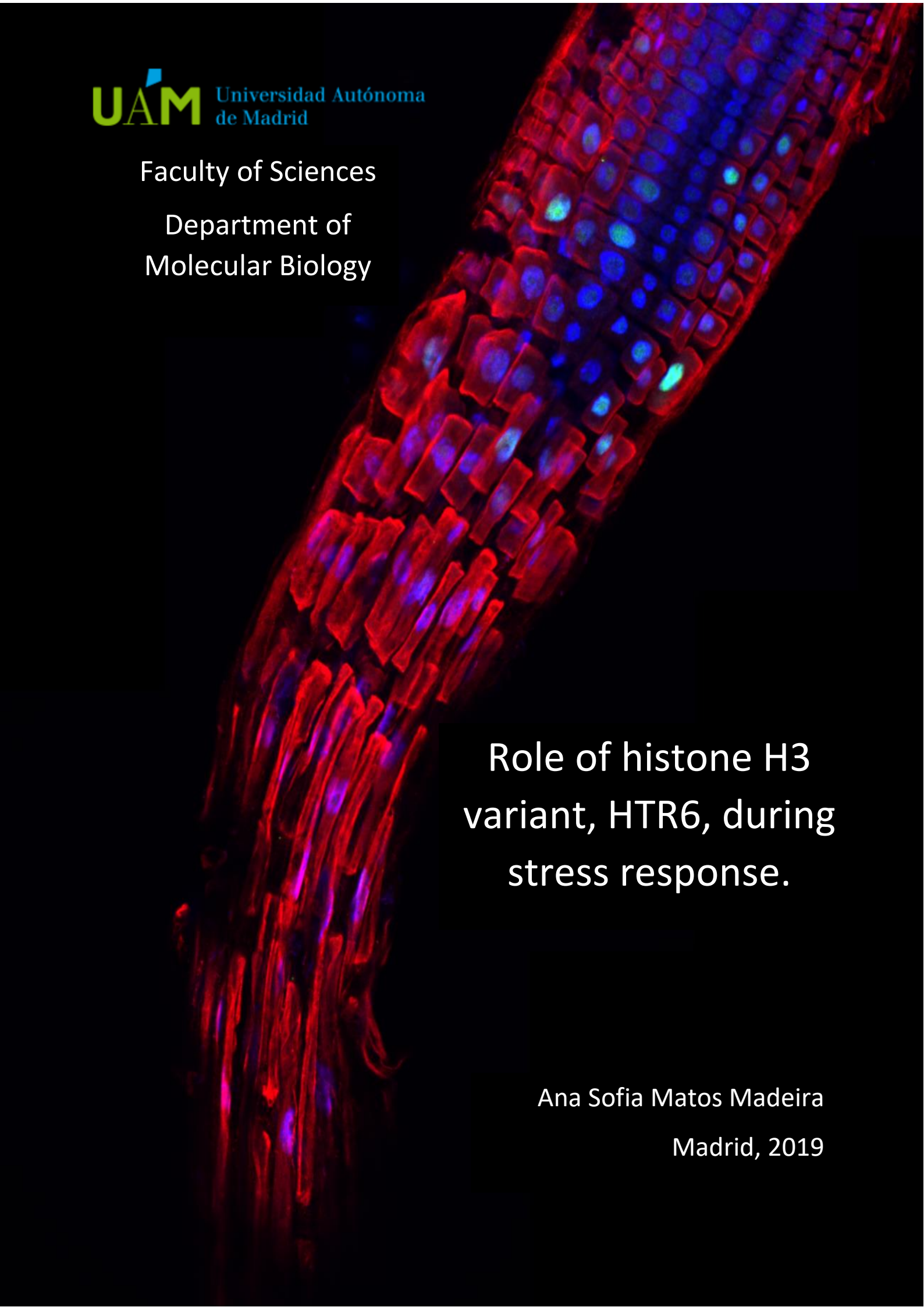
Universidad Autónoma de Madrid

Faculty of Sciences

Department of Molecular Biology



Role of histone H3 variant, HTR6, during stress response.

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Table of contents

Abbreviations

A

A-Alanine

ABA- Abscisic acid

APC/C - Anaphase-promoting complex/cyclosome

Aphi- aphidicolin

ARR2- ARABIDOPSIS RESPONSE REGULATOR 2

ASF1- Anti Silencing Factor 1

ATRX- α-thalassemia X-linked mental retardation process

B

BCA- bicinchoninic acid assay

Bp- base pair

BR- Brassinosteroids

BrdU- 5-bromo-2'-deoxyuridine

BSA- bovine serum albumin

C

CABIN1- Calcineurin Binding protein 1

CAF1- CHROMATIN ASSEMBLY FACTOR1

CAK- CDK ACTIVATING KINASES

CCS52- CELL CYCLE SWITCH 52

CDC6- CELL DIVISION CYCLE 6

CDC45- CELL DIVISION CYCLE 4

CDKs- Cyclin-dependent kinases

cDNA- complementary DNA

CDT1- CDC10 DEPENDENT TRANSCRIPTION1

CEI- cortex/endodermal initial cells

CEID- CEI daughter cells

ChIP- Chromatin Immunoprecipitation

CKIs- cyclin-dependent kinase inhibitors

CLF- CURLY LEAF

CTAB - Cetyl trimethylammonium bromide

CYCs- Cyclins

D

DAPI- 4′,6-diamidino-2-phenylindole

Daxx- death domain-associated protein 6

DHSs- DNAse I hypersensitive sites

DNA- Deoxyribonucleic Acid

DMSO- Dimethyl sulfoxide

DRB- 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole

E

EDTA- Ethylenediamine tetraacetic acid

EdU- 5-ethynyl-2-deoxyuridine

EMF2- EMBRYONIC FLOWER2

ETG1- E2F TARGET GENE 1

F

F-Phenylalanine

FAS1- FASCIATA1

FAS2- FASCIATA2

FDR- False Discovery Rate

FEN1- FLAP STRUCTURE-SPECIFIC ENDONUCLEASE 1

FIE- FERTILIZATION INDEPENDENT ENDOSPERM

FIS2- FERTILIZATION INDEPENDENT SEED2

FRAP- Fluorescence recovery after photobleaching

G

G-Glycine

G1 phase- Gap 1 phase

G2 phase- Gap 2 phase

GFP- Green Fluorecent Protein

H

H-Histidine

HATs- histone acethyltransferases

HDMs- histone demethylates

HRP- Horseradish peroxidase

HS- Horse Serum

HTR-Histone Three Related

HTR- HISTONE THREE RELATED

HTR12- CenH3

HPY2- HIGH PLOIDY2

H2O2 - Hydrogen peroxide

I

I-Isoleucine

ILP1- INCREASED LEVEL OF POLYPLOIDY 1

K

KIP- KINASE INTERACTING PROTEIN

KRPs- KIP-RELATED PROTEIN

L

L-Leucine

LB- Lysogeny broth

LIG1- LIGASE 1

M

M-CDK- mitotic-CDK

M-phase - Mitotic phase

MCM- MINICHROMOSOME MAINTENANCE

MEA- MEDEA

MES- 2-(N-Morpholino)ethanesulfonic acid hydrate

MG132- N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-DLeucinamide

MSA- M specific activator

MSI1–5- MULTICOPY SUPRESSOR OF IRA 1–5

MSS – Murashige and Skoog Sucrose

MTSB- Microtubules stabilizing

MYB3R1- MYB3 PROTEIN

N

N- Asparagine

NADPH- adenine dinucleotide phosphate hydrogen

NAP1- NUCLEOSOME ASSEMBLY PROTEIN-1

O

O2- - Superoxide radical

ORCs- ORIGIN RECOGNITION COMPLEX

ORIs- Origin of replication

P

PBS- Phosphate buffered saline

PBST- Phosphate buffered saline with Tween-20

PCNA- PROLIFERATING CELL NUCLEAR ANTIGEN

PCR- Polymerase chain reaction

PIN- PIN-FORMED

PIPES- piperazine-N,N′-bis(2-ethanesulfonic acid)

PTMs- Post-translational modifications

PLT- PLETHORA

PMSF- Phenylmethylsulfonyl fluoride

PRC2- Polycomb Repressive Complex 2

PWMs- Position weight matrices

Q

qPCR- Real time quantitative PCR

QC- Quiescent center

R

R-arginine

RAM- the root apical meristem

RFC- REPLICATION FACTOR C

RNA- Ribonucleic acid

ROI- Regions of interest

ROS- Reactive oxygen species

RPA- REPLICATION PROTEIN A

Rpm- revolutions per minute

RT_PCR- Reverse Transcriptase PCR

RT- Room Temperature

S

S-Serine

S-phase- DNA synthesis phase

SCR- SCARECROW

SDS- sodium dodecyl sulfate

SDS-PAGE- sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SHR- SHORTROOT

SHY2- SHORT HYPOCOTYL2

SIM- SIAMESE

SMR- SIAMESE-RELATED

SUMO- small ubiquitin-like modifier

SWN- SWINGER

T

T- Threonine

T-DNA- Transfer DNA

TE- Tris-EDTA

TEs- transposable elements

TF- Transcription factors

TSS- Transcription Start Site

TTS- Transcription termination site

U

UBN- Ubinuclein

UPBEAT1- UPB1

V

V-Valine

VRN2- VERNALIZATION2

W

WOX5- WUSCHEL-RELATED HOMEOBOX5

Y

Y- Tyrosines

Abstract

Chromatin organization and function are essential for development and growth of all organisms as well as for their response to environmental challenges. Histones are key to channel intra- and extracellular signals and modulate chromatin dynamics, ultimately influencing genome functions. This is primarily achieved by post-translational modifications of histones and the exchange of canonical by variant histones. Here, we have sought to define the role of *Arabidopsis thaliana* HTR6, a histone H3 variant of unknown function.

We found that HTR6 expression is induced in plants exposed to abscisic acid (ABA), a hormone that is highly involved in several types of abiotic stresses, such as to high salt. A spatiotemporal expression analysis in ABA-treated roots unveiled that HTR6 is expressed in the transition/elongation zone, where cells switch from the mitotic cell cycle to the endocycle and increase their nuclear ploidy. HTR6 expression is largely excluded from the root apical meristem as well as also from the differentiated zone. Moreover, HTR6 expression is confined to external cell layers, such as epidermis and cortex. At the cellular level, we found that HTR6 is mainly expressed during the S-phase in endocycling cells. Interestingly, its incorporation into chromatin relies on the HIRA and DEK3 chaperones and is independent of CAF-1. Consistent with this, HTR6 is a euchromatic histone deposited in a DNA replication-independent manner.

This is a feature shared with the histone H3.3 variant. However, HTR6 possesses unique amino acid residues, with F41 being a striking one because it is shared with the canonical H3.1. We found that F41 residue is important to increase HTR6 turnover, as revealed by the increased amount of HTR6 carrying a F41Y mutation. This is similar to the role of the F41 of H3.1 in euchromatic regions. Other changes in the C-terminal region of HTR6 to the amino acids present in H3.3, such as N63I, V124I or A132G, are sufficient to increase HTR6 accumulation. In particular, the A132 residue of HTR6 seems important for the proteasome-mediated degradation of HTR6 beyond the transition zone.

HTR6 plays a crucial role in restricting growth in response to ABA and salt, as demonstrated by the larger root length, root apical meristem and higher cortical cell number of the *htr6-1* loss of function seedlings. Since the ploidy level of *htr6-1* root nuclei remains unaffected, HTR6 might play a role in controlling the boundary between the root apical meristem and the transition/ elongation zone in response to stress. Furthermore, our transcriptomic analysis showed that the genes that failed to be activated in the *htr6-1* mutant early after ABA treatment (10 minutes), are involved in cell wall biogenesis genes and lipid metabolism, and encode members of the TCP family of transcription factors. Furthermore, several LEA family genes failed to be downregulated in the *htr6-1* mutant.

Together, our studies have uncovered a key role of HTR6, a previously uncharacterized histone H3 variant, in the early response of Arabidopsis to the abiotic stress.

Resumen

La organización y la función de la cromatina son esenciales para el crecimiento y desarrollo de los organismos, y para su respuesta a cambios ambientales. Las histonas canalizan las señales intra y extracelulares para modular la dinámica de la cromatina y las funciones del genoma. Esto se consigue principalmente mediante modificaciones post-traduccionales de las histonas y la sustitución de las formas canónicas por sus variantes. En este trabajo hemos querido definir el papel de HTR6, una variante de la H3 de *Arabidopsis thaliana* de función desconocida.

La expresión de HTR6 se induce tras exposición a ácido abscísico (ABA), una hormona involucrada en respuesta a estrés abiótico. El análisis espacio-temporal en raíces tratadas con ABA reveló que HTR6 se expresa entre las zonas de transición y elongación, donde las células inician los ciclos de endorreplicación e incrementan la ploidía. No se ha detectado expresión de HTR6 ni en el meristemo apical de la raíz ni en la zona de diferenciación. Asimismo, la expresión de HTR6 está restringida a las capas celulares externas (epidermis y córtex). A nivel celular, HTR6 se expresa durante la fase S de células en endorreplicación. Su incorporación a cromatina depende de las chaperonas HIRA y DEK3, siendo independiente de CAF-1. Así, HTR6 es una histona presente en eucromatina, depositada de manera independiente a la replicación del ADN.

Esta es una característica compartida con la variante H3.3. Sin embargo, HTR6 posee aminoácidos específicos, siendo F41 de los más interesantes ya que se encuentra en H3.1. F41 es importante para su eliminación del nucleosoma, ya que la mutación F41Y aumenta su permanencia en cromatina, de manera similar a la función de F41 de H3.1 en eucromatina. Otros cambios en la región C-terminal de HTR6 a los presentes en H3.3, como N63I, V124I o A132G, son suficientes para incrementar la acumulación de HTR6. El residuo A132 de HTR6 parece tener un papel en la degradación de HTR6 por el proteasoma una vez pasada de la zona de transición.

HTR6 es crucial para restringir el crecimiento de la planta en respuesta a ABA y sal, como demuestra la mayor longitud de la raíz y de su meristemo apical y por el mayor número de células corticales en plántulas mutantes con pérdida de función en *htr6-1*. Dado que la ploidía nuclear en raíces de *htr6-1* no está afectada, HTR6 podría desempeñar un papel en el establecimiento de los límites entre el meristemo apical de la raíz y la zona de transición/elongación en situaciones de estrés. Además, nuestros análisis transcriptómicos mostraron que entre los genes que el mutante *htr6-1* es incapaz de activar en respuesta a ABA (10 minutos), se encuentran genes implicados en la biogénesis de la pared celular y del metabolismo lipídico, y genes que codifican algunos factores de transcripción de la familia TCP. Igualmente, varios miembros de la familia LEA han perdido la regulación negativa en el mutante *htr6-1*.

En conjunto, nuestro estudio ha puesto de manifiesto un papel importante de la histona HTR6, una variante de la histona H3 de función desconocida hasta ahora, en la respuesta temprana a estrés abiótico en Arabidopsis.

1.Introduction

Developmental processes are fundamental for multicellular organism to reach its full maturity. Formation of distinct tissues and organs is dependent on cell growth, proliferation and differentiation and those developmental programs have evolved as a result of the interplay between genetically inherited instructions and the influence of external environmental factors. Epigenetic events are also key players in these interactions and can modulate genome function in a heritable way without changes in the DNA sequence. Therefore, understanding how environmental clues influence genome function and dynamics through epigenetic events is of primary importance. Due to the fact that plants are immobile organisms, they have to cope with any external adversity that arises, which makes them exceptional models to study how organisms tolerate and respond to environmental variations.

1.1 *Arabidopsis thaliana* as a model

Arabidopsis thaliana is a small flowering plant that is widely distributed around the world, with a major presence in Europe (Fig. 1). Arabidopsis was discovered in Germany, in the XVIth century, by Johannes Thal. In the laboratory, Arabidopsis is one of the most used models in research as it allows combining genetics, genomics, cellular and molecular approaches. From a basic research point of view, Arabidopsis offers many advantages:

Figure 1. Image depicting the typical phenotype of a flowering *Arabidopsis Thaliana* (http://www.90ways.com).

- Arabidopsis genome (125Mb) comprises 5 chromosomes extensively mapped after having been sequenced in 2000.
- Its life cycle is fast, allowing researchers to obtain a numerous progeny in 6 weeks.
- The methods to transform Arabidopsis, by *Agrobacterium tumefaciens*, are efficient, quick and easy.
- It is also a great model to generate and select mutants, including many genes that are viable in Arabidopsis but not in other organisms. Furthermore, there are various stock centers that distribute mutant lines to the scientific community.
- The resources used to grow plants are economically sustainable since the requirements are only water, air, light, and a few minerals.

1.2 The root

One vital function of the root is the uptake of nutrients and water to the plant as well as to provide structural support to the aerial part The past 30 years' studies performed in Arabidopsis have highlighted the importance of roots as an excellent organ to study growth and development.

1.2.1 Structure and organization

Organogenesis in plants is a postembryonic process and the well-organized structure of the root allows effortless distinction of the different tissues, cell types and developmental stages (Petricka *et al.*, 2014). The Arabidopsis root is formed by concentric cell layers that form functional tissues (Du and Scheres, 2018). The fate of the distinct cell types and tissue layers is acquired in the root apical meristem (RAM) from a subset of initial cells, stem cells, which provide the basis for growth, regeneration, and development of the root. In the RAM, stem cells are surrounding the quiescent center (QC) cells that play crucial functions in maintaining stem cell identity by preventing stem cells to differentiate (van den Berg *et al.*, 1997) (Fig. 2).

The orchestration of root organogenesis is primarily mediated by transcription factors regulated by phytohormones (Drisch and Stahl, 2015). Specifically, homeodomain containing WUSCHEL-RELATED HOMEOBOX5 (WOX5) is considered one of the central transcription factors implicated in QC cell fate maintenance by negatively regulating promoters of cell division and proliferation genes like CYCD3;3 and CDF4 (Heyman *et al.*, 2013; Forzani *et al.*, 2014; Kong *et al.*, 2015; Drisch and Stahl, 2015). Other family of transcription factors, AP2-domain PLETHORA (PLT), also act as regulators of QC identity and stem cell activity in embryonic and adult meristem. PLT proteins display gradient distributions obeying auxin patterns, where the PTL levels are higher at the stem cell niche and decrease as the cells become differentiated (Aida *et al.*, 2004; Galinha *et al.*, 2007). Thus, PTLs control meristem size by coordinating cell division and differentiation (Mahonen *et al.*, 2014; Blilou *et al.*, 2005; Petersson *et al.*, 2009).

Stem cells below the QC produce the columella root cap while epidermis and lateral root cap are derived from lateral stem cells (Fig. 2). Together, columella, lateral root cap and epidermis constitute the external surface that primarily protect the root from environmental adversities (Jalean *et al.*, 2014). Columella cells also function in gravity sensing. Columella stem cells are maintained by CLE40-ACR4/CLV1 pathway that negatively regulate WOX5 in the root apical meristem which is essential to restrict WOX5 expression to the QC (Stahl *et al.*, 2009; Kong *et al.*, 2015). Epidermis cells encompass two types of cells, atrichoblast and trichoblasts. Trichoblasts cells undergo changes in plasma membrane and cell wall to produce root hairs in the elongation zone of the root (Baluška *et al.*, 2000). The root hairs are crucial for water and nutrients absorption and attachment of the plant to the soil (Ryan *et al.*, 2001).

Other stem cells denominated cortex/endodermal initial cells (CEI) and subsequent CEI daughter cells (CEID) are generating endodermal and cortex precursors (Petricka *et al.*, 2014) (Fig. 2). This sequential asymmetric division required for cortex and endodermis formation is dependent on the GRAS-transcription factors SHORTROOT (SHR) and SCARECROW (SCR) function (Sabatini *et al.*, 2003). SHR activates the expression of SCR in CEI cells and together activate CYCD6;1

that consequently promotes the activation of cell cycle genes (Sozzani *et al.*, 2010). Curiously, WOX5 expression also requires SHR and SCR (Sarkar *et al.*, 2007) and it was demonstrated that SHR/SCR, in parallel with PLT, act to sustain QC identity and stem cell homeostasis in the Arabidopsis root (Aida *et al.*, 2004). The function of cortex in plant growth and physiology is not so clear as for other types of tissues. The importance of endodermis has been highlighted in many studies demonstrating that this tissue layer act as a central regulator of intracellular signals involved in root growth and tissue patterning. Moreover, early in development, endodermal cells acquire the Casparian strips, a cell wall structure that functions as an impermeable apoplasmic barrier to water and nutrients (Miyashima and Nakajima, 2011).

Finally, plant vasculature, which is organized in a central cylinder or stele, derived from the set of vascular initials stem cells proximal to the QC (Dolan *et al.*, 1993; Scheres *et al.*, 1994; Ohashi-Ito and Fukuda, 2010). The central cylinder is composed by the xylem, phloem and procambial cells. Water and nutrients are transported along the xylem while the photosynthates flow through phloem cells (Jalean *et al.*, 2014). Lastly, the pericycle layer surrounds the plant vasculature and is the layer that gives rise to lateral roots (Du and Scheres, 2018).

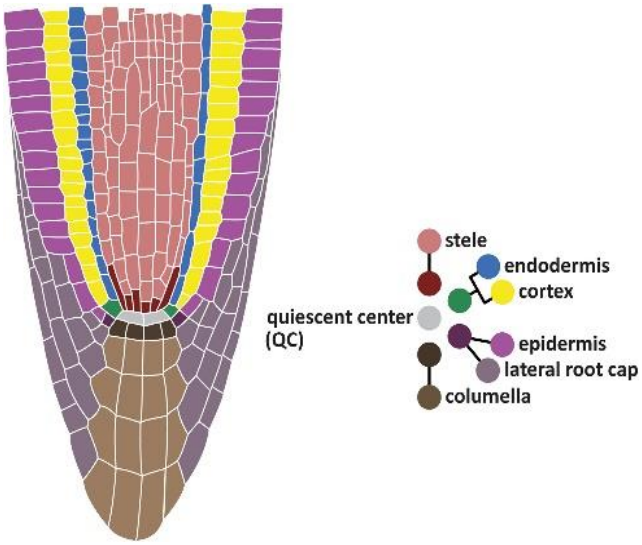

Figure 2. Root organization and stem cell niche. The various root tissues are represented on the left in different color codes; Columella: brown; Lateral root cap: light purple; Epidermis: dark pink; Cortex: yellow; Endodermis: blue; Stele: pink. The QC cells and stem cells (stem cell niche) are shown on the right in distinct colors, depicting also the possible differentiation pathways undertaken by each cell type that originates from the QC; QC cells: Light grey; Columella initials: dark brown; Lateral root cap and epidermis initials: purple; CEI: green; vascular initials: Maroon (Rahni *et al.*, 2016).

1.3 Developmental program along the root

Along the longitudinal axis, the primary root is mainly divided into three distinct developmental domains: meristematic zone, elongation zone and differentiation zone (Dolan *et al.*, 1993) (Fig. 3) that also reflect the temporal evolution of cells within the root (Pavelescu *et al.*, 2018). New cells provenient from the stem cell niche in meristematic zone undergo consecutive divisions until they become part of the elongation zone where the cells increase in length and DNA content. The boundary between the meristematic and elongation zones is denominated transition zone. Transition zone is a region where root cells undergo cytoarchitectural rearrangements to prepare

for rapid cell elongation and where cells switch from the mitotic cycle to the endocycle, where cells replicate DNA during S phase and slap mitosis, thereby increasing ploidy (Edgar *et al.*, 2014; Kong *et al.*, 2018) (Fig. 3).

This border defines the meristem size that is determined by the balance between cell division and differentiation and is mediated by a correct balance of phytohormones (Beemster and Baskin, 1998; Moubayidin *et al.*, 2010). The phytohormones auxin and cytokinin are considered the master coordinators of cell proliferation and differentiation. Auxin form a gradient along the meristem, mediated by AUX1/LAX (Auxin resistant1/Like AUX1) and PIN-FORMED (PIN), carriers that are polarly localized in cell membranes. According to this gradient, auxin is present in higher amounts in the stem cell niche and sequentially decrease near the transition zone (Tanaka *et al.*, 2006). Several studies indicate that auxin acts on multiple targets which control cell proliferation (Roudier *et al.*, 2003; Hartig and Beck, 2005; Blilou *et al.*, 2005; Takatsuka and Umeda, 2014). On the contrary, cytokinin promotes cell differentiation in the transition zone by enhancing the expression of SHORT HYPOCOTYL2 (SHY2) gene which negatively regulates the PIN genes (Taniguchi *et al.*, 2007; Dello Ioio *et al.*, 2008; Tian *et al.*, 2002; Blilou *et al.*, 2005). Conversely, high amounts of auxin drive the degradation of the SHY2 protein (Dello Ioio *et al.*, 2008). In analogy to auxins, gibberellins also promote cell division in the meristem by repressing cytokinin responsive transcription factors that induce SHY2 (Moubayidin *et al.*, 2010). On another side, brassinosteroids (BR), promotes elongation via high BZR1 accumulation in nuclei of hair epidermal cells, antagonizing the auxin effect (Chaiwanon and Wang, 2015; Wei and Li, 2016).

Parallel to phytohormones, reactive oxygen species (ROS) generated by the cellular redox reactions such as respiration and photosynthesis, also participate in the cell proliferation/differentiation balance (Singh *et al.*, 2016). Thus, the superoxide radical (O_2^-) accumulates in the meristem because it is required for cell proliferation, while hydrogen peroxide (H_2O_2) is mainly present in the elongation zone where differentiation is induced (Tsukagoshi *et al.*, 2010). This balance is mainly regulated by transcription factors like UPBEAT1 (UPB1) and Myb36 (Tsukagoshi *et al.*, 2010; Liberman *et al.*, 2015; Fernandez-Marcos *et al.*, 2017). In the transition zone, there is an overlap of both types of ROS which highlight the idea of transition zone behaving as an oscillatory zone that may act as a kind of command center (Baluška and Mancuso, 2013). In the elongation zone, cells undergo rapid growth through water uptake, accompanied by vacuole expansion and irreversible cell wall extension (Cosgrove, 1993; Dolan and Davies, 2004). Recent evidences indicate that histone H3.1/H3.3 balance along the root is also associated with proliferation/differentiation domains. Cell proliferation potential in the RAM is strongly related with high amounts of histone H3.1 while differentiation along the root is associated with replacement of canonical H3.1 by variant H3.3 (Otero *et al.*, 2016).

Finally, cells achieve their maturity in the differentiation zone, presenting specialized characteristics and functions. In this stage, where cells are no longer elongating, new structures appear, like root hairs from epidermal trichoblast cells and the casparian strip (Petricka *et al.*, 2014).

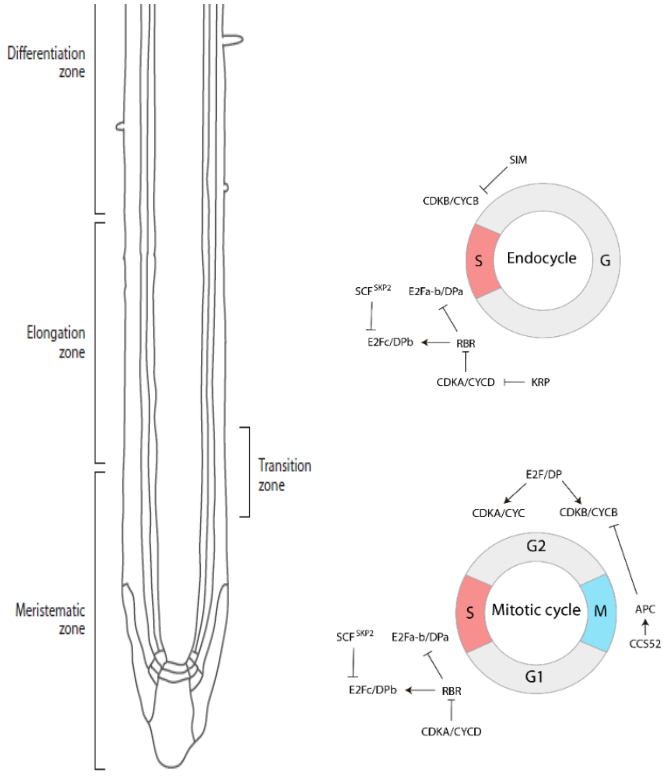

Figure 3. Root developmental domains and cell cycle. The left part of the image depicts the three main developmental regions of the root, which are the meristem, elongation and differentiation zones. The transition zone between meristem and elongation is also represented. The meristematic zone is characterized by the presence of proliferative cells encompassing a mitotic cycle (bottom right) while the transition and elongation zone cells are under endocycle (upper right). The main regulators of each phase of the cell cycle are also represented on the right part of the image. Adapted from Gutierrez, 2009, Geng *et al.*, 2013 and Edgar *et al.*, 2014.

1.2.3 Mitotic Cell cycle

The production of new cells during proliferation is essential for organogenesis in plants. Cell number increases in a population by passing through the mitotic cell cycle that consists of four distinct phases: Gap 1 phase (G1 phase), DNA synthesis phase (S phase), Gap 2 phase (G2 phase) and mitotic phase (M phase) (Fig. 3). In sum, the S phase encompasses DNA duplication (replication) and assembly of chromatin required to package genomic DNA, while the M phase allows chromosome and cytoplasmatic segregation to the two daughter cells through nuclear (karyokinesis) and cell (cytokinesis) division, respectively. During the gap phases, oscillatory transcription waves of cell cycle phase-specific genes are activated (Breeden, 2003) and cellular homeostasis and correct DNA replication are monitorized, enabling DNA repair and cell cycle surveillance mechanisms to determine if check-point activation is necessary (Waterworth *et al.*, 2011; Velappan *et al.*, 2017). The transition of the distinct phases of cell cycle is controlled by periodic phosphorylations and inhibitory phosphorylations/de-phosphorylation of cyclin-dependent kinases (CDKs) in combination with different cyclins (CYCs) (Fig. 3). This oscillatory post-translational regulation of CDK/CYC complexes is synchronized by activators like CDK ACTIVATING KINASES (CAK) and by inhibitors such KINASE INTERACTING PROTEIN

(KIP)-RELATED PROTEIN (KRPs) (Vandepoele *et al.*, 2002; Menges *et al.*, 2005). In contrast with the described reversible modifications, spatial and temporal irreversible degradation of CDKs and CYCs is mediated by the ubiquitin-proteasome system, a proteolysis mechanism that assures the strict unidirectionality of the cell cycle (Bassermann *et al.*, 2014).

The G1 phase is characterized by cell growth, organelle duplication and the preparation of the necessary machinery for the replication process that occurs during the S-phase. Early in G1, the ORIGIN RECOGNITION COMPLEX (ORCs) binds to the chromatin to mark potential replication start sites (ORIs). Then, the ORI licensing process is completed when components of pre-replicative complex, such as CDC6 (CELL DIVISION CYCLE6), CDT1 (CDC10 DEPENDENT TRANSCRIPTION1) and the helicases MCM (MINICHROMOSOME MAINTENANCE, MCM2 to MCM7) complex are recruited to the ORC bound sites (Masai *et al.*, 2010). Although licensing occurs at many genomic sites, only a subset of ORIs will specifically fire during S phase (Costas *et al.*, 2011; Sanchez *et al.*, 2012; Mechali *et al.*, 2013; MacAlpine and Almouzni, 2013; Mojardin *et al.*, 2013; Desvoyes *et al.*, 2014). The features leading to ORI specification are not yet completely understood in multicellular eukaryotes. The most efficient ORIs are those firing more often inside a population and, in plants, just like in animals, those ORIs seem to be associated with highly active transcribed genes, especially in stretches of GC rich regions and clusters of GGN trinucleotides (Costas *et al*., 2011; Sequeira-Mendes *et al*., 2019). Also in heterochromatin, the most densely compacted regions of the genome, ORIs colocalize with transposable elements (TEs) that contain higher GC content (Vergara *et al*., 2017).

Transcriptional waves are also an important point of control in the cell cycle, allowing the availability of specific cell cycle regulators only when their activity is required. During G1, CDKA/CYCD complex is activated by CAK pathway. In an active form, CDKA/CYCD complex triggers successive phosphorylations that inactivate RBR repressive action leading to E2Fa–b/DP release (Gutierrez *et al.*, 2002; Magyar *et al*., 2005; Berckmans and De Veylder, 2009) (Fig. 3). The Arabidopsis E2Fa/DP and E2Fb/DP promote the G1/S transition by inducing transcriptional activation of target genes required for S-phase that contain the E2F box motifs. On the contrary, E2Fc/DP is a transcriptional repressor that also forms a complex with RBR to promote a repressive activity (Velappan *et al.*, 2017). At the S/G1 transition, the E2Fc/DP in complex with RBR are also phosphorylated by CDKA/CYCD complex and subsequentially degraded by the proteasome through ubiquitin E3 ligase SCF^{SKP2} system (del Pozo *et al.*, 2006).

During the S phase of the cell cycle, the chromatin (DNA complexed with histones) is faithfully duplicated. Only a subgroup of ORIs is activated, although not all selected ORIs fire at the same time, obeying a temporal program of activation throughout the S-phase. Gene rich regions with

active epigenetic marks fire early in the S-phase while condensed regions, enriched in repetitive elements and repressive epigenetic marks, are activated later (Leonard and Méchali, 2013; Cadoret *et al.*, 2008; Hansen *et al.*, 2010; Eaton *et al.*, 2011, Dellino *et al.*, 2013; Fragkos *et al.*, 2015). Additional factors, including CDC45 (CELL DIVISION CYCLE 45), and the GINS complex, DNA polymerases A (α), D (δ) and E (ε) complexes, PCNA (PROLIFERATING CELL NUCLEAR ANTIGEN), RFC (REPLICATION FACTOR C), RPA (REPLICATION PROTEIN A), FEN1 (FLAP STRUCTURE-SPECIFIC ENDONUCLEASE 1) and LIG1 (LIGASE 1) are sequentially assembled to the pre-replicative complexes to permit DNA replication (Sanchez *et al.*, 2012; Sequeira-Mendes and Gutierrez., 2015). The beginning of S-phase is marked by the activation of MCM helicases, which is dependent on CDKs. Together with the rest of the replication machinery, they initiate the process of replication. To avoid re-licensing and re-replication of DNA, CDT1 and CDC6 are phosphorylated by CDKs, evicted from the chromatin and degraded by the ubiquitin proteasome pathway (Petersen *et al.*, 2000; Sugimoto *et al.*, 2008).

Throughout replication, new nucleosomes must be assembled behind the replication fork in order to maintain chromatin structure and histone post-translational marks. New nucleosomes are formed in DNA by the CHROMATIN ASSEMBLY FACTOR1 (CAF1) which mediate the deposition of one $(H3–H4)_2$ tetramer followed by the incorporation of two H2A–H2B dimers by the NUCLEOSOME ASSEMBLY PROTEIN-1 (NAP1) (Groth *et al.*, 2007; MacAlpine and Almouzni, 2013).

The G2/M transition is mediated by the cooperation between CDKA-CYC (associated with cyclin A-,D- and specially B) and CDKB-CYC (Inzé and De Veylder, 2006). CDKB expression is dependent of E2F (Boudolf *et al.*, 2004) and both CDKA- and CDKB-CYC complexes are regulated by CAK activity (Fig. 3). Together, CDKA- and CDKB-CYC complexes are responsible for the phosphorylation of several targets necessary for mitosis. In addition, G2 progression requires a new transcriptional wave, dependent on M specific activator (MSA) recognizing TFs, to produce the elements essential for mitosis (Gutierrez, 2009; Desvoyes *et al*, 2014).

During G2, a crucial checkpoint assesses the chromatin integrity before the segregation of the genetic material. In the case of DNA damage or replication stress, WEE1 kinase inhibits the activity of CDKs through phosphorylation, provoking a cell cycle arrest in G2 (De Schutter *et al.*, 2007). In addition, some chromatin related events take place in G2 such as the deposition of histone H3 variant CENH3 at the centromeric regions (Lermontova *et al.*, 2006) and the increase of H3K4me3 histone modifications in G2 expressed target genes (Xiao *et al.*, 2005; Zhu *et al.*,2005; Desvoyes etal., 2014).

Four main steps compose mitosis: prophase, metaphase, anaphase and telophase. All the machinery is prepared during G2 to start chromosome condensation at prophase. The compaction of all genetic material in mitotic chromosomes requires profound chromatin changes. During this process, phosphorylation of specific residues present in the histone H3 N-terminal tail occurs, an event that is conserved among animals and plants. Thus, H3T3ph, H3S10ph, H3T11ph, and H3S28ph are the most crucial phosphorylation events that leads to chromatin compaction (Manzanero *et al.*, 2000; Houben *et al.*, 2007; Rossetto *et al.*, 2012). In parallel, a transcription shut down is observed together with a decrease of acetylation events in both H3 (K18 and K23) and H4 (K5, K8, K12 and K16) (Bonenfant *et al.*, 2007). At metaphase, the chromosomes are positioned at the cell plate to assure that sister chromatids are correctly spared during anaphase. The multi-subunit E3 ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C) is a major regulator of mitotic progression by triggering metaphase-to anaphase transition and exit from mitosis. APC/C promotes the degradation of cyclins, like CYCB, by the 26S proteasome (Weingartner *et al.*, 2003). In plants, CDC20 and CELL CYCLE SWITCH 52 (CCS52) activate the APC/C and provide substrate specificity (Eloy *et al.*, 2015) (Fig. 3). At telophase, there is a rebuilding of structures and components, in order to form the two interphase nuclei and chromosome decompaction occurs. Cell division finishes with cytokinesis where all cytoplasmic components are equally distributed into the two daughter cells and a new cell wall is synthesized (Gutierrez, 2009).

1.2.4 Endocycle

In plant, differentiation processes are often associated with endoreplication. In the transition zone of the root, cells switch from proliferation to differentiation as a response to developmental stimuli. This process is accompanied by a change from the mitotic cell cycle to the endocycle, where the cells duplicate the chromatin and DNA content without passing through division (Edgar *et al.*, 2014). Along the root, several rounds of endoreplication cycles occur, increasing the levels of genomic ploidy (from 2C to 4C, 8C, 16C). During the endocycle, cyclin-dependent kinase inhibitors (CKIs) play a crucial function by regulating CDKs and cyclins that control the cell cycle (Veylder *et al*, 2001; Li *et al*., 2019) (Fig. 3). Regulators of G2/M transition are the most affected factors. Cyclin A, involved in G2/M transition, is repressed by transcription factor ILP1 (INCREASED LEVEL OF POLYPLOIDY 1) that consequently attenuates mitotic-CDK (M-CDK) activity (Imai *et al*, 2006). Cyclin D3;1 is also depleted during this process (Dewitte *et al.*, 2003). CDKB1 activity (S–G2 M-CDKs) is suppressed by a plant specific CKI SIAMESE (SIM) (Churchman *et al.*, 2006). SIM inhibits Cyclin B expression by blocking the transcription factor MYB3 PROTEIN (MYB3R1) (Edgar *et al.*, 2014). Additionally, to SIM, SMR (SIAMESE-RELATED) gene family members also exert functions as mitotic cell cycle inhibitors that promotes endoreplication (Li *et al.*, 2019).

As mentioned before, cytokinins influence meristem size and the switch to endocycle. The ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2), activated by cytokinins, stimulates CCS52A1 expression. Subsequently, CCS52A1 activates the APC/C complex to stop division by degradation of mitotic regulators (Takahashi *et al.*, 2013) In agreement, down-regulation of CCS52A decreases the cotyledon ploidy level. CCS52A expression is negatively repressed by E2Fe/DEL1 in Arabidopsis and *e2fe/del1* mutants present increased levels of ploidy highlighting the function of CCS52 in endoreplication (Lammens *et al.*, 2008). Endoreplication is also affected by G1/S phase elements like E2F-RBR complexes. It has been described that decreased levels of RBR stimulates both cell division and endocycle progression, (Park *et al*, 2005; Desvoyes *et al*., 2006; Borghi *et al.*, 2010) possibly by E2FA-DPA release. The overexpression of this complex promotes endoreplication (Magyar *et al*., 2005; De Veylder *et al*., 2002). Interestingly, some components of pre-RC, like CDC6 and CDT1, endorses endocycle progression, whereas others like MINICHROMOSOME MAINTENANCE (MCM)-interacting protein and ETG1 (E2F TARGET GENE 1) prevent the early switch to endoreplication (Castellano *et al.*, 2001; Castellano *et al.*, 2004; Takahashi *et al*., 2008). Some elements are also implicated in endocycle onset prevention in the meristematic region, which is the case of a small ubiquitin-like modifier (SUMO) E3 ligase and HIGH PLOIDY2 (HPY2), that induces the expression of cell division regulators (Petricka *et al.*, 2012).

In summary, the main role of endocycle has not been completely uncovered yet. However, the evidences suggest that endoreplication might be advantageous to increase gene expression and metabolic output in order to support plant growth and development. Furthermore, these insights could also be the reasons why distinct environmental stresses, biotic or abiotic, lead to prematurely switch to endocycle.

1.3 Abiotic stress

Plants, as other organisms, are influenced by external environment conditions during their life cycle. Abiotic factors like water, salinity, temperature, light and chemical composition of the soil deeply influence growth and developmental programs. Variations of any of these conditions alter homeostasis leading to physiologic stress, also designated as abiotic stress (Singh and Laxmi, 2015). Plants have developed strategies to deal with such adversities. However, rapid climate change is producing high rates of abiotic stress that affects global crop production and geographic distribution of plants in different ecosystems. These evidences highlight the importance of studying the plant response mechanisms to abiotic stress (Fedoroff *et al.*, 2010). The most common abiotic stresses are due to drought, heat, cold, osmotic pressure, nutrient deficiency, and excess of salt or toxic metals, e.g. aluminum, in the soil (Zhu, 2016). The complexity of the response generated is dependent on the nature and duration of the abiotic stress (Cramer *et al.*,

2011). Nevertheless, pleiotropic events reveal many interactions and cross-talks between pathways triggered by the diverse stresses (Takahashi *et al.*, 2004). Most molecular responses to abiotic stress culminate in transcriptome, metabolome and proteome alteration that can ultimately lead to phenotypical changes, changing plant architecture (Cramer *et al.*, 2011).

Hormone imbalance provokes physiologic changes that resemble to abiotic stress. Interplay between signal transduction pathways mediated by phytohormones is essential for balancing growth and stress resistance (Bechtold and Field, 2018). This flexibility permits rapid reorganization of developmental, physiological and metabolic programs in response to environmental stress (Amtmann and Armengaud, 2009; Cutler *et al*, 2010). Specifically, abcisic acid (ABA) is considered one of the central regulator hormones, and its pathway is triggered in response to several types of abiotic stresses.

ABA was discovered in 1960 as a weak acid that hindered growth by accumulating in abscising cotton fruit (Liu and Carns, 1961). Nowadays, the role of ABA in growth and developmental programs has been deciphered, revealing crucial functions in seed dormancy/germination, embryo maturation, stomatal regulation, meristem size definition, senescence, floral induction, and responses to environmental stresses (Finkelstein, 2013). Plants under environmental stress conditions, such as drought, high salinity, cold (abiotic stresses) overproduces ABA. First, ABA signaling pathway activation is important for stomatal closure regulation to avoid plant dehydration (Pacifici *et al.*, 2015). Then, high ABA amounts, induced both by external application of ABA or by osmotic stress, influence root growth rate (Finkelstein *et al.*, 2002; Li *et al.*, 2017). A wide variety of genes are activated by ABA under these conditions, indicating that ABA is crucial to mediate adaptive responses in order to restore plant homeostasis (Micol-Ponce *et al.*, 2015). In the entire plant, seedling growth and lateral root formation are affected. ABA represses primary root growth and formation of lateral roots (Wasilewska *et al.*, 2008) by repressing auxins and auxins transport regulators like AUX1/LAX and carriers PIN proteins (He *et al*., 2012; Yang *et al*., 2014; Promchuea, *et al*., 2017). ROS production also increases under high concentrations of ABA, that consequently increase the inhibitory effect on auxin regulators by activation of NADPH oxidases, thus suppressing primary root development (Sun *et al*., 2018). At cellular level, transcriptome alterations encompass synthesis of new proteins, modifications in metabolic pathways, variations in ion uptake, free radical scavenging and cell cycle adjustments (Bhattacharjee and Saha, 2014). Several studies indicate that an arrest in cell cycle is produced by high ABA concentrations by influencing crucial cell cycle regulators such as CDT1 and CYCB1 (Castellano *et al*., 2004; Xu *et al.*, 2010). Regulation of root growth under osmotic stress conditions comprises a hormonal crosstalk network between cytokinin, ethylene and auxin that involves ABA-dependent and ABA-independent pathways (Yoshida *et al.*, 2014; Rowe *et al.*, 2016).

1.4 Chromatin

Chromatin structure was first observed approximately 45 years ago in an electron microscope (Olins and Olins, 1973). The first description compared chromatin structure with "beads on a string". The repeating spherical structures observed are the functional units of chromatin, the nucleosomes, that are formed by an octamer of histones (two dimers of H2A-H2B histones together with a tetramer of H3-H4 histones) wrapped by approximately 147 bp of DNA (Thomas and Kornberg, 1975) (Fig. 4). Additionally, an extra histone, H1, bind nucleosomes and is important for nucleosome interactions to form a higher order structure, the chromatin fiber (Luger *et al.*, 1997). The compaction status of chromatin was first recognized by Heitz (1928) and varies from accessible chromatin (euchromatin), enriched in active genes, to highly condensed chromatin (heterochromatin), containing repetitive regions and transcriptionally silenced genes (Sun *et al.*, 2001; Fransz *et al.*, 2003). The nuclear distribution of chromatin also differs between nuclear regions, with euchromatin domains being localized in the center of the nucleus while heterochromatin is confined to the nuclear periphery (DeRisi *et al.*, 1997; Solovei *et al.*, 2004; De Nooijer *et al.*, 2009) (Fig. 4).

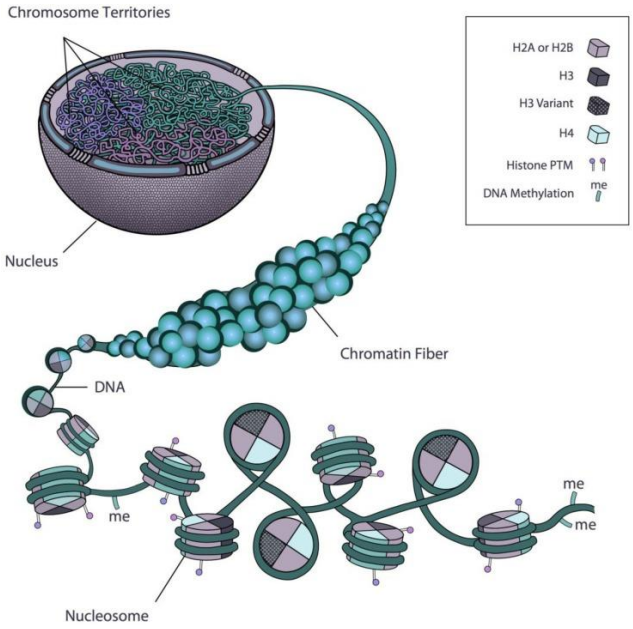

Figure 4. Chromatin structure in the nucleus. The main regulators of chromatin, like histone families, post-translational modifications (PTM) and DNA methylation are represented here, depicting the basic units that compose the chromatin fiber. The image depicts how the compaction of the chromatin fiber allows the genetic material to be stored and organized inside the nucleus (Rosa and Shaw, 2013).

The structural and functional regulation of chromatin implies an extraordinary combinatorial complexity. Firstly, canonical histones present in the nucleosomes can be replaced by isoforms, which are variants of the same family (Talbert and Henikoff, 2010). Secondly, histones can suffer multiple post-translational modifications at numerous residues. Thirdly, DNA can be also modified by addition of methyl groups at cytosines present in CG dinucleotides or CHG and CHH sequences, where H is A, C, or T (Zemach *et al.*, 2010; Saze *et al.*, 2011; Feng *et al.*, 2010). These characteristics, together with the activity of nucleosomes remodeling complexes, mediate

chromatin dynamics, a key regulator of vital cellular processes like transcription, replication, DNA repair and recombination, which ultimately affect cell proliferation, growth and development (Rosa and Shaw, 2013; Otero *et al.*, 2014; Sequeira-Mendes and Gutierrez, 2016).

1.4.1 Histones: The H3 family

Canonical histones are integrated in the nucleosome mainly during the synthesis of new DNA that occurs at the S phase of the cell cycle (Henikoff and Ahmad 2005). Except for the H4 family, histone families encompass variants, that can replace canonical histones at several points during the cell cycle, in association with transcription, DNA replication, repair or recombination (Malik and Henikoff, 2003; Bernstein and Hake, 2006; Ingouff and Berger, 2010; Zhu *et al.*, 2013; Otero *et al.*, 2014; Rutowicz *et al.*, 2015). Despite similarities, canonical and variant histones differ in some amino acids residues and properties, dictating different characteristics to chromatin.

Histone 3 family is one of the most studied and complex of all the histone families. In Arabidopis, this family encompasses a group of fifteen genes, called HISTONE THREE RELATED (HTR), which encode H3 proteins (http://www.chromdb.org/) with very distinct properties. (Ingouff *et al.*, 2010; Otero *et al.*, 2014; Jiang and Berger, 2017). CenH3 (HTR12) is essential to centromeric region formation, enabling the assembly of kinetochores that are crucial for proper chromosome segregation along the process of mitosis (Ravi *et al.*, 2011; Fukagawa and Earnshaw, 2014) CenH3 structure differs from the other H3 substantially, especially at N-terminal tail, allowing this particular histone to be modified with unique epigenetic marks for the specification of centromer positioning through subsequent generations (Rosa and Shaw., 2013).
The canonical H3.1 is encoded by 5 intronless genes (HTR1, HTR2, HTR3, HTR9 and HTR13) while the variant H3.3 by 3 genes (HTR4, HTR5 and HTR8). H3.1 and H3.3 dynamics and properties are incredibly different, in spite of that the amino acid sequences differ only in 4 residues at positions (H3.1: A31-F41-S87-A90 / H3.3: T31-Y41-H87-L90). In animals there are two histones H3.1 and H3.2 homologues to the H3.1 in plants, which differ with H3.3 in positions 31, 87, 89 and 90 (Waterborg and Robertson, 1996; Ingouff and Berger, 2010; Shi *et al.*, 2011) (Fig. 5).

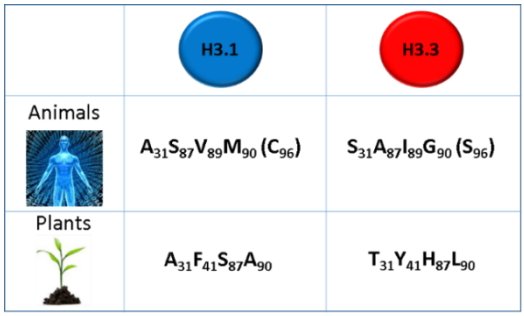

Figure 5. Amino acid residue differences between H3.1 and H3.3 histones in animals and plants.

Curiously, the presence of Phenalanine at position 41 appeared first in ferns and is unique to plants (Lu *et al.*, 2018). The difference between animal and plant H3 point to an independent origin but with convergent evolution.

During the S phase of cell cycle, the histone H3.1 is incorporated all over the genome in a DNA replication dependent manner by the Chromatin Assembly Factor-1 (CAF1) chaperone. Then, in euchromatic regions, H3.3 replaces H3.1 by a process that is dependent on transcription. Thus, H3.1 is enriched at heterochromatin regions, the chromocenters, that comprises pericentromeric regions formed by repetitive sequences and transposable elements (Fransz *et al.*, 2002.; Stroud *et al.*, 2012; Wollmann *et al.*, 2012; Simon *et al.*, 2015) (Fig. 9). Silenced genes and their regulatory domains outside the pericentromeric regions form small patches of heterochromatin that also contain H3.1. It is known that H3.1 peaking domains correlate with DNA methylation sites (CG, CHG, and CHH, where H = A, T or C) and epigenetic marks that promote silencing, like H3K9me2, H3K27me3 and H3K27me1 (Stroud *et al.*, 2012). Contrary to H3.1, H3.3 is deposited throughout the cell cycle by the chaperone HIRA in actively transcribed genes. In Arabidopsis, H3.3 peaks towards the 3´end of the genes, protecting gene bodies from DNA methylation that is crucial for high transcription rates (Wollmann *et al.*, 2017). As in animals, Arabidopsis H3.3 is associated with active marks, like H3K4me3 and H3K36me3, and is correlated with RNA Pol II occupancy and H2B ubiquitylation (Stroud *et al.*, 2012; Wollmann *et al.*, 2012). Likewise, H3.3 is enriched in telomeres and subtelomere regions, indicating that, in many higher eukaryotes, telomeric chromatin exhibit euchromatic features, despite consisting of repetitive DNA sequences (Vaquero-Sedas and Vega-Palas, 2013).

A recent study emphasizes the importance of H3.1/H3.3 ratio in different cell populations along the root axis (Otero *et al.*, 2016). Cells with elevated proliferation rates located at the most apical part of the meristem contain higher levels of H3.1, whereas at the upper part of meristem H3.1 is evicted early in G2, just before the transition to the endocycle. These cells are characterized by having longer G2 phases with lower H3.1/H3.3 ratio. Then, at the elongation zone the H3.1/H3.3 ratio increases during endoreplication and H3.1 is once more evicted when cells fully differentiate (Otero *et al.*, 2016).

The histone H3 family also comprises other variants that influence chromatin status and transcription programs. HTR10 encodes a H3 variant that substantially differs from H3.1 and H3.3 in many amino acid residues. HTR10 is important for chromatin remodeling in germ cells, specifically in sperm cells present in mature pollen, being evicted from the genome after fertilization (Ingouff *et al.*, 2007). HTR14 is also present in pollen but, it is expressed in the vegetative nucleus and not in sperm cells. Interestingly, HTR14 together with HTR6 and HTR15 are considered H3 “unusual” or rare histones because they share features with both H3.1 and H3.3

regarding their amino acidic sequence. Nevertheless, in normal conditions, no transcripts of these unusual histones are detected in seedlings (Ingouff *et al.*, 2010). Finally, HTR7 and HTR11 are considered pseudogenes (Ingouff *et al.*, 2010).

1.4.2 Histones post translational modifications

The histone protein structure includes the histone folding domain, formed by three α-helices (α1, α2, and α3) intercalated by two loops (L1 and L2) and unfolded structures, the N- and C-terminal tails (Luger *et al.*, 1997). The N- and C-terminal tails extend out of the nucleosome and can be posttranslationally modified serving as a platform for interaction with a variety of protein and adding another layer of regulation (Jenuwein and Allis, 2001; Turner, 2002, Ho and Crabtree, 2010). Histone posttranslational modifications (PTMs) can be heritable and transmitted, propagating epigenetic information along generations at particular *loci* in the genome (Leatham-Jensen *et al.*, 2019). The most typical modifications occurring in histones are acetylation, phosphorylation, methylation and ubiquitination (Bannister and Kouzarides, 2011) (Fig. 6). All kind of PTM are highly dynamic and regulated by effectors that can add, substitute or remove the modification.

Lysines are usually targets of histone acethyltransferases (HATs) that catalyse the transfer of an acetyl group from the cofactor acetyl CoA to the ε-amino group of lysine (Hodawadekar and Marmorstein, 2007). Both in animals and plants, lysine acetylation occurs at K9, K18, K23 and specially at K14 of H3 promoting transcription by weakening the interactions between histones and DNA permitting a higher nucleosome turnover (Johnson *et al.*, 2004). On the other hand, histone deacetylases (HDAC) are potential transcriptional repressors, acting by removing acetyl group and restoring the positive charge of the lysine, endorsing in this way histone-DNA interactions (Bannister and Kouzarides, 2011).

Histone phosphorylation is a fundamental regulator of the cell cycle, including mitosis and replication, DNA repair and recombination (Moraes and Casas-Mollano, 2014). Phosphorylation mainly relies on the transfer of a phosphate group from ATP to the hydroxyl group of Serines (S), Threonines (T) and Tyrosines (Y), regardless of whether the residue is on the N-terminal or in the core part of the histone. This process is mediated by kinases and, like acetylation, significantly influences chromatin structure by altering histone charges. Histone phosphorylation is also a reversible process, meaning that phosphatases have the power to revert the modification (Bannister and Kouzarides, 2011). In plant histones, phosphorylation occurs predominantly in the following residues: H3T3, H3T6, H3S10, H3T11, H3S28, and H3Y41 (Moraes and Casas-Mollano, 2014). Most of those phosphorylations are related with condensation and cohesion processes, during mitosis (Houben *et al.* 2007; Kaszas and Cande 2000; Karimi-Ashtiyani *et al.*, 2015).

Lysines and arginines are common targets of methylation. Histone methylation consists of the addition of one, two or three methyl groups by methyltransferases. Histone methylation does not alter the charge of the histone protein (Bannister and Kouzarides, 2011). On the other side, histone demethylates (HDMs) are responsible for the removal of methyl groups (Shi *et al.*, 2004; Tsukada *et al.*, 2006; Chang *et al.*, 2007). Methylation has complex consequences because depending on the residue and number of methyl groups added, it can be associated either with transcriptional activation or repression (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Turner, 2002; Johnson *et al.*, 2004). Specifically, H3K4me2/3 and H3K36me2/3 are associated with active transcription and are marks commonly enriched in H3.3, whereas H3K27m1/2/3 and H3K9me2/3 are implicated in silencing and mainly present in H3.1 (Rosa and Shaw, 2013; Johnson *et al.*, 2004). One of the most studied modification is the H3K27me3 that has a crucial role in repression of Polycomb regulated developmental genes and maintenance of cellular identity (Kinoshita *et al.*, 2001; Chanvivattana *et al.*, 2004; Schubert *et al.*, 2005).

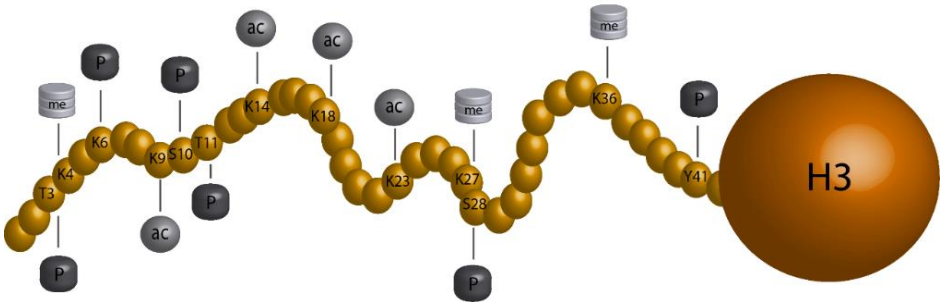

Figure 6. Histone H3 most typical N-terminal post- translational modifications. Small brown beads correspond to N-terminal amino acids and the larger bead is representative of H3 core. The number in each smaller bead indicate the residue position and the letter the correspondent amino acid (T-threonine; K-Lysine; S-serine; Y-tyrosine). Phosphorylation is represented by P (black); methylation corresponds to me (light grey) and Acetylation is indicated as ac (dark gray).

In Arabidopsis, Polycomb Repressive complex 2 (PRC2), is composed by three E(z) homologues [CURLY LEAF (CLF), SWINGER (SWN), and MEDEA (MEA)], a single Esc homologue [FERTILIZATION INDEPENDENT ENDOSPERM (FIE)], three Su(z)12 homologues [EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2), and FERTILIZATION INDEPENDENT SEED2 (FIS2)], and five p55 homologues [MULTICOPY SUPRESSOR OF IRA 1–5 (MSI1–5)] (Goodrich *et al.*, 1997; Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999; Chanvivattana *et al.*, 2004; Hennig *et al.*, 2005). Although distinct complexes are formed depending on the developmental transitions, PRC2 complexes are always able to deposit H3K27me3 (Zhang *et al.*, 2007a; Bouyer *et al.*, 2011).

1.5 Chaperones

Histone chaperones guide the spatial and temporal deposition/eviction of histones in chromatin (Mattiroli *et al.*, 2015; Lu *et al.*, 2018). Thus, by promoting nucleosome assembly/disassembly in

chromatin during replication, transcription and repair, chaperones are considered fundamental pieces of the epigenetic regulation process (Zhu *et al.*, 2013).

1.5.1 CAF1

CAF-1 complex is responsible for H3.1-H4 deposition into DNA in a process dependent on replication (Fig. 7). CAF-1 complex is composed by three subunits, which in plants, are FASCIATA1 (FAS1) the larger subunit (p150), the p60 corresponds to FASCIATA2 (FAS2) and the p48 homologue is MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Smith and Stillman, 1989; Kaya *et al.*, 2001; Hennig *et al.*, 2003; Polo and Almouzni, 2006; Reyes, 2006; Otero *et al.*, 2014). Besides being part of CAF-1 complex, MSI1 is also part of the PRC2, essential for plant development (Kohler *et al.*, 2003a; Schonrock *et al.*, 2006a). In addition, MSI1 establishes interactions with RBR1 protein, which is a key player in cell cycle progression (Ach *et al.*, 1997). The wide range of interactions reflects the importance of MSI1 for cell survival but it also makes it difficult to delineate the exact function of MSI1 as part of CAF-1 complex. The interaction of CAF-1 with H3.1-H4 is mediated through the FAS1 subunit that also promotes the connection of CAF-1 with Proliferating cell nuclear antigen (PCNA) (Zhang *et al.*,2016). PCNA functions as a mediator that attracts CAF-1 to the replication forks (Zhang *et al.*,2016). On another side, FAS2 interacts with ASF-1 mediating the transfer of H3.1-H4 dimers from ASF-1 to CAF-1. Both *fas1* and *fas2* mutants are not lethal, nonetheless, these mutations cause pleotropic effects and plants exhibit genomic instability, impaired cell cycle progression and premature cell differentiation, with increased ploidy levels that ultimately results in disorganized meristems and reduced fertility (Kaya *et al.*, 2001; Exner *et al.*, 2006; Ramirez-Parra and Gutierrez, 2007; Kirik *et al.*, 2006; Ono *et al.*, 2006; Schonrock *et al.*, 2006; Mozgova *et al.*, 2010; Abascal *et al.*, 2013). Recently, it has also been described that telomere are shortened in these mutants, and that the number of ribosomal DNA clusters is reduced (Mozgova *et al.*, 2010; Muchova *et al.*, 2015; Havlova *et al.*, 2016; Pavlistova *et al.*, 2016; Varas *et al.*, 2017; Muñoz-Viana *et al.*, 2017).

1.5.2 HIRA

The HIRA chaperone constitutively deposits H3.3-H4 in actively transcribed regions of the genome functioning as a crucial element in chromatin organization (Fig. 7). Like in animals, HIRA forms a complex with Anti Silencing Factor 1 (ASF1), Calcineurin Binding protein 1 (CABIN1) and Ubinuclein (UBN) 1 and 2 (Tagami *et al.*, 2004; Balaji *et al.*, 2009; Nie *et al.*, 2014). A null HIRA mutant, *hira-1*, exhibits reduced genome-wide distribution of H3.3 and nucleosome occupancy disruption at euchromatic and heterochromatic loci (Duc *et al.*, 2015). As a consequence, *hira-1* displays pleiotropic defects in vegetative development, presenting seedling growth retardation, cotyledons serration, and reduced fertility (Nie *et al.*, 2014; Duc *et al.*, 2015). This contrasts with animals, where HIRA null mutants are not viable. Triple homozygous mutant

ubn1;ubn2;cabin1, but not single mutants, exhibited a phenotype resembling *hira-1*, a further evidence that all elements are needed for the correct function of the complex (Nie *et al.*, 2014). In animals, it has been shown that HIRA complex binds H3.3 through UBN, which recognizes specifically the H3.3 amino acid Gly90, present in the core of the histone (Ricketts *et al.*, 2015; Lu *et al.*, 2018). HIRA also interacts with ASF1 and an analysis of *asf1a;asf1b* mutants indicated that ASF1 interacts both with HIRA and CAF-1 but in an independent way. HIRA has a crucial function in chromatin dynamics not only because it is responsible for the replacement of H3.1 by H3.3 in euchromatin but also because it participates in developmental reprogramming and environmental adaptation processes. For example, protoplast formation is characterized by the dedifferentiation of the cells and a wide transcriptome reprogramming, promoted by HIRA-mediated H3.3 incorporation. In vegetative tissues, a response to abiotic stress induces transcriptome alterations where HIRA seems to play a role, as genes down-regulated in *hira-1* mutant are related to environmental stress response (Nie *et al.*, 2014).

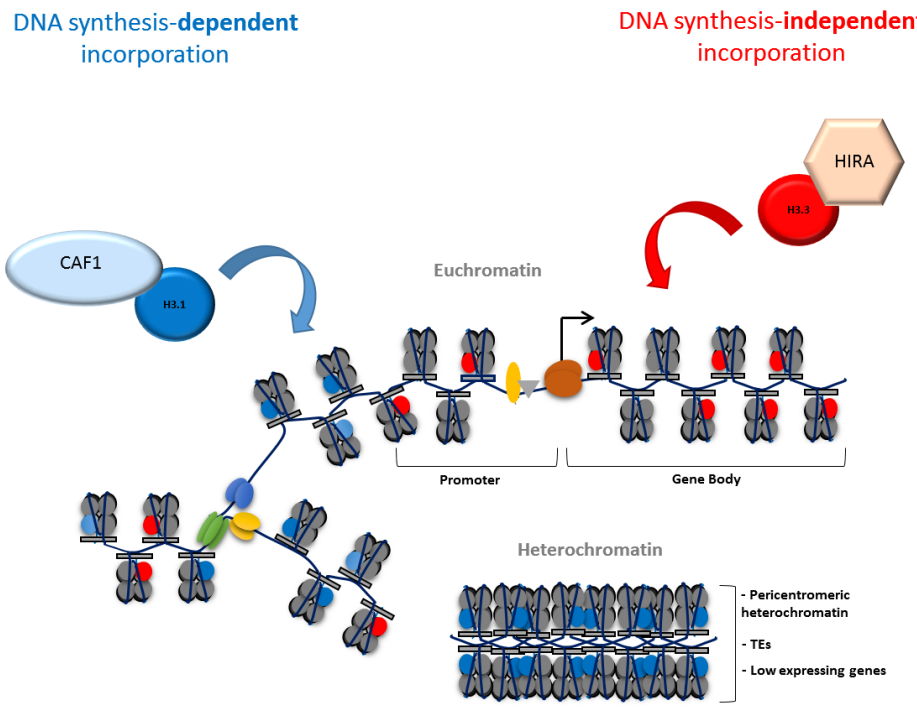

Figure 7. Histone H3.1 and H3.3 incorporation into the DNA by CAF-1 and HIRA. Histone H3.1 is incorporated by the chaperone CAF1 during DNA replication. At regions of active genes, H3.1 is then replaced by H3.3 that is deposited by HIRA chaperone, a process which is cell cycle independent. Thus, H3.1 is enriched in heterochromatic regions like pericentromeric chromatin, transposable elements (TEs) and low expressed genes.

1.5.3 Other chaperones

In eucariotes, apart from CAF-1 and HIRA, there are other histones chaperones that also can incorporate histones H3. ASF1 c can bind H3.1-H4 or H3.3-H4 dimers escorting the complex from the cytoplasm to the nucleus (Zhu *et al.*, 2011). Then, H3.1-H4-ASF1 and H3.3-H4-ASF1

complexes interact both with CAF-1 and HIRA, respectively (Tagami *et al.*, 2004; Zhu *et al.*, 2011). In mammals, ATRX (α-thalassemia X-linked mental retardation process) together with Daxx (death domain-associated protein 6) facilitate H3.3 deposition at telomeres (Goldberg *et al.*, 2010; Lewis *et al.*, 2010). Although, no DAXX homologue has been identified in Arabidopsis. A recent study has uncovered that ATRX functions in partnership with HIRA at a genome wide scale and that in an atrx mutant H3.3 occupancy at 45S ribosomal DNA loci is severely affected (Duc *et al.*, 2017). Finally, DEK chaperones also incorporate H3 histones and, in animals, is implicated in many processes such as heterochromatin integrity, DNA replication, DNA double-strand break repair, mRNA splicing and transcriptional regulation. In Arabidopsis, four DEK proteins exist and their function is related to the regulation of nucleosome occupancy, transcription and salt stress tolerance (Waidmann *et al.*, 2014).

Altogether, these insights reinforce the importance of chromatin dynamics during growth and developmental processes, showing that a delicate interplay between the many types of histone variants, chaperones and posttranscriptional modifications at specific loci and at different time points are of fundamental importance not only for the correct development of the organism but also for its adaptation to different stimuli.

2.Aim

The chromatin landscape has an important function in genome regulation. Histones are key modulators of chromatin status that influences genomic function and dynamics. Thus, deciphering how these elements are regulated during the development and in response to environmental changes is of primary importance. Thus, the global aim of this work is to understand the role of histone H3 variant HTR6 in *Arabidopsis thaliana*. The objectives that we propose are:

1. Study the regulation of histone HTR6 expression.

2. Determine the spatiotemporal dynamics of HTR6 at the cellular an organ level in response to abiotic stress.

3. Define the function of HTR6 during abiotic stress.

3. Material and Methods

3.1 Material

3.1.1 Bacterial strains

Escherichia coli, stain DH5α, was used for all cloning procedures. Growth was performed LB medium at 37 ºC with desired antibiotics. Gateway empty vectors were amplified using DB3.1 strain that grows at 37 ºC. Plant transformation was performed using *Agrobacterium tumefaciens*, strain C58C1.

3.1.2 Plant ecotypes, growth conditions and selection

Arabidopsis thaliana, Columbia ecotype (Col-0) was used to transform with Agrobacterium to generate transgenic plants. Plants were grown in an incubator at 21 ºC and 60 % moisture under long day conditions (16-hours light, 8-hours darkness) and in 1 % or 0.8 % agar (Duchefa) MSS plates (pH 5.7) supplemented with MES (Sigma), 1 % sucrose (Duchefa) and vitamins (Duchefa). To select transformed plants and transgenerational inheritance, the medium was supplemented with antibiotics or drugs, as needed.

3.1.3 Plant drug treatments

Different treatments were carried out to induce HTR6 expression. Seedlings were exposed to ABA (Sigma) or NaCl (Merck). Treatment time varied between 10 min to 1 day, as indicated along the results.

In order to study HTR6 degradation, 5-days-old plants were treated during 4h with different proteasome inhibitors, 100 µM MG132 (Peptide Institute) and 0.5 µM epoxomicin (Peptide Institute), 50 µM Bortezomib (Sellectchem), in presence of 50 µM of ABA.

Plants were treated during 2 hours with 0.24 µg/ml of aphidicolin (Aphi) (Sigma) to inhibit replication process or with 0.2 mM of 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) (Sigma) to impair transcription in presence of 50 µM of ABA.

3.1.4 Plants lines

The transgenic plants generated are specified in Table 3.1 indicating the promoter, gene and tag. The T-DNA insertion lines used in this study are indicated in Table 3.2. The cell cycle phases marker plants, pCDT1a::CDT1aCFP pHTR13::HTR13Cherry pCyCB1.1::CycB1.1YFP, pHTR5::HTR5-mRFP (H3.3) and pHTR13::HTR13-mRFP (H3.1) was also used in this study.

Table 3.1 Transgenic plants generated

Promoter	Gene	Tag
p*HTR6*	*HTR6*	*G3GFP*

p*HTR6*	*HTR6*	*4xMYC*
p*HTR6*	*HTR6S6T*	*G3GFP*
p*HTR6*	*HTR6H11T*	*G3GFP*
p*HTR6*	*HTR6F41Y*	*G3GFP*
p*HTR6*	*HTR6N63I*	*G3GFP*
p*HTR6*	*HTR6V124I*	*G3GFP*
p*HTR6*	*HTR6A132G*	*G3GFP*

Table 3.2 T-DNA insertion lines

Mutant names	Description
abi4-1	Finkelstein, 1994
abi5-1	Finkelstein, 1994
snrk2.2/snrk2.3	Fujii *et al*., 2007
pyr1 pyl1 pyl2 pyl4 pyl5 pyl8	Gonzalez-Guzman *et al*., 2012
clf29	Xu and Shen, 2008
fas2-4	Exner *et al*., 2006
hira-1	Nie *et al*., 2014
dek3-2	Waidmann *et al.*, 2014
htr6-1	-

3.2 Molecular biology techniques

3.2.1 Molecular cloning

Accuprime Pfx DNA Polymerase (Live Technologies) was used to amplify genomic fragments of *HTR6* promoter and gene, without termination codon. Primer pairs contained the attB sites for GATEWAY cloning (Invitrogen) (Table 3.3). PCR products were purified by Wizard SV Gel and PCR Clean-UP system (Promega) or NZYGel pure (nzytech) and recombined into pDONR221 (Invitrogen) using BP Clonase II (Gateway technology, Invitrogen). Clones were analyzed by restriction enzymes and confirmed by sequencing. The resulting entry clones were then transferred into the plant destination vectors (Nakagawa *et al*., 2007) by LR reaction (Gateway technology, Invitrogen).

Destination vectors

pGWB450: Gateway binary vectors for plant transformation containing a region to insert gene fused with G3GFP at C-terminal. Resistance: Bacteria-spectinomycin; Plant-Kanamycin

pGWB416: Gateway binary vectors for plant transformation containing a region to insert gene fused with 4xMyc at C-terminal. Resistance: Bacteria-spectinomycin; Plant-Kanamycin

pGWB453: Gateway binary vectors for plant transformation containing a region to insert gene fused with mRFP at C-terminal. Resistance: Bacteria-spectinomycin; Plant-Kanamycin

All destination vectors were provided by Tsuyoshi Nakagawa from the Research Institute of Molecular Genetics, Matsue, Japan.

Table 3.3 Primers used for cloning

Primer name	Sequence
HTR6attb_F	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**AGCAAACATTACGGGGGTTA
HTR6attb_R	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CAGCACGCTCTGCACGAATCC

3.2.2 Site-directed mutagenesis

Site direct mutagenesis was used to introduce point mutations leading to amino acid changes. Plasmid containing the gene of interest, pDONOR 221 p*HTR6*::*HTR6*, was amplified by Pfu Turbo DNA polymerase (Agilent Technologies). Primers used in the amplification contained the desired mutation and were designed following the kit instructions (QuikChange® Site-Directed Mutagenesis Kit) (Stratagene). Primer sequences and Pfu Turbo polymerase amplification conditions are given in Table 3.4. After amplification, non-mutated parental DNA template was digested by DpnI that recognizes parental methylated strands. Then, plasmids were transformed in the DH5α strain, amplified, purified and transferred into the plant destination vectors (detailed in 3.2.1 Molecular cloning).

Table 3.4 Primers used for site-directed mutagenesis

Primer name	Sequence
FHTR6F41Y_F	CTCACCGTTACCGTCCTGGAACCGTC
RHTR6F41Y_R	GGACGGTAACGGTGAGGTTTCTTGAC
FHTR6S6T_F	CGAAGCAAACCGCGAGAAAATCACACG
RHTR6S6T_R	CTCGCGGTTTGCTTCGTACGAGCCATG
FHTR6H11T_F	GAAAATCAACCGGAGGAAAAGCTCCG
RHTR6H11T_R	CCTCCGGTTGATTTTCTCGCGGATTG
HTR6N63I_F	GAGTTGTTGATCCGTAAACTTCCATTC
HTR6N63I_R	CAAGTTTACGGATCAACAACTCTGTGC
HTR6V124I_F	CCTAAAGATATTCAATTGGCAAGAAGG
HTR6V124I_R	GCCAATTGAATATCTTTAGGCATAATGG
HTR6A132G_F	GCAAGAAGGATTCGTGGAGAGCGTGC
HTR6A132G_R	GCACGCTCTCCACGAATCCTTCTTGC

3.2.3 Extraction of genomic DNA and genotyping

Roots, leaves or seedlings were collected in tubes containing glass beads and were frozen with liquid nitrogen or in dry ice. Frozen tissues were ground in Silamat S5 (Ivoclar Vivadent) for 8 seconds and 200 µl of extraction buffer (0.14 M d-Sorbitol, 0.22 M Tris-HCl pH 8, 0.022 M EDTA pH 8, 0.8 M NaCl, 0.8 % CTAB, 1 % n-Lauroylsarcosine) were added to each sample. The samples were incubated at 65 ºC for 10 min with shaking (600 rpm). One volume of chloroform was added and the lysates were centrifuged at 18000 x g for 7 min. The supernatant (aqueous phase) was transferred to a fresh eppendorf tube and DNA precipitation was carried out with 200 µl of isopropanol for 10 min (room temperature). After centrifugation at 18000 x g during 15 min, the DNA pellet was washed with 70 % ethanol and air dried before being resuspended with 50 µl of water or 1x TE (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0). Genotyping was performed by PCR using NZYTaq II DNA polymerase (Nzytech) and the primers are listed in Table 3.5.

Table 3.5 Primers used for genotyping

Primer name	Sequence
hira-F-genot	GAGAGTCACTGTTTTGGCTGG
hira-R-genot	CTACTAAAATTTGAGGCCGGG
WiscDsLox	AACGTCCGCAATGTGTTATTAAGTTGTC
DEK-salk137152-F	GGTTGGATTACCGTCATTCG
DEK-salk137152-R	CTCTTTTCTGGTGGGCTCTG
Salk LBb1.3	ATTTTGCCGATTTCGGAAC
Sail LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
fas2-4-F-genot	ATGATGCAGGAGGTGAGCTT
fas2-4-R-genot	TTCGAAAGAAAGACGGCAAT
fas2-4-O5	CAACACTCAACCCTATCTCGG

3.2.4 RNA extraction

RNA was extracted from leaves; roots or seedlings of *Arabidopsis Thaliana*. First, tissues were frozen in liquid nitrogen in tubes containing glass beads and homogenized using a Silamat S5 (Ivoclar Vivadent) for 6 seconds. Samples were ground for another 6 seconds after Trizol extraction (1 ml per 50-100 mg of tissue) (Invitrogen) and left at room temperature for 5 min. Chloroform was used in the proportion of 0.2 ml per ml of Trizol to extract total RNA by centrifugation at 20000 x g for 15 min at 4 ºC. The upper phase was collected and transferred to a new tube and RNA was precipitated using 20 µg of glycogen and 500 µl of isopropanol during 10 min at -20C. Then, the pellet obtained by centrifugation (10 min at 4 ºC) was washed with 75 % ethanol, air dried for 5 min and resuspended in 44 µl of nuclease free water. Contaminating DNA was eliminated by DNase I treatment carried out during 20 min at 37 ºC by addition of 0.1

U/µl DNase I (Roche). The enzyme was then inactivated by EDTA and heat and RNA was extracted with phenol:chloroform:isoamyalcohol (25:24:1). RNA was precipitated with 20 µg of glycogen (Roche), 1/10 3 M sodium acetate pH 5.2 and 2.5 volumes of 100 % ethanol, washed with 75 % ethanol, air-dried and resuspended in nuclease free water.

RNA quantification: RNA was quantified in a Nanodrop (Thermo Scientific) and RNA integrity was assessed by 1 % agarose gel electrophoresis. Gel was loaded with 500 ng-1000 ng of RNA.

RNA extraction for RNAseq

RNA was extracted from roots of wt and *htr6-1* mutant in 2 different conditions: MSS or MSS complemented with ABA for 10 min. (50 µM). RNA extraction for RNAseq experiments (Macrogen) was performed using the total RNA mini kit (IBI Scientific) following the manufacture´s protocol. The library preparation of the RNA extracts was performed in Macrogen.

3.2.5 Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to convert mRNA in complementary DNA (cDNA). RNA was extracted and purified as described above and 0.5-1 µg of total mRNA was reverse-transcribed to cDNA with SuperScript III (Invitrogen) by using oligo-dT as primers. A negative control without RT (RT-) was also included to assess the absence of genomic DNA in the sample. All cDNA samples were treated with RNase H to remove RNA, diluted 5 times and use as template for qPCR.

3.2.6 Quantitative real-time PCR (qPCR)

cDNA obtained after RT-PCR was quantified by qPCR by using GoTaq Master Mix (Promega) according to the manufacturer´s instructions. The amplification was performed in 384 well plates in an ABI Prism 7900HT machine (Applied Biosystems) using the primers pairs listed in Table 3.6. Serial dilutions of cDNA were used to set standard curves in order to assess primers efficiency. Relative expression of histone genes was calculated after normalization with housekeeping genes as reference, such as GAPC2, and fold change was calculated relative to Col-0.

Calculations:

$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$

$\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{control}}$

$2^{-\Delta\Delta Ct}$

Table 3.6 Primers used for qPCR to measure mRNA *HTR6* levels

Primer name	Sequence
HTR6-F-endo	AAGAAGGATTCGTGCAGAGC
HTR6-R-endo	CGACAAAGCAAAAACAATCG
HTR6cDNA_F	CTTTGGCGATTTTTCTCTCTG
HTR6cDNA_R	CGTGTGATTTTCTCGCGGAT
GAPC2_F	TTGCTCCTCTTGCCAAGGTTA
GAPC2_R	GGACAGTGGTCATGAGTCCC

3.2.7 Protein extraction

Plants expressing tagged versions of histones (HTR6-Myc) were used to evaluate protein expression levels. Seedlings (1-4 g) were grown during 5 days in MSS medium and half of material was transferred to MSS plate containing the desired treatment during 4 hours. Aerial part and roots of seedlings were frozen separately in liquid nitrogen. Plant tissues were ground with pestle in a mortar with liquid nitrogen until the material became a fine powder. Collected powder was resuspended in 10 ml of extraction buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 % Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail for plant extracts (Sigma)) per each gram of material to lyse the chloroplasts. Then, nuclei were released by passing the samples through the douncer homogenizer with tight and loose pestles. Extract was filtered twice, first by using a 70 µm strainer and then a 40 µm strainer to obtain a clean nuclei suspension. After centrifugation at 4 ºC, 3000 x g, for 20 min, nuclei were resuspended in 150 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 % SDS, 1 mM PMSF, 1x protease inhibitor cocktail for plant extracts (Sigma)) during 30 min to solubilize nuclear proteins. DNA was disrupted and samples homogenized by 10 cycles of sonication (30 seconds on/30 seconds off) performed in a Bioruptor®. Centrifugation at 4 ºC during 15 min at 20000 x g was used to separate soluble from insoluble proteins. Soluble proteins were quantified with the BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific). The amount of protein used for Western Blot was between 50 µg to 100 µg depending of the experiment. Desired amount of protein extracts was diluted in 1x Laemmly to SDS denature proteins and boiled for 5 min. Then, each sample was fractionated by SDS-PAGE electrophoresis in a 14 % Tris-glycine polyacrylamide gel.

3.2.8 Western Blotting

Proteins in the gel were transferred to a methanol-activated Immobilon-P membrane (0.45 µm, Millipore) for 90 min at 250 mA. Membrane blocking was carried out for 30 min with 5 % non-fat milk in PBST (1x PBS, 0.1 % Tween-20) and then incubated overnight with the primary antibody against α-Myc (clone 4A6 Millipore; 1:3000) at 4 ºC. After three washes with PBS-T (10 min each) the membrane was incubated with the secondary antibody for 1h (Amersham ECL

rabbit IgG HRP linked (GE Healthcare Life Science) diluted 1:10000)). After three washes with 1x PBS-T for 10 min, the membrane was treated with the Immobilon WB Chemiluminescent Kit for HRP substrate (Millipore) during 5min to detect the proteins and membrane was exposed to photographic film.

3.2.9 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was used to reveal H3K27me3 and H3K4me3 enrich in *HTR6* gene and promoter with and without ABA treatment. ChIP was carried out according to published procedures (Desvoyes *et al*., 2018). Briefly, 7-days-old seedlings (wt) were grown in MSS media and then, half of the samples (4 g) were transfected to MSS containing ABA (50 µM) during 4 h. Roots from both conditions were harvested and crosslinked in 50 ml falcon tubes containing 1 % formaldehyde in PBS. Infiltration was performed by 3 rounds (2min On, 1 min OFF) of vacuum (85000 Pa) and the raction stopped by addition of glycine (125mM) with further 5 min of vacuum infiltration. Three washes were carried out with milliQ water and then, roots were frozen in N_2 liquid and kept at -80 ºC. Plant material was ground with liquid nitrogen. Each gram of sample was ressuspended in 10 ml of extraction buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 % Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail for plant extracts (Sigma)). In order to increase the yield of nuclei released, the samples were passed twice with loose and tight pestle in a douncer homogeneizer. After sequential filtration through 100 µm and 40 µm strainer, the nuclei were collected by centrifugation for 20 min at 3000 x g and 4 ºC. The nuclei pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 % SDS, 1 mM PMSF, 1x protease inhibitor cocktail for plant extracts (Sigma)) and incubated for 15–30 min on a rotary shaker at 4 ºC to release chromatin. Chromatin fragmentation was carried out at high power mode for 30 cycles (30 seconds on/30 seconds off) in a Bioruptor® to obtain 100-500 bp sized DNA fragments. Soluble and insoluble fractions were separated by centrifugation during 5 min at 19000 x g and 4 ºC.

Chromatin fragmentation was evaluated before proceeding with chromatin immunoprecipitation. An aliquote (30-60 µl) of soluble chromatin was transferred to another tube to reverse the cross-links, overnight with 200 mM NaCl at 65 ºC with shaking in a Thermomixer (Eppendorf). RNA was digested with 0.1 U/µl RNase A/T1 mix (Thermo Scientific) during 1 h at 37 ºC with agitation. Then, proteins were removed with 50 µg/ml of proteinase K for 2 h at 37 ºC. Sonicated DNA was extracted with one volume of phenol:chloroform:isoamylalcohol (25:24:1) using phase lock gel tubes (3 Prime). The mix in phase lock tubes was centrifuged at 19000 x g for 5 min at room temperature. DNA was precipitated with 20 µg/ml glycogen, 1/10 of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold ethanol. After washing with 75 % ethanol, the DNA pellets were resuspended in nuclease free water. DNA was quantified using Quibit dsDNA High Sensitivity

assay kit (Life technologies). Assessment DNA fragments size was carried out by agarose (1 %) gel electrophoresis.

The rest of soluble chromatin was transferred to a new tube and diluted ten times in ChIP dilution buffer (16.7 mM Tris–HCl pH 8.0, 167 mM NaCl, 1.2 mM EDTA, and 1.1 % Triton X-100, 1 mM PMSF and 1x protease inhibitor cocktail) to reduce the SDS concentration up to 0.1 % maximum (1 ml of diluted chromatin was used per ChIP). Chromatin preclearing was performed during 1 h at 4 ºC with washed beads (Santo Cruz Biotechnology; 30 µl of protein G plus agarose beads per 1 ml of diluted chromatin) to eliminate contaminants. The supernatant was collected after spinning down the beads at 1000 xg and 4 ºC during 5 min. An input of 10 % was taken and kept (-20 ºC) and 1ug of pre-cleared chromatin was used per ChIP reaction.

Antibodies used in each ChIP are indicated in the following Table 3.7.

Table 3.7 Antibodies used in each ChIP

Antibody name	Concentration	Product reference
Anti-total H3	2 µg	Abcam ab1791
Anti-rat IgG	2 µg	Abcam ab6703
Anti-H3K4me3	3 µg	Abcam ab8580
Anti-H3K27me3	5 µg	Abcam ab6002

Antibody incubation was carried out overnight at 4 ºC in a rotating wheel. Next day, chromatin was incubated during 2 h at 4 ºC in a rotating wheel with 50 µl pre-washed protein G Plus agarose beads to pull down the immune complexes. Beads were submitted to successive washes with four different buffers:

- Low salt wash buffer (20 mM Tris–HCl pH 8.0, 2mM EDTA, 150mM NaCl, 0.1 % SDS, and 1 % Triton X-100, 1mM PMSF and 1x protease inhibitor cocktail for plant tissues).

- High salt wash buffer (20 mM Tris–HCl pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.1 % SDS, and 1 % Triton X-100, 1 mM PMSF and 1x protease inhibitor cocktail for plant tissues).

- LiCl wash buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1 % Igepal CA-630, and 1 % sodium deoxycholate).

- TE buffer (10 mM Tris–HCl pH 8.0, and 1 mM EDTA, 1 mM PMSF and 1x protease inhibitor cocktail for plant tissues).

For each wash, first a quick wash was performed and then samples were incubated for 5 min with washing buffer at 4 ºC with rotation. To spin down the beads, tubes were centrifuged for 2 min at 1000 x g at 4 ºC, and then the supernatant was discarded. Immune complexes were eluted from the beads by incubation twice the samples with 200 µl of pre-warmed elution buffer at 65 ºC

under agitation for 15 min (freshly prepared; 1 % SDS, 0.1 M NaHCO3). The input volume was adjusted to 400 µl with elution buffer. Cross link reversion was performed as described previously. DNA was precipitated with 20 µg/ml glycogen, 1/10 of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold ethanol. Washed with 75 % ethanol, and resuspended in 1x TE. ChIP was evaluated by quantitative real time PCR.

Quantification of samples obtained from ChIP was done by qPCR using a standard curve of genomic DNA with known concentrations as reference. Primers are listed in the *Table* 3.8. Each sample was first normalized by the input and then against total H3 content.

Table 3.8 Primers used for qPCR of ChIP samples.

Primer name	Sequence
ChIP_HTR6_Pair1_F	acaacattaaaagctttggcgatt
ChIP_HTR6_Pair1_R	agaatctgagagcttagaaactat
ChIP_HTR6_Pair2_F	TGGCTCGTACGAAGCAATCC
ChIP_HTR6_Pair2_R	CTTGACTCCTCCGGTAGTCG
ChIP_HTR6_Pair3_F	CCAAAAGAGCACAGAGTTGTTG
ChIP_HTR6_Pair3_R	GAAATCTTGAGCGATTTCACGA
ChIP_HTR6_Pair4_F	GCTGCGGAGGCATATTTGGT
ChIP_HTR6_Pair4_R	AGCACGCTCTGCACGAATCC

3.3 Cell biology techniques

3.3.1 Confocal microscopy

Root of 5 to 6 days old plants were dipped in 10 µM FM4-64 (Life technologies) to stain plasma membranes images and were acquired using LSM710 or LSM800 confocal microscope (Zeiss). GFP signal was detected after excitation with 488nm laser and mRFP with 561nm laser.

For fluorescence live imaging microscopy seedlings were grown for 5 days, transferred to a P35 glass bottom dish (MatTek) and covered with a peace of MSS agar 1 % supplemented with or without ABA (50 µM). Images were acquired with a LSM800 inverted confocal microscope (Zeiss) every 30 min.

Measure of fluorescent intensity of confocal images were proceeded in ImageJ v2.0.0-rc/59. In the case of images with Z-stack, fluorescence was measured after applied Z-stack maximum projection. For live imaging experiments, registration of images was realized with the StackReg plugin. Fluorescent intensity was measured as the integrity density of a determined ROI and statistical analysis was performed using Prism V5.0a.

3.3.2 Meristem cortex cells count and measure

Seven days-old roots from wt and *htr6-1* mutants were grown in MSS medium or in MSS medium complemented with ABA (50 µM) for the last 3 days. The cortex cells length and number of the wt and *htr6-1* roots in both conditions were measured in cortex cell file from the QC until the first elongated cell. The measurements were carried out in ImageJ v2.0.0-rc/59 and statistical analysis in the Prism V5.0a

3.3.3 Immunohistochemical assays

Five day old seedlings were treated with 50 µM of ABA during 4h in liquid MSS. In experiments were cells undergoing S-phase were to be counted, at the end of the treatment with ABA, cells for were labelled for 10 min with 50 µM of the thymidine analog, EdU (5-ethynyl-2-deoxyuridine; Life Technologies), protected from the light. After washing once with MSS, the seedlings were fixed with 4 % of paraformaldehyde (Sigma) diluted in MTSB buffer (100 mM PIPES pH 6.9, 10 mM EGTA, 10 mM $MgSO_4$). The solution was infiltrated 20 min under vacuum (8 inches Hg) and protected from the light. Four washes of 10 min were performed with 1x MTSB buffer followed by four washes with PBS (two washes of 10 min and two washes of 5 min) and usually one wash of 5 min with H_2O. Seedlings were placed on charged slides (Superfrost plus-Thermo scientific) and dried overnight protected from the light. A hydrophobic region around the seedlings was generated with pap-pen and then roots were rehydrated with 1x MTSB buffer for 10 min at RT. Cell wall permeabilization was carried out with a driselase solution (20 mg / ml in 1x MTSB) during 45 min at 37 ºC in a humid chamber. After four 5 min washes with PBS, membrane was permeabilized by incubating the root tips with 1x MTSB, 10 % DMSO, 3 % NP40 for 1 hour. Roots were washed four times with PBS during 5 min and then the blocking was accomplished with 3 % BSA, 10 % Horse Serum (HS) in 1x PBS for 1 hour at 37 ºC in a humid chamber. The roots were then incubated with a primary antibody (Table 3.9) diluted in 1 % BSA, 10 % HS, 0.1 % Tween-20 and 1x PBS for 1 hour or overnight at 4 ºC. Three washes with 3 % BSA-PBS were performed before the incubation with the secondary antibody (Table 3.9) diluted in 1 % BSA, 10 % HS, 0.1 % Tween-20 and 1x PBS for 1 hour. Three washes of 5 min with 1x PBS were carried out. Roots labelled with EdU were submitted to one extra step. EdU detection was carried out for 30 min, following the manufacturer's instructions of Invitrogen Click-iT® imaging kit 647. Then, nuclei were stained for 15 min with 10 µg / ml of DAPI in PBS. Three washes with PBS and one with H_2O were executed before the root tips were covered with mounting media, Mowiol 4-88 and coverslips.

Table 3.9 Antibodies used in Immunohistochemical assays

Primary antibody	Dilution	Product reference	Secondary antibody	Dilution	Product reference
Anti-GFP	1:1000	A6455 Life Technologies	Goat anti-rabbit-488	1:500	A11034 Life Technologies
Anti-H3K9me2	1:1000	Abcam 1220	Donkey Anti-mouse 647	1:500	A-31571 Thermo Fisher
Anti-H3K27me1	1:1000	Milipore 07-448	Donkey Anti-mouse 647	1:500	A-31571 Thermo Fisher
Anti-H3K27me3	1:1000	Abcam ab6002	Donkey Anti-mouse 647	1:500	A-31571 Thermo Fisher
Anti-H3K4me3	1:1000	Abcam ab8580	Donkey Anti-mouse 647	1:500	A-31571 Thermo Fisher

3.3.4 Cell cycle progression assay

In order to compare the cell cycle progression in presence or absence of ABA, 5 day-old seedlings were labelled with two thymidine analogs, EdU (200 μM) and BrdU (200 μM) (5-bromo- 2-deoxyuridine; Sigma), to sequentially mark cells under S-phase. Each analog labelling was carried out for 30 min, separated with increasing chase time period with thymidine (200 μM) (0h-2h), in order to see cell cycle progression. Immunodetection was performed as described previously in section 3.3.5. The DNA was mildly digested with 0.003 U/μl DNase I RNase free (Roche) during 1.5 h at 37 ºC in order to allow the anti-BrdU antibody access BrdU-labeled DNA. DNaseI was inactivated with several washes of ice-cold EDTA-PBS 8 mM. Posterior steps of immunodetection, Edu detection and DAPI staining were performed as in 3.3.5 (Immunohistochemical assay). The antibodies used in this assay are listed in table 3.10.

Table 3.10 Antibodies used in cell cycle progression assay

Primary antibody	Dilution	Product reference	Secondary antibody	Dilution	Product reference
Anti-GFP	1:1000	A6455 Life Technologies	Goat anti-rabbit-488	1:500	A11034 Life Technologies

Anti-BrdU	1:200	347580 Becton Dickinson	Donkey anti-mouse-555	1:500	A31570 Life Technologies

3.3.5 Fluorescence recovery after photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) method was used to compare histones dynamics in vivo. Images were adquired on a Zeiss LSM800 confocal microscope. We used 488 nm laser for excitation of GFP. After selection of the area of interest a stack of 15 to 20 pre-bleach images (pinhole 2.5 µm, 512 x 512 pixels) was acquired with a 1 µm step. Plants were prepared as described in confocal microscopy (3.3.1). After selecting the area of interest, two images were acquired and fluorescence was measured. Then, we selected a ROI inside the nucleus and photobleached it with the laser at maximum power, 100 %. After photobleaching, series of images were acquired every 5 minutes to measure the fluorescence recovery. Image processing and data analysis were executed with ImageJ Fiji v2.0.0-rc/59 and according to Rosa (2018) specifications.

3.3.6 Flow cytometry of roots

Flow cytometry was used to measure ploidy in roots after 3 days of treatment with ABA (50 µM) comparing roots exposed to stress (50 µM of ABA). Five mm of 7 day old plants were cut and chopped inside a petri dish in 500 µl of cold Galbraith buffer (45 mM MgCl2, 30 mM Sodium Citrate, 20 mM 4-MOPS pH 7.0; 0.1 % Triton X-100) to liberate nuclei. Samples were filtrated through a 30 µm filter to eliminate cell debris and avoid clogging of the flow cytometer. The collected nuclei were stained with 2 µg/ml of DAPI and analyzed with a FACSCanto II flow cytometer (Becton Dickinson). We used detectors that detect DAPI (FL7) at logarithmic scale and light scattered forward (FSC-A) to distinguish populations. The singlets we discriminated from aggregates by using FL8 detectors in a linear mode, a histogram against FL7-A was created to calculate the percentage of nuclei with 2C, 4C, 8C and 16C DNA content. 10000 events were count to measure ploidy of each type of sample (stresses and non-stressed). All this analysis was executed in FlowJo software.

3.4 Bioinformatic analysis

3.4.1 *In silico* promoter analysis

The chromatin states (Sequeira-Mendes *et al.*, 2014) and DNaseI cleavage pattern footprints (Sullivan *et al*., 2015) were analyzed in IGB genome browser. TF-binding site were obtained by scanning the motifs along 2.9 kb upstream sequences. Position weight matrices (PWMs) for TF-binding sequences (Franco-Zorrilla *et al*., 2014; Mathelier *et al*., 2014; Weirauch *et al*., 2014) were used to scan Arabidopsis promoter sequences using RSAT (Turatsinze *et al*., 2008).

3.4.2 RNA-seq data analysis

The quality analyses of the sequencing of RNA extracts was performed over reads using FastQC software (Kim *et al*., 2015). Then, the reads were aligned against *A. thaliana* reference genome (TAIR10) using Hisat2 aligner (Robinson *et al.*, 2011). The htseq-count software (Anders *et al.*, 2014) was used to count the reads mapping of each feature and the differential expression analysis was performed using Deseq2 (Love *et al.*, 2014), an R software package. The gene expression data has been graphically shown in Volvano plot. The volcano plot shows the statistical significance, as the negative log10 of the False Discovery Rate (FDR) with adjusted p-value, combined with the log2 of the fold change between wt in ABA treatment for 10 min and *htr6-1* mutants in ABA treatment for 10 min.

4. Results

4.1 Characterization of *HTR6* histone.

4.1.1 *HTR6* share features with H3.1 and H3.3

The histone H3 family encompasses 15 Histone Three Related (HTR) genes, including genes that encode the canonical H3.1 and variant H3.3. Previous studies have shown that histone H3.1 and variant H3.3 have different properties, are differently distributed along the genome and are deposited by distinct machinery (Stroud *el al*., 2012; Otero *et al*., 2016). Interestingly, those divergent features result from variations in only 4 amino acid residues at position 31, 41, 87 and 90 (Fig. 4.1 A).

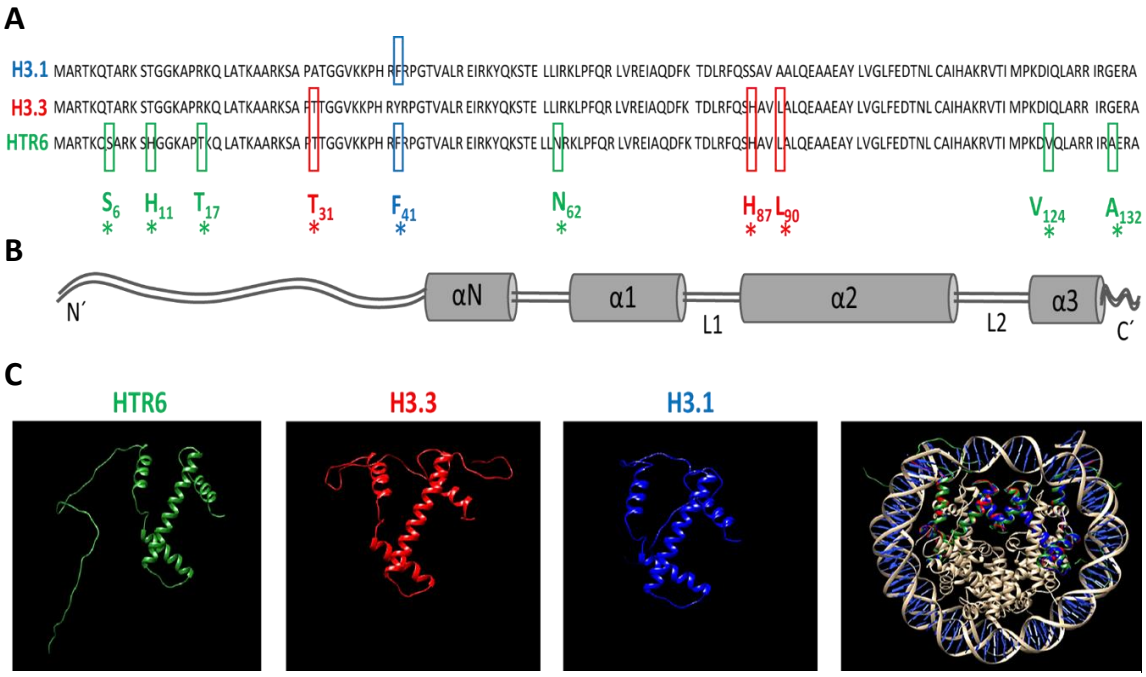

Figure 4.1. Structure of HTR6, H3.1 and H3.3 proteins. **(A)** Alignment of Arabidopsis histones H3.1 and H3.3 amino acid sequences with histone *HTR6*. The amino acids that are specific of *HTR6* are highlighted in green. The four residues that diverge in H3.1 and H3.3 are represented in blue and red, respectively, depending on which homology these amino acids share with *HTR6*. **(B)** Schematic representation of histone H3 secondary structure. The N´terminal is represented by a tail preceding the αN helix while the C´terminal tail is after the α3 helix. Straight lines represent the loops and the cylinders are alpha helices. The scale of the picture is in correspondence with residue positions in A. **(C)** *HTR6* structure prediction by the UCSF Chimera based on the H3.1 and H3.3 human nucleosomes crystallography models (Tachiwana *et al*., 2011). Protein Data Bank accession number: 3AV1 (H3.1) and 3AV2 (H3.3). *HTR6*: green; H3.3: red; H3.1: blue.

A protein sequence blast of H3 family (WU-BLAST 2.0) (data not shown) indicated that other histone variants, share characteristics with H3.1 and with H3.3, regarding protein length and composition. Curiously, an H3 variant, *HTR6*, showed a high similarity with both H3.3 (94% protein homology) and H3.1 (93% protein homology), differing in only 7 and 9 residues, respectively. In fact, from the 4 residues that diverge between H3.1 and H3.3, *HTR6* shares the position T31, H87 and L90 with H3.3 and F41 with H3.1 (Fig. 4.1 A). Moreover, there are

residues uniquely present in *HTR6* ($S_6T_{17}N_{62}A_{132}$) and some amino acids that differ from H3.1 and H3.3, but are also found in other H3 variants (H_{11} and V_{124}), such as HTR14. These similarities and dissimilarities to canonical H3.1 and variant H3.3 lead the scientific community to consider *HTR6* as an "unusual" histone due also to the fact that its function was unknown.

Figure 1 B represents the H3 secondary structure, which is formed by the N-terminal tail that connects with the αN helix, three alpha helices (α1–α3) speared by short loops (L1 and L2), and the short C-terminal tail. The core structure of the histone includes the region from the beginning of αN helix until the end of α3 helix (Luger *et al*., 1997). The amino acids shown in the primary structure (Fig. 4.1 A) are in scale with the correspondent position in the secondary protein structure (Fig. 4.1 B). We used UCSF Chimera program to predict the tertiary and quaternary structure of *HTR6* using as a reference the X-ray structure of human H3.1 and H3.3 nucleosomes (3AV1and 3AV2) (Tachiwana *et al*., 2011; Fig. 4.1 C). Human H3.1 and H3.3 proteins have a high homology with the corresponding ones present in Arabidopsis (97 % and 96% match for each one, respectively (BLASTp suit). Despite the slight differences found, our predictions indicate that *HTR6* might adopt a structure similar to both human H3.1 and H3.3, which also suggests analogous interactions inside the nucleosome. The αN helix is still predicted to be located at the DNA entry/exit site of the nucleosome (Luger *et al*., 1997; Arents *et al*.,1991) while C- and N- terminal tails are unstructured (Fig. 4.1 C). Nonetheless, many of *HTR6* residues ($S_6H_{11}T_{17}A_{132}$) that differ from H3.1 and H3.3 are located in its N and C-terminal. Since the different residues can undergo a plethora of distinct post-translational modifications (PTMs), influencing protein function and chromatin landscape regulation, it is conceivable that the unique amino acids of *HTR6* could be fundamental to play new functions for this unusual H3 variant, as discussed later.

4.1.2 *In silico* analysis of *HTR6* gene and promoter

HTR6 (AT1G13370) is localized in the chromosome 1 (from 4587780 to 4588661) of Arabidopsis thaliana in the Crick minus strand (-). The *HTR6* gene encompasses 3 exons separated by two introns and encode a 136 amino acids protein (Fig. 4.2).

Analysis of a compilation of genomic features in the *Arabidopsis thaliana* genome allowed to define 9 different chromatin states (Sequeira-Mendes, *et al.*, 2014). State 5, as well as state 4, is characterized by high levels of H3K27me3 deposited by PRC2 proteins. In fact, our analysis showed that state 4 is prevalent downstream of the *HTR6* gene. Looking at the promoter side of the *HTR6* gene, 280bp chromatin upstream of the TSS, is covered by state 2. This state is characterized by the presence of the repressive modification H3K27me3 but also by the presence of active marks (H3K4me3). All these data indicate that *HTR6* does not seem to be a particularly

active gene but, nonetheless, they also point to the possibility that, under appropriate stimuli, activation can be triggered.

Around 0.7Kb upstream of *HTR6*, there is another gene called *DUF1218* which encode a protein with a function in sodium/hydrogen exchanger. *DUF1218* gene is covered by state 3 and 1, two sates that are enriched in active marks and depleted of Polycomb. The clear barrier between the repressive state *of HTR6* and active states of *DUF1218*, suggests that the *HTR6* promoter is confined to the 0.7 kb intergenic region that separates these two genes.

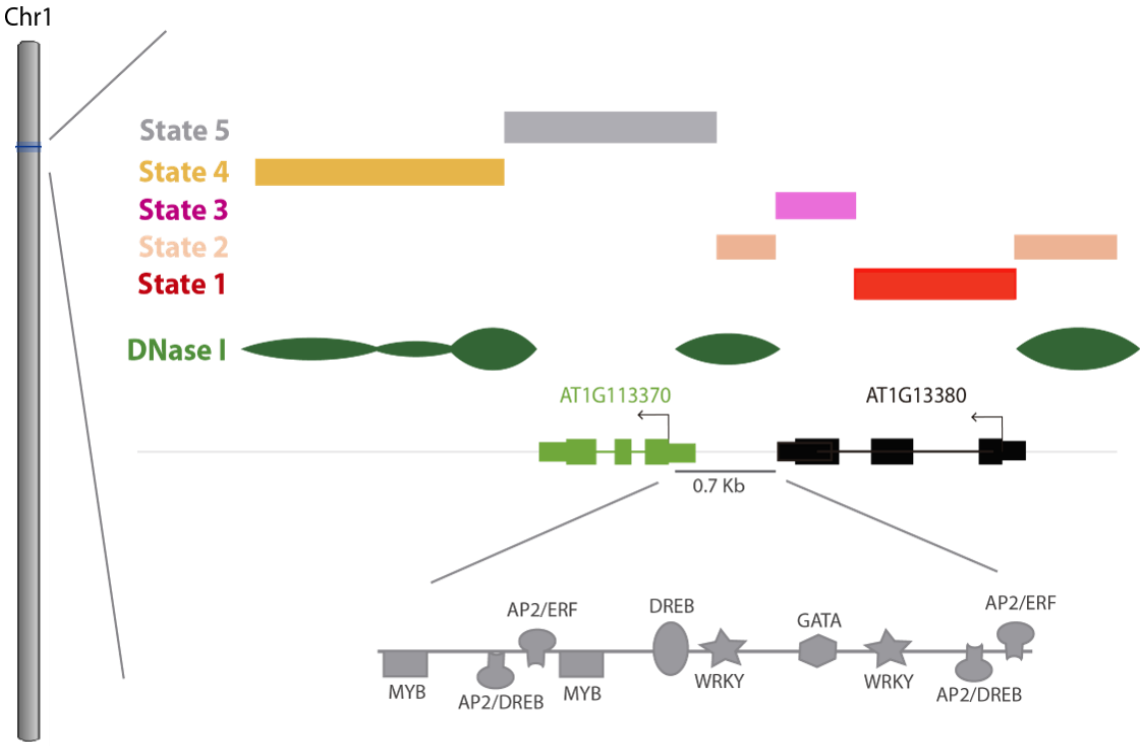

Figure 4.2. Genomic landscape of the *HTR6* gene in chromosome 1. The *HTR6* gene is represented in light green while the upstream gene is in black. Squares inside the genes spared by lines are exons and introns, respectively. The arrows represent the direction of transcription. Chromatin states encompassing different genomic elements are symbolized by boxes with different colors: state 1- active gene (red); state 2- proximal promoters (salmon); state 3- 5´end of genes (pink); state 4- intergenic (dark yellow); state 5- PcG (grey) (Sequeira-Mendes *et al.*, 2014). DNase I digestion pattern is shown in dark green (Sullivan *et al.*, 2014). The predicted TF binding sites located in the 0.7 Kb upstream region of the HTR6 gene are represented in grey. This analysis was carried out with the IGB genome browser. Note that states 6 to 9 are not present in the window analyzed.

In order to identify the most important regulatory regions and to define promoter length we analyzed a DNase I hypersensitive sites (DHSs) map generated for 7 days-old Arabidopsis seedlings (Sullivan, *et al.*, 2014). DNase I endonuclease recognizes and cleaves accessible chromatin. TF occupancy triggers an atypical DNaseI cleavage pattern, called footprint, allowing the identification of trans-acting factors occupancy regions. According to the DNase I map, the intergenic region between the *HTR6* and *DUF1218* gene covered by state 2 and 5 is enriched in cis- acting regulatory elements (Fig. 4.2), supporting the notion that the *HTR6* promoter might

encompass the upstream 0.7Kb. TFs are essential regulators of gene expression that preferentially recognize specific DNA consensus sequences, also known as cis regulatory elements (Todeschini *et al*., 2014). Based on cis- regulatory element screenings, we scored which TFs could potentially bind the 0.7kb region upstream of *HTR6*. TFs that respond to osmotic stress such as MYB, AP2/ERF, DREB, WRKY and GATA showed the higher scores, which means that they are more prone to bind to the *HTR6* promoter. Putative binding sites of higher score TFs are represented in Fig. 4.2 and Table 4.1. This suggests that *HTR6* as a potential role in abiotic stress response.

Table 4.1. Potential position of predicted TF binding sites in the *HTR6* 0.7 Kb upstream region.

TF family	**Position upstream of *HTR6* TSS**	**TF family**	**Position upstream of *HTR6* TSS**
Myb	-18	WRKY	-209
AP2/DREB	-150	GATA	-248
Myb	-153	WRKY	-384
DREB	-207	AP2/DREB	-680

4.2 Regulation of *HTR6* expression

4.2.1 *HTR6* expression is induced by abiotic stress

High salt or drought provoke an osmotic stress that trigger ABA response in plants (Boudsocq and Laurière, 2005). In order to check if *HTR6* expression is induced after osmotic stress, we analyzed *HTR6* transcript levels in wild type (wt) Columbia (Col-0) seedlings after exposure to an abiotic stress mimicked by a treatment of seedlings with high concentrations of NaCl (140 mM) or ABA (50 µM). Expression of *HTR6* increased after plants were submitted to stress conditions during 4 h (Fig. 4.3 A), supporting the *in silico* results that suggest that *HTR6* might have a role in the abiotic stress response. Strikingly, high concentrations of ABA caused higher effects on the accumulation of mRNA, resulting in a 5-fold increase in the expression of *HTR6*, which was two times the value of *HTR6* expression increase achieved with salt exposure (Fig. 4.3 A).

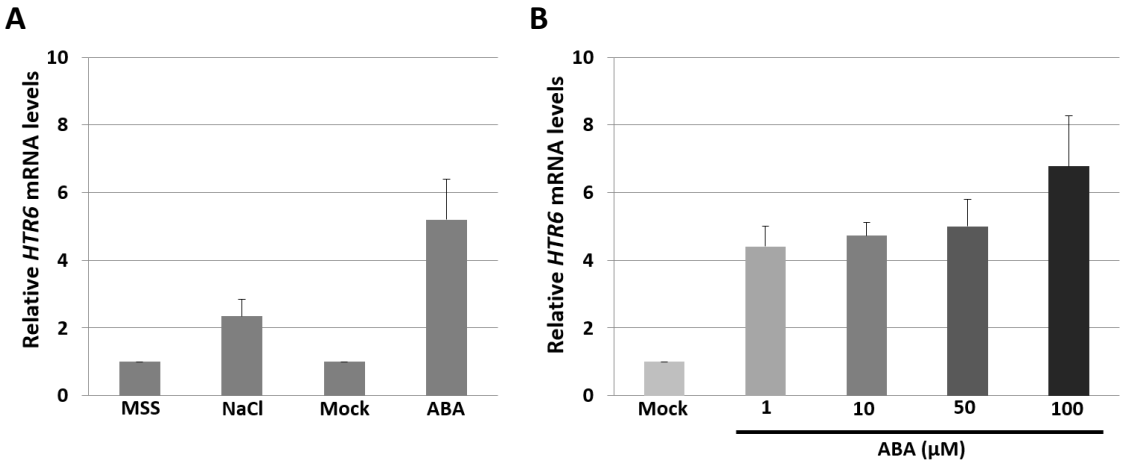

Figure 4.3. *HTR6* expression after abiotic stress exposure. A) *HTR6* expression in 7 days-old wt (Col-0) seedlings after 4hours exposition to stress conditions (140 mM of NaCl or 50 µM of ABA) versus controls (MSS and Mock). B) Effect of different exogenous ABA concentrations on *HTR6* expression in 7 days-old seedlings (4 hours treatment). Mock samples were seedlings treated with methanol (where ABA is dissolved) during the same period of time. Error bars represent standard errors of the mean based on 2 biological replicates.

It is postulated that treatment with different ABA concentrations can produce distinct responses in plants (Ghassemian *et al.*, 2000). Concentrations below 1µM stimulate plant growth while higher concentrations have the opposite effect, restricting plant growth (Ghassemian *et al.*, 2000). Thus, we compared *HTR6* expression levels in plants treated with four different ABA concentrations, varying from 1µM up to 100µM. As it can be seen in Fig. 4.3 B, *HTR6* expression increased in a dose-dependent manner reaching higher values in plants exposed to 100µM ABA,. Unless otherwise stated, we used a concentration of 50 µM ABA in the subsequent experiments, that produced significant *HTR6* expression without drastically affecting plant growth.

4.2.2 *HTR6* expression is mainly induced in roots

Abiotic stress triggers responses in several plant tissues (Dinneny *et al.*, 2008). In Arabidopsis, expression of *H1.3* histone is induced in various tissues and organs in response to abiotic stress, except in stomata cells, where it is constitutively expressed, even in absence of stress (Rutowicz *et al.*, 2015). Based on this example, we wondered if *HTR6* could be differentially expressed in the shoot and root. We found that, upon ABA treatment, higher expression values of *HTR6* were detected in roots (3.7 fold increase) when compared to the shoot part of the seedling (2.2 fold change) (Fig. 4.4 A). No differences were detected in *HTR6* expression in mock conditions comparing shoots and roots.

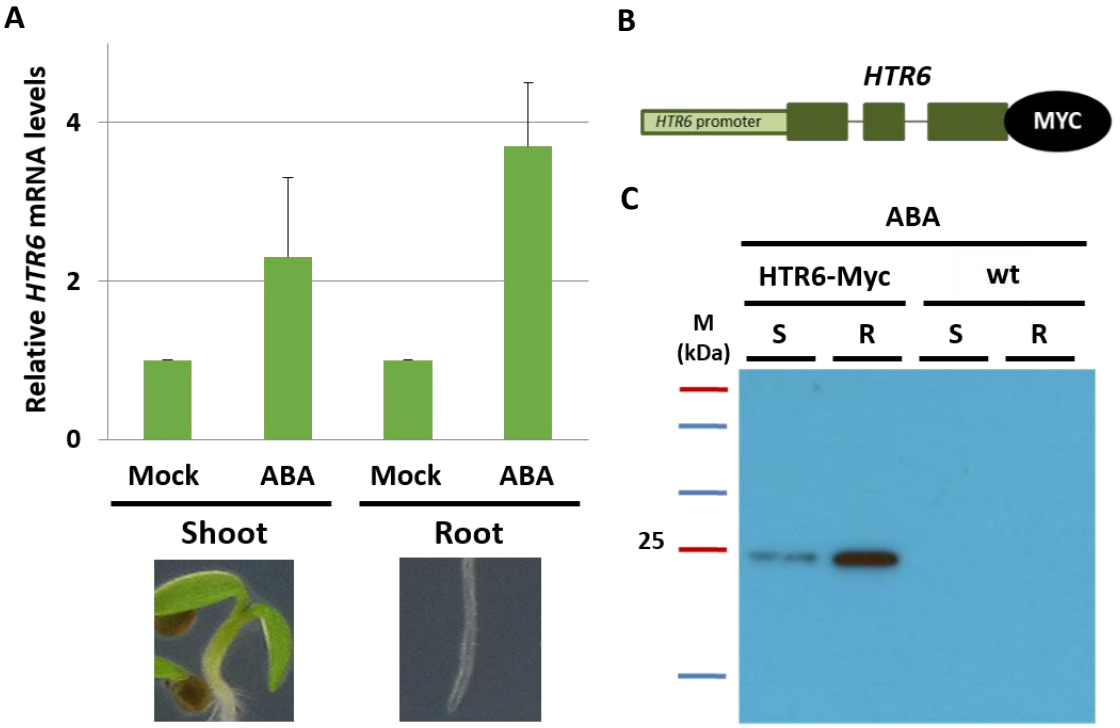

Figure 4.4. Expression of *HTR6* in roots and shoots. A) *HTR6* RNA expression level in shoots and roots with and without ABA. Error bars represent standard errors of the mean based on 2 biological replicates. B) Scheme representing the construct (~2.5 Kb) used to generate transgenic plant expressing *HTR6*-Myc. Light green box represents the promoter; dark green boxes represent exons and the lines represent introns. C) Western blot analysis of shoot (S) and roots (R) nuclear extracts of Col-0 and *HTR6*-Myc expressing plants, after ABA treatment (50 µM during 4 hours).

To determine whether the *HTR6* gene activation after stress exposure is translated into increasing levels of protein we carried out the analysis of *HTR6* protein levels by western blot. In the absence of an antibody that specifically recognizes *HTR6*, we generated a transgenic plant expressing *HTR6* fused to a MYC tag under the regulation of its native promoter (Fig.4.4 B). Anti-Myc antibody was used to detect *HTR6*-Myc protein by western blot. Western blot of shoot and root nuclear extract, is consistent with the mRNA levels, showing a higher level of *HTR6* being induced in roots than in shoots.

4.2.3 Disruption of ABA signaling pathway impairs *HTR6* induction under stress conditions.

As mentioned before (Results section 4.2.1), exogenous ABA application in the media affects plant growth (Ghassemian *et al*., 2000). To better establish the effect of external application of ABA on root growth we measured root length of plants grown with and without ABA (50 µM).

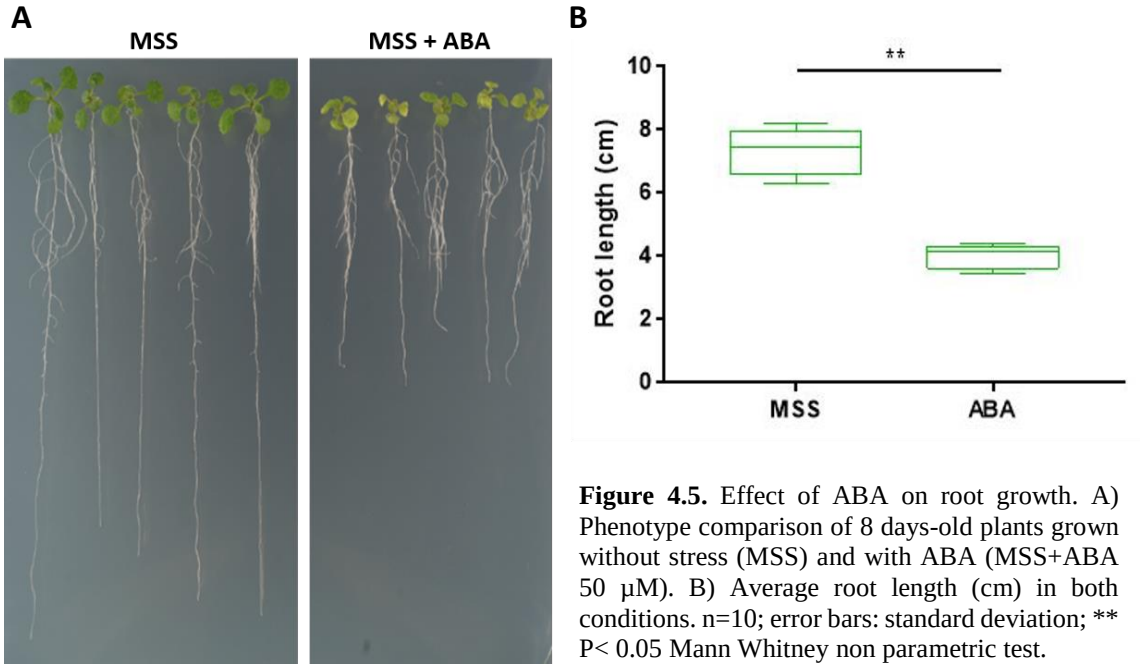

Figure 4.5. Effect of ABA on root growth. A) Phenotype comparison of 8 days-old plants grown without stress (MSS) and with ABA (MSS+ABA 50 µM). B) Average root length (cm) in both conditions. n=10; error bars: standard deviation; ** P< 0.05 Mann Whitney non parametric test.

As it is shown in Fig. 4.5, after 8 days, plants exposed to ABA showed a restricted growth when compared to plants grown in MSS (Fig. 4.5A). Roots under ABA treatment grew significantly less, reaching a total length of 4 cm on average, while plant roots without treatment were able to reach a mean length value of 7.5 cm (Fig. 4.5B). Although for this experiment leaf size was not quantified, it is possible to visualize on Fig. 4.5A that leaves of ABA treated plants seem to be smaller and decolored when compared to plants grown in MSS.

The ABA signaling pathway encompasses several elements, including specific receptors, such as PYR/PYL/RCAR, kinases and transcription factors that bind promoters of target genes at the end of the activation pathway (Fig. 4.6A). In order to determine the extent to which ABA is required for *HTR6* induction we evaluated the *HTR6* expression levels in mutants of distinct elements of the ABA signaling pathway. First, we investigated the *HTR6* expression in two ABA insensitive (abi) transcription factor mutants, *abi4-1* and *abi5-1*. Loss of function of these genes implies that their target genes are no longer activated after ABA exposure. We observed that after ABA treatment, levels of *HTR6* slightly increase in abi mutants, indicating that *HTR6* might not be a direct target of ABI4 or ABI5 (Fig. 4.6 B).

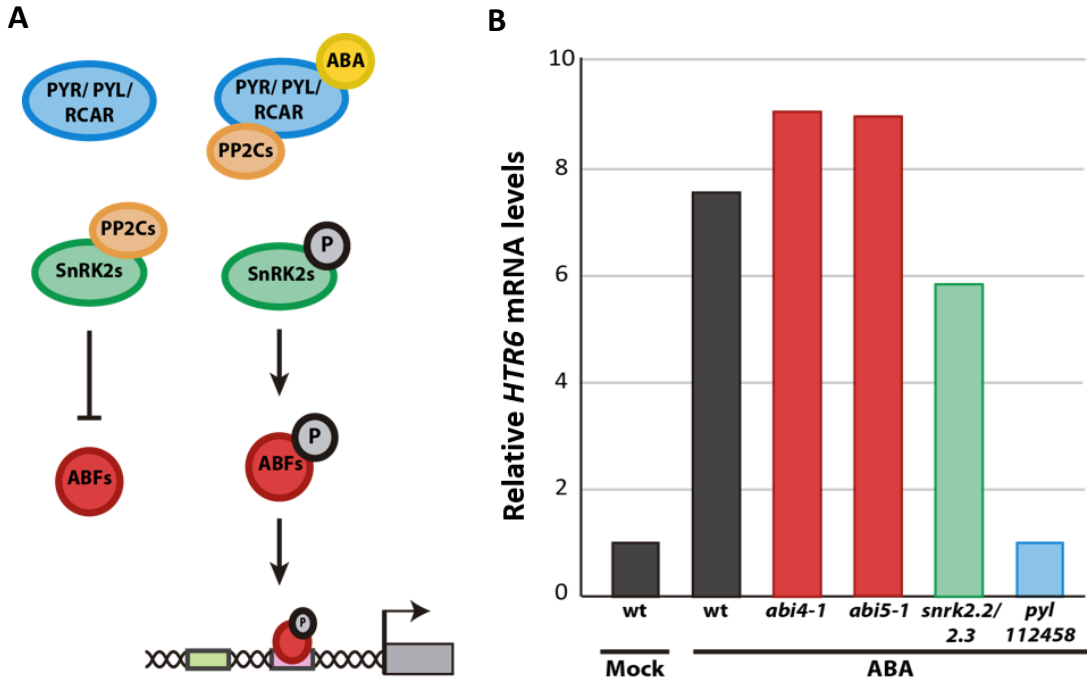

Figure 4.6. Influence of ABA pathway elements on *HTR6* expression. A) Simplified scheme of the ABA signaling pathway in the absence (left side) and presence (right side) of ABA. In the absence of ABA, PPC2s inactivates subclass III SnRK2s kinases, which impair the activation of ABA responsive genes. In the presence of ABA, the PYR/PYL/RCAR receptors suppress PP2Cs phosphatase activity, allowing the activation of subclass III SnRK2s kinases. SnRK2s subsequently phosphorylate AREB/ABFs transcription factors, which recognize ABRE elements in the genome, activating ABA responsive genes. Adapted from (Fernando and Schroeder, 2015). B) Relative *HTR6* expression levels in different ABA pathway mutants using wt as reference. The *pyl 112458* sextuple mutant is ABA-insensitive impaired in six PYR/PYL ABA receptors *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8*.

Upstream in the ABA response pathway, a group of kinases denominated, sucrose nonfermenting-1 (SNF1) related protein kinase 2s (SnRK2s), have an important role in the phosphorylation events that are essential for the progression of the signal (Fig. 4.6 A). We analyzed the *HTR6* mRNA levels in a double mutant of *SnRK2*s kinases, *snrk2.2/snrk2.3*. *HTR6* induction in this mutant was slightly weaker compared to wt (Col-0), suggesting that these factors could be implicated in *HTR6* regulation (Fig. 4.6 B). However, other SnRK2 kinases exist in the signaling pathway and might also be participating in *HTR6* induction. We also determined that *HTR6* induction was completely impaired in plants deficient in ABA perception due to the mutation of six ABA receptors, *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* (abbreviated as 112458; Fig. 4.6 B). Altogether, these results demonstrate that *HTR6* induction is dependent on ABA.

4.2.4 Chromatin environment changes in the *HTR6* gene and promoter during abiotic stress

The study of chromatin states (Sequeira-Mendes *et al.*, 2014) indicates that *HTR6* promoter is in part covered by a repressive epigenetic mark, H3K27me3, that is deposited by the Polycomb complex, PRC2. Furthermore, marks associated with active transcription, such as H3K4me3, are not frequent in this region. Nonetheless, this chromatin landscape study was performed in

seedlings (10-day-old) grown without stress. Exposing seedlings to an environmental stress could completely alter chromatin leading to new scenarios. Thus, to determine whether alterations in the chromatin landscape occur upon exposure to ABA, we performed a Chromatin Immunoprecipitation assay (ChIP) in roots of plants either untreated or treated with 50µM ABA. We used antibodies against the repressive mark, H3K27me3, and the active mark, H3K4me3 (Fig. 4.7).

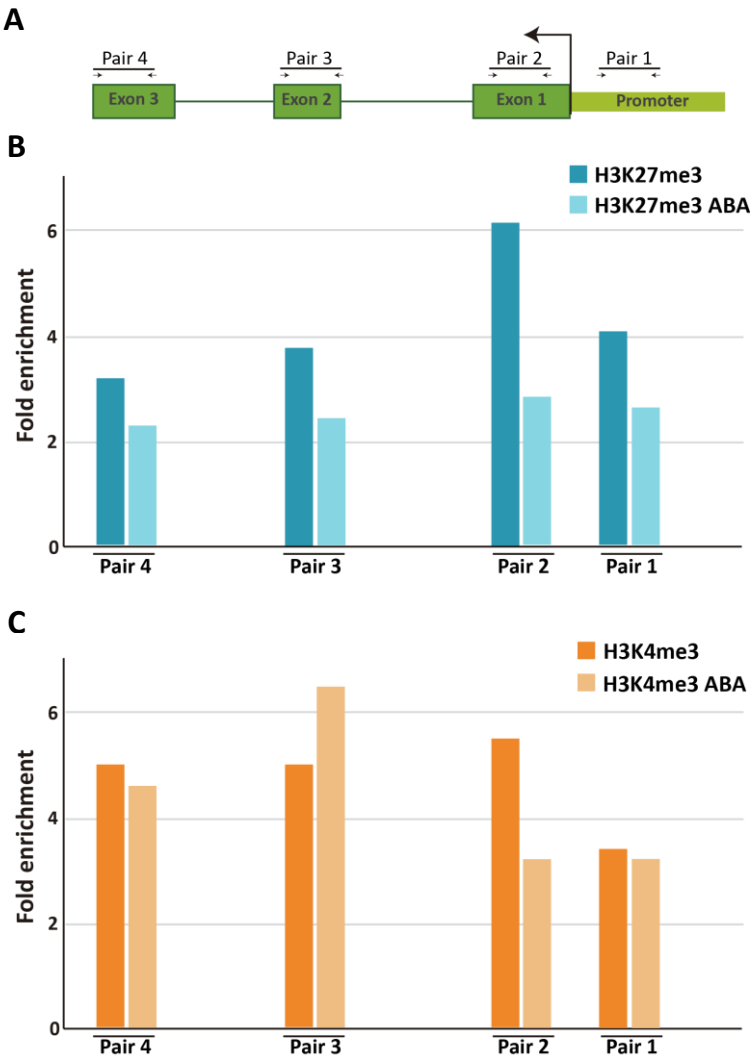

Figure 4.7. Chromatin landscape alterations in the *HTR6* gene and proximal promoter after ABA treatment (50 µM during 4h) in 7 days-old roots. A) Schematic representation of the *HTR6* gene and the regions analyzed. Dark green boxes represent the exons; lines between exons represent the introns; light green boxes represents the proximal promoter; arrow indicates transcription orientation; pair 1 to 4 indicate the regions analyzed by qPCR. B) ChIP analysis of H3K27me3 (repressive mark). C) ChIP analysis of H3K4me3 (active mark).

For this analysis, different regions of *HTR6* locus were selected (Fig. 4.7A). Levels of active mark H3K4me3 were largely maintained, after ABA treatment, in the majority of the regions analyzed, with the exception of a slight increase detected in exon 2 (Pair 3) and a decrease at the beginning of the gene (Pair 2 at Exon 1) (Fig. 4.7C). On the contrary, we observed a general decrease in the enrichment of H3K27me3 all over the *HTR6* locus. Altogether, these results point towards a change in the chromatin environment of *HTR6* gene after ABA exposure, which seems to be associated with a decrease of the repressive marks deposited by the PRC2 complex.

To further investigate if *HTR6* is a target of Polycomb complex, we evaluated the levels of *HTR6* transcription in a mutant of CURLY LEAF (CLF) subunit, *clf29* (Fig. 4.8). CLF, together with SWINGER and MEA, are subunits of the PRC2 complex that contain a SET domain responsible for histone H3 methylation on lysine 27. The *clf29* allele is a null mutant, meaning that no protein or transcript is detected in the plant, however the mutant phenotype is weak due to the presence of other functional methyltransferase such as SWINGER (SWN) (Wang *et al*., 2016).

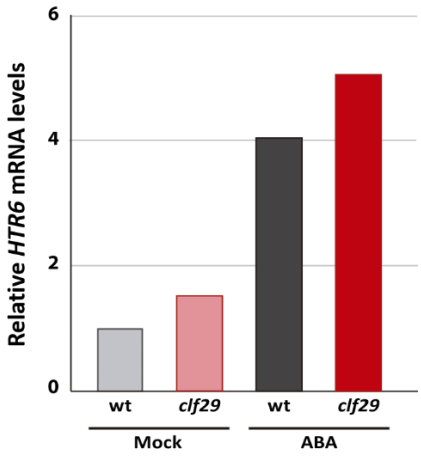

Figure 4.8. *HTR6* expression levels in wt and *clf29* mutant in roots of 5 day-old seedlings, in the presence and absence of ABA (50 µM).

As shown in Fig. 4.8, in absence of ABA, *HTR6* transcript level is slightly higher in the *clf29* mutant compared to wt (Col-0), although, the difference is not significant. After ABA treatment, *HTR6* is also induced at higher level in the *clf29* mutant, however, the difference is still not significant. Thus, we can conclude that i) derepression in the single *clf29* mutant is not effective probably because of the presence of other methyltransferase such as SWN that is able to deposit H3K27me3, and ii) if the transcription factors responsible for ABA response are not activated, the reduction of H3K27me3 alone is not enough to significantly induce *HTR6* expression.

4.3 HTR6 Dynamics

4.3.1 Spatio-temporal dynamics of HTR6 during abiotic stress along the root

It has been postulated that ABA response in plants is initiated very early after the exposition to an abiotic stress (Kollist *et al.*, 2019). We found that *HTR6* expression in roots is induced by ABA, but the kinetics of the induction has not yet been uncovered yet. Thus, we measured relative *HTR6* mRNA levels in 7 days-old wt roots at different times after ABA treatment (Fig. 4.9). As a control we also measured expression levels of HTR6 in samples growing without ABA (Mock). *HTR6* expression increased gradually until reaching a peak at 4 h, and then slightly decrease, although, without diminishing to basal levels (Fig. 4.9). Interestingly, during the first thirty minutes after stress induction, *HTR6* mRNA levels increased to almost the double compared to the untreated control, suggesting an early role in abiotic stress response.

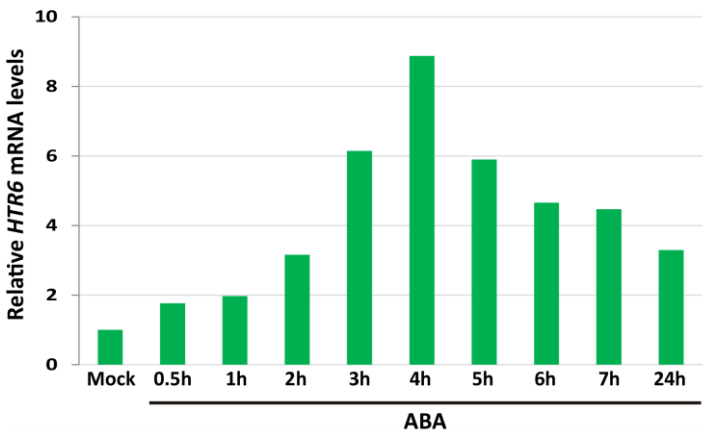

Figure 4.9. *HTR6* expression time course during ABA (100 µM) treatment.

To further clarify the spatio-temporal dynamics of HTR6 along the root, we generated transgenic plants carrying HTR6-GFP fusion protein under the control of its native promoter (Fig. 4.10 A). In general, nuclei containing HTR6-GFP were detected only after ABA exposure (Fig. 4.10 B). Nuclei with high fluorescence level were visualized after 3 hours of treatment, however, as soon as 1 h after ABA exposure, we could already detect few nuclei containing a low HTR6 signal. Despite we demonstrated that *HTR6* mRNA level reached a maximum after 4 h treatment and decreased afterwards (Fig. 4.9) the accumulation of HTR6-GFP protein seems to be stable from 3 h to 24h after ABA treatment (Figure 4.10 B).

The anatomical organization of the Arabidopsis root allows to identify easily the different developmental domains as well as the distinct tissues that constitute the roots (Petricka *et al.*, 2014). To determine HTR6 distribution along the root we studied in detail 5 days-old roots treated with ABA for 4h by confocal microscopy (Fig.4.11), as this time point previously gave us the highest *HTR6* expression levels. We observed that HTR6 is mainly present in a subset of cells at the transition/elongation zone and in the lateral root cap (Fig. 4.11).

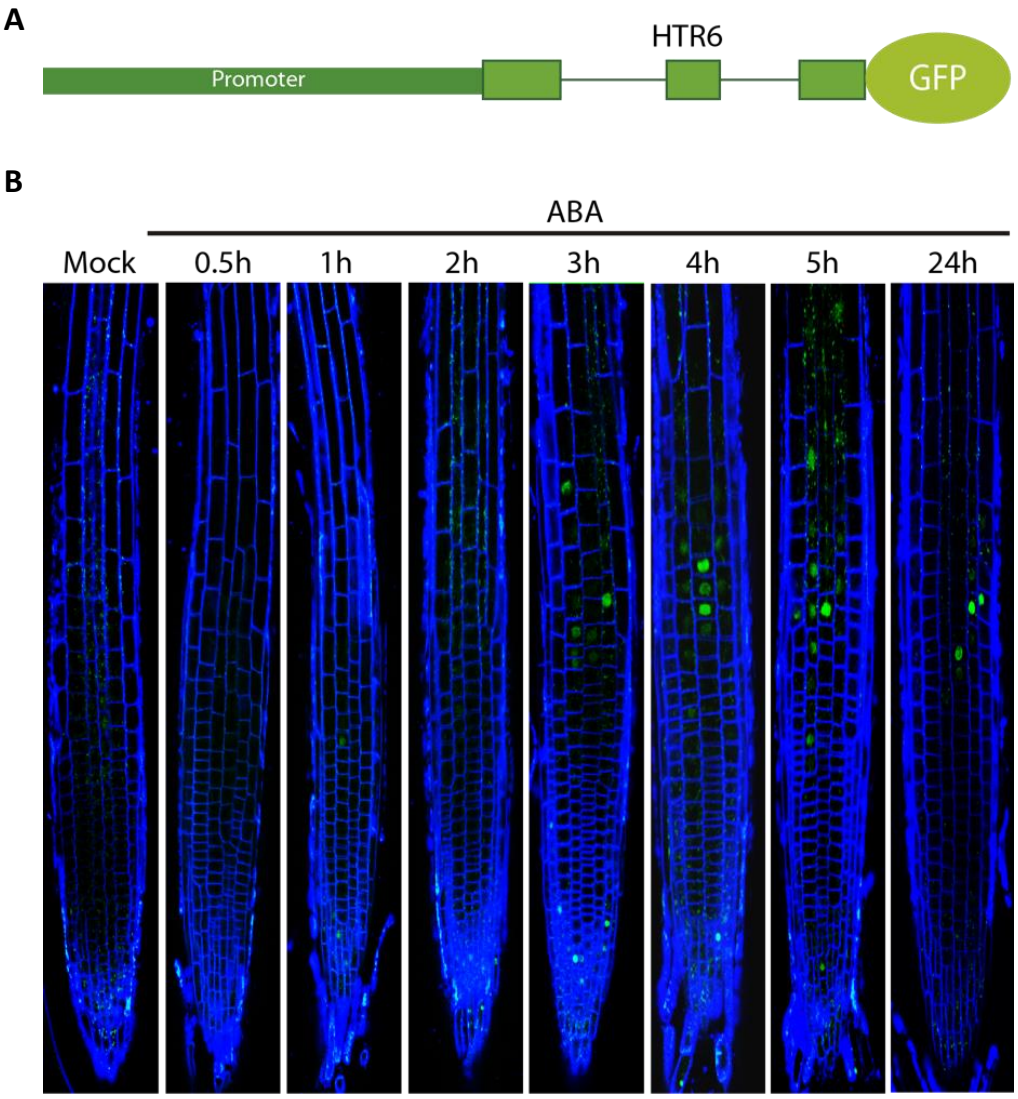

Figure 4.10 Spatio-temporal induction of *HTR6* during abiotic stress along the root. A) Schematic representation of the transgene construct including *HTR6* promoter and gene fused with GFP. Dark green boxes represent the exons; lines between exons represent the introns; light green box represents the proximal promoter; GFP tag is represented by an oval. B) HTR6 expression pattern along the root at different time point of ABA exposure.

However, HTR6 is induced in the transition zone/elongation zone but absent either in the proliferative cells (Fig. 4.11) and in the differentiation zone (data not shown). Furthermore, it is detected only in the most external tissues, such as root cap, epidermis and cortex, which suggests that these tissues could have a particular role during the stress response.

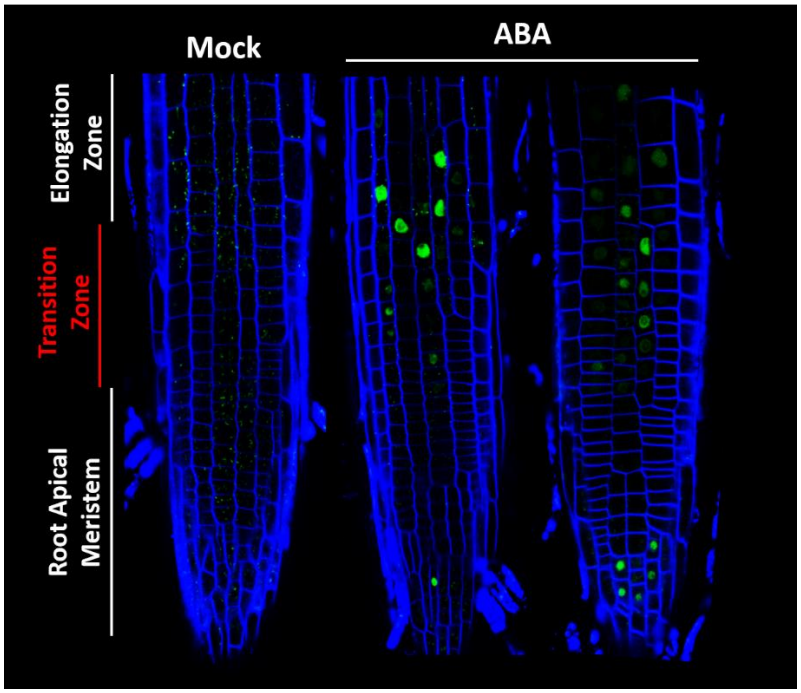

Figure 4.11 HTR6-GFP expression pattern in the epidermis along the different root developmental domains (Root apical meristem, transition zone and elongation zone) after plants being exposed to 4 hours of ABA (50 µM).

We, then, visualized HTR6-GFP by live imaging in 5 days-old seedlings to monitor HTR6 dynamics. First we exposed seedlings expressing HTR6-GFP to ABA (50 µM) for 3h and, then we imaged the roots every 30 min until 7.5h of ABA exposure (Fig. 4.12).

During the course of the experiment we could detect nuclei that start to accumulate HTR6 showing that the loading process occurred in a short window of ~30 min and that HTR6-GFP reach a maximal intensity after ~1h (Fig. 4.12, white arrowhead). Degradation is not so fast and occurs in a range of 2 to 3h (Fig. 4.12, red arrowhead). We were not able to detect incorporation and degradation of HTR6 in single nucleus, which make difficult to estimate how long HTR6 is maintained in the nucleus. However, we found that some nuclei have a relatively high level of HTR6-GFP during the 7.5h of the experiment indicating that the protein is present, at least, for that period of time (Fig. 4.12, blue arrowhead).

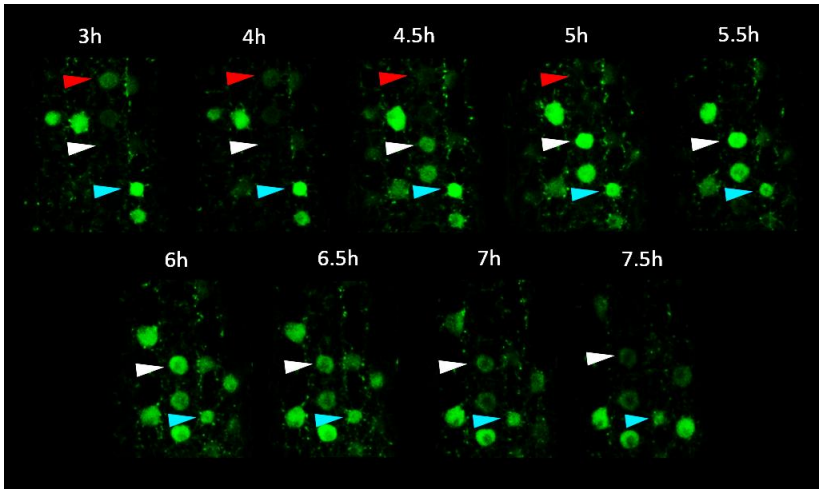

Figure 4.12. Live imaging visualizing HTR6 dynamics in a root treated with ABA (50 µM) during 3 to 7.5h. White arrowhead point to cell that *de novo* incorporating HTR6. Red arrowhead marks *HTR6* degradation; and blue arrowhead indicates nuclei that maintain *HTR6* level during the course of the experiment.

4.3.2 Degradation of *HTR6* is dependent of the proteasome complex

A lot of intracellular proteins are degraded by the ubiquitin-proteasome system (Etlinger and Golberg, 1977). In order to assess if it is also the case for HTR6, we treated HTR6-GFP expressing plants with different proteasome inhibitors, such as bortezomib, MG132 and epoxomicin, that impair the catalytic site of 26S proteasome inhibiting its function. HTR6-GFP plants were treated with ABA with and without Bortezomib (Bonvini et al., 2007) or MG132 plus epoxomicin during 4h. In both cases we could detect an accumulation of HTR6 in presence of the inhibitors (Fig. 4.13 A) with more HTR6 per nuclei and more nuclei containing HTR6 (Fig. 4.13 B). Bortezomib treated plants accumulate more HTR6 than MG132 + Epoxomicin treated plants (80 nuclei/root with a mean fluorescence intensity of ~70.000 versus ~60 nuclei/root with a mean fluorescence intensity of ~60.000 respectively), significantly higher than plants treated only with ABA (~25 nuclei/root with a mean fluorescence intensity of ~30.000) (Fig. 4.13 B). These experiments showed that bortezomib seems to be a more efficient inhibitor than the combination MG132 + epoxomicin and unequivocally indicate that 26S proteasome target HTR6 for degradation.

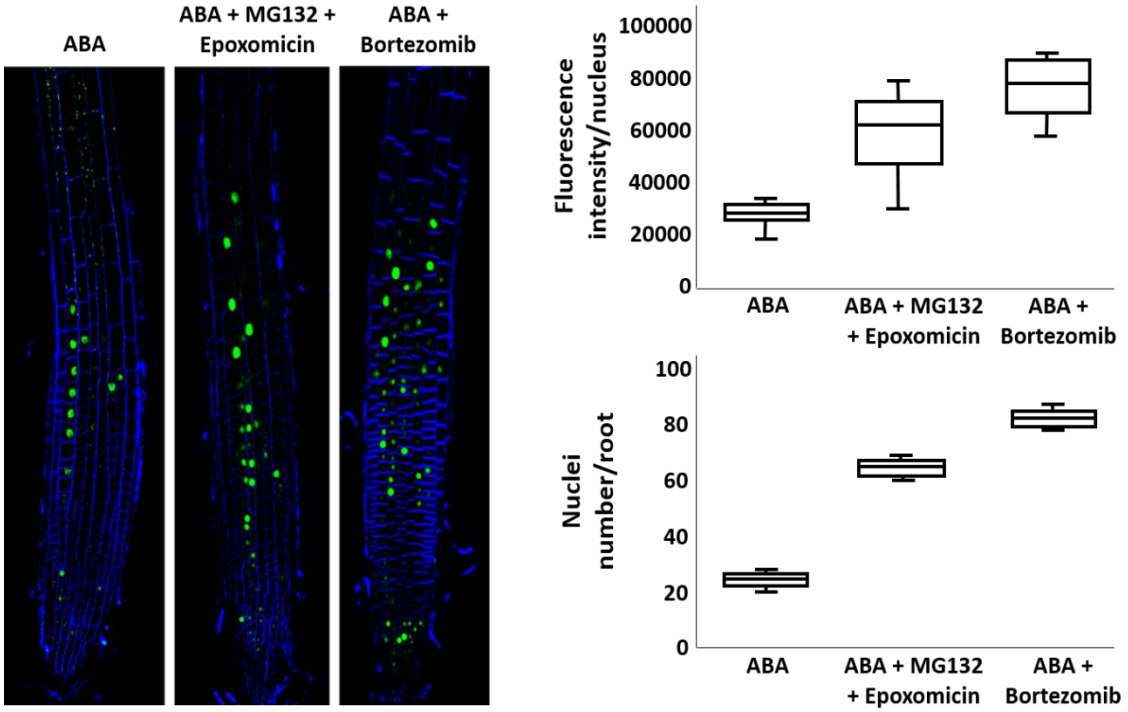

Figure 4.13. HTR6 expression pattern after treatment with ABA and proteasome inhibitors for 4h. A) *HTR6* expression pattern along the root in the different treatments. B) Number of nuclei/root and fluorescence intensity of the nuclei expressing *HTR6*-GFP. n=15

4.3.3 HTR6 dynamics during endocycle

HTR6 is not constitutively present in all cells of the root and even inside the region of expression there is only a subset of cells that contain the histone at any given time. This suggests that HTR6 could be cell cycle regulated, being present only in specific phases of cell cycle. Interestingly, we observed that the majority of cells containing HTR6 are located in the transition zone, where the

switch from mitotic cell cycle to endocycle occurs, generating polyploid cells (De Veylder *et al*., 2011; Edgar *et al*., 2014). Thus, we sought to identify which percentage of cells containing HTR6 were under S-phase. To address this question we labeled cells with a thymidine analog, EdU, which is incorporated into newly synthesized DNA and allows the detection of cells in S-phase. After treating the seedlings with ABA for 4 hours we labeled them with EdU during the last 30 minutes (Fig.4.14 A) and checked the colocalization of HTR6 and EdU by immunofluorescence and Click chemistry respectively.

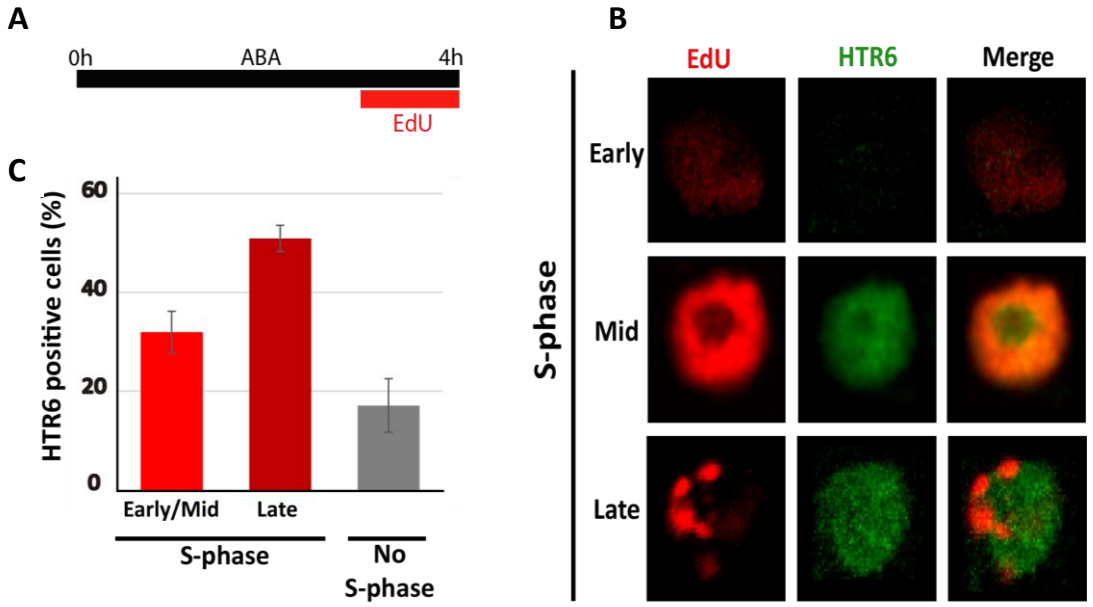

Figure 4.14 HTR6 expression during S-phase in endocycling cells. A) Schematic representation of the experimental design. Roots were treated with 50 µM of ABA during 4h and labeled with EdU during the last 30 min. B) Distribution of cells expressing HTR6 depending on their EdU pattern. Cells in S-phase were divided in early/mid and late depending of the pattern observed in C. C) EdU and HTR6 patterns in the nuclei along the S-phase.

We found that ~ 82 % of cells that contained HTR6 were in S-phase (Fig.4.14 B). According to the EdU pattern, the S-phase can be divided in 3 stages: early, mid and late (Masai *et al*., 2010; Dvořáčková *et al.*, 2018; Fig.4.14 B). Homogenous staining of EdU in the whole nuclei, with eventually lower signal in the nucleolus, marks cells in early and mid-S phases and corresponds to the period when euchromatin is duplicated. Whereas, during the late S phase, heterochromatin is duplicated, and the EdU pattern can be recognized as dotted structures observed in the periphery of the nucleus (chromocenters). HTR6 is present in very low amounts in nuclei that are in early S-phase while amounts of HTR6 in mid S-phase nuclei are high. Our results indicated that most of cells expressing HTR6 are in late S phase (50 %), but strikingly, HTR6 is intensively present in euchromatin and absent in chromocenters (Fig.4.14 B and C). Altogether, these results suggest that maximum levels of HTR6 are reached during the mid-S phase and are maintained in chromatin beyond the S-phase.

Nonetheless, we wondered how the mitotic cell cycle and the endocycle are affected in roots exposed to ABA. To address this, we quantified cells in S-phase in the proliferation zone and in the transition zone in the presence and absence of 50 µM ABA. Total nuclei were visualized after DAPI staining. In general, roots treated with ABA presented a reduced number of replicating cells (Fig. 4.15). However, the highest difference was seen in the RAM, that contained 39% of replicating cells in absence of ABA, and only 21% in the presence of ABA. In the elongation region, roots exposed to ABA have also less endoreplicating cells (~28%), than untreated roots (~36%). These data suggest that ABA have an effect on cell cycle progression.

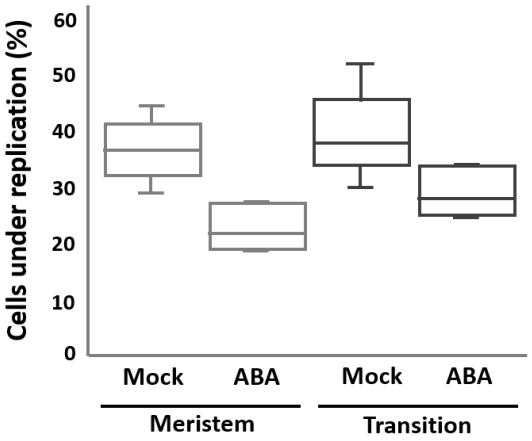

Figure 4.15 Average number of replicating cells in meristematic and transition regions after 4h ABA exposure and EdU labeling during the last 30 min. These regions were analyzed in presence and absence (Mock) of 50 µM ABA for 4 hours. n_{Mock}=10; n_{ABA}=8

To assess whether ABA treatment affects S-phase progression we used an experimental strategy that relies on the use a double pulse labeling with two thymidine analogs. First, we pulsed-labeled cells with EdU (30 min) and then with BrdU (30 min) leaving different chase times, while maintaining cells in the presence or absence of ABA for the entire experiment (Fig, 4.16 A). When no chase time was allowed in between the two pulses (Fig. 4.16 A, left) almost all nuclei are labelled with the two analogs (orange nuclei). Separating the two pulses allowed cells to progress during the endocycle and, at the time of the second pulse, some of them have left the S-phase and are no longer labeled (red nuclei) whereas others have entered the S-phase and are now labeled with the second analog (yellow nuclei; Fig. 4.16 A). Those still remaining in S-phase will incorporate the second analog (orange). Therefore, the rate of decrease in the amount of doubly-labeled nuclei (orange) is a proxy of S-phase progression.

In the absence of ABA, we observed a gradual decrease in the amount of doubly-labeled nuclei with increasing chase times (80%, 55%, 36% with 0, 1 and 2h chase, respectively; Fig. 4.16 C). A concomitant increase in cells entering S-phase (yellow nuclei; 7%, 18%, 32% with 0, 1 and 2h chase, respectively.) and in cells leaving S-phase (red nuclei, 11%, 27%, 32% with 0, 1 and 2h chase, respectively). Treatment with ABA produced a severe effect on S-phase progression since we observed both a decreased reduction rate in the amount of doubly-labeled nuclei (98%, 67%, 62% with 0, 1 and 2h chase) and a decreased amount of nuclei entering (1%, 14%, 18%) or leaving

S-phase (1 %, 19%, 20%). These results indicate that in the presence of ABA cells suffered a significant delay in S-phase progression.

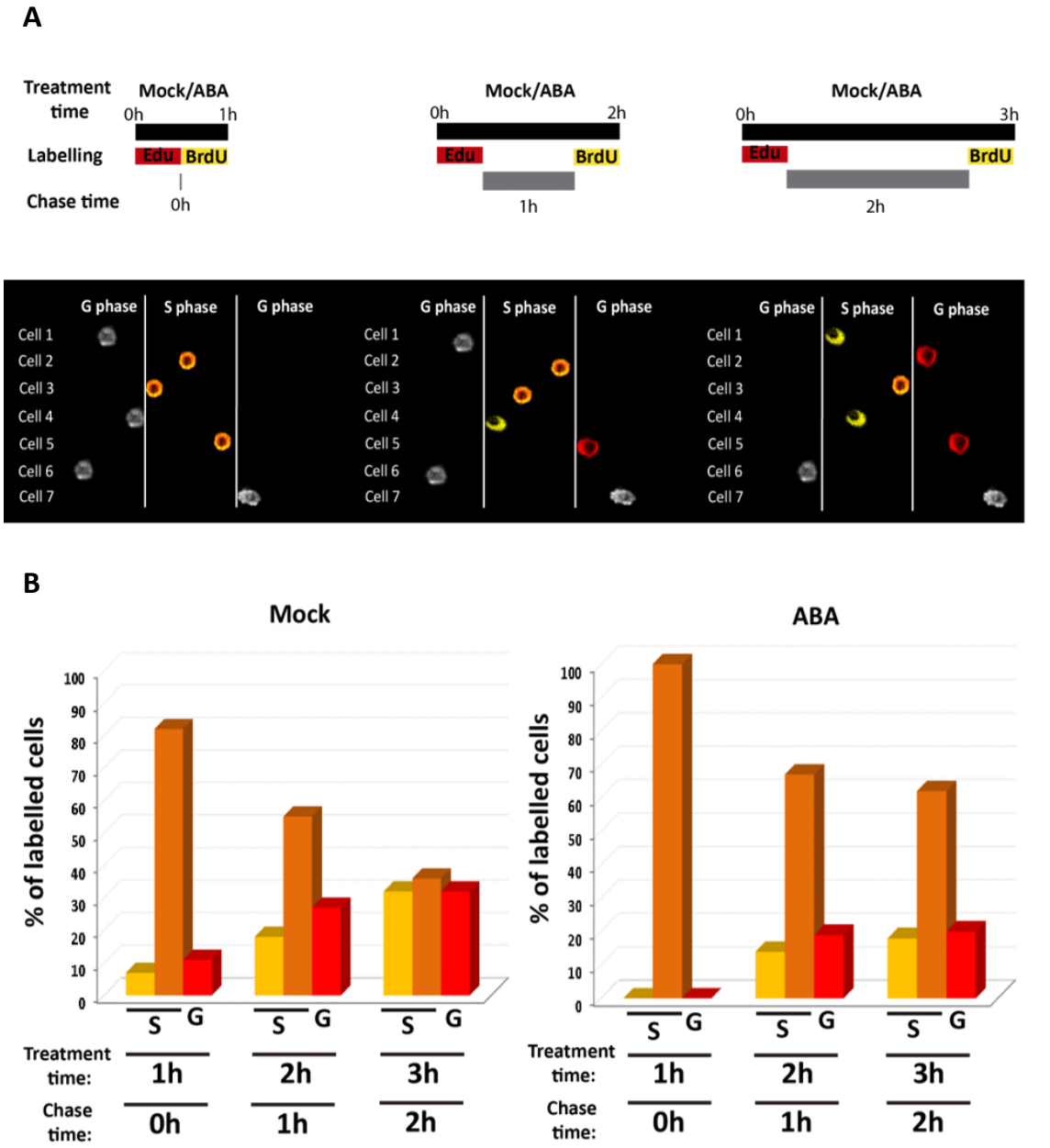

Figure 4.16 S-phase progression of root cells treated with and without ABA. A) Schematic representation of the experimental design. Roots were treated with ABA (50 µM) for distinct periods of time (from 1h to 3h) and a double pulse of 30 min was carried out at the beginning of the experiment, with EdU (red) and at the end with BrdU (yellow). Thymidine was used in the different chase times between pulses (from 0h to 2h). Controls without ABA (mock) were also used. B) Percentage of labeled cells in each phase analyzed. Cells entering in S-phase are labeled with BrdU only (yellow), the cells that leave S-phase are marked with EdU only (red) and those still in S-phase are marked with both analogs (orange).

To further decipher the effect of ABA on cell cycle, we analyzed a reporter line expressing cell cycle phases markers that identify cells in G1 (CDT1a-CFP), in S-G2 (H3.1-mCherry) and in G2/M (CYCB1;1-YFP). Confocal live imaging of these roots showed that cells in the meristem and in the transition/elongation zone are differently affected by ABA. In the root meristem, during the 5h time course of ABA treatment the number of cells in G1 (blue cells) increases and concomitantly, the number of cell in S-G2 (red cells) decreases indicating that ABA arrest the cell cycle in G1 (Fig. 4.17). However, in the transition/elongation zone, we observed a progressive decrease of CDT1a-CFP labeling cells in G phase, while the histone H3.1, which is incorporated in S-phase, is maintained. These data are in agreement with our previous observations that S-phase is slowed down in endoreplicating cells and interestingly suggest that mitotic cycle and endocycle are differently regulated by ABA.

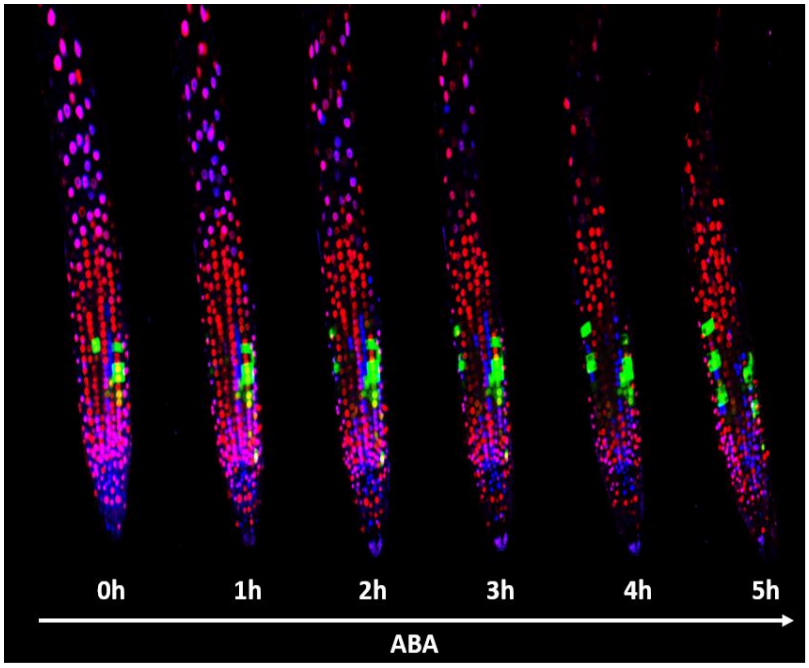

Figure 4.17 Time course of cell cycle progression in root treated with ABA. Root expressing cell cycle markers, treated with ABA (50 µM), were followed for 5h using live imaging. Images were taken every 20 minutes. CDT1a was used as a G1 marker (Blue); CYCB1;1 was used as a G2/M marker (green); H3.1 histone (red) is incorporated during S-phase and maintained during G2.

4.3.4 HTR6 is present in euchromatin and co-localizes with active transcriptional marks.

Eukaryotic cell nuclei possess a spatial-temporal organization that enables to discern the different chromatin territories of euchromatin and heterochromatin (Guo and Fang, 2014). As previously mentioned, euchromatin, which is the "open" chromatin that is associated with transcription, is localized in the central domain of the nucleus, while the constitutive heterochromatin (Heitz, 1928), the condensed chromatin associated with silent regions, is mainly organized in chromocenters in the periphery of the nucleus (Sun *et al.*, 2001; Fransz *et al.*, 2003).

As described earlier, immunolocalization of HTR6 in EdU labeled roots unveiled that HTR6 seems to be present in euchromatin and absent in heterochromatin (Fig.4.14B). To further characterize HTR6 distribution inside the nucleus, we performed an immunohistochemical assay

to detect post-translational modifications of H3 associated with different chromatin regions. H3K9me2 and H3K27me1 are marks of constitutive heterochromatin, enriched in repetitive regions and TEs. Fig. 4.18 shows that HTR6 does not colocalize with neither of these marks. Although, some abiotic stresses, like heat, led to chromocenter disruption (Pecinka *et al.*, 2010), our analysis led us to infer that ABA treatment does not disturb chromocenter organization.

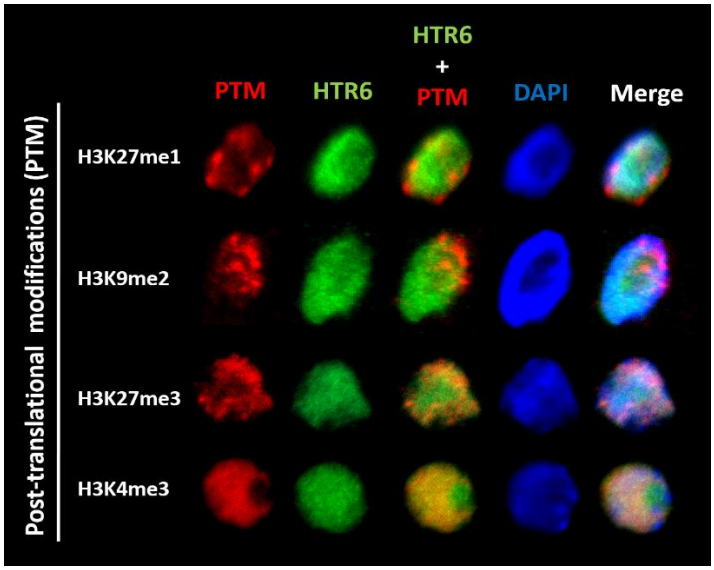

Figure 4.18 Immunohistochemical assay of nuclei to visualize HTR6-GFP and chromatin marks of histone H3. Roots were treated with ABA (50 µM) during 4 hours.

The H3K27me3 mark is a repressive mark present in facultative heterochromatin, enriched in silenced Polycomb-regulated genes mainly related with development programs. The patterns of HTR6 and H3K27me3 do not completely overlap (Fig. 4.18). The H3K4me3 mark, which is present in actively transcribed regions, colocalizes with HTR6 suggesting that this histone could have a role in transcriptional activation. In all the scenarios analyzed, HTR6 binding regions overlap with regions stained by DAPI which indicates that HTR6 is bound with chromatin in nuclei.

4.3.5 HTR6 has a higher turnover than H3.1 and H3.3.

After incorporation of H3.1 into chromatin in a replication dependent manner during the S-phase of the cell cycle, it is maintained and therefore enriched in heterochromatin regions. On the contrary, H3.3 is constitutively expressed deposited in a cell cycle-independent manner and is associated with actively transcribed regions (Stroud *et al.*, 2012; Otero *et al.*, 2016). Our previous results indicate that HTR6 has similarities with both H3.3 and H3.1, as it is mainly present in S-phase, such as H3.1, and is associated with histone marks typical of actively transcribed regions, such as H3.3.

To further compare HTR6 with H3.1 and H3.3, we crossed p*HTR6*::HTR6-GFP with pHTR5::HTR5-mRFP (H3.3) and pHTR13::HTR13-mRFP (H3.1). We observed that the presence of HTR6 in the nucleus does not modify the patterns of both H3.1 and H3.3, coexisting

in the same nucleus (Fig. 4.19). As expected, there is a higher overlap in the distribution patterns of the HTR6 and H3.3.

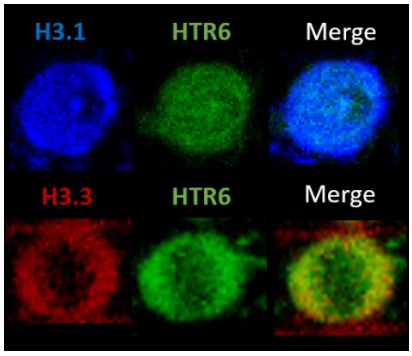

Figure 4.19 Immunolocalization of HTR6 (green), H3.1 (blue) and H3.3 (red) in cells from the transition zone of the root.

To further characterize the features of HTR6 compared to H3.3 and H3.1 we measured the dynamics of those proteins by using Fluorescence recovery after photobleaching (FRAP) assays.

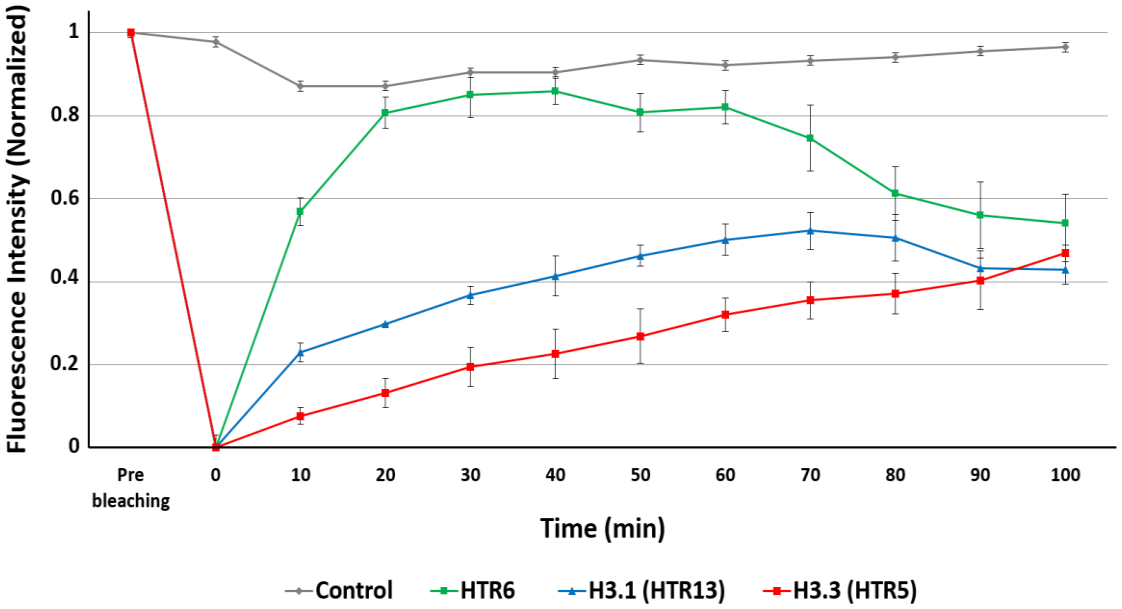

Figure 4.20 Quantitative analysis of FRAP assay measuring the turnover of histones HTR6-GFP, H3.1-GFP and H3.3-GFP during ABA treatment (50µM). Measured nuclei were located in the transition/elongation region of root. Unbleached HTR6-GFP are represented in grey, HTR6-GFP in green, H3.1-GFP in blue and H3.3-GFP in red.

A typical FRAP experiment consists of three phases. First, the initial fluorescence intensity is measured, then, a high power laser pulse is used to photobleach the fluorophore in the region of interest (ROI) (in our case, the entire nucleus), and finally the recovery of the fluorescence in that region is measured in a time-course series of image captures, allowing the measurement of protein turnover (Rosa *et al*., 2018). Protein turnover is defined as the time required for newly synthesized non-bleached protein to exchange with the bleached proteins. If the protein is dynamic and newly synthesized, the fluorescence intensity increases until it reaches a plateau. The time needed to reach this plateau is considered the Tmax.

After bleaching, HTR6 (green) needs less time to reach the maximum recovery of fluorescence levels (Tmax= 20 min) when compared with H3.1 (blue) (Tmax= 70 min) or H3.3 (red) (H3.3 Tmax superior to 100 min) indicating that it has a fast recovery. Moreover, HTR6 was able to reach almost the initial fluorescence while H3.1 recovered only ~50% of its initial fluorescence (Fig. 4.20). This difference suggests that many bleached H3.1-GFP proteins are not being substituted by new H3.1-GFP, consistent with their exclusive deposition during replication. Furthermore, after reaching the plateau the levels of fluorescence of both HTR6 and H3.1 start to decrease highlighting the cell cycle dependent dynamics of these proteins. Although the plateau of H3.3 recovery is not reached within the time of the experiment, we observed a gradual exchange of newly synthesized proteins. To summarize, it seems that HTR6 has a much higher turnover than H3.1 and H3.3, representing a unique property of HTR6.

4.3.6 HTR6 dynamics is severely affected by transcriptional impairment

To define whether HTR6 turnover is dependent of DNA replication, transcription or both we carried out FRAP experiments in the presence of specific inhibitors. Aphidicolin is a drug that leads to replication fork stalling and DRB inhibits the passage of RNA polymerase II from the initiation to the elongation transcription state. We first confirmed the drug efficiency inhibiting DNA replication and transcription by EdU and EU labeling, respectively and showed that the incorporation of both analogs is completely abolished (Fig. 4.21).

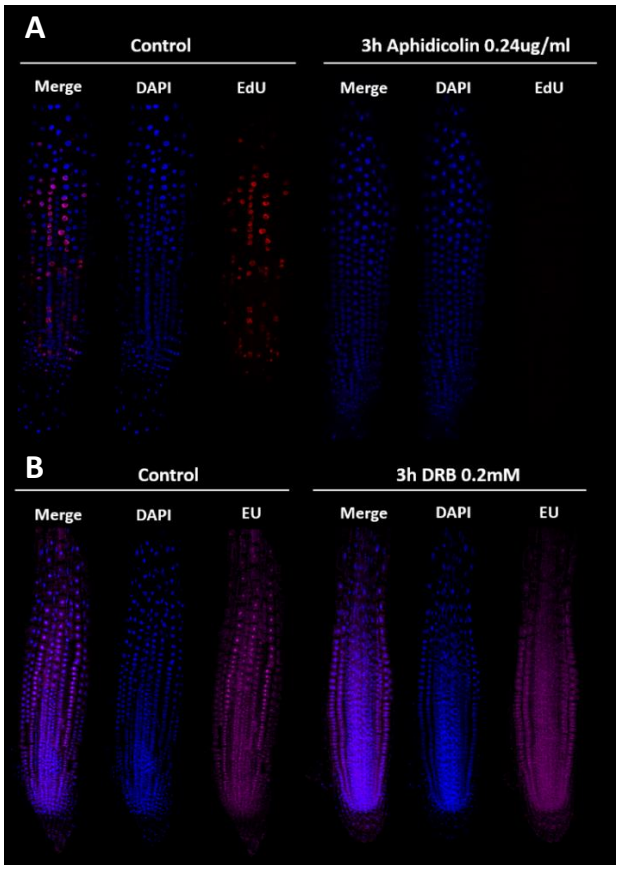

Figure 4.21 Effect of aphidicolin and DRB on replication and transcription in wt roots. A) Col-0 plants were incubated during 3h with and without 0.24µg/mL of aphidicolin and pulse labeled with EdU the last 15 min. B) Col-0 plants were incubated during 3h with and without 0.2mM of DRB during 3h and labeled with EU the last 2h.

We then measured by FRAP the dynamics of HTR6 in presence of these two drugs. As seen in Fig. 4.22, both treatments affected the recovery of HTR6-GFP fluorescence, indicating that the inhibition of either processes, replication and transcription, contributed to a reduction in HTR6 dynamics. However, transcription inhibition abolished almost completely HTR6 turnover. This strongly reinforces the idea that HTR6 deposition could be related with the transcription process, a feature also shared with H3.3.

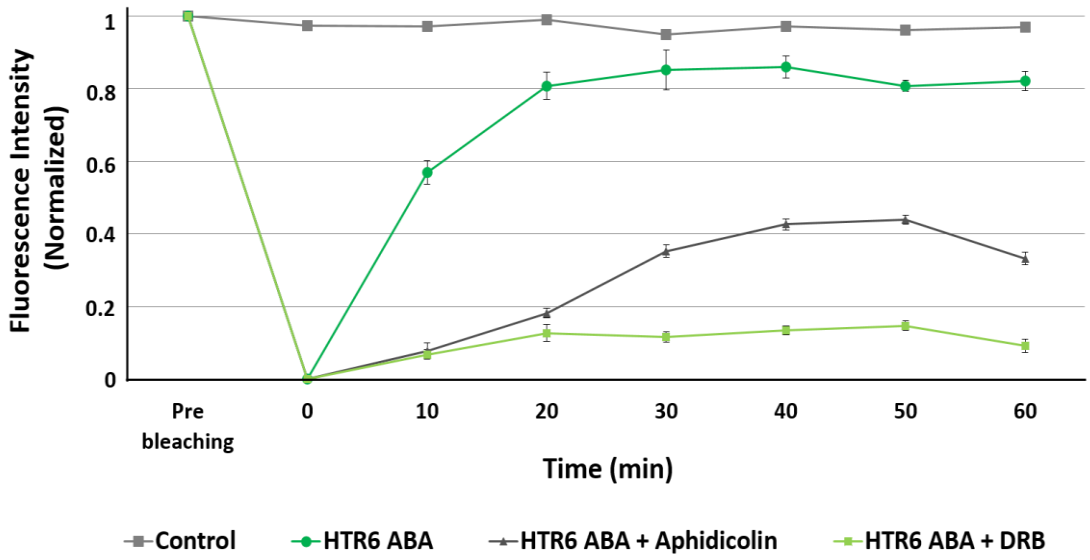

Figure 4.22 Quantitative analysis of FRAP assay to determine the dependence of HTR6 dynamics on DNA replication and transcription. Plants were treated with ABA (50µM) for 3 hours and aphidicolin (0.24µg/mL) or DRB (0.2 mM) were added during the last 2 hours and imaged were acquired with a confocal microscope every 10 min. Unbleached HTR6-GFP are represented in grey; HTR6-GFP treated with ABA is shown in dark green; ABA+Aphi is shown in dark grey; ABA +DRB is shown in light green

4.3.7 HTR6 deposition is independent of CAF-1

CAF-1 complex, which is composed of the FAS1, FAS2 and MSI1 subunits, is responsible for H3.1 incorporation in a DNA replication-dependent manner (Smith and Stillman, 1989; Kaya et al., 2001; Hennig et al., 2003; Polo and Almouzni, 2006; Reyes, 2006). A null mutant of the FAS2 subunit, *fas2-4*, is viable but displays a phenotype with short roots, due to a reduction in the elongation zone and severely disturbed cellular and functional organization of the root meristem (Leyser, 1992; Kaya *et al*., 2001; Exner *et al*., 2006; Ramirez-Parra and Gutierrez, 2007). The levels of heterochromatin are also reduced in this mutant and there is an increased amount of H3.3 at these domains to compensate for H3.1 decrease (Schonrock *et al*., 2006; Otero *et al*., 2016).

FRAP experiments indicated that HTR6 has a higher turnover than H3.1, although, both are predominantly present at S-phase in endocycling cells. Furthermore, despite the fact that transcription inhibition had a strong effect on *HTR6* dynamics, replication impairment also causes a decrease of HTR6 turnover. To determine whether HTR6 deposition depends on CAF-1 we

expressed pHTR6::HTR6-GFP in a *fas2-4* mutant background. We observed that the presence of HTR6-GFP at the transition zone of *fas2-4* roots treated with ABA, demonstrating that HTR6 incorporation occurs in a CAF-1 independent-manner (Fig 4.23).

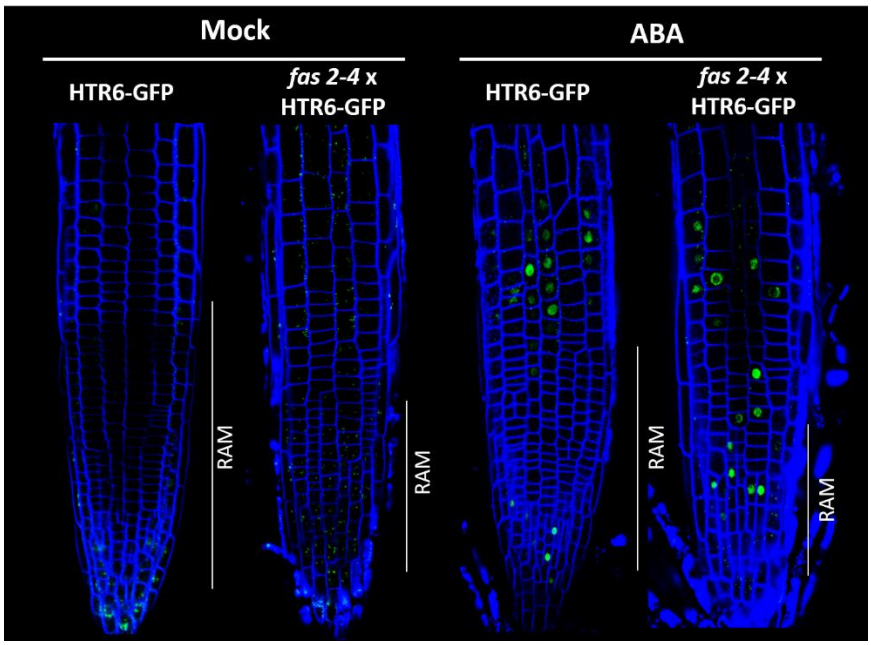

Figure 4.23 Expression patterns of HTR6 in *fas2-4* mutant background with and without ABA treatment (50 µM, 4h).

But contrary to what occurs with H3.3 that is incorporated in heterochromatin in *fas* mutants, we could not detect a change in HTR6 distribution to compensate for the H3.1 decrease.

4.3.8 HTR6 incorporation is dependent of HIRA and DEK

HIRA, together with Ubinuclein (UBN) and Calcineurin Binding protein 1 (CABIN1), are elements of HIR complex that deposits H3.3 into chromatin throughout the whole cell cycle (Ray-Gallet *et al*., 2002; Tagami *et al*., 2004; Duc *et al.*, 2015). Depletion of HIRA, leads to a genome-wide reduction of H3.3 (Goldberg *et al*., 2010; Pchelintsev *et al*., 2013) and pleiotropic phenotypic defects. According to our previous findings, HTR6 deposition was affected when transcription process was impaired. Since HIRA deposits H3.3 in a transcription dependent manner (Nie *et al.*, 2014), we sought to determine whether HIRA is incorporating HTR6.

We crossed p*HTR6*::*HTR6*:GFP plants with the *hira-1* knockout mutant and analyzed the incorporation of HTR6 after ABA treatment. We observed a high reduction of HTR6-GFP signal in the transition/elongation region of the root but HTR6-GFP could still be detected in the root cap (Fig. 4.24). These results indicated that incorporation of HTR6 is HIRA dependent in the transition zone of the root but that another chaperone is responsible for the loading in the root cap cells.

In Arabidopsis, histone H3 can also be incorporated by other chaperones. DEK3 is an example of protein with chaperone activity that was shown to interact with histones H3 and H4 (Waidmann

et al., 2014). Moreover, DEK3 was also identified as a regulator of stress tolerance. Null mutants of DEK3, *dek3-2* are also viable but presented defects on nucleosome occupancy and chromatin accessibility (Waidmann *et al*., 2014).

To determine if DEK3 interferes with HTR6 deposition we expressed HTR6-GFP in a *dek3-2* mutant background. Similarly, to what we observed in the *hira-1* mutant, after 4h of ABA treatment, the lack of DEK3 also led to a reduction of HTR6-GFP deposition in the transition/elongation zone and in the root cap, indicating that DEK might be responsible for HTR6 incorporation in both types of cells (Fig. 4.24).

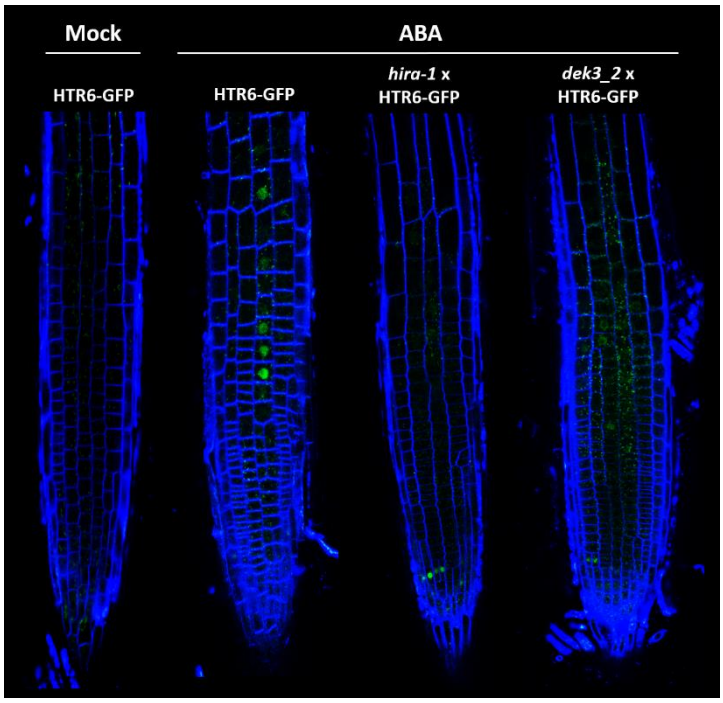

Figure 4.24 Expression pattern of HTR6 in *hira-1* and *dek3-2* mutant background during ABA treatment (50 µM, 4h).

Although both HIRA and DEK are important for HTR6-GFP deposition, there is no compensation between them, suggesting that these chaperones work in the same pathway.

4.3.9 Specific residues have a higher impact in HTR6 dynamics

As mentioned before, H3.1 and H3.3 differ in only four amino acids and that is sufficient to completely alter protein dynamics and function. HTR6 possesses several other residues that are unique to this variant and differ from H3.1 and H3.3 (Fig.4.1 A). To determine the contribution of each of these amino acids to HTR6 function and dynamics, we changed them to the corresponding amino acid in H3.3 (Fig. 4.25 A). The resulting point mutants were expressed in plants under the control of the native HTR6 promoter. In general, we observed that alteration of residues in the C-terminal tail and in the core structure of the protein have a higher effect on HTR6 dynamics than those in the beginning of N-terminal tail (Fig. 4.25 B).

Many residues of the N-terminal tail of H3 are prone to suffer post translational modifications (Jenuwein and Allis, 2001; Turner, 2002, Ho and Crabtree, 2010). HTR6 harbors a serine at

position 6, which was substituted by a threonine. HTR6S6T-GFP protein distribution along the root did not severely change, although, some nuclei seem to contain higher amounts of HTR6. On the contrary, substitution of histidine at position 11 by a threonine decreases the number of cells containing HTR6H11T-GFP.

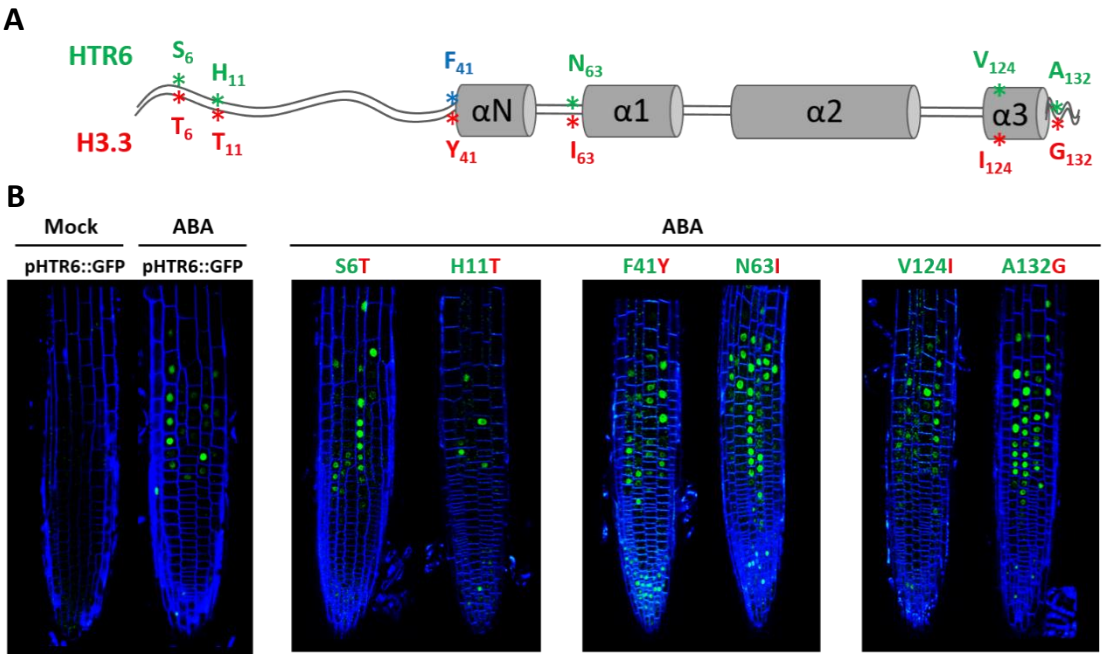

Figure 4.25 Effect of point mutations in HTR6-GFP expression. A) Schematic representation of the modified residues along HTR6 to the correspondent in H3.3. B) Expression of HTR6-GFP containing S6T, H11T, F41Y, N63I, V124I and A132G point mutations after the plants were exposed to 50 µM of ABA for a period of 4h. Roots expressing HTR6-GFP without point mutations were used as control.

Residue F41 is unique and conserved among plants (Lu *et al*., 2018). The phenylalanine at position 41 is an amino acid that HTR6 shares only with H3.1. Recently, a study claimed that the F41Y substitution in H3.1 leads to accumulation of this protein at actively transcribed regions. HTR6 is already present in active transcribed regions, although, the F41Y change in HTR6 led to an increase of the number of nuclei containing HTR6F41Y-GFP in the transition/elongation region, as well as in the root cap. This gave us support to speculate that F41 could be important for HTR6 replacement by other histone or that tyrosine at this position could be phosphorylated as in animals, (Moraes and Casas-Mollano, 2014) generating a more stable protein. Changing the asparagine by an isoleucine at position 63 resulted also in a higher accumulation of HTR6N63I-GFP at the transition/elongation region. Nonetheless, the protein could still be degraded in the upper part of the root elongation zone.

The A132G substitution results in protein accumulation along the root with a pattern similar to roots treated with proteasome inhibitors (Fig. 4.13), suggesting that G132 is important for HTR6

degradation by proteasome. A similar effect was observed for V124I change, although the accumulation level was reduced compared with the HTR6A132G-GFP.

4.4 Function of HTR6

4.4.1 DNA replicative stress response is independent of HTR6

Replication stress caused by DNA damage or fork stalling triggers mechanisms to detect and repair DNA lesions (Dona and Scheid, 2015). Some histones are involved in DNA damage detection, as is the case of γH2AX that is recruited to signal the DNA damage response (Charbonnel *et al*., 2011). Both histones H3.1 and H3.3 along with their respective chaperones, CAF-1 and HIRA, have a role in the DNA repair process (Dona and Scheid, 2015). ABA treatment of young seedlings causes genomic instability that leads to DNA damage response activation (Roy and Das, 2017). Since the induction of HTR6 is ABA dependent and the protein is mainly present in S-phase nuclei, we hypothesized that HTR6 could have a role in DNA damage detection or DNA lesion repair. To test this hypothesis, we treated HTR6-GFP plants with two drugs, aphidicolin and hydroxyurea (HU), for 4 hours, without adding ABA, to induce DNA replication stress. As mentioned before, aphidicolin blocks DNA polymerase α leading to fork stalling and hydroxyurea has a slowdown effect on the forks by diminishing the dNTP pool. DNA replication stress provoked by HU and aphidicolin does not induce HTR6 expression (Fig. 4.26) suggesting that HTR6 function is not related with DNA damage response.

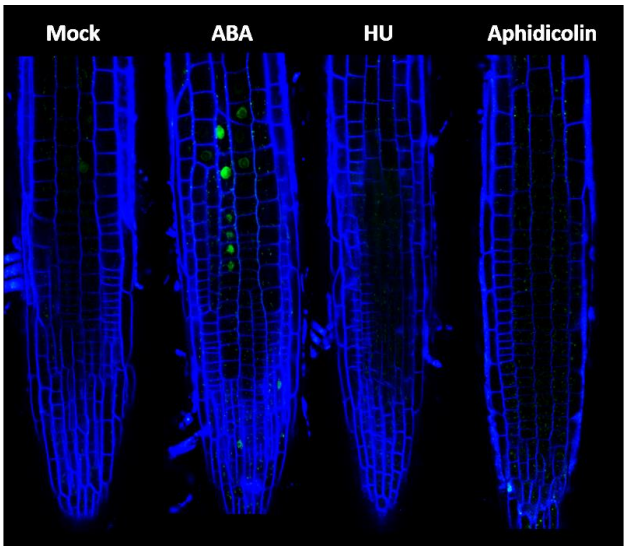

Figure 4.26 Expression of HTR6-GFP in the roots under DNA replication stress conditions. Roots were exposed to 4 hours of treatment with HU (1mM) and aphidicolin (0.24 µg/mL). Plants treated with ABA or with methanol (Mock) were used as positive and negative controls, respectively.

4.4.2 Identification of *htr6* mutant lines

We identified, in the stock collections, a T-DNA line, generated by SAIL (SAIL_698_F01c), with the purpose of obtaining an *htr6* mutant. We first characterized the line by sequencing the *HTR6* genomic region and found that the SAIL_698_F01c line contains an insertion in the third exon of *HTR6* (Fig. 4.27 A).

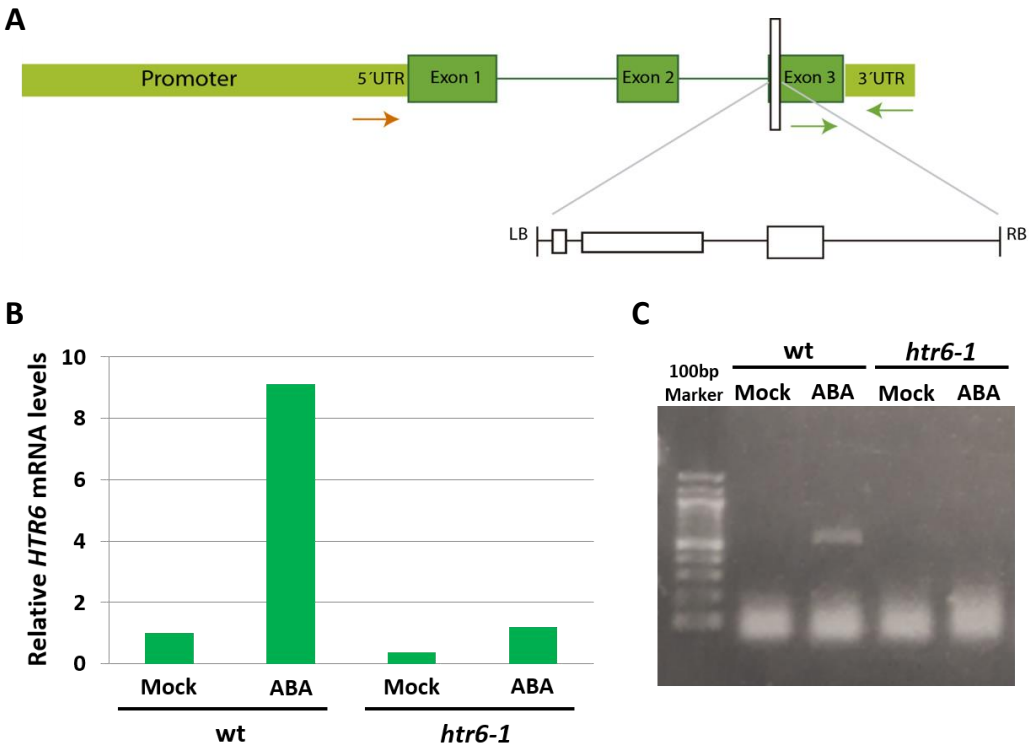

Figure 4.27 Characterization of *HTR6* T-DNA insertion line, SAIL_698_F01c. A) Schematic representation of the *HTR6* gene with the T-DNA insertion in the third exon (*htr6-1*). Primers used in panels B and C are also represented. B) qPCR measurement of *HTR6* mRNA levels in wt and *htr6-1* mutant (SAIL *HTR6*_698_F01c), with and without ABA (50 µM) treatment for 4 hours. The primers pair used in this approach is represented in A (green). C) Semiquantitative analysis of *HTR6* mRNA levels. Orange primer represented in A was used as forward primer and green primer annealing at 3´UTR was used as reverse primer.

Then, *HTR6* mRNA levels were measured in roots of wt and SAIL_698_F01c line (*htr6-1*), with and without ABA treatment. Less transcripts of *HTR6* gene were obtained in SAIL_698_F01c (*htr6-1*) line when compared with wt in both mock and ABA conditions (Fig. 4.27 B). To assess if T-DNA insertion in the third exon impairs the formation of a full *HTR6* transcript, we performed a PCR analysis of mRNA using primers located at the beginning and at the end of the gene. Under ABA treatment, *HTR6* transcripts were only detected in the wt plants, indicating that the SAIL_698_F01c T-DNA line is a *htr6* knockout mutant (Fig. 4.27 C), that we named *htr6-1*.

4.4.3 *htr6-1* mutant roots present higher growth rates than wild type in abiotic stress conditions

The lack of a protein function has, very often, a consequence in plant phenotype. We began by comparing the root length of *htr6-1*and wt plants after various treatments. No differences in root growth were observed between wt and *htr6-1* plants grown in MSS and or mock treated (Fig. 4.28). But *htr6-1* mutant grew more than wt in the presence of ABA (50 µM) and NaCl (140mM), with higher differences noticed in the case of ABA exposure (Fig.4.28). In the presence of ABA, the differences between the mutants and wt can be detected at day 2 of growth, with *htr6-1* roots

growing approximately 1.7x times more than wt. Similar results were observed in the presence of NaCl (Fig.4.28).

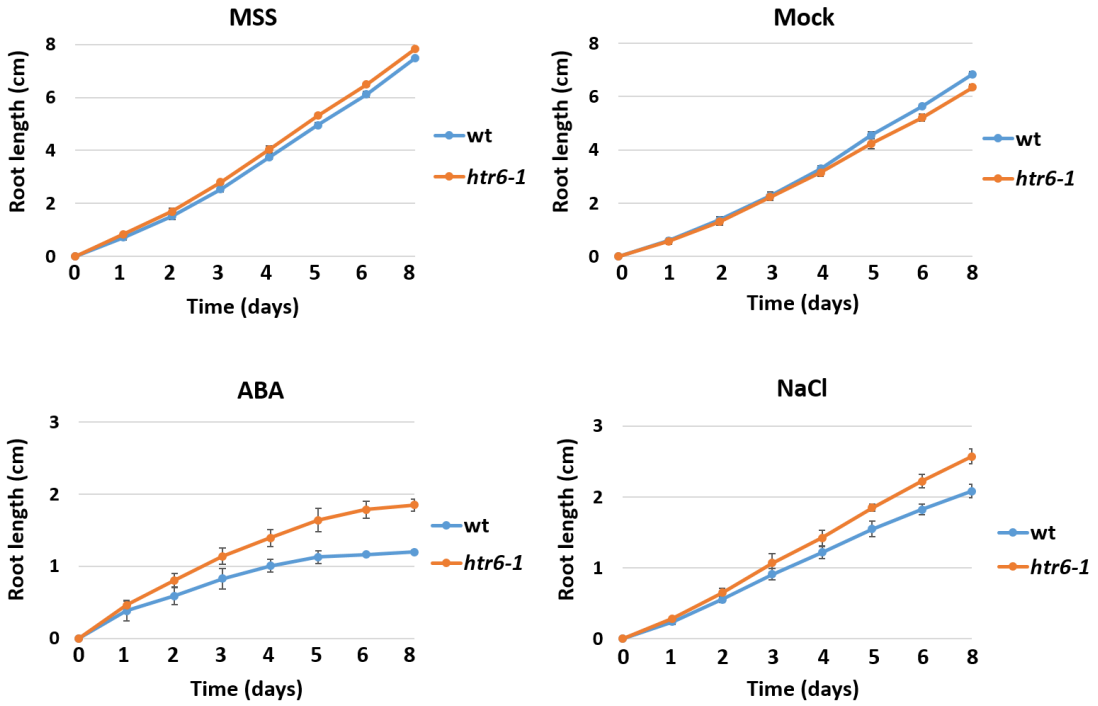

Figure 4.28 Role of HTR6 in root growth under abiotic stress. 4 day-old plants were transferred to control plates containing MSS or MSS + methanol (Mock), MSS + ABA (50 µM) and MSS + NaCl (140 mM). Measurements were performed every day at the same time during 7 days. n_{wt}=8, $n_{HTR6\text{-}1}$=8

Altogether, these results suggest that HTR6 is a root growth repressor when ABA signaling pathway is triggered under abiotic stress conditions.

The total root length depends on the combination of cell proliferation and elongation (Ivanov, 1997). First, we wondered if root length differences observed between wt and *htr6-1* were due to distinct proliferation rates or different cell growth rates in the meristem. To assess this, we measured the length and number of cortical cells from the QC up to the elongation zone in 7 days-old roots after ABA treatment for 3 days.

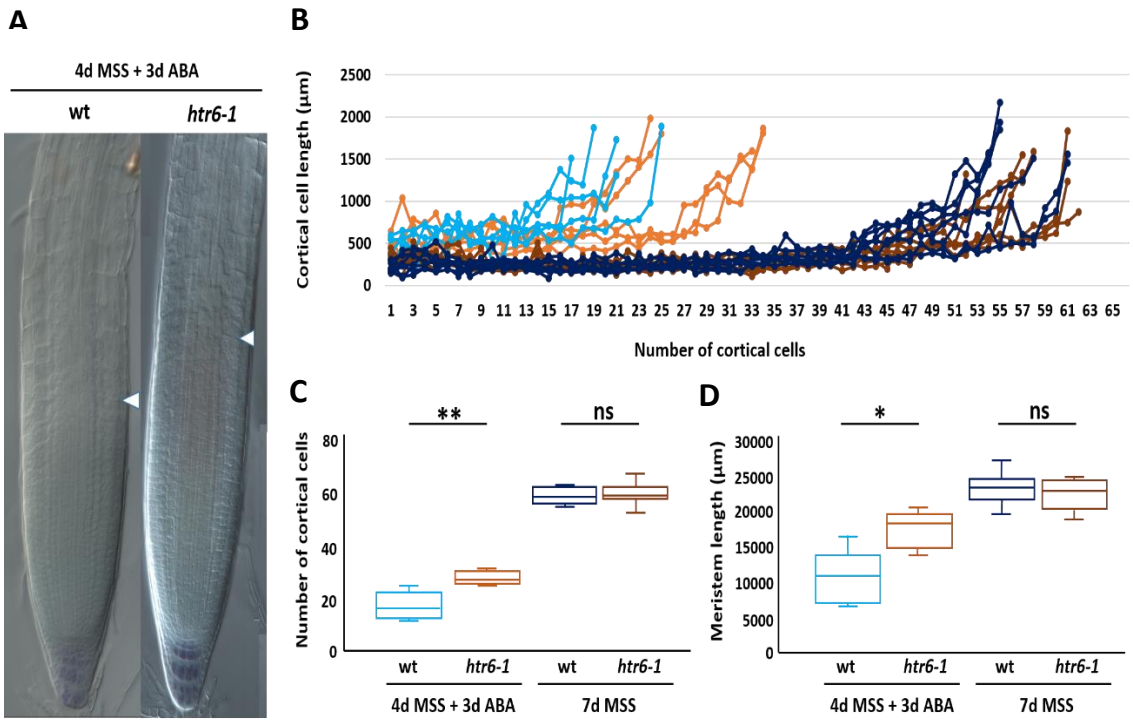

Figure 4.29 Size of the root meristem of 7 days old *htr6-1* mutant and wt (Col-0) with or without ABA (50 µM) treatment for 3 days. A) RAM nomarsky brightfield images of wt and *htr6-1* plants after ABA exposure. B) Cortical cells length from the QC to the transition zone of wt and htr6-1 treated or not with ABA. C) Number of cortical cells from the QC to the transition zone in wt and *htr6-1* roots. D) Meristem length from the QC to the transition zone in wt and *htr6-1* roots. One way ANOVA, Welch´s t test was used as statistic test. $n_{wt\ ABA}$=5, n_{wt} =7, $n_{htr6\text{-}1\ ABA}$=5; $n_{htr6\text{-}1}$=7;* and ** corresponds to a p-value of $p<0.05$.

We found that the RAM of the *htr6-1* mutant contained a significant higher number of cortical cells than wt after ABA treatment (Fig. 4.29 B and C). As a consequence, the total meristem size of *htr6-1* mutants was also longer (Fig. 4.29 A and D). On the contrary, no significant differences in RAM size were observed between *htr6-1* mutants and wt grown for 7 days in absence of ABA (Fig 4.29 B-D). Altogether, these results suggest that HTR6, under stress conditions, plays a role in controlling the RAM size and the exit from the proliferation domain to the transition zone.

Taking into account that HTR6 is mainly expressed in cells undergoing endoreplication, we sought to investigate if the DNA content along the root was altered in the *htr6-1* mutant. No significant differences were found in the ploidy profile comparing wt with *htr6-1* (Fig. 4.30). After 3 days of ABA treatment, we noticed a slight increase in 8C cells in both wt and *htr6-1*, indicating that ABA exposure independently of HTR6 increases slightly endoreplication in the root.

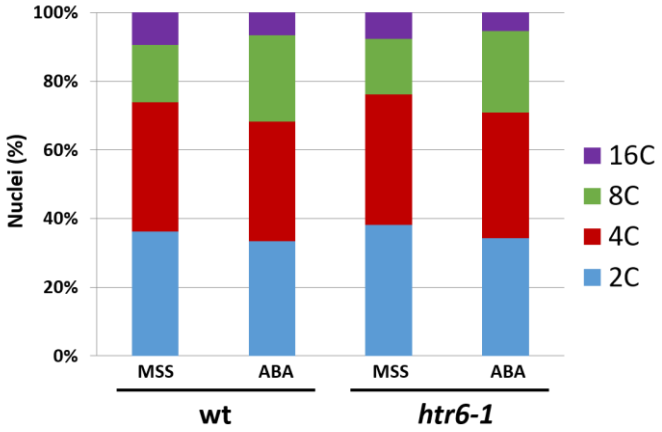

Figure 4.30 Ploidy profile of cells of wt and *htr6-1* roots grown in MSS or treated with ABA (50 µM) for 3 days. Ploidy is indicated in the image by the different color codes (2C in blue, 4C in red, 8C in green and 16C in purple).

4.4.4 Abnormal transcriptomic response of *htr6-1* in ABA

We showed that loss of HTR6 causes differential growth rates when roots were treated with ABA. Phenotypic differences are usually associated with transcriptomic changes. In order to identify HTR6 ABA dependent responsive genes, we performed an RNA Seq. To determine which genes are affected by the loss of HTR6, we carried out a RNA-sequencing analysis of wt and *htr6-1* roots that were treated with ABA for 10 min. At this stage, we have focused on short time treatment with the aim of identifying primary targets of HTR6.

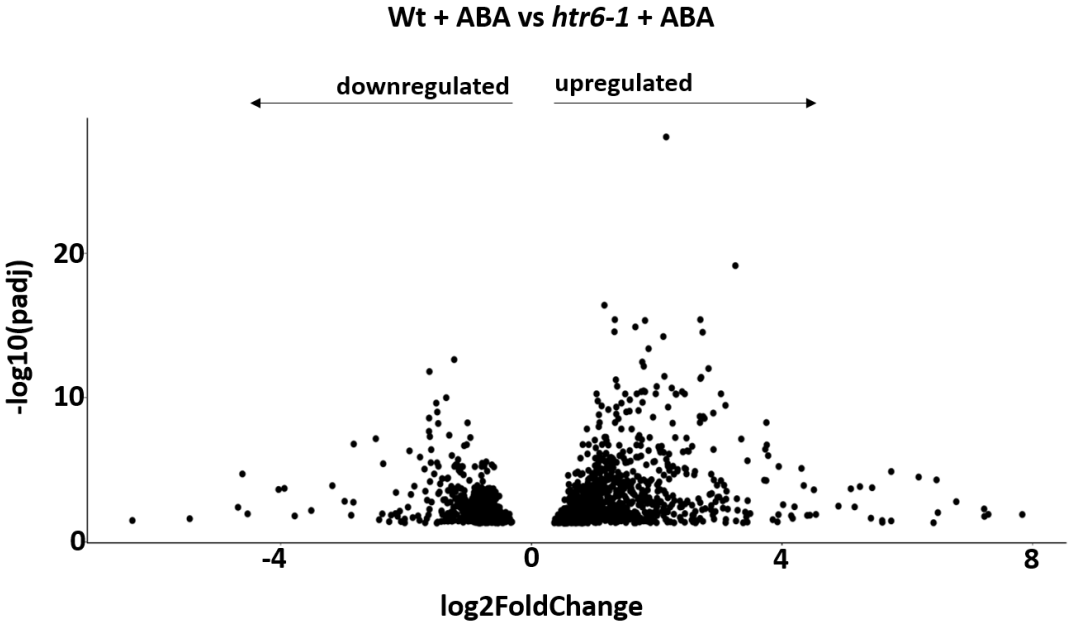

Figure 4.31 Volcano plot showing genes that are significantly up-regulated or down-regulated when comparing wt and *htr6-1* mutant, after 10 min of treatment with ABA.

Our preliminary analysis indicated that 1327 genes are misregulated in the *htr6-1* mutant after 10 min of ABA treatment (q-value<0.05). We perform a Volcano plot to better identify and separate

genes that were up (916 genes) and down-regulated (411 genes) based on fold-change levels (Fig. 4.31). Among the top upregulated genes, we found that HTR6 is important to stimulate genes encoding proteins related with lipids and fatty acids metabolism and the TCP family. On the contrary, the presence of HTR6 decrease the expression of genes encoding proteins involved in late embryogenesis, such LEA genes, or related with abiotic stress response. Curiously, genes involved in cell wall organization are enriched in both up and downregulated groups of genes. Altogether, these results indicate that HTR6 expression promoted by ABA treatment cause transcriptome variations in a short period of time that could have consequences in cell metabolism, structure and environmental adaptation.

5. Discussion

5.1 HTR6 as a player in abiotic stress response

Both in plants and animals, histone variants and post-translational modifications add another level of complexity to nucleosome organization and function by influencing its stability and compaction differently from the canonical isoforms (Kamakaka and Biggins, 2005; Otero *et al.*, 2014; Kawashima *et al.*, 2015). This combinatorial complexity has an impact on chromatin status, impinging on vital processes like genome replication, gene expression, repair and recombination. Together, these events are essential for organism development, growth and adaptation to environmental stimuli, allowing the response to different stresses.

Among the four types of core histones that compose the nucleosome (H2A, H2B, H3 and H4), H2A and H3 are the families that comprise more variants (Talbert and Henikoff, 2010). In Arabidopsis, among the H3 family, the crucial role of histones H3.1 and H3.3 along the plant growth and development has been established. (Ingouff *et al.*, 2010; Stroud *et al.*, 2012; Otero *et al.*, 2016; Wollmann *et al.*, 2017). The difference in 4 amino acids residues between H3.1 and H3.3 causes high impact on their distribution, function and dynamics. Inside the histone H3 family, we have identified that histone variant, HTR6, shares characteristics with H3.1 and H3.3 regarding amino acidic sequence. Furthermore, modeling of HTR6 protein also indicated structural similarities with H3.1 and H3.3. Therefore, this raised further questions about regulatory and functional aspects of HTR6.

To gain further insights on this matter, we first evaluated the chromatin environment in the genomic region of HTR6. The *in silico* analysis of the *HTR6* locus indicated that this region is covered by chromatin state 5, 4 and 2. One common feature of these chromatin states is that they are enriched in H3K27me3, in particular state 5 and 4, that is a mark typically associated with transcriptional repressed genes (Sequeira-Mendes *et al.*, 2014). Nonetheless, state 2, present in the proximal promoter, is mainly enriched in active marks such as H3K4me3. This, together with the fact that the promoter region is devoid of chromatin state 1, corresponding to highly open chromatin, and devoid of higher repressive states such state 8 and 9, predicted well the inducible gene activation by external stimuli such as abiotic stress. The ChIP and RNA expression experiments we realized with roots of plants grown in normal conditions confirmed the validity of our *in silico* analysis showing that *HTR6* is mainly covered by the H3K27me3 mark and repressed in absence of stress. According to this, we observed that treating seedlings with ABA lead to a stimulation of *HTR6* transcription and ChIP results indicated that, under these conditions, the levels of repressive mark H3K27me3 diminished along the *HTR6* genomic region, while the levels of the transcriptional active mark H3K4me3 increased.

The trimethylation of H3K27 is catalyzed by the SET domain methyltransferase of the Polycomb Repressive Complex 2 (PRC2) and covers mainly the facultative heterochromatin, affecting 25%

of Arabidopsis genes (Gan *et al.*, 2015). It has been described that the PRC2 repression pathway is essential to control, among others, flowering time by silencing of flowering regulators such as FLOWERING LOCUS C (FLC) and FLOWERING LOCUS T (FT) (Jiang *et al.*, 2008). It is also important to mention that this particular histone modification is reversible because the H3K27me3 mark can be removed by a group of histone lysine demethylases (KDM) containing JUMONJI (JMJ) proteins (Gan *et al.*, 2015). Additionally, H3K27me3 could also serves as an epigenetic mark that promoted the binding of the LIKE HETEROCHROMATIN PROTEIN-1 (LHP1) that subsequently is implicated in the recruitment of other complex, PRC1, promoting chromatin compaction and transcription inhibition and blocking chromatin remodeling (King *et al.*, 2002; Francis *et al.*, 2004; Turck *et al.*, 2007; Zhang *et al.*, 2007). We believe that genes with roles in abiotic stress response should be maintained silenced to not interfere with the normal functionality and development of the organism. However, the repressed state of these genes should be reversible, allowing a switch to an active state when the organism is under stress. Our findings that *HTR6* is mostly covered with H3K27me3 strongly suggest that this gene could have a role in the response to external factors, being poised for activation only when required by the organism.

Interestingly, the MSI1 protein present in all PRC2 complexes is a crucial subunit of CAF-1 complex and interacts with the Retinoblastoma-related protein RBR1 (Ach *et al.*, 1997; Exner *et al.*, 2006; Jullien *et al.*, 2008). Pleiotropic phenotypes were observed in the *msi1-cs* knockdown mutant and a transcriptomic analysis revealed that a group of ABA-responsive genes are specifically up-regulated in the mutant and not in the plants where RBR pathway, CAF1 and PRC2 complexes are misregulated. (Alexandre *et al.*, 2009). Surprisingly, *HTR6* gene belongs to that group of genes that are up-regulated in *msi1-cs* mutant (Alexandre *et al.*, 2009), reinforcing the idea that HTR6 plays some role during the abiotic stress response. A different study claims that MSI1 acts with HISTONE DEACETYLASE 19 (HDA19) in the repression of the ABA receptors, PYL4, PYL5 and PYL6 (Mehdi *et al.*, 2016). Thus, it is also possible that in absence of stress *HTR6* is repressed by the action not only of MSI1 but also by HDA19 histone as the PYLs receptors.

It has been found that BLISTER (BLI) protein interacts with PRC2 and together they repress ABA-responsive target genes, emphasizing the relevance of the PRC2 complex in the repression of stress-responsive genes. Recent studies in Arabidopsis revealed that PRC2 can also be recruited by telomere-repeat-binding factors (TRBs). In a subset of genes, the interaction of TRBs with CLF/SWN subunits seems to be a mechanism essential for H3K27me3 deposition (Zhou *et al.*, 2018). Interestingly, *HTR6* is up-regulated in a TRB triple mutant, *trb1-2*, *trb2-1*, and *trb3-2* (*trb1/2/3*) but not in the BLI mutant, *bli* (Kleinmanns *et al.*, 2017). All together, these studies showed that mechanisms to repress ABA target genes are very diverse and may depend on PRC2

interacting partners Furthermore, it is possible that the activation of those genes could also be different depending on the type of stress.

In vegetative tissues, the CLF mutant, *clf29*, results in the up-regulation of hundreds of H3K27me3-associated genes (Liu *et al.*, 2016; Wang *et al.*, 2016; Bellegarde *et al.*, 2018). Evaluation of *HTR6* expression levels in the *clf29* mutant, in absence of stress, revealed only a small but not significant increase of the mRNA compared to the wild type. This indicates 1- that diminishing the level of H3K27me3 seem not be sufficient to activate the gene, however we haven´t measured if there was a change in H3K27me3 levels in *HTR6* genomic region in *clf29* mutant plants and/or 2- that the methyltransferase SWINGER (SWN), that also can be a member of PRC2 complex, can compensate for the absence of CLF.

ABA biosynthesis and signaling is triggered by a wide variety of abiotic stresses such as low temperature, high salinity, UV-radiation, nutrient deficiencies, and heavy metal toxicity (Cutler *et al.*, 2010; Hauser *et al.*, 2011; Iyer-Pascuzzi *et al.*, 2011; Zhu, 2016). ABA signaling encompasses many elements from the receptors involved in starting the signaling cascade to the transcription factors ultimately responsible for activating the target genes. Thus, ABA signaling culminates in the induction of several genes that are needed for plant survival during abiotic stress.

We observed that *HTR6* expression is increased in a wide range of ABA concentrations in a dose dependent manner. Absence of *HTR6* transcripts in the sextuple mutant of ABA receptors (*pyr1 pyl1 pyl2 pyl4 pyl5 pyl8*) suggests that induction of *HTR6* requires the activation of the ABA signaling pathway. SnRK2 family is composed by kinases that are essential for ABA signaling pathway (Cutler *et al.*, 2010; Umezawa *et al.*, 2010). Despite of the existence of 8 SnRK2s family members, disruption Snrk2.2 and Snrk2.3 causes a decrease of *HTR6* transcripts but we cannot discard that other Snrk2 also participate in the activation pathway (Boudsocq *et al.*, 2007). Altogether, these results demonstrate that *HTR6* expression is dependent on ABA signaling. Nevertheless, we were not able to identify the transcription factors responsible for *HTR6* expression but found that ABI4 and ABI5 were not involved. Given the high number of factors that are activated in an ABA-dependent pathway, it is possible that there is a redundant function between TFs. The ABRE TFs and AREB/ABF TFs, are considered the crucial players on ABA-dependent pathway in vegetative tissues (Finkelstein and Lynch, 2000; Umezawa *et al.*, 2011; Fernando and Schroeder, 2015; Singh and Laxmi, 2015), but we could not identify perfect ABRE neither AREB/ABF consensus motifs in the HTR6 promoter. However, other TFs belonging to MYC, MYB, NF-Y or WRKY are also activated (Abe *et al.*, 2003). Our *in silico* analysis of the *HTR6* promoter indicated that putative binding sites for these TFs are present in the promoter and could potentially regulate *HTR6* expression.

In osmotic stress, provoked by high salinity or drought, in addition to TFs induced by ABA signaling, there are some TFs that are induced in an ABA-independent pathway. AP2/ERF, DRE and DREB are some examples of those TFs with essential functions in plant osmotic stress tolerance (Chen *et al.*, 2010; Shang *et al.*, 2010; Yoshida *et al.*, 2014). The *HTR6* promoter also encompasses *cis*-elements that can be recognized by those TFs. Several studies reveal a crosstalk between elements of ABA-dependent and independent pathways, which emphasizes the complexity of abiotic stress response (Singh and Laxmi, 2015; Liu *et al.*, 2018). Recently, a transcriptome analysis comparing seedlings exposed to prolonged drought stress that activates both ABA dependent and independent pathways, and exogenous ABA treatment revealed that they were very similar (Liu *et al.*, 2018). Surprisingly, *HTR6* transcripts was not detected in neither ABA dependent or independent pathway response group. This might due to the use in this study of three-week-old plants whereas we have observed that *HTR6* induction occurs principally in a small region of the root apex. Furthermore, ABA application was carried out by spraying plants, which could differently influence ABA response in the root.

HTR6 was also expressed after seedlings were exposed to high salt concentrations. High concentrations of NaCl inhibits root growth, however, we detect that the lack of HTR6 attenuates the root growth inhibition provoked by the salt, indicating that HTR6 is a component involved in salt stress response. A spatio-temporal transcriptome analysis was performed on roots exposed to salt during 1h to 48h (Geng *et al.*, 2013). Our analysis of these data sets showed a peak of *HTR6* expression after 3 hours of salt exposure (Fig. 5.1), which is in full agreement with our results. Furthermore, in this study, it was shown that HTR6 expression was confined to cells in the epidermis and root cap. After 4 hours of ABA treatment we also detected HTR6 at the epidermis and lateral root cap cells. Interestingly, these tissues are the most external ones, which suggests that are the first ones to sense and respond to the exogenous stresses (Zhu, 2016). It has been observed that epidermis cells are first to perceive osmotic and ionic changes triggering rescue mechanisms (Sánchez-Calderón *et al.*, 2013).

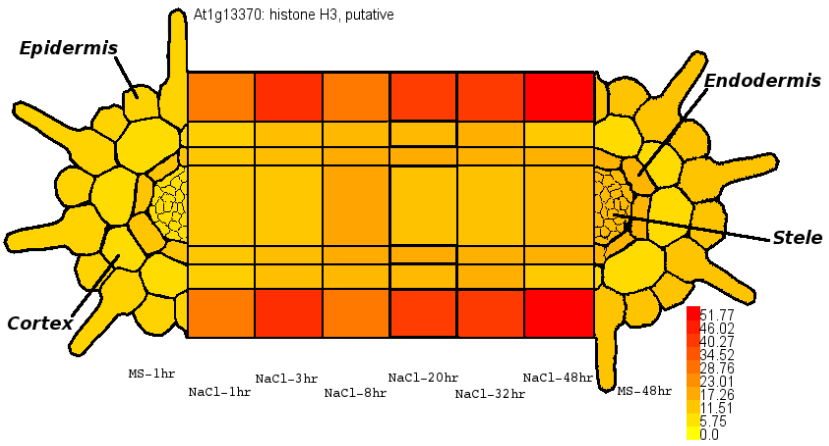

Figure 5.1 Spatio-temporal expression pattern of *HTR6* (AT1G13370) during the salt response. Picture taken from Arabidopsis eFP browser (http://dinnenylab.dpb.carnegiescience.edu). (Geng *et al.*, 2013)

Altogether, multiple evidences suggest that histone variant HTR6 is participating in the abiotic stress response. Similar to HTR6, in primates, a new histone H3 variant, H3.Y, has been identified and increases after stress, such as starvation and high cellular density. Nonetheless, H3.Y is also present at low levels in certain brain regions and malignant tissues (Wiedemann *et al.*, 2010). In Arabidopsis, other histones variants were also identified to respond to abiotic stresses, such as H1.3 and H2A.Z.

The linker DNA binding histone, H1 family, encompasses a histone variant, H1.3, that responds to stresses, such as drought, low light and exposure to ABA (Rutowicz *et al.*, 2015). Nonetheless, even without stress exposure, H1.3 is highly expressed in guard cells and is required for stomatal function. In stress conditions, H1.3 is present in other tissues, both in shoots and roots coexisting with the other H1.1 and H1.2 histones variants. The function of HTR6 in the areal part of the plant is unexplored yet, however, we detected small amounts of HTR6 in the shoot after ABA treatment.

The H2A.Z histone plays a dual role since it promotes transcription when it is located at the transcription start sites (TSS) but restricts gene expression when it is enriched within the gene body (Coleman-Derr and Zilberman, 2012). H2A.Z occupancy is inversely correlated with DNA methylation, which is a typical mark of gene bodies of highly transcribed genes (Zilberman *et al.*, 2008). Loss of function of H2A.Z or components of the SWRI chaperone complex mainly induces misexpression of genes associated with response to environmental stresses, such as cold and heat response (Lazaro *et al.*, 2008; Coleman-Derr and Zilberman, 2012). During these responses, some genes are rapidly upregulated or downregulated (Kumar and Wigge, 2010) confering a fast adaptation of plants to an adverse environment.

Regarding the H2A family, it has been described that phosphorylated variant γH2A.X is essential to signal DNA damage and in the recruitment of factors intervening in DNA repair (Amiard *et al.*, 2010). Unlike γH2A.X, HTR6 seems to be dispensable for DNA replicative stress response despite of being present during the S-phase of endocycling cells. Nonetheless, the hypothesis that replication arrest could influence HTR6 by blocking its expression or incorporation is not discarded.

5.2- Molecular basis of HTR6 dynamics during abiotic stress response

The adaptation of immobile plants to environmental stress is strongly related with gene expression adjustments (Yaish, 2017). Signaling pathways comprising plant hormones, such as ABA, connect environmental stress detection with activation of transcription factors, which in turn bind to the promoter regions of target genes to modulate their expression. This transcriptional reprogramming requirement also depends on changes in the chromatin landscape that is achieved by the coordination of several epigenetic events (Asensi–Fabado *et al*., 2017).

Nucleosome formation initiates with the association of a tetramer of H3-H4 histones with DNA and then the core structure ties up with the integration of two dimers of H2A-H2B histones. Histone chaperones, responsible for spatial and temporal deposition/eviction of histones in chromatin, are highly conserved in plants and animals (Polo and Almouzni, 2006; Mattiroli *et al*., 2015; Lu *et al*., 2018). Formation of nucleosomes containing H3.1-H4 tetramers is mediated by CAF-1 (FAS1, FAS2, MSI1 subunits) chaperone in a DNA replication-dependent manner. We detected that although the majority of cells containing HTR6 are in S-phase of endocycling cells, HTR6 incorporation was not disrupted in plants lacking CAF-1, indicating that HTR6 deposition is DNA replication-independent.

On the other side, HIRA chaperone as part of the HIR complex deposits H3.3-H4 tetramers into chromatin. In Arabidopsis, loss of HIRA causes reduced genome-wide loading of H3.3, nucleosome occupancy disruption and defects in vegetative development (Nie *et al*., 2014; Duc *et al*., 2015). Our results showed that HTR6 incorporation is impaired in the *hira-1* mutant, suggesting that HIRA incorporates HTR6. In vegetative tissues, HIRA seems to play a role in abiotic stress response, as genes downregulated in the *hira-1* mutant are related to environmental stresses (Nie *et al*., 2014). In humans, the interaction of H3.3 with HIR complex is mediated by UBN1 that recognizes the amino acid residue G90 of H3.3 (Ricketts, *et al*., 2015). In plants, it has been also demonstrated that residues H87 and L90 are important for the correct H3.3 deposition (Shi *et al*., 2011; Lu *et al*., 2018). The substitution of H3.3 residues H87 and L90 by the corresponding residues found in H3.1, S87 and A90, impaired H3.3 nucleosome assembly into the rDNA (Shi *et al*., 2011). HTR6 shares residues H87 and L90 with H3.3 which is a further evidence that HIR complex could also be incorporating HTR6. In animals, the stress-responsive histone variant H3.Y is also deposited by HIRA in transcriptionally active regions and, as expected, the chaperone recognition site motif, G90, is conserved (Kujirai *et al*., 2016; Zink *et al*., 2017).

Besides HIRA, we also found that the DEK chaperone also has a role in HTR6 incorporation. DEK3 has the ability of binding histones and changing the superhelical structure of DNA in vitro by interaction with Topoisomerase 1α. Both in Arabidopsis and in animals, DEK3 can modulate transcription by altering DNA accessibility or by recruiting distinct chromatin regulators (Sammons *et al*., 2006; Gamble and Fisher, 2007; Kappes *et al*., 2011; Waidmann *et al*., 2014). Furthermore, it has been found that reduced levels of DEK3 in the *dek3-2* mutant confers higher salt tolerance in Arabidopsis (Campillos *et al*., 2003; Sawatsubashi *et al*., 2010). Consistent with these data, we found that the *htr6-1* mutant also showed higher salt tolerance.

A striking observation is that HIRA and DEK3 cannot substitute for each other to incorporate HTR6, as shown by the reduced HTR6 levels in each *hira-1* and *dek3-2* mutants. This may suggest

that both could function in the same pathway. It could be possible that DEK is binding HTR6 before HIRA in the pathway, since it was shown that DEK3 specifically copurified with histones H3 and H4 but not with H2A and H2B, demonstrating that DEK3 does not associate with fully assembled nucleosomes (Waidmann *et al*., 2014). Alternatively, it is also possible that ABA signaling in these mutants is impaired, which could prevent HTR6 expression. Thus, HTR6 mRNA expression levels should be quantified in *hira-1* and *dek3-2* mutants.

During the genome replication process, H3.1 is inserted all over the genome by CAF-1, both in euchromatin and heterochromatin. After replication, H3.1 is replaced by H3.3 in regions that are actively transcribed in a process mediated by HIRA. Likewise, HTR6 deposition seems to be transcription related because stalling of transcription elongation with DRB decreased the levels of HTR6 incorporation. However, we do not discard the possibility that DRB is inhibiting the synthesis of new HTR6-GFP transcripts preventing, in this way, its turnover. Inhibition of DNA replication with aphidicolin also affected HTR6 turnover, although to a lesser extent. We believe that HTR6 is replacing H3.1 after replication in order to establish the transcriptional status of HTR6 target genes. Thus, if DNA replication is slowed down after aphidicolin treatment, the replacement by HTR6 is also going to be slowed down, resulting in a decreased HTR6 turnover. This claim is consistent with the finding that HTR6 is predominantly present during S-phase of endocycling cells. Based on the EdU labeling pattern, we can distinguish that euchromatin, containing active genes, is replicated in the early and mid-stages of S-phase while heterochromatin, enriched in silent regions, is replicated in the late stage. Our immunolocalization studies demonstrated that HTR6 is excluded from the heterochromatin regions and colocalize with a mark associated with actively transcribed regions, H3K4me3. In line with this, we observed that despite the fact that the majority of cells containing HTR6 are in the late stage of S-phase, the HTR6 distribution pattern only coincides with euchromatin.

The residence time of HTR6 in chromatin is shorter than that of H3.3, as expected, since H3.3 is constitutively expressed and HTR6 is not. Taking into account our FRAP results, HTR6-GFP protein recovered faster from bleaching but then, the fluorescence dropped much earlier than H3.1, which is also not constitutively expressed. Accordingly, in stress conditions, the histone variant H1.3, despite of being stimulated in more tissues, also display a higher turnover comparing with H1.1 and H1.2., which might indicate that, in general, these stress-inducible histones are less stable in order to promote a chromatin state change that is only temporal and rapidly reversible. We observed, from live imaging, that HTR6 is rapidly loaded to the chromatin and then is maintained in high amounts during ~1h. It is possible that HTR6 target genes are only active/repressed for a short period of time. Similar to H2A.Z, it is likely that HTR6 could function as an activator or a repressor depending on the target. Some studies reported that unstable nucleosomes containing H2A.Z and H3.3 are very often present at TSSs (Jin and Felsenfeld, 2007;

Jin *et al.*, 2009). It is possible that some nucleosome core particles contain both H2A.Z and HTR6 simultaneously, although HTR6 function in cold and heat stresses has not yet been explored. A co-IP of both histones from mononucleosomal preparations would be a useful approach to address this question. Another interesting experiment would be to determine if heterotypic nucleosomes could be formed containing both HTR6 and H3.3.

After 1h of incorporation, the nuclear HTR6 amount reached a maximum, but then the protein is slowly evicted, despite of being maintained during 4 to more than 7h. It could be possible that small amounts of the protein could be retained as a "memory" to maintain a specific chromatin environment in its target genes along the elongation region. Nonetheless, before cells rise the differentiation zone, proteasome seems to completely degrade HTR6.

The unstructured N-terminal tail of the histones is usually a platform for a high range of post-translational modifications that influence histone function and distribution (Jenuwein and Allis, 2001; Turner, 2002, Ho and Crabtree, 2010). Even the difference in one single amino acid could completely change protein interactions, leading to distinct dynamics and functions. This is the case of the alanine (A) at position 31 of H3.1. The presence of a T in H3.3 instead of an A residue prevents interaction of H3.3 with the methyltransferases, ATXR5 and ATXR6, which mediate the monomethylation of lysine 27 in H3.1 (Jacob *et al*., 2014). In Arabidopsis, H3K27me1, as well as H3K9me2, are typical marks of constitutive heterochromatin that are only present in H3.1 (Johnson *et al*., 2004; Stroud *et al*., 2012). These marks colocalize with repressive DNA methylation patterns allowing the maintenance of the genomic regions in a silenced state. As in H3.3, HTR6 has a T at position 31, which avoids the binding of ATXRs enzymes, preventing the deposition of the H3K27me1 mark and this is consistent with the absence of HTR6 from heterochromatin. Additionally, a mass spectrometry analysis in Arabidopsis demonstrated that repressive mark H3K27me3, mediated by Polycomb complex, is also present in H3.1 and excluded from H3.3 (Johnson *et al*., 2004). Our immunolocalization analysis showed that HTR6 and H3K27me3 do not completely overlap, indicating that HTR6 also seems to be excluded from facultative heterochromatin enriched in H3K27me3. Nonetheless, it is possible that some of HTR6 targets are silenced by Polycomb previous to the stress induction. Other example of the importance of the type of amino acid residues to confer histone unique characteristics was observed for H3.Y histone. The presence of unique amino acids in the core and C-terminal domain of H3.Y, prevent DAXX/ATRX chaperone, but not HIRA, binding, despite that H3.Y contains the G90 residue, which was previously identified as crucial for both HIRA and DAXX/ATRX chaperones recognize H3.3 histone (Zink *et al*., 2017). This suggests that HTR6 specific residues could confer unique properties and dynamic to the histone to prevent or benefit the binding of specific chaperones or PTMs effectors.

We carried out extensive efforts to isolate enough amount of HTR6-Myc in order to detect HTR6 PTMs by western blot with specific antibodies or by Mass spectrometry. Unfortunately, so far, we were unable to purify sufficient material for those analyses. Some studies described similar technical difficulties claiming that only ~1% of total histones correspond to the epitope-tagged transgenic H3 proteins (Lu *et al*., 2018). Similarly, a previous study using GFP-tagged H3.1 and H3.3 in Drosophila also showed that each of the fusion proteins constituted <0.5% of the total H3 in cells (Schwartz and Ahmad, 2005). We also faced similar problems when attempting to obtain good quality ChIP DNA to decipher the genomic HTR6 target regions. This highlights the fact that further optimization of the techniques for purifying this histone is needed, to decipher the complete set of post translational modifications (PTMs) that can occur in the HTR6 histone and how those HTR6 specific marks correlate with the genomic targets of this histone.

In addition to the T31, the similarities between HTR6 and H3.3 are evident, differing only in 7 amino acidic residues ($S_6H_{11}T_{17}F_{41}N_{62}V_{124}A_{132}$). HTR6 has three residues in the N-terminal tail, S6, H11 and T17, that differ from both H3.3 and H3.1 ($T_6T_{11}R_{17}$). In Arabidopsis, T6 and T11 are usually phosphorylated to promote chromosomal condensation in mitosis and meiosis (Houben *et al*. 2005; Caperta *et al*. 2008; Ashtiyani *et al*. 2011; Moraes and Casas-Mollano, 2014; Kniewel *et al*., 2017). Despite that both S and T can be phosphorylated, the substitution of S by T at position 6, leads to the increase of HTR6 levels. On the contrary, the substitution of H11 by a T makes the HTR6H11T-GFP be present in fewer nuclei. This could be because T11 is evicted from the cells or because H is also involved in HTR6 incorporation. T11ph in other H3 histones is usually necessary to chromosome preparation for mitosis, once that HTR6 is present in endocycle cells, it is possible that T11 do not benefit the presence of HTR6. Interestingly, another stress responsive histone of the H3 family, HTR14, also has a H at position 11, and is not expressed in mitotic cells either (data not published). To date, we have not yet obtained plants expressing HTR6 with T17 substituted by an arginine (R), the corresponding residue in H3.3. It is known that R17 can be methylated, and this is a mark that is associated with transcriptional activation (Schurter *et al*., 2001). It is important to highlight that alteration of these residues could not only influence the potential PTMs on these residues but also the interactions with nuclear proteins as we described earlier for the substitution of A31T in H3.1 and H3.3 respectively.

The residue at position 41 is of particular relevance because it differs between H3.1 (F41) and H3.3 (Y41). Interestingly, the F at position 41 is specific of vascular plants (Waterborg, 2012; Cui, 2015). HTR6 shares F41 residue with H3.1. We observed that F41 substitution causes changes in HTR6 dynamics. The HTR6F41Y-GFP accumulates in more cells of the epidermis/cortex in the transition/elongation zone and also in the root cap. This mutation, in H3.1 provoked that the histone was no longer evicted from the euchromatin suggesting that F41 is required for an efficient exchange of H3.1 with other variants, e.g. H3.3 or HTR6 (Lu *et al*., 2018).

It is possible that the same occurs for HTR6F41Y-GFP and that the mutant has a reduced capacity to be exchanged explaining why we observed more positive cells in the transition domain. Nevertheless, it seems that this mutant can be degraded efficiently by the proteasome because no protein accumulated in the differentiation zone of the root. Moreover, in humans, Y41 in H3.3 is phosphorylated by Janus kinase (JAK), which prevents the binding of heterochromatin protein HP1α, promoting an active transcription environment (Dawson *et al*., 2009). A JAK homolog has not been identified in plants, but we cannot discard that other kinases could phosphorylate HTR6F41Y-GFP. Furthermore, phosphorylation introduces additional negative charges to the proteins that could influence histone turnover. In this sense, residue 41, which is located just at the DNA entry/exit region of the nucleosome, could directly influence wrapping and unwrapping dynamics of the nucleosome before and after phosphorylation (Fig 5.2; Bowman and Poirier, 2015).

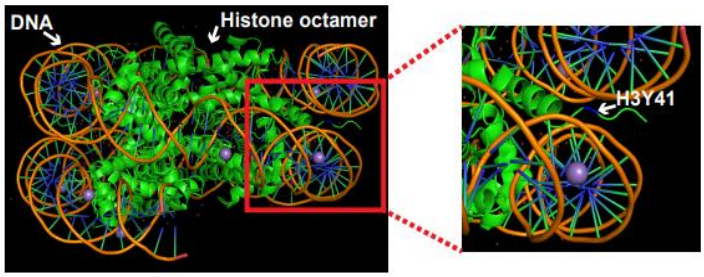

Figure 5.2 Visualization of H3Y41 in the nucleosome. H3Y41 is highlighted in blue inside the right panel. The accession number of the nucleosome structure data is Protein Data Bank (PDB): 2CV5. (Lu *et al.*, 2018)

Curiously, all the point mutations attempted in the core region of the protein and C-terminal (N63I, V124I and A132G), lead to expression of HTR6 in more nuclei. Particularly, the A132G change led to a HTR6 expression pattern in the roots very similar with the one obtained after treatment with proteasome inhibitors, which suggests that residues at the C-terminals could be important for the protein degradation.

Further experiments will be necessary to analyze the phenotypic consequences of expressing these and other HTR6 mutants in the *htr6-1* background.

5.3- Role of HTR6 in root development after abiotic stress

Using a reporter line expressing cell cycle phase markers, we found that proliferating and endocycling cells behaves differently in response to ABA. In the root meristem, CDT1a, used as a G1 marker, is expressed in more cells after ABA treatment indicating that cells are arrested in G1. However in the transition zone, CDT1a, that label G phase cells, is reduced. We consider two possible hypothesis: 1- the delay of the S phase progression observed in presence of ABA could modify the balance between cells in S and G phase in the transition domain. 2- previous studies showed that CDT1a is a target of ABA and downregulated in the whole seedling after ABA treatment (Castellano *et al.*, 2004). This effect may be specific of the transition zone. Strikingly,

an ABA-responsive factor, GEM specifically expressed in the transition and differentiation zones of the root, interacts with CDT1a (Caro *et al*., 2007, Mauri *et al*., 2016). It is tempting to speculate that it participates in CDT1a inhibition at the endocycling region. Possibly, this could also lead to a pre-RC assembly impairment, explaining why there is a delay in the S-phase. Nonetheless, there are many elements of DNA replication machinery that are important for a proper ABA signaling. The disruption of DNA polymerases, such as DNA pol alpha, delta or epsilon, lead to abnormalities in abiotic stress response (Micol-Ponce *et al.*, 2015; Johnson *et al.*, 2015; Gutzat and Mittelsten, 2012; Probst and Mittelsten Scheid, 2015).

The boundary between proliferation and differentiation is mainly regulated by phytohormones and ROS (Beemster and Baskin, 1998; Moubayidin *et al.*, 2010; Singh *et al.*, 2016). In Arabidopsis, the auxin:cytokinin ratio is critical in determining the rate of root growth (Dello *et al.*, 2008; Moubayidin *et al.*, 2010; Rowe *et al.*, 2016). An imbalance of any of these hormones results in meristem size deregulation. Osmotic stress increases ABA levels in seedlings, which consequently limits auxin transport via PIN1, causing lower auxin accumulation and leading to a reduction in meristem size and reduced root growth (Rowe *et al.*, 2016). Furthermore, osmotic stress also increases the levels of DELLA in an ABA-dependent manner, contributing also to meristem size reduction (Ubeda-Tomás *et al.*, 2008; Achard *et al.*, 2009). Lastly, it has been described that ABA also promotes ROS production provoking a decrease in auxin accumulation and consequently resulting in inhibition of PLETHORA1 (*PLT1*) and *PLT2* expression (Yang *et al.*, 2014). Thus, it seems that ABA creates an environment in the transition zone that causes the reduction of meristem size and promotes the switch to elongation. Curiously, the transition zone is considered as an oscillatory zone that may act as a kind of command center (Baluška and Mancuso, 2013).

In the transition zone of the root cells have stopped dividing and switched to the endocycle. As a consequence of repeated endocycle rounds, the genomic ploidy increases from 2C to 4C, 8C and 16C (Edgar *et al.*, 2014). Besides ABA, stress conditions such as high salinity, low pH, temperature and ion starvation, promote a premature switch to the endocycle and cell expansion leading to shorter meristems. Whether this constitutes an advantage is not very clear but the endocycle could be important for plants, allowing them to cope with environmental stress because it stimulates the metabolic activity, and thus could lead to a more efficient stress response. This may explain why the endocycle is mainly observed in species growing in variable environments (Barow, 2006; Scholes and Paige, 2015) and that possess a high metabolic activity (Bhosale *et al.*, 2018; Bhosale *et al.*, 2019). In tomato, it was demonstrated that endoploidy increased the amount of ribosomal RNA, RNA polymerase II abundance and gene transcript levels (Bourdon *et al.*, 2012). Endoreplication is also common in lower invertebrates, arthropods and mammals (Fox and Duronio, 2013). In lower invertebrates, endoreplication is most often associated with

increased cell size, and it is believed to be a crucial determinant of adult body size (Flemming *et al.*, 2000; Bhosale *et al.*, 2019).

The endocycle process is accompanied by a rapid growth through water uptake, vacuole and cell expansion, which is essential for plant cells to elongate and differentiate (Cosgrove, 1993; Dolan and Davies, 2004). Recent studies indicate that the endocycle is a potential driver of cell wall modifications by increasing the number of gene copies and the amount of cell wall proteins (Bhosale *et al.*, 2019). Our comparative transcriptome analysis of wt and *htr6-1* mutant plants after a very short ABA treatment revealed that the presence of HTR6 induces the expression of cell wall modifying genes, presumably to prepare cells for the often massive cell enlargement following cell cycle exit. Furthermore, roots lacking HTR6 and exposed to ABA display longer meristems that contain more cortical cells. This means that, despite the fact that the repressive effect of ABA on the root meristem is maintained, the effect is attenuated by the lack of HTR6 in *htr6-1* mutant plants, reinforcing the idea that presence of HTR6 at the transition domain participates in the inhibition of meristem growth. *HTR6* expression is confined to the transition/elongation region where high rates of cell expansion occur. Vacuolar expansion and water uptake are major forces driving cell growth, although, under exogenous ABA treatment or other stress conditions, e.g. salt, the level of water uptake is compromised (Zhu, 2002). Therefore, it is possible that other players are necessary to stimulate transcriptome changes related to cell wall metabolism.

Mutations in endocycle regulators, such as SIM and SMR1, have also consequences on cell wall biosynthesis and lead to a decrease in pathogen resistance (Wang *et al.*, 2014; Hamdoun *et al.*, 2016). This highlights the link between cell polyploidy, cell wall modifications and a crosstalk between biotic and abiotic stresses. It would be interesting to determine if plants with disrupted endocycle, as is the case of *sim srm1* mutants, would be able to trigger *HTR6* after ABA treatment. Thus, we would be able to infer if HTR6 stimulation is indirectly dependent of cell cycle regulators.

Besides, cell wall modifiers, the presence of HTR6 also altered the expression of other genes, such as those involved in lipid transport, catabolism and systemic acquired resistance, including GDSL-type esterases/lipases. A recent study revealed that GDSL-type lipase proteins showed diverse expression patterns during abiotic and biotic stress responses (Lai *et al.*, 2017). Some of these genes also showed altered expression patterns during geminivirus infection, which suggests that HTR6 could potentially have a role in biotic stress, an aspect that would be attractive to investigate in the future.

The late embryogenesis abundant (LEA) proteins belong to the group of genes repressed by HTR6. The function of LEA genes in vegetative plant tissues is largely unknown, despite the fact

that it has been reported a possible role in desiccation tolerance and that they are present in a high variety of cell compartments (Candat *et al.*, 2014). Nevertheless, a link between HTR6 function and LEA proteins has yet to be uncovered. As mentioned before, this emphasizes our theory that HTR6 could function in a similar way as H2A.Z, behaving as an activator or repressor depending where the histone is incorporated in the target gene region.

Altogether, our results suggest that HTR6 might have an important function as an epigenetic factor necessary to reprogram chromatin structure of specific genomic regions associated with genes necessary for abiotic stress response (Fig. 5.3).

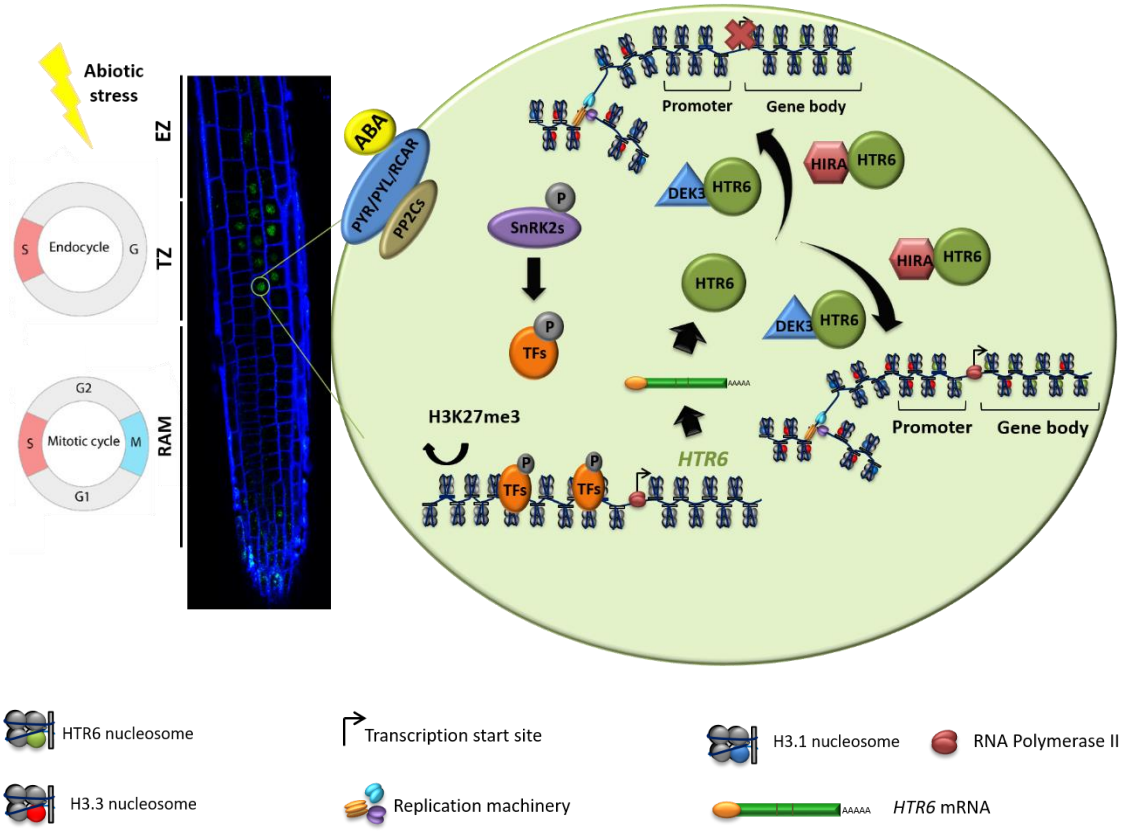

Fig. 5.3 Model of HTR6 dynamics and function in Arabidopsis root. Under abiotic stress, HTR6 is specifically expressed in a set of epidermal and cortical cells at the transition/elongation zone. The normal repressed status of HTR6 gene, characterized by high levels of H3K27me3, is relieved and HTR6 expression is induced by TFs dependent on ABA signaling. During S-phase but independently of DNA replication, HTR6 is incorporated in the nucleosomes by HIRA and/or DEK3 in euchromatin, e.g. promoter, TSS or gene body regions of genes. This induces chromatin remodeling that lead to modulation of target genes transcription. Finally, this promotes a premature switch from cell proliferation to cell differentiation, making the meristem shorter.

6. Conclusions
Conclusiones

1. *HTR6* encodes a histone H3 variant and is transiently induced in *Arabidopsis thaliana* plants exposed to abscisic acid (ABA) or NaCl, with a peak of expression after 4 hours of exposure.

2. The promoter region of *HTR6* contains the repressive mark *H3K27*me3. ABA treatment leads to an alteration of the chromatin landscape at the *HTR6* locus, decreasing the levels of H3K27me3.

3. In roots, the HTR6 expression domain is restricted to the transition/elongation zone, where cells are endocycling, and to the external tissues, such as epidermis and cortex. It is also detected in the root cap.

4. The majority of cells containing HTR6 are undergoing the S-phase of the endocycle but HTR6 incorporation is independent of CAF-1 indicating that is DNA replication-independent.

5. HTR6 is deposited into chromatin by the HIRA or DEK chaperones in transcriptionally euchromatic regions, enriched in H3K4me3, likely through the conserved H87 and L90 residues that mediates HIRA-histone binding in H3.3. HTR6 is excluded from heterochromatin, consistent with the presence of T31, which in H3.3, prevents deposition of heterochromatin mark H3K27me1 by ATRX5/6.

6. Unlike H3.1 and H3.3, HTR6 is a target of proteasome degradation.

7. Some of the residues unique to HTR6 ($S_6N_{63}V_{124}A_{132}$) compared with H3.3, increase HTR6 turnover. This may be enhanced by the presence of F41, conserved in H3.1 where it favors H3.1 exchange.

8. HTR6 is a factor necessary to reprogram chromatin structure in specific genomic regions, promoting transcription of cell wall modifiers and lipid metabolism but repressing expression of several LEA genes.

9. Plants lacking HTR6 exhibit higher tolerance to both ABA treatment and high salt concentrations. *htr6-1* mutants also displayed larger meristems, containing a few more cells, after being exposed to ABA treatment for several days. This highlights that HTR6 is an important component that contributes for proper abiotic stress response in *Arabidopsis thaliana* root.

1. *HTR6* codifica una variante de histona H3 que se induce de forma transitoria en plantas de *Arabidopsis thaliana* expuestas a ácido absicíco (ABA) o NaCl, con un pico de expresión a las 4 horas de exposición.

2. La región promotora de *HTR6* contiene la marca represiva H3K27me*3*. El tratamiento con ABA altera su distribución en el locus de *HTR6*, disminuyendo los niveles de H3K27me3.

3. El dominio de expresión de HTR6 en raíces está restringido a la zona de transición/elongación, donde las células desarrollan los endociclos, y a los tejidos externos, como la epidermis y a corteza. También se detecta en la cofia.

4. La mayoría de las células que contienen HTR6 se encuentran en la fase S del endociclo, pero la incorporación de HTR6 es independiente de CAF-1, lo que indica que es independiente de la replicación del ADN.

5. HTR6 es depositado en la cromatina por las chaperonas HIRA o DEK en regiones transcripcionalmente eucromáticas, enriquecidas en H3K4me3, probablemente a través de los residuos conservados H87 y L90 que median la unión HIRA-histona en H3.3. HTR6 está excluido de la heterocromatina, lo que concuerda con la presencia de T31, que en H3.3 impide la deposición de la marca de heterocromatina H3K27me1 por ATRX5/6.

6. A diferencia de H3.1 y H3.3, HTR6 se degrada por el proteasoma.

7. Algunos de los residuos únicos de HTR6 ($S_6N_{63}V_{124}A_{132}$) coincidentes con H3.3, aumentan la dinámica de HTR6. Esto puede aumentarse por la presencia de F41, que está conservada en H3.1 donde favorece el intercambio de H3.1.

8. HTR6 es un factor necesario para reprogramar la estructura de la cromatina en regiones genómicas específicas, promoviendo la transcripción de modificadores de la pared celular y el metabolismo de los lípidos, pero reprimiendo la expresión de varios genes LEA.

9. Las plantas que carecen de HTR6 exhiben una mayor tolerancia tanto al tratamiento con ABA como a altas concentraciones de sal. Los mutantes *htr6-1* poseen meristemos más grandes, con mayor número de células, tras tratamiento con ABA durante varios días. Por tanto, HTR6 es un importante componente que contribuye a la respuesta adecuada al estrés abiótico en la raíz de *Arabidopsis thaliana*.

7. References

7. References

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