
Mojica, F.J.M., Dez-Villaseor, C., Garca-Martnez, J., Soria, E., 2005. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. Journal of Molecular Evolution 60, 174–182. https://doi.org/10.1007/s00239-004-0046-3
Mojica, F.J.M., Ferrer, C., Juez, G., Rodríguez-Valera, F., 1995. Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. Molecular Microbiology 17, 85–93. https://doi.org/10.1111/j.1365-2958.1995.mmi_17010085.x
Mojica, F.J.M., Juez, G., Rodriguez-Valera, F., 1993. Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Molecular Microbiology 9, 613–621. https://doi.org/10.1111/j.1365-2958.1993.tb01721.x
Monfreda, C., Ramankutty, N., Foley, J.A., 2008. Farming the planet: 2. Geographic distribution of crop areas, yields, physiological types, and net primary production in the year 2000: GLOBAL CROP AREAS AND YIELDS IN 2000. Global Biogeochemical Cycles 22, n/a-n/a. https://doi.org/10.1029/2007GB002947
Moody, J.D., Levy, S., Mathieu, J., Xing, Y., Kim, W., Dong, C., Tempel, W., Robitaille, A.M., Dang, L.T., Ferreccio, A., Detraux, D., Sidhu, S., Zhu, L., Carter, L., Xu, C., Valensisi, C., Wang, Y., Hawkins, R.D., Min, J., Moon, R.T., Orkin, S.H., Baker, D., Ruohola-Baker, H., 2017. First critical repressive H3K27me3 marks in embryonic stem cells identified using designed protein inhibitor. Proceedings of the National Academy of Sciences 114, 10125–10130. https://doi.org/10.1073/pnas.1706907114
Morbitzer, R., Romer, P., Boch, J., Lahaye, T., 2010. Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors. Proceedings of the National Academy of Sciences 107, 21617–21622. https://doi.org/10.1073/pnas.1013133107
Morgante, C.V., Brasileiro, A.C.M., Roberts, P.A., Guimaraes, L.A., Araujo, A.C.G., Fonseca, L.N., Leal-Bertioli, S.C.M., Bertioli, D.J., Guimaraes, P.M., 2013. A survey of genes involved in *Arachis stenosperma* resistance to *Meloidogyne arenaria* race 1. Functional Plant Biology 40, 1298. https://doi.org/10.1071/FP13096
Moscou, M.J., Bogdanove, A.J., 2009. A Simple Cipher Governs DNA Recognition by TAL Effectors. Science 326, 1501–1501. https://doi.org/10.1126/science.1178817
Msanne, J., Lin, J., Stone, J.M., Awada, T., 2011. Characterization of abiotic stress-responsive *Arabidopsis thaliana* RD29A and RD29B genes and evaluation of transgenes. Planta 234, 97–107. https://doi.org/10.1007/s00425-011-1387-y

Murovec, J., Pirc, Ž., Yang, B., 2017. New variants of CRISPR RNA-guided genome editing enzymes. Plant Biotechnology Journal 15, 917–926. https://doi.org/10.1111/pbi.12736
Murray, J.D., Maga, E.A., 2016. Genetically engineered livestock for agriculture: a generation after the first transgenic animal research conference. Transgenic Research 25, 321–327. https://doi.org/10.1007/s11248-016-9927-7
Mwadzingeni, L., Shimelis, H., Dube, E., Laing, M.D., Tsilo, T.J., 2016. Breeding wheat for drought tolerance: Progress and technologies. Journal of Integrative Agriculture 15, 935–943. https://doi.org/10.1016/S2095-3119(15)61102-9
Najm, F.J., Strand, C., Donovan, K.F., Hegde, M., Sanson, K.R., Vaimberg, E.W., Sullender, M.E., Hartenian, E., Kalani, Z., Fusi, N., Listgarten, J., Younger, S.T., Bernstein, B.E., Root, D.E., Doench, J.G., 2017. Orthologous CRISPR–Cas9 enzymes for combinatorial genetic screens. Nature Biotechnology 36, 179–189. https://doi.org/10.1038/nbt.4048
Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. Frontiers in Plant Science 5. https://doi.org/10.3389/fpls.2014.00170
Napoli, C., Lemieux, C., & Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. The Plant Cell, 2(4), 279–289. http://doi.org/10.1105/tpc.2.4.279
Newell-McGloughlin, M., 2008. Nutritionally Improved Agricultural Crops. PLANT PHYSIOLOGY 147, 939–953. https://doi.org/10.1104/pp.108.121947
Nickson, T.E., 2008. Planning Environmental Risk Assessment for Genetically Modified Crops: Problem Formulation for Stress-Tolerant Crops. Plant Physiology 147, 494–502. https://doi.org/10.1104/pp.108.118422
Nicol, J.M., Turner, S.J., Coyne, D.L., Nijs, L. den, Hockland, S., Maafi, Z.T., 2011. Current Nematode Threats to World Agriculture, in: Jones, J., Gheysen, G., Fenoll, C. (Eds.), Genomics and Molecular Genetics of Plant-Nematode Interactions. Springer Netherlands, Dordrecht, pp. 21–43. https://doi.org/10.1007/978-94-007-0434-3_2
Nielsen, A.A., Voigt, C.A., 2014. Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. Molecular Systems Biology 10, 763–763. https://doi.org/10.15252/msb.20145735
Notani, N.K., Joshi, V.P., Kanade, R.P., 1984. Genetic transformation in bacteria. Journal of Biosciences 6, 525–533. https://doi.org/10.1007/BF02703903
Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M.O., Jin, Y.-W., Lee, E.-K., Loake, G.J., 2016. Plant cell culture strategies for the production of natural products. BMB Reports 49, 149–158. https://doi.org/10.5483/BMBRep.2016.49.3.264
O'Geen, H., Ren, C., Nicolet, C.M., Perez, A.A., Halmai, J., Le, V.M., Mackay, J.P., Farnham, P.J., Segal, D.J., 2017. dCas9-based epigenome editing suggests acquisition of histone methylation is not sufficient for target gene repression. Nucleic Acids Research 45, 9901–9916. https://doi.org/10.1093/nar/gkx578
O'Geen, H., Yu, A.S., Segal, D.J., 2015. How specific is CRISPR/Cas9 really? Current Opinion in Chemical Biology 29, 72–78. https://doi.org/10.1016/j.cbpa.2015.10.001
Oh, S.-J., 2005. Arabidopsis CBF3/DREB1A and ABF3 in Transgenic Rice Increased Tolerance to Abiotic Stress without Stunting Growth. Plant Physiology 138, 341–351. https://doi.org/10.1104/pp.104.059147
Okada, M., Kanamori, M., Someya, K., Nakatsukasa, H., & Yoshimura, A. (2017). Stabilization of Foxp3 expression by CRISPR-dCas9-based epigenome editing in mouse primary T cells. Epigenetics and Chromatin, 10(1), 1–17. http://doi.org/10.1186/s13072-017-0129-1
Oladosu, Y., Rafii, M.Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H.A., Miah, G., Usman, M., 2016. Principle and application of plant mutagenesis in crop improvement: a review. Biotechnology & Biotechnological Equipment 30, 1–16. https://doi.org/10.1080/13102818.2015.1087333
O'Malley, R.C., Barragan, C.C., Ecker, J.R., 2017. A User's Guide to the Arabidopsis T-DNA Insertional Mutant Collections 22.
Oosterhuis, D.M., n.d. Stress Physiology in Cotton 175.
Osakabe, Y., Osakabe, K., Shinozaki, K., Tran, L.-S.P., 2014. Response of plants to water stress. Frontiers in Plant Science 5. https://doi.org/10.3389/fpls.2014.00086
Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.-S.P., 2013. Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. Journal of Experimental Botany 64, 445–458. https://doi.org/10.1093/jxb/ers354
Pagès, V., Fuchs, R.P., 2002. How DNA lesions are turned into mutations within cells? Oncogene 21, 8957–8966. https://doi.org/10.1038/sj.onc.1206006
Palle, S.R., Campbell, L.M., Pandeya, D., Puckhaber, L., Tollack, L.K., Marcel, S., Sundaram, S., Stipanovic, R.D., Wedegaertner, T.C., Hinze, L., Rathore, K.S., 2013. RNAi-mediated Ultra-low gossypol cottonseed trait: performance of transgenic lines under field conditions. Plant Biotechnology Journal 11, 296–304. https://doi.org/10.1111/pbi.12013
Pandey, G., Sharma, N., Pankaj Sahu, P., Prasad, M., 2016. Chromatin-Based Epigenetic Regulation of Plant Abiotic Stress Response. Current Genomics 17, 490–498. https://doi.org/10.2174/1389202917666160520103914

Park, J.-J., Dempewolf, E., Zhang, W., Wang, Z.-Y., 2017a. RNA-guided transcriptional activation via CRISPR/dCas9 mimics overexpression phenotypes in Arabidopsis. PLos One 12, e0179410. https://doi.org/10.1371/journal.pone.0179410

Park, J.-J., Dempewolf, E., Zhang, W., Wang, Z.-Y., 2017b. RNA-guided transcriptional activation via CRISPR/dCas9 mimics overexpression phenotypes in Arabidopsis. PLos One 12, e0179410. https://doi.org/10.1371/journal.pone.0179410

Pasini, M.E., Bertolotto, F., Fasano, P., 2010. The role of models in science: an experience with Drosophila. Procedia - Social and Behavioral Sciences 2, 1164–1168. https://doi.org/10.1016/j.sbspro.2010.03.166

Patel, S.J., Sanjana, N.E., Kishton, R.J., Eidizadeh, A., Vodnala, S.K., Cam, M., Gartner, J.J., Jia, L., Steinberg, S.M., Yamamoto, T.N., Merchant, A.S., Mehta, G.U., Chichura, A., Shalem, O., Tran, E., Eil, R., Sukumar, M., Guijarro, E.P., Day, C.-P., Robbins, P., Feldman, S., Merlino, G., Zhang, F., Restifo, N.P., 2017. Identification of essential genes for cancer immunotherapy. Nature 548, 537–542. https://doi.org/10.1038/nature23477

Perry, E.D., Ciliberto, F., Hennessy, D.A., Moschini, G., 2016. Genetically engineered crops and pesticide use in U.S. maize and soybeans. Science Advances 2, e1600850–e1600850. https://doi.org/10.1126/sciadv.1600850

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research, 29(9), 45e–45. http://doi.org/10.1093/nar/29.9.e45

Phillips, R.L., 2009. Ronald L. Phillips, in: Wolf Prize in Agriculture. World Scientific, pp. 931–986. https://doi.org/10.1142/9789812835857_0038

Piatek, A., Ali, Z., Baazim, H., Li, L., Abulfaraj, A., Al-Shareef, S., Aouida, M., Mahfouz, M.M., 2015a. RNA-guided transcriptional regulation *in planta* via synthetic dCas9-based transcription factors. Plant Biotechnology Journal 13, 578–589. https://doi.org/10.1111/pbi.12284

Piatek, A., Ali, Z., Baazim, H., Li, L., Abulfaraj, A., Al-Shareef, S., Aouida, M., Mahfouz, M.M., 2015b. RNA-guided transcriptional regulation *in planta* via synthetic dCas9-based transcription factors. Plant Biotechnology Journal 13, 578–589. https://doi.org/10.1111/pbi.12284

Pombo, A., Dillon, N., 2015. Three-dimensional genome architecture: players and mechanisms. Nature Reviews Molecular Cell Biology 16, 245–257. https://doi.org/10.1038/nrm3965

Poss, Z.C., Ebmeier, C.C., Taatjes, D.J., 2013. The Mediator complex and transcription regulation. Critical Reviews in Biochemistry and Molecular Biology 48, 575–608. https://doi.org/10.3109/10409238.2013.840259

Prado, J.R., Segers, G., Voelker, T., Carson, D., Dobert, R., Phillips, J., Cook, K., Cornejo, C., Monken, J., Grapes, L., Reynolds, T., Martino-Catt, S., 2014. Genetically Engineered Crops: From Idea to Product. Annual Review of Plant Biology 65, 769–790. https://doi.org/10.1146/annurev-arplant-050213-040039

Purugganan, M.D., Fuller, D.Q., 2009. The nature of selection during plant domestication. Nature 457, 843–848. https://doi.org/10.1038/nature07895

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. Cell 152, 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022

Radzisheuskaya, A., Shlyueva, D., Müller, I., Helin, K., 2016. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucleic Acids Research 44, e141–e141. https://doi.org/10.1093/nar/gkw583

Ran, F.A., Hsu, P.D., Lin, C.-Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y., Zhang, F., 2013. Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Cell 154, 1380–1389. https://doi.org/10.1016/j.cell.2013.08.021

Rech, E.L., Vianna, G.R., Aragão, F.J.L., 2008. High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nature Protocols 3, 410–418. https://doi.org/10.1038/nprot.2008.9

Rittié, L., Perbal, B., 2008. Enzymes used in molecular biology: a useful guide. Journal of Cell Communication and Signaling 2, 25–45. https://doi.org/10.1007/s12079-008-0026-2

Rivera, A.L., Gómez-Lim, M., Fernández, F., Loske, A.M., 2012. Physical methods for genetic plant transformation. Physics of Life Reviews 9, 308–345. https://doi.org/10.1016/j.plrev.2012.06.002

Romer, P., Hahn, S., Jordan, T., Strauss, T., Bonas, U., Lahaye, T., 2007. Plant Pathogen Recognition Mediated by Promoter Activation of the Pepper Bs3 Resistance Gene. Science 318, 645–648. https://doi.org/10.1126/science.1144958

Rusk, N., 2011. TALEs for the masses. Nature Methods 8, 197–197. https://doi.org/10.1038/nmeth0311-197

Sadowski, I., Ma, J., Triezenberg, S., Ptashne, M., 1988. GAL4-VP16 is an unusually potent transcriptional activator.

Sahito, A., Baloch, Z.A., Mahar, A., Otho, S.A., Kalhoro, S.A., Ali, A., Kalhoro, F.A., Soomro, R.N., Ali, F., 2015. Effect of Water Stress on the Growth and Yield of Cotton Crop (<i>Gossypium hirsutum</i> L.). American Journal of Plant Sciences 06, 1027–1039. https://doi.org/10.4236/ajps.2015.67108

Sakuraba, Y., Kim, Y.-S., Han, S.-H., Lee, B.-D., Paek, N.-C., 2015. The Arabidopsis Transcription Factor NAC016 Promotes Drought Stress Responses by Repressing *AREB1* Transcription through a Trifurcate Feed-Forward Regulatory Loop Involving NAP. The Plant Cell 27, 1771–1787. https://doi.org/10.1105/tpc.15.00222
Saleh, A., Al-Abdallat, A., Ndamukong, I., Alvarez-Venegas, R., Avramova, Z., 2007. The Arabidopsis homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus. Nucleic Acids Research 35, 6290–6296. https://doi.org/10.1093/nar/gkm464
Schiml, S., Puchta, H., 2016. Revolutionizing plant biology: multiple ways of genome engineering by CRISPR/Cas. Plant Methods 12. https://doi.org/10.1186/s13007-016-0103-0
Schneider, A., Chatterjee, S., Bousiges, O., Selvi, B.R., Swaminathan, A., Cassel, R., Blanc, F., Kundu, T.K., Boutillier, A.-L., 2013. Acetyltransferases (HATs) as Targets for Neurological Therapeutics. Neurotherapeutics 10, 568–588. https://doi.org/10.1007/s13311-013-0204-7
Schrader, TJ, 2003. In Encyclopedia of Food Sciences and Nutrition (Second Edition),
2003
Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., Goodrich, J., 2006a. Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. The EMBO Journal 25, 4638–4649. https://doi.org/10.1038/sj.emboj.7601311
Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., Goodrich, J., 2006b. Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. The EMBO Journal 25, 4638–4649. https://doi.org/10.1038/sj.emboj.7601311
Secretariat of the Convention on Biological Diversity (Ed.), 2000. Cartagena Protocol on Biosafety to the Convention on Biological Diversity: text and annexes. Secretariat of the Convention on Biological Diversity, Montreal.
Sen, G.L., Blau, H.M., 2006. A brief history of RNAi: the silence of the genes. The FASEB Journal 20, 1293–1299. https://doi.org/10.1096/fj.06-6014rev
Serrano, L., 2007. Synthetic biology: promises and challenges. Molecular Systems Biology 3. https://doi.org/10.1038/msb4100202
Setlow, J.K., Hollaender, A., 1979. Genetic Engineering: Principles and Methods Volume 1.
Sgouras, D.N., Athanasioul, M.A., Beal, G.J., Fisherl, R.J., Blair, D.G., Mavrothalassitis, G.J., n.d. ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation 13.
Shahmuradov, I.A., Umarov, R.K., Solovyev, V.V., 2017. TSSPlant: a new tool for prediction of plant Pol II promoters. Nucleic Acids Research gkw1353. https://doi.org/10.1093/nar/gkw1353
Shamim, Z., Rashid, B., ur Rahman, S., Husnain, T., 2013. Expression of drought tolerance in transgenic cotton. ScienceAsia 39, 1. https://doi.org/10.2306/scienceasia1513-1874.2013.39.001
Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N.G., Palmer, A.E., Tsien, R.Y., 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nature Biotechnology 22, 1567–1572. https://doi.org/10.1038/nbt1037
Sherratt, D., 2001. Bacteria rule!: The start of a new era in bacterial microbiology? EMBO reports 2, 175–176. https://doi.org/10.1093/embo-reports/kve057
Sheth, R.U., Yim, S.S., Wu, F.L., Wang, H.H., 2017. Multiplex recording of cellular events over time on CRISPR biological tape. Science 358, 1457–1461. https://doi.org/10.1126/science.aao0958
Shimizu, Y., Bhakta, M.S., Segal, D.J., 2009. Restricted spacer tolerance of a zinc finger nuclease with a six amino acid linker. Bioorganic & Medicinal Chemistry Letters 19, 3970–3972. https://doi.org/10.1016/j.bmcl.2009.02.109
Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology 6, 410–417. https://doi.org/10.1016/S1369-5266(03)00092-X
Shipman, S.L., Nivala, J., Macklis, J.D., Church, G.M., 2017a. CRISPR–Cas encoding of a digital movie into the genomes of a population of living bacteria. Nature 547, 345–349. https://doi.org/10.1038/nature23017
Shipman, S.L., Nivala, J., Macklis, J.D., Church, G.M., 2017b. CRISPR–Cas encoding of a digital movie into the genomes of a population of living bacteria. Nature 547, 345–349. https://doi.org/10.1038/nature23017
Shipman, S.L., Nivala, J., Macklis, J.D., Church, G.M., 2016. Molecular recordings by directed CRISPR spacer acquisition. Science 353, aaf1175. https://doi.org/10.1126/science.aaf1175
Shmakov, S., Abudayyeh, O.O., Makarova, K.S., Wolf, Y.I., Gootenberg, J.S., Semenova, E., Minakhin, L., Joung, J., Konermann, S., Severinov, K., Zhang, F., Koonin, E.V., 2015. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Molecular Cell 60, 385–397. https://doi.org/10.1016/j.molcel.2015.10.008
Sikora, P., Chawade, A., Larsson, M., Olsson, J., Olsson, O., 2011. Mutagenesis as a Tool in Plant Genetics, Functional Genomics, and Breeding. International Journal of Plant Genomics 2011, 1–13. https://doi.org/10.1155/2011/314829
Silva, G.H., 2004. Analysis of the LAGLIDADG interface of the monomeric homing endonuclease I-DmoI. Nucleic Acids Research 32, 3156–3168. https://doi.org/10.1093/nar/gkh618

Silvente, S., Sobolev, A.P., Lara, M., 2012. Metabolite Adjustments in Drought Tolerant and Sensitive Soybean Genotypes in Response to Water Stress. PLoS ONE 7, e38554. https://doi.org/10.1371/journal.pone.0038554

Silver, M. L, Mouse genetics, concepts and applications *Oxford University Press 1995 Adapted for the Web by: Mouse Genome Informatics The Jackson LaboratoryBar Harbor, Maine November 2001 Revised August 2004, January 2008*

Singh, D., & Laxmi, A. (2015). Transcriptional regulation of drought response: a tortuous network of transcriptional factors. Frontiers in Plant Science, 6(October), 1–11. http://doi.org/10.3389/fpls.2015.00895

Smith, J., Grizot, S., Arnould, S., Duclert, A., Epinat, J.-C., Chames, P., Prieto, J., Redondo, P., Blanco, F.J., Bravo, J., Montoya, G., Pâques, F., Duchateau, P., 2006. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nucleic Acids Research 34, e149–e149. https://doi.org/10.1093/nar/gkl720

Song, G., Jia, M., Chen, K., Kong, X., Khattak, B., Xie, C., Li, A., Mao, L., 2016. CRISPR/Cas9: A powerful tool for crop genome editing. The Crop Journal 4, 75–82. https://doi.org/10.1016/j.cj.2015.12.002

Sridha, S., Wu, K., 2006. Identification of *AtHD2C* as a novel regulator of abscisic acid responses in Arabidopsis. The Plant Journal 46, 124–133. https://doi.org/10.1111/j.1365-313X.2006.02678.x

Stoddard, B.L., 2011. Homing Endonucleases: From Microbial Genetic Invaders to Reagents for Targeted DNA Modification. Structure 19, 7–15. https://doi.org/10.1016/j.str.2010.12.003

Su, L.-C., Deng, B., Liu, S., Li, L.-M., Hu, B., Zhong, Y.-T., Li, L., 2015a. Isolation and characterization of an osmotic stress and ABA induced histone deacetylase in *Arachis hygogaea*. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00512

Su, L.-C., Deng, B., Liu, S., Li, L.-M., Hu, B., Zhong, Y.-T., Li, L., 2015b. Isolation and characterization of an osmotic stress and ABA induced histone deacetylase in *Arachis hygogaea*. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00512

Taketo, A., n.d. DNA transfection of *Escherichia coil* by electroporation 7.

Tanenbaum, M.E., Gilbert, L.A., Qi, L.S., Weissman, J.S., Vale, R.D., 2014. A Protein-Tagging System for Signal Amplification in Gene Expression and Fluorescence Imaging. Cell 159, 635–646. https://doi.org/10.1016/j.cell.2014.09.039

Tang, N., Zhang, H., Li, X., Xiao, J., Xiong, L., 2012. Constitutive Activation of Transcription Factor OsbZIP46 Improves Drought Tolerance in Rice. PLANT PHYSIOLOGY 158, 1755–1768. https://doi.org/10.1104/pp.111.190389

Tang, X., Lowder, L.G., Zhang, T., Malzahn, A.A., Zheng, X., Voytas, D.F., Zhong, Z., Chen, Y., Ren, Q., Li, Q., Kirkland, E.R., Zhang, Y., Qi, Y., 2017. A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. Nature Plants 3, 17018. https://doi.org/10.1038/nplants.2017.18

Tardieu, F., n.d. Drought perception by plants Do cells of droughted plants experience water stress? 12.

Taylor, N.J., Fauquet, C.M., 2002. Microparticle Bombardment as a Tool in Plant Science and Agricultural Biotechnology. DNA and Cell Biology 21, 963–977. https://doi.org/10.1089/104454902762053891

Terns, R.M., Terns, M.P., 2014. CRISPR-based technologies: prokaryotic defense weapons repurposed. Trends in Genetics 30, 111–118. https://doi.org/10.1016/j.tig.2014.01.003

Tezara, W., Mitchell, V.J., Driscoll, S.D., Lawlor, D.W., 1999. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP 401, 4.

Thakore, P.I., D'Ippolito, A.M., Song, L., Safi, A., Shivakumar, N.K., Kabadi, A.M., Reddy, T.E., Crawford, G.E., Gersbach, C.A., 2015. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. Nature Methods 12, 1143–1149. https://doi.org/10.1038/nmeth.3630

The ENCODE Project Consortium, 2012. An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74. https://doi.org/10.1038/nature11247

Thorstensen, T., Grini, P.E., Aalen, R.B., 2011. SET domain proteins in plant development. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1809, 407–420. https://doi.org/10.1016/j.bbagrm.2011.05.008

Thurtle-Schmidt, D.M., Lo, T.-W., 2018. Molecular biology at the cutting edge: A review on CRISPR/CAS9 gene editing for undergraduates: A Review on CRISPR/CAS9 Gene Editing for Undergraduates. Biochemistry and Molecular Biology Education 46, 195–205. https://doi.org/10.1002/bmb.21108

Tian, G., Cheng, L., Qi, X., Ge, Z., Niu, C., Zhang, X., Jin, S., 2015. Transgenic Cotton Plants Expressing Double-stranded RNAs Target *HMG-CoA Reductase* (*HMGR*) Gene Inhibits the Growth, Development and Survival of Cotton Bollworms. International Journal of Biological Sciences 11, 1296–1305. https://doi.org/10.7150/ijbs.12463

Tian, X., Ruan, J., Huang, J., Fang, X., Mao, Y., Wang, L., Chen, X., Yang, C., 2016a. Gossypol: phytoalexin of cotton. Science China Life Sciences 59, 122–129. https://doi.org/10.1007/s11427-016-5003-z

Tian, X., Ruan, J., Huang, J., Fang, X., Mao, Y., Wang, L., Chen, X., Yang, C., 2016b. Gossypol: phytoalexin of cotton. Science China Life Sciences 59, 122–129. https://doi.org/10.1007/s11427-016-5003-z

Tie, F., Banerjee, R., Stratton, C.A., Prasad-Sinha, J., Stepanik, V., Zlobin, A., Diaz, M.O., Scacheri, P.C., Harte, P.J., 2009. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. Development 136, 3131–3141. https://doi.org/10.1242/dev.037127
Tiwari, S., Lata, C., Chauhan, P. S., Prasad, V., & Prasad, M. (2017). A Functional Genomic Perspective on Drought Signalling and its Crosstalk with Phytohormone-mediated Signalling Pathways in Plants. Current Genomics, 18(6), 469–482. http://doi.org/10.2174/1389202918666170605083319
Todaka, D., Shinozaki, K., Yamaguchi-Shinozaki, K., 2015. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. Frontiers in Plant Science 6. https://doi.org/10.3389/fpls.2015.00084
Tolmasky, M.E., Alonso, J.C. (Eds.), 2015. The Agrobacterium Ti Plasmids, in: Plasmids: Biology and Impact in Biotechnology and Discovery. American Society of Microbiology, pp. 295–313. https://doi.org/10.1128/microbiolspec.PLAS-0010-2013
Touchette, B.W., Iannacone, L.R., Turner, G.E., Frank, A.R., 2007. Drought tolerance versus drought avoidance: A comparison of plant-water relations in herbaceous wetland plants subjected to water withdrawal and repletion. Wetlands 27, 656–667. https://doi.org/10.1672/0277-5212(2007)27[656:DTVDAA]2.0.CO;2
Triezenberg, S.J., Kingsbury, R.C., McKnight, S.L., 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes & Development 2, 718–729. https://doi.org/10.1101/gad.2.6.718
Udvadia, A.J., Linney, E., 2003. Windows into development: historic, current, and future perspectives on transgenic zebrafish. Developmental Biology 256, 1–17. https://doi.org/10.1016/S0012-1606(02)00083-0
Ueki, S., Magori, S., Lacroix, B., Citovsky, V., 2013. Transient Gene Expression in Epidermal Cells of Plant Leaves by Biolistic DNA Delivery, in: Sudowe, S., Reske-Kunz, A.B. (Eds.), Biolistic DNA Delivery. Humana Press, Totowa, NJ, pp. 17–26. https://doi.org/10.1007/978-1-62703-110-3_2
Ullah, A., Sun, H., Yang, X., Zhang, X., 2017. Drought coping strategies in cotton: increased crop per drop. Plant Biotechnology Journal 15, 271–284. https://doi.org/10.1111/pbi.12688
Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., Yamaguchi-Shinozaki, K., 2000. Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences 97, 11632–11637. https://doi.org/10.1073/pnas.190309197
Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., Gregory, P.D., 2010. Genome editing with engineered zinc finger nucleases. Nature Reviews Genetics 11, 636–646. https://doi.org/10.1038/nrg2842
Vain, P., 2007. Thirty years of plant transformation technology development. Plant Biotechnology Journal 5, 221–229. https://doi.org/10.1111/j.1467-7652.2006.00225.x
Veluthambi, K., Ream, W., Gelvin, S.B., 1988. Virulence genes, borders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens.* Journal of Bacteriology 170, 1523–1532. https://doi.org/10.1128/jb.170.4.1523-1532.1988
Verma AS, Agrahari S, Rastogi S, Singh A. Biotechnology in the Realm of History. *Journal of Pharmacy and Bioallied Sciences*. 2011;3(3):321-323. doi: 10.4103/0975-7406.84430.
Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., Zhu, J.-K., 2006. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. The Plant Journal 45, 523–539. https://doi.org/10.1111/j.1365-313X.2005.02593.x
Viana, A.A., Fragoso, R.R., Guimarães, L.M., Pontes, N., Oliveira-Neto, O.B., Artico, S., Nardeli, S.M., Alves-Ferreira, M., Batista, J.A., Silva, M.C., Grossi-de-Sa, M.F., 2011. Isolation and functional characterization of a cotton ubiquitination-related promoter and 5'UTR that drives high levels of expression in root and flower tissues. BMC Biotechnology 11, 115. https://doi.org/10.1186/1472-6750-11-115
Vojta, A., Dobrinić, P., Tadić, V., Bočkor, L., Korać, P., Julg, B., Klasić, M., Zoldoš, V., 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucleic Acids Research 44, 5615–5628. https://doi.org/10.1093/nar/gkw159
Wagner, T.A., Liu, J., Puckhaber, L.S., Bell, A.A., Williams, H., Stipanovic, R.D., 2015. RNAi construct of a cytochrome P450 gene CYP82D109 blocks an early step in the biosynthesis of hemigossypolone and gossypol in transgenic cotton plants. Phytochemistry 115, 59–69. https://doi.org/10.1016/j.phytochem.2015.02.016
Wally, O., Punja, Z.K., 2010. Genetic engineering for increasing fungal and bacterial disease resistance in crop plants. GM Crops 1, 199–206. https://doi.org/10.4161/gmcr.1.4.13225
Waltz, E., 2016. Gene-edited CRISPR mushroom escapes US regulation. Nature 532, 293–293. https://doi.org/10.1038/nature.2016.19754
Wang, H., La Russa, M., Qi, L.S., 2016. CRISPR/Cas9 in Genome Editing and Beyond. Annual Review of Biochemistry 85, 227–264. https://doi.org/10.1146/annurev-biochem-060815-014607
Wang, K. (Ed.), 2006. Agrobacterium protocols, 2nd ed. ed, Methods in molecular biology. Humana Press, Totowa, N.J.

Wang, N.N., 2005. The GUS reporter-aided analysis of the promoter activities of Arabidopsis ACC synthase genes AtACS4, AtACS5, and AtACS7 induced by hormones and stresses. Journal of Experimental Botany 56, 909–920. https://doi.org/10.1093/jxb/eri083

Wani, S.H., Dutta, T., Neelapu, N.R.R., Surekha, C., 2017. Transgenic approaches to enhance salt and drought tolerance in plants. Plant Gene 11, 219–231. https://doi.org/10.1016/j.plgene.2017.05.006

Wieczorek, A. M. & Wright, M. G. (2012) History of Agricultural Biotechnology: How Crop Development has Evolved. Nature Education Knowledge 3(10):9

Wilson, R.C., Doudna, J.A., 2013. Molecular Mechanisms of RNA Interference. Annual Review of Biophysics 42, 217–239. https://doi.org/10.1146/annurev-biophys-083012-130404

Witcombe, J.., Hollington, P.., Howarth, C.., Reader, S., Steele, K.., 2008. Breeding for abiotic stresses for sustainable agriculture. Philosophical Transactions of the Royal Society B: Biological Sciences 363, 703–716. https://doi.org/10.1098/rstb.2007.2179

Wong, N., Liu, W., Wang, X., 2015. WU-CRISPR: characteristics of functional guide RNAs for the CRISPR/Cas9 system. Genome Biology 16. https://doi.org/10.1186/s13059-015-0784-0

Wu, H.-Y., Liu, K.-H., Wang, Y.-C., Wu, J.-F., Chiu, W.-L., Chen, C.-Y., Wu, S.-H., Sheen, J., Lai, E.-M., 2014. AGROBEST: an efficient Agrobacterium-mediated transient expression method for versatile gene function analyses in Arabidopsis seedlings. Plant Methods 10, 19. https://doi.org/10.1186/1746-4811-10-19

Wunderlich, S., Gatto, K.A., 2015. Consumer Perception of Genetically Modified Organisms and Sources of Information. Advances in Nutrition 6, 842–851. https://doi.org/10.3945/an.115.008870

Xu, H., Xiao, T., Chen, C.-H., Li, W., Meyer, C.A., Wu, Q., Wu, D., Cong, L., Zhang, F., Liu, J.S., Brown, M., Liu, X.S., 2015. Sequence determinants of improved CRISPR sgRNA design. Genome Research 25, 1147–1157. https://doi.org/10.1101/gr.191452.115

Xu, X., Tao, Y., Gao, X., Zhang, L., Li, X., Zou, W., Ruan, K., Wang, F., Xu, G., Hu, R., 2016. A CRISPR-based approach for targeted DNA demethylation. Cell Discovery 2. https://doi.org/10.1038/celldisc.2016.9

Yadav, B.C., Veluthambi, K., Subramaniam, K., 2006. Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. Molecular and Biochemical Parasitology 148, 219–222. https://doi.org/10.1016/j.molbiopara.2006.03.013

Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. TRANSCRIPTIONAL REGULATORY NETWORKS IN CELLULAR RESPONSES AND TOLERANCE TO DEHYDRATION AND COLD STRESSES. Annual Review of Plant Biology 57, 781–803. https://doi.org/10.1146/annurev.arplant.57.032905.105444

Yan, H., Opachaloemphan, C., Mancini, G., Yang, H., Gallitto, M., Mlejnek, J., Leibholz, A., Haight, K., Ghaninia, M., Huo, L., Perry, M., Slone, J., Zhou, X., Traficante, M., Penick, C.A., Dolezal, K., Gokhale, K., Stevens, K., Fetter-Pruneda, I., Bonasio, R., Zwiebel, L.J., Berger, S.L., Liebig, J., Reinberg, D., Desplan, C., 2017. An Engineered orco Mutation Produces Aberrant Social Behavior and Defective Neural Development in Ants. Cell 170, 736-747.e9. https://doi.org/10.1016/j.cell.2017.06.051

Yan, P., Shen, W., Gao, X., Li, X., Zhou, P., Duan, J., 2012. High-Throughput Construction of Intron-Containing Hairpin RNA Vectors for RNAi in Plants. PLoS ONE 7, e38186. https://doi.org/10.1371/journal.pone.0038186

Yang, H., Wu, J.-J., Tang, T., Liu, K.-D., Dai, C., 2017. CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in Brassica napus. Scientific Reports 7. https://doi.org/10.1038/s41598-017-07871-9

Yang, Y., Li, L., Qu, L.-J., 2016. Plant Mediator complex and its critical functions in transcription regulation: Plant Mediator complex and its function. Journal of Integrative Plant Biology 58, 106–118. https://doi.org/10.1111/jipb.12377

Yoshida, T., Fujita, Y., Maruyama, K., Mogami, J., Todaka, D., Shinozaki, K., Yamaguchi-Shinozaki, K., 2015. Four *A rabidopsis* AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress: Transcriptional regulation in SnRK2 signalling. Plant, Cell & Environment 38, 35–49. https://doi.org/10.1111/pce.12351

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K., 2010a. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. The Plant Journal 61, 672–685. https://doi.org/10.1111/j.1365-313X.2009.04092.x

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K., 2010b. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. The Plant Journal 61, 672–685. https://doi.org/10.1111/j.1365-313X.2009.04092.x

Younis, A., Siddique, M.I., Kim, C.-K., Lim, K.-B., 2014. RNA Interference (RNAi) Induced Gene Silencing: A Promising Approach of Hi-Tech Plant Breeding. International Journal of Biological Sciences 10, 1150–1158. https://doi.org/10.7150/ijbs.10452
Yu, L.-H., Wu, S.-J., Peng, Y.-S., Liu, R.-N., Chen, X., Zhao, P., Xu, P., Zhu, J.-B., Jiao, G.-L., Pei, Y., Xiang, C.-B., 2016. *Arabidopsis EDT1* / *HDG11* improves drought and salt tolerance in cotton and poplar and increases cotton yield in the field. Plant Biotechnology Journal 14, 72–84. https://doi.org/10.1111/pbi.12358
Yue Y, Zhang M, Zhang J, Tian X, Duan L, et al. (2012) Overexpression of the *AtLOS5* gene increased abscisic acid level and drought tolerance in transgenic cotton. J Exp Bot 63: 3741–3748.
Zalatan, J.G., Lee, M.E., Almeida, R., Gilbert, L.A., Whitehead, E.H., La Russa, M., Tsai, J.C., Weissman, J.S., Dueber, J.E., Qi, L.S., Lim, W.A., 2015. Engineering Complex Synthetic Transcriptional Programs with CRISPR RNA Scaffolds. Cell 160, 339–350. https://doi.org/10.1016/j.cell.2014.11.052
Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., Koonin, E.V., Zhang, F., 2015. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell 163, 759–771. https://doi.org/10.1016/j.cell.2015.09.038
Zetsche, B., Strecker, J., Abudayyeh, O., Gootenberg, J., Scott, D., Zhang, F., 2017.A Survey of Genome Editing Activity for 16 Cpf1 orthologs.
Zhang, C., Wohlhueter, R., Zhang, H., 2016. Genetically modified foods: A critical review of their promise and problems. Food Science and Human Wellness 5, 116–123. https://doi.org/10.1016/j.fshw.2016.04.002
Zhang, F., Cong, L., Lodato, S., Kosuri, S., Church, G.M., Arlotta, P., 2011. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nature Biotechnology 29, 149–153. https://doi.org/10.1038/nbt.1775
Zhang, X., Cai, X., 2013. Climate change impacts on global agricultural water deficit: MORE WATER NEEDED FOR AGRICULTURE? Geophysical Research Letters 40, 1111–1117. https://doi.org/10.1002/grl.50279
Zhang, N., McHale, L., Finer, J., 2015. Isolation and characterization of “GmScream” promoters that regulate highly expressing soybean (Glycine max Merr.) genes Plant Science 241 (2015) 189–198 http://dx.doi.org/10.1016/j.plantsci.2015.10.010
Zhang, X., Wang, Jingman, Cheng, Q., Zheng, X., Zhao, G., Wang, Jin, 2017. Multiplex gene regulation by CRISPR-ddCpf1. Cell Discovery 3, 17018. https://doi.org/10.1038/celldisc.2017.18
Zhao, J., Gao, Y., Zhang, Z., Chen, T., Guo, W., Zhang, T., 2013. A receptor-like kinase gene (GbRLK) from Gossypium barbadense enhances salinity and drought-stress tolerance in Arabidopsis. BMC Plant Biology 13, 110. https://doi.org/10.1186/1471-2229-13-110
Zheng, T., Hou, Y., Zhang, P., Zhang, Z., Xu, Y., Zhang, L., Niu, L., Yang, Yi, Liang, D., Yi, F., Peng, W., Feng, W., Yang, Ying, Chen, J., Zhu, Y.Y., Zhang, L.-H., Du, Q., 2017. Profiling single-guide RNA specificity reveals a mismatch sensitive core sequence. Scientific Reports 7, 40638. https://doi.org/10.1038/srep40638
Zhu, Y.-N., Shi, D.-Q., Ruan, M.-B., Zhang, L.-L., Meng, Z.-H., Liu, J., Yang, W.-C., 2013. Transcriptome Analysis Reveals Crosstalk of Responsive Genes to Multiple Abiotic Stresses in Cotton (*Gossypium hirsutum L*.). PLoS ONE 8, e80218. https://doi.org/10.1371/journal.pone.0080218
Ziemienowicz, A., 2014. Agrobacterium-mediated plant transformation: Factors, applications and recent advances. Biocatalysis and Agricultural Biotechnology 3, 95–102. https://doi.org/10.1016/j.bcab.2013.10.004
Zischewski, J., Fischer, R., Bortesi, L., 2017. Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. Biotechnology Advances 35, 95–104. https://doi.org/10.1016/j.biotechadv.2016.12.003
Zuberi, A., Misba, L., Khan, A.U., 2017. CRISPR Interference (CRISPRi) Inhibition of luxS Gene Expression in E. coli: An Approach to Inhibit Biofilm. Frontiers in Cellular and Infection Microbiology 7. https://doi.org/10.3389/fcimb.2017.00214
Zupan, J., Muth, T.R., Draper, O., Zambryski, P., 2000. The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. The Plant Journal 23, 11–28. https://doi.org/10.1046/j.1365-313x.2000.00808.x

List of Publications

1) Arraes et al. BMC Plant Biology (2015) 15:213 DOI 10.1186/s12870-015-0597- RESEARCH ARTICLE

Implications of ethylene biosynthesis and signaling in soybean drought stress tolerance

Fabricio Barbosa Monteiro Arraes[1,2], Magda Aparecida Beneventi[1,2], Maria Eugenia Lisei de Sa[2,4], **Joaquin Felipe Roca Paixao**[2,3], Erika Valeria Saliba Albuquerque[2], Silvana Regina Rockenbach Marin[5], Eduardo Purgatto[6], Alexandre Lima Nepomuceno[5] and Maria Fatima Grossi-de-Sa[2,7*]

2)

Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with an Histone Acetyl Transferase

Joaquin Felipe Roca Paixão[1,2*§], François-Xavier Gillet [1*], Thuanne Pires Ribeiro[1], Caroline Bournaud[1], Isabela Tristan Lourenco-Tessutti[1], Janice Almeida-Engler[2] and Maria Fatima Grossi-de-Sa

3)

Elucidating the functional role of Arabidopsis Fimbrins in the actin cytoskeleton rearrangements occurring in root-knot nematode feeding sites

Tijs Ketelaar[1*], Nathalia Rodiuc[2,3*], **Joaquin Felipe Roca Paixão**[3*], Gilbert Engler[2], Olivier Pierre[2], Sophie Pagnota[4], Tiago Gonçalves[2,5], Cristiano Lacorte[6], Andrei Smertenko[7], Maria Fatima Grossi de Sa[3†] and Janice de Almeida Engler[2†§]

4)

Simultaneous improved tolerance to drought stress and *M. Incognita* infection in transgenic cotton

Joaquin Felipe Roca Paixão, Maria Eugenia Lisei de Sa, Regina Amorim, Leonardo Pepino and Maria Fatima Grossi-de-Sa.