EPIGENETIC REGULATORS HDA19 AND ARP6 INFLUENCE TOMATO DEVELOPMENT

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Summary

Tomato as a fleshy fruit is one of the most important components of healthy diets, providing unique and critical contributions to food security. Fleshy fruit in comparison with dry, or dehiscent, fruit undergo a range of changes in chemistry and physiology, including synthesis and accumulation of characteristic pigments, evolution of aroma volatiles and modifications of texture with the final goal of spreading seeds through the attraction of animal vectors. Recently, increasing evidence has indicated that the regulatory network of tomato development include not only hormonal and genetic regulation but also epigenetic modulations. Substantial advances have been achieved in understanding DNA methylation, which plays a critical role as an important developmental regulatory component. However, in addition to DNA methylation, histone post-translational modifications (HPTMs) and histone variants can influence chromatin structure and gene expression. Among HPTMs, histone acetylation and deacetylation are biological processes considered crucial in plant growth and development through facilitation of chromatin relaxation and gene transcription regulation. Among histone variants, H2A.Z is the most evolutionary conserved and can impacts multiple processes, including transcription, DNA repair and response to environmental stresses. The ATP-dependent chromatin-remodeling complex SWR1 controls H2A.Z replacement in the nucleosome. However, HPTMs and nucleosome histone variants are not as well documented as DNA methylation in tomato plant development. Given that, our work has focused on the identification and the functional characterization, using amiRNA silenced mutants, of tomato Histone Deacetylase 19 (*HDA19*) and Actin Related Protein 6 (*ARP6*). The former is a histone deacetylase (HDACs) belonging to the RPD3 family and it has been shown to control flowering time, germination and seed set reduction in Arabidopsis. The latter is one of the subunits of the SWR1 complex and for that reason has been widely used to study the effects of H2A.Z depletion from chromatin. Arabidopsis plants defective in ARP6 exhibit global reduction in size, curly leaves, altered inflorescence and flower morphology, and early flowering.

Our phenotypic analysis showed that HDA19 influences fruit size, ethylene production and carotenoids accumulation. In addition, HDA19 impacts on seeds set and is therefore necessary for embryo development. Conversely, ARP6 has a role on the vegetative development of tomato. It also influence germination and early seedlings development. Further, we showed that ARP6

contribute to plant tolerance to salt and heat stress in tomato. Taken together our data suggest a clear involvement of epiregulators HDA19 and ARP6 during reproductive and vegetative development of tomato, respectively.

1. Introduction

1.1 Tomato: a model for fruit development and ripening

Fruit formation is a developmental process unique to flowering plants. It occurs following fertilization that stimulates the growth of carpels in simple fruits (Giovannoni, 2001; Seymour et al., 2013). Fleshy fruit in comparison with dry, or dehiscent, fruit has the peculiarity to change in color, texture, taste, and flavor during maturation for attracting animal vectors that consume them, thus liberating the seeds and dispersing them in an efficient way (Tiffney, 2004). Tomato as well as other fleshy fruits are composed of an epidermis, a thick pericarp and placental tissues surrounding the seeds (Fig. 1).

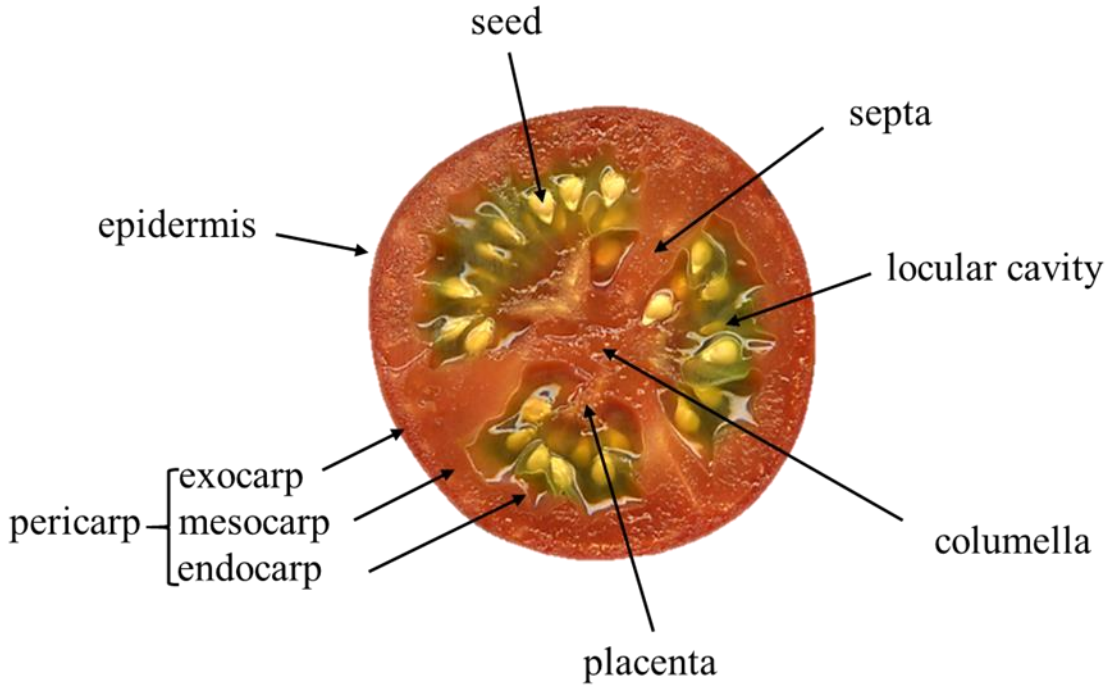

Figure 1. Transverse section of a tomato fruit cv Ailsa Craig

The fruit setting is established during and soon after fertilization and can be divided in two distinct processes, the development and the ripening (Fig. 2).

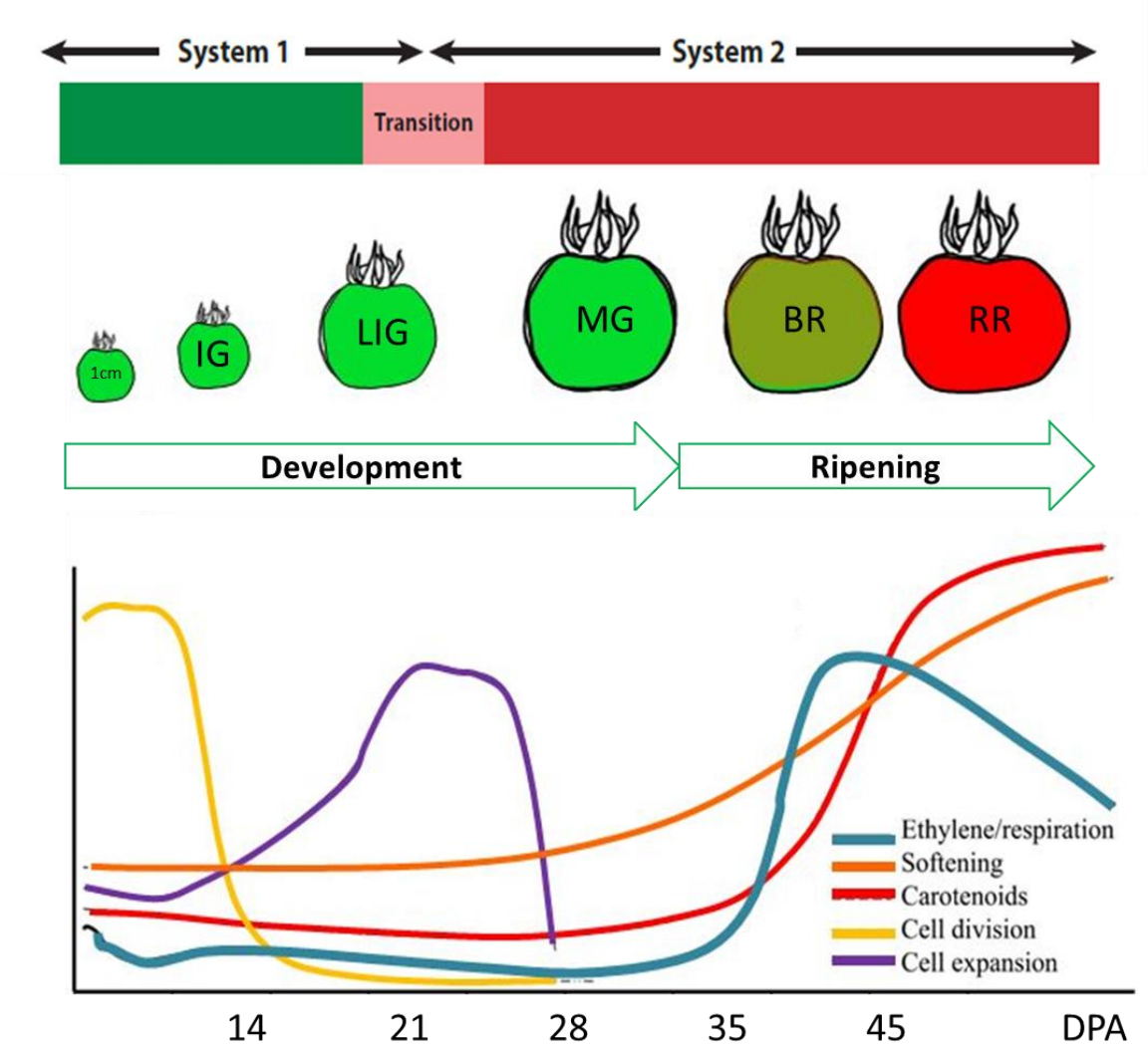

Figure 2. Tomato growth can be divided in two different processes: development and ripening. The former is a period of intense cell division and expansion. The latter is a period in which respiration, ethylene synthesis, fruit softening, and carotenoid accumulation increase. In addition, tomato development can fall into two different system depending on whether exogenous somministration of ethylene inhibit (system 1) or promote (system 2) the ripening. Days Post Anthesis (DPA) can vary substantially among cultivars. The time line shown would be for a medium-/large-fruit cultivar. IG=immature green; LIG=late immature green; MG=mature green; BR=Breaker; RR=red ripe. Modified from Giovannoni 2004 and Giovannoni et al. 2017.

The development involves cell division and expansion of the ovary tissues. Cell divisions both periclinal and anticlinal start when the ovary is 1 mm in diameter with 10 cell layers, approximately 2 days post anthesis (DPA). By 4 DPA the fruit is 1.5 mm in diameter and has 30 cell layers. At 7–8 DPA, cell expansion becomes evident and the cell layer number increases to 35 at the apex of the fruit and 20 at the equator. Cell division stops by 10–13 DPA, also called Immature Green

stage (IG), and cell expansion progresses at a dramatic rate until approximately 30 DPA, when the fruit reaches a diameter of 1.5–2 cm, also called Mature Green stage (MG) (Pabon-Mora and Lytt, 2011). Cell expansion is responsible for the increase in fruit size, with cell sizes reaching 0.5 mm in diameter in the pericarp of some tomato varieties (Chevalier et al. 2011). At this stage of development cells enlarge up to 20-fold (Cong et al., 2002), due to multiple rounds of endo-reduplication with DNA contents as high as 256C in mature fruit (Bergervoet et al., 1996). After growth has finished, the ripening phase starts with the Breaker stage (Br) and involves rapid chemical and structural changes that determine fruit aroma, color, texture, and biochemical composition of the fully mature fruit (Red ripe stage=RR). During this process there is no change in fruit size and shape (Tanksley 2004). The ripening process is the last phase and climacteric the ripening process involves a dramatic increase in respiration associated with an ethylene burst (Fig. 2). Ethylene biosynthesis proceeds at a low level during development (System 1), but at the onset of ripening it becomes autocatalytic (System 2) (Fig. 2). Interestingly, ethylene application can promote early ripening only once the fruit has achieved the competence to respond. Ethylene provided before this competence does not promote the ripening and can even delay it. This observation is the basis of the physiological distinction between system 1 and system 2 ethylene responses; in the former, ethylene has an inhibitory effect on ripening and in the latter, it has a positive effect (Giovannoni et al., 2017) (Fig. 3).

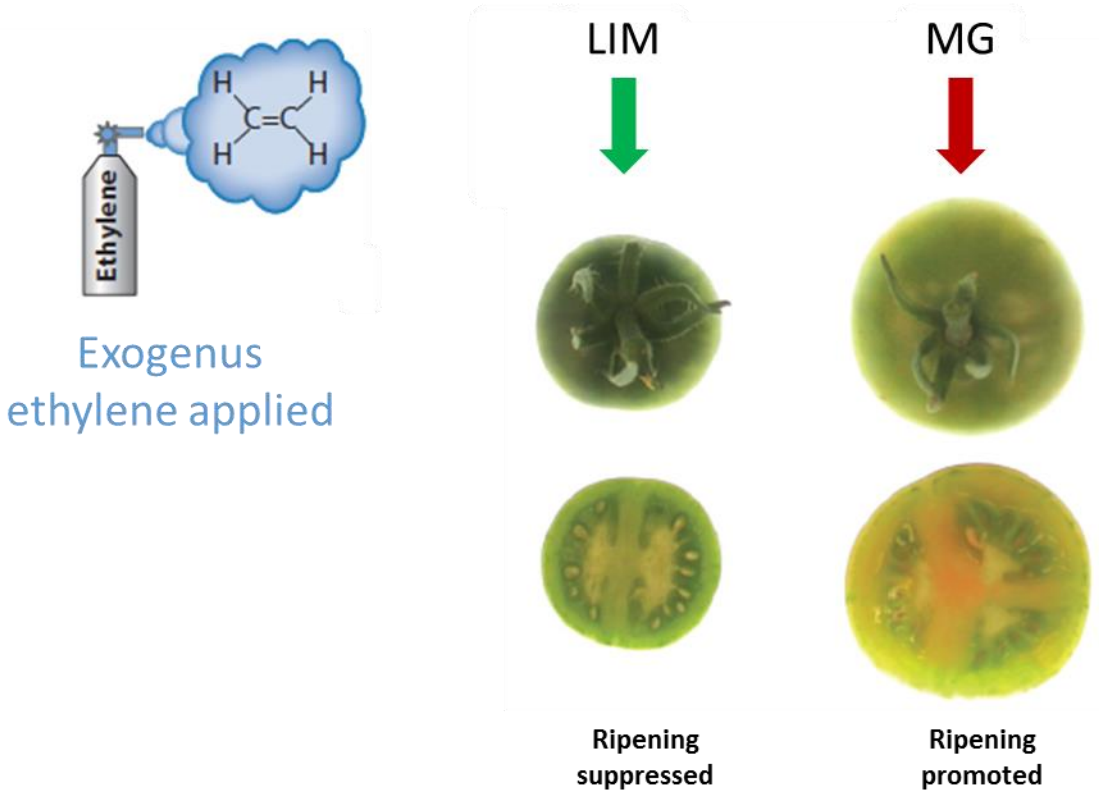

Figure 3. The physiological responses of ripening fruit to exogenous ethylene fall into two categories, known as system 1 and system 2, where the former represses ripening and the latter promotes it. The transition occurs between the LIM and MG stages in which underlying molecular changes render the fruit tissue competent to ripen in response to ethylene. Immature fruits (i.e., those at the IM or LIM stage) are defined as such because their seeds are not fully developed. MG fruits are full size and their seeds are mature (viable), but the fruits themselves have not yet begun climacteric respiration or increased endogenous ethylene production. Application of exogenous ethylene promotes ripening in MG but not IM or LIM fruit. Modified from Giovannoni et al. 2017.

During the transition from Late Immature Green (LIG) to Mature Green (MG) stage the seeds become fully developed and capable of germination, and the locule tissue transitions from firm to a jelly-like consistency. Seeds themselves may provide signals to the maternal fruit tissues indicating that embryo development is complete and seed dispersal mechanisms can be implemented (Giovannoni et al., 2017). On the other hand, although seeds are logical sources of initial ripening signals, many fruits can be seedless (parthenocarpic) and can be able to ripen in the absence of seed development. This observation do not necessarily mean that seeds are not sources of ripening signals; rather, fruits may have regulatory systems selected to confer maturation in the absence of seed development.

The pathway of ethylene biosynthesis is now well understood and the major steps involve the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and then by ACC oxidase (ACO) to ethylene (Alexander and Grierson, 2002). A major point of regulation for ethylene synthesis occurs at the level of ACS transcription (Klee and Giovannoni, 2011).

Table 1. List of transcription factors involved in tomato ripening

Gene	Locus	References	Function
RIN-MADS	Solyc05g012020	Vrebalov et al. 2002; Ito et al. 2008; Martel et al 2011	ripening TF
CNR-SPL	Solyc02g077920	Manning et al. 2006; Chen et al.2015	ripening TF
TAGL1	Solyc07g055920	Giovannoni et al., 2017	ripening TF
TAG1	Solyc02g071730	Pnueli et al. 1994; Pan et al. 2010; Gimenez et al. 2016	ripening TF
FUL1	Solyc06g069430	Bemer et al. 2012; Seymour et al., 2013; Fujisawa et al. 2014;	ripening TF
FUL2	Solyc03g114830	Bemer et al. 2012; Seymour et al., 2013; Fujisawa et al. 2014;	ripening TF
NOR-NAC	Solyc10g006880	Tigchelaar et al. 1973; Martel et al. 2011; Osorio et al. 2011	ripening TF
AP2a	Solyc03g044300	Karlova et al. 2014; Klee and Giovannoni, 2011; Giovannoni 2017	ripening TF
NR	Solyc09g075440	Klee and Giovannoni, 2011; Giovannoni et al. 2017	ripening TF
GLK2	Solyc10g008160	Powell et al. 2012; Nguyen et al 2014; Giovannoni et al. 2017	ripening TF
ACO	various 1	Alexander and Grierson, 2002	ethylene
ACS2	Solyc01g095080	Klee and Giovannoni, 2011	ethylene
ACS4	Solyc05g050010	Klee and Giovannoni, 2011	ethylene
LeETR1	Solyc12g011330	Klee and Giovannoni, 2011	ethylene
LeETR2	Solyc07g056580	Klee and Giovannoni, 2011	ethylene
LeETR4	Solyc06g053710	Klee and Giovannoni, 2011	ethylene
LeETR5	Solyc11g006180	Klee and Giovannoni, 2011	ethylene
LeETR6	Solyc09g089610	Klee and Giovannoni, 2011	ethylene
LeETR7	unavailable	Klee and Giovannoni, 2011	ethylene
EIN3-like	Solyc01g009170	Giovannoni et al. 2017	ethylene
EBF1	Solyc12g009560	Pech et al. 2011 (book)	ethylene

EBF2	Solyc08g060810	Pech et al. 2011 (book)	ethylene
PG2a	Solyc10g080210	Giovannoni et al., 2017	cell wall
PMEU1	Solyc03g123630	Dumville et al., 2003	cell wall
PL	Solyc03g111690	Uluisik et al. 2016	cell wall
ZDS	Solyc01g097810	Fantini et al. 2013	carotenoid
CRTISO	Solyc10g081650	Enfissi et al.2017	carotenoid
PSY1	Solyc03g031860	Bartley et al., 1992; Fray and Grierson, 1993	carotenoid
LCBY	Solyc04g040190	Bartley et al., 1992; Fray and Grierson, 1993;Ronen et al., 1999	carotenoid
Z-ISO	Solyc12g098710	Aoki et al., 2010; Fantini et al. 2013	carotenoid

There are at least eight characterized *ACS* genes in tomato and three additional identified in the tomato genome sequence (Tab. 1), each with a distinctive tissue and stimulus specificity. Four *ACO* genes were characterized in tomato and three additional genes were found in the genome sequence. Even though ACO activity is not limiting, certain *ACO* genes are ethylene inducible, particularly in ripening fruits. *ACO1* is the most highly induced *ACO* during ripening and its antisense prevents ethylene synthesis and ripening. Antisense genes targeting *ACS* and *ACO* are highly effective in reducing ethylene synthesis and delaying ripening (Klee and Giovannoni, 2011).

Signal transduction is also a critical aspect of ethylene action. In this regard there are seven ethylene receptor genes (*LeETR1*, *LeETR2, NR, LeETR4*, *LeETR5, LeETR6*, and *LeETR7*). Five of these receptors have been shown to bind ethylene while two, LeETR6 and LeER7, were not tested. Based on gene and protein structures, the ethylene receptors are divided into subfamily 1 and subfamily 2. The subfamily 1 members have the highest similarity to histidine kinases, whereas the subfamily 2 members have diverged and acquired serine kinase activities (Moussatche and Klee, 2004). Reduced expression of either subfamily 2 receptor gene, *LeETR4* or *LeETR6*, results in substantially increased ethylene sensitivity. Antisense plants with greatly reduced expression of either of these two receptors show phenotypes consistent with a constitutive ethylene response, including significantly earlier fruit ripening (Klee and Giovannoni, 2011). This enhanced ethylene sensitivity can be restored to wild type by overexpression of the subfamily 1 receptor NR. LeETR4, LeETR6 and NR expression increases significantly at the onset of fruit ripening and these three

receptor genes are by far the most highly expressed in ripening fruits. The dominant *Nr* (*Never-ripe)* mutant is one of the earliest known tomato fruit ripening mutants. *Nr* fruits do not ripen, even when exposed to ethylene. Flowers do not senesce or abscise following fertilization and seedlings are not responsive to ethylene, indicating that this mutation confers ethylene insensitivity throughout the plant (Lanahan 1994). The lack of *Nr* ripening confirms the essentiality of ethylene perception for ripening. Loss-of-function for any of the other receptors has no effect on ethylene sensitivity or ripening behavior (Kevany et al., 2007). Other genes involved in the tomato ethylene signaling pathway are indicated in Table 1. Among those, the ethylene-inducible transcription factors EIN3s and ERFs that activate ethylene responsive genes at the bottom of the signaling cascade. In concert with ethylene signaling a relatively small number of transcription factors regulate ripening (Giovannoni et al. 2017) (Tab. 4). The first such gene to be characterized was a SEPALLATA clade (E-class) MADS-box transcription factor gene that is partially deleted in the ripening inhibitor (*rin*) mutant (Vrebalov et al. 2002; Ito et al. 2008; Martel et al 2011). RIN-MADS activity contributes to the expression of hundreds of ripening-related genes, such as genes necessary for ethylene biosynthesis and perception (ACS- and ACO encoding genes), for carotenoid flux (PHYTOENE SYNTHASE 1 (PSY1) and LYCOPENE β-CYCLASE (LCYB)) and multiple cell wall–integral and carbohydrate-modifying proteins that shape the textural properties of the ripe fruit. A SQUAMOSA PROMOTER BINDING–LIKE PROTEIN (SPL) gene resides at the *Colorless non-ripening* (Cnr) locus and is necessary for manifestation of ripening (Manning et al., 2006). The CNR-SPL protein is required for RIN-MADS to interact with promoters of the ripening genes it regulates (Martel et al., 2011). A NAC-domain protein underlying the tomato nonripening (nor) locus is also essential for ripening, as defined by complete ripening inhibition in the homozygous nor/nor mutant in a manner that is both phenotypically and physiologically similar to the *rin* mutant (Martel et al., 2011; Osorio et al., 2011). Additional components of ripening regulatory network include TOMATO AGAMOUS-LIKE1 (TAGL1), APETALA2a (AP2a), and FRUITFULL (FUL1 and FUL2) (Table 1). Tomato fruit ripening-related TFs have recently been reviewed by Giovannoni et al., (2017), Karlova et al., (2014) and Seymour et al., (2013) and are reported in Table 1.

The most obvious ripening-related changes are alterations in fruit color due to the accumulation of pigments such as carotenoids and anthocyanins. In tomato, carotenoids accumulation occurs as the thylakoid membranes in the chloroplast break down and the plastids become chromoplasts.

Several nuclear genes encoding enzymes involved in the biosynthesis of carotenoids are highly transcribed at the beginning of ripening (Bramley, 2013). The best studied of these gene is phytoene synthase (*PSY1*) that catalyzes the first step in the carotenoid biosynthetic pathway. Phytoene is used as the precursor for the formation of the red pigment lycopene and down-regulation of *PSY1* abolishes normal carotenoid accumulation (Bartley et al., 1992; Fray and Grierson, 1993). Ripening involves other processes such as softening of the fruit tissues to facilitate seed dispersal (Isaacson et al 2009; Saladie et al 2007). This biological process involves a cell wall remodeling with changes in the texture of fruit guided by the expression of a large number of genes. In tomato, more than 50 cell wall structure related genes are expressed during fruit development or ripening (Tomato Genome Consortium, 2012). Thanks to the well-known network of transcriptional and hormonal regulators and to the availability of a high quality tomato genome sequence the tomato fruit has emerged as the preeminent model for study of fruit ripening and ethylene control of developmental processes.

1.2 Epigenetic regulation of fruit development and ripening in tomato

Recently, increasing evidence has indicated that the regulatory network of tomato fruit development and ripening include epigenetic modulations (Giovannoni et al., 2017). Epigenetic information is mainly based on DNA methylation and histone modifications that collectively determine the state of chromatin structure. They regulate gene expression by affecting transcription factor binding and activity or, conversely, being affected by transcription factors that recruit chromatin remodelling proteins during fruit ripening and development (Kaufmann et al., 2010). DNA methylation is a crucial reversible mark consisting in the addition of a methyl group to the carbon 5 of cytosine (5MeC). In plants DNA methylation occurs at cytosine residues in all DNA contexts (CG, CHG and CHH, where H represents A, C or T). In particular, cytosines in all sequence contexts can be de novo methylated through the well-known RNA-directed DNA methylation pathway (RdDM), in which 24-nt siRNAs guide the DNA methyltransferase domains rearranged methyltransferase 2 (DRM2) to methylate target loci (Zhong et al., 2013). DNA methylation can be maintained during replication; mCG and mCHG are maintained by the DNA methyltransferases DNA methyltransferase 1 (MET1) and chromomethylase 3 (CMT3), respectively, whereas mCHH is maintained by CMT2 and RdDM (Zhong et al., 2013; Kawakatsu

et al., 2017). Plants can also actively demethylate DNA through the activity of DNA Glycosylase-Lyases, the so-called DEMETER-Like DNA demethylases (DMLs), that remove methylated cytosine and replaced it by a non-methylated cytosine (Liu et al. 2015). A first evidence about the role of DNA methylation in tomato fruit ripening was provided by the discovery of *Cnr* mutant. As reported in the previous paragraph, *Cnr* is a rare example of an epiallele, in that it results from heritable hypermethylation which manifests in drastically reduced transcriptional activity (Manning et al., 2006).

Another epiallele has been reported in the wild species *S. pennellii*. Indeed, a gene encoding a 2-methyl-6-phytylquinol methyltransferase underlying a quantitative trait locus (QTL) for vitamin E was shown to be associated with differential methylation (Quadrana et al., 2014). Zhong et al. (2013) confirmed the role of DNA methylation during the fruit ripening by treating tomato fruits with 5-azacytidine (5 AzaC), an exogenous compound which is an inhibitor of DNA methylation. Indeed, after a treatment of 17 days post anthesis (DPA), tomato berries resulted in premature fruit ripening. The whole-genome bisulfite sequencing performed in four stages of fruit development, from immature to ripe, showed that, after injecting 5-azacytidine, more than 50,000 regions (representing 1% of the tomato genome) are differentially methylated. Remarkably, the degree of methylation of promoter regions decreased progressively along fruit development (Zhong et al., 2015). These included genes encoding proteins involved in carotenoid accumulation [PHYTOENESYNTHASE (PSY1) and 1,5-CIS-ZETA-CAROTENE ISOMERASE (SIGLA)], in ethylene synthesis (ACO1 and ACS2), in fruit softening [POLIGALATTURONASE (PG) and PECTINMETHYLESTERASE (PMEU1)], and in several previously mentioned transcription factors such as RIPENING INHIBITOR (RIN), NON-RIPENING (NOR), COLORLESSNON-RIPENING (CNR), and TAGL1. In addition, Chen and collaborators (2015) unravel that SlCMT3 silencing results in reduction of DNA methylation and enhanced key ripening TFs (i.e. *LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1*) gene expression as well as the expression of genes involved in the biosynthesis (*SlACS1*, *SlACS2*, *SlACS4* and *SlACO1*) and signal transduction (*SlEBF1* and *SlEBF2*) of the ripening hormone ethylene.

Likewise, the importance of DNA demethylation in regulating fruit ripening was suggested in the study of Teyssier et al. (2008) who showed a 30% decrease of the global DNA methylation levels in tomato pericarp, but not in locular tissues, during tomato fruit maturation. More recently, Liu et

al. (2015) highlighted the crucial relation between DNA demethylation and fruit ripening mediated by the tomato DML2. In particular, RNAi *SlDML2* knockdown results in ripening inhibition, via hypermethylation, of the expression of genes encoding ripening transcription factors. This gene was further repressed in the *Cnr* and *nor* mutants, but not in the *rin* mutant. *SlDML2* is responsible for the demethylation of as many as 29,764 genomic regions (Lang et al., 2017). The authors also suggested that *SlDML2* is necessary for the activation of hundreds of ripening-related genes, such as *RIN*, and genes involved in ethylene and pigment synthesis and cell wall hydrolysis. Genes in the carotenoid biosynthesis pathway, including *PSY1*, *Z-ISO*, *ZDS*, and *CRTISO*, were hypermethylated and silenced in the *sldml2* mutant. Many other genes known to be important for fruit ripening including *PG2a* and *PL* which are involved in cell wall degradation, *ACS*, *ACO*, and *ETR* (which are involved in ethylene biosynthesis or signaling) were hypermethylated and silenced in the mutants. Another study on tomato *DDB1*, coding for a key component of the CUL4-based ubiquitin E3 ligase complex, suggested that this protein plays an important role in controlling genes related to the organ size, growth habit and photosynthesis in an epigenetic manner (Liu et al., 2012; Tang et al., 2012). Transgenic tomato plants overexpressing an alternatively spliced DDB1 transcript displayed reduced organ size (flowers, seeds and fruits) and a decrease in DNA methylation level at the *WEE1* gene, a negative regulator of cell division. Reduced DNA methylation in the *WEE1* promoter was shown to be correlated with high expression levels of this gene in the transgenic plants, likely leading to growth arrest of the fruits (Liu et al., 2012; Tang et al., 2012). Notably, some of the phenotypes such as reduced organ size and high shoot branching observed in transgenic plants overexpressing the DDB1 splicing variant are independent of the presence of the transgene in subsequent generations, thus indicating an epigenetically control and transmission over generations (Liu et al., 2012).

In addition to DNA methylation, histone post-translational modifications (HPTMs) can influence chromatin structure and gene expression (Kouzarides, 2007; Berr et al., 2011). HPTMs depend on a wide range of enzymes and include the phosphorylation, methylation, acetylation and ubiquitination of various amino acids mainly in the histone tails. More than 60 residues, especially on H3 and H4 histones, were identified as substrate for HPTMs by mass spectrometry (Fig. 4). So far, four major chromatin states, corresponding to specific combinations of 11 different HPTMs and DNA methylation, have been determined in Arabidopsis that are preferentially associated with active or repressed genes, intergenic regions and transposons. In addition, some marks seem

preferentially associated to specific chromatin states. For example, histone acetylation is preferentially linked to gene expression whereas H3K9me2 seems to correlate with constitutive heterochromatin; tri-methylation of lysine 27 (H3K27me3) is associated with gene repression (Roudier et al., 2011).

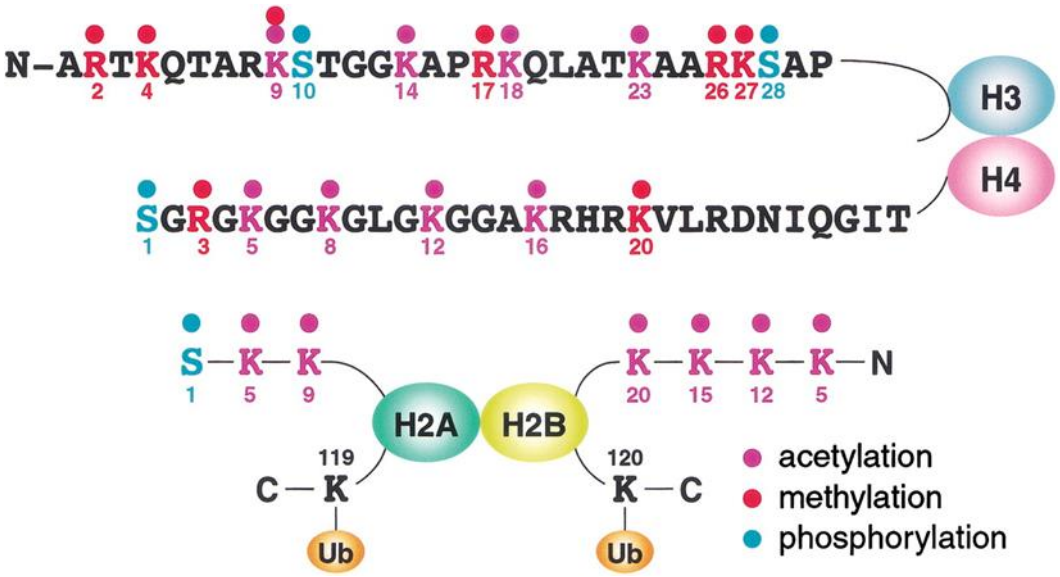

Figure 4. Major post-translational modifications on H3, H4, H2A and H2B histones. In red the methylation on arginine and lysine. In purple, acetylation on lysine. In blue, phosphorylation on threonine and in yellow ubiquitations on lysines 119 and 120. Modified from Zhang and Reinberg, 2001.

The expression pattern of histone modifiers, including histone deacetylases (HDACs), histone acetyltransferase (HATs), or histone methyltransferases (HMTs) reported in a range of fleshy fruits including apple (Janssen et al., 2008), citrus (Xu et al., 2015), grape (Aquea et al., 2010, 2011; Almada et al., 2011) and tomato (Cigliano et al., 2013; Zhao et al., 2014) suggests a function of HPTMs in the regulation of fruit development.

In tomato, histone modifications during fruit development are not as well documented as DNA methylation and even less during the fruit ripening. The polycomb group (PcG) proteins are involved in a repressive function via trimethylation of histone H3 on lysine 27. Three different polycomb-repressive complexes (PRCs) have been identified, called PRC1, PRC2 and PhoRC. The proteins belonging to PRC2 are the best studied in plants. PRC2 complex is composed of 4

different core proteins named Enhancer of Zeste [E(Z)], Extra sex combs (Esc), Suppressor of Zeste 12 [Su(z)12] and p55 (Teyssier et al. 2008). In tomato, two Enhancer of Zeste, *SlEZ1* and *SlEZ2*, preferentially expressed at early stages of fruit development during the cell division phase (Aiese Cigliano et al. 2013), are involved in controlling the carpels number and the carpels initiation (How Kit et al., 2010; Boureau et al., 2016). *SlEZ2* RNAi lines are characterized by modifications of fruit shape, color and cuticle deposition (Boureau et al., 2016). Another PcG component, *MULTICOPY SUPPRESSOR OF IRA1 (SlMSI1*) through chromatin remodeling inhibits fruit ripening by negatively regulating a large set of fruit-ripening genes (Liu et al., 2016).

1.3 Histone acetylation and deacetylation: Role of *HDA19*

In eukaryotes, histone acetylation and deacetylation are biological processes considered among the major factors influencing plant growth and development through facilitation of chromatin relaxation and gene transcription regulation (Waterborg, 2011). Histone acetylation is a dynamic and reversible process carried out by histone acetylases (HATs) and erased by histone deacetylases (HDACs). Histone acetylation has the potential to unfold chromatin since it neutralizes the basic charge of the lysine (Kouzarides, 2007), whereas HDACs, removing the acetyl groups added by HATs, reset the chromatin structure for the transcription. Furthermore, HDACs and HATs can function in protein complexes as transcriptional co-repressors and co-activators (Utley et al., 1998; Clayton et al., 2006; Yang and Seto, 2007) or associated with chromatin remodelers as modulators of the accessibility of DNA to different machineries. HATs and HDACs are classified into different families that are generally conserved in eukaryotes, including yeast, animals, and plants (Aiese Cigliano et al., 2013). Plant HATs include: (1) HAG for GCN5-related N-terminal acetyltransferases superfamily, (2) HAM for MYST superfamily, (3) HAC for CREB-binding protein (CBP) family, (4) HAF for TATA binding protein-associated factor (TAFII250) family. Plant HDACs are grouped into three families: the Reduced Potassium Deficiency 3 (RDP3/HDA1), the Sirtuin 2 (SIR2) and the HD-tuins (HD2). The first family is the most present throughout eukaryotes and is the most widely studied (Hollender and Liu, 2008). HD2 family originally determined in maize (Lusser et al., 1997) appears to be unique to plants and unrelated to the other families (Pandey et al. 2002). SIR2 family includes the homologous proteins to the yeast Silent Information Regulator 2 (Sir2), which is a nicotinamide adenine dinucleotide (NAD)-

dependent enzyme (Frye, 2000). In the past decades, several HDACs were purified and characterized especially in model plants. In Arabidopsis, HDACs belonging to the RPD3 Class 1 emerged as crucial players in reproductive processes including flowering, gametophyte development, embryogenesis and seed germination (Yu et al., 2011; Aiese Cigliano et al., 2013, Guo et al., 2016; Zhao et al., 2016; Van Zanten et al., 2014) as well as in responses to environmental cues (Haak et al., 2017). RPD3/HDA1 class include among others *HDA19* (Pandey et al., 2002). Several findings highlighted *HDA19* requirement in the reproductive development. Mutations in *AtHDA19* induced delayed flowering, flower abnormalities and seed set reduction in Arabidopsis (Tian et al., 2003). Tian and colleagues (2003) in a loss of function *athda19* mutant have evidenced abortive seed development. An elevated transcription of several seed maturation genes accompanied by an enrichment of histone acetylation at their promoters was found in *athda19* seedling. Moreover, *HDA19* was reported to form multi protein complexes with SCARECROW-LIKE15 (SCL15) (Gao et al., 2015) and HIGH-LEVEL EXPRESSION OF SUGAR_INDUCIBLE GENES2-LIKE1 (HSL1) (Zhou et al., 2013), both driving the repression of seed specific gene expression. Genetic analyses revealed that the homozygous *hsl1 hda19* double mutant is embryonic lethal, thereby suggesting that *HDA19* and *HSL1* play a vital role during embryogenesis. Wang and colleagues (2013) showed a decreased seed dormancy in *hda19* mutant as well as in *snl1snl2* double mutant characterized by loss-of-function of SWI-INDIPENDENT3 (SIN3)-LIKE1 (SNL1) and SNL2. They demonstrated that *SNL1* interacts with *HDA19* in a protein complex required to promote seed dormancy through the transcription modulation of genes involved in ethylene and ABA pathways. Seeds of *snl1* and *snl1snl2* release more ethylene and have a markedly reduced ABA content than the WT. *HDA19* is also required for ABA dependence of seed germination. *hda19* mutant is hypersensitive to ABA during germination, indeed a low percentage of germination was shown by *hda19* seeds treated with ABA (Chen and Wu, 2010). Furthermore, the Histone Deacetylation Complex I (Perrella et al., 2013) is associated with *HDA19* as part of the same histone deacetylase complex (Mehdi et al., 2015). *Hdac1* knockout phenocopied *hda19* with respect to ABA sensitive germination (Perrella et al., 2013). The authors of this study speculate that HDAC1 could stabilize the histone deacetylase complex and/or its association with the chromatin. In Arabidopsis, *HDA19* physically interacts with the transcription factor APETALA2 (AP2) negatively regulating multiple floral organ identity genes (Krogan et al., 2012). *AtAP2* is also involved in seed development and regulation of seed

size (Jofuku et al, 2005). In tomato the AP2 homolog (*SlAP2a*) has been elucidated as a major regulator of tomato fruit ripening. RNAi repression of *SlAP2* results in fruits that ripen earlier, overproduce ethylene, and have altered carotenoid levels (Chung et al., 2010; Karlova et al., 2011). The demonstrated regulatory role of *HDA19* in reproductive processes and the evidence that the tomato *HDA19* homolog is expressed in buds and is up regulated in fruit at 1 cm stage, breaker and red ripe stages (Aiese Cigliano et al., 2013) (Fig. 5) suggest that *SlHDA19* likely play an important role in tomato fruit development and ripening.

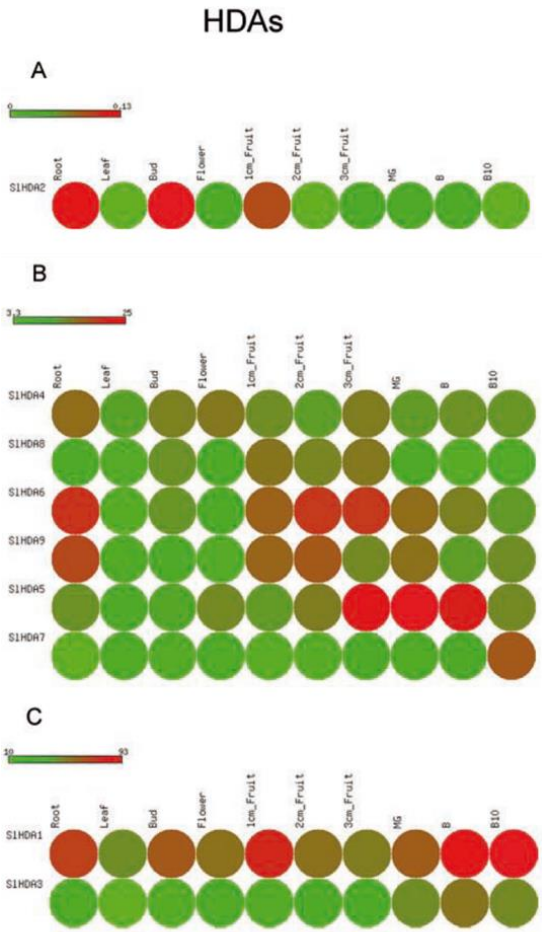

Figure 5. Expression profiles of tomato HDACs with low (A), middle (B) and high expression (C) in different organs and developmental stages. SlHDA1 corresponding to SlHDA19 is among the most expressed HDACs during the fruit development and ripening. Expression values are measured as reads per kilobase of exon model per million mapped reads (RPKM). Modified from Aiese Cigliano, 2013

1.4 Histone variants: Role of *ARP6*

Plant histone family contains a number of variants with small differences in amino acid sequence and structure, resulting in changes in affinities for DNA or histone binding proteins. The most characterized histone variants belong to the H3 and H2A families (Probst and Mittelsten Scheid, 2015). In Arabidopsis histone H3 is present in two variants: H3.1 and H3.3 which differ by four amino acids (Shi et al., 2011). Histone H2A variants are instead H2A.X, H2A.Z, and H2A.W. The histone H2A family encompasses the largest number of variants, with the histone H2A family member Z (H2A.Z) being the most evolutionarily conserved (Papamichos-Chronakis et al. 2011). H2A.Z function in multiple processes, including transcription, DNA repair, response to environmental stresses (Coleman-Derr and Zilberman, 2012; Malik and Henikoff, 2003; Jarillo and Pineiro, 2015; Sura et al. 2017; Haak et al. 2017). H2A.Z deposition/removal is controlled by the ATP-dependent chromatin-remodeling complex SWR1 (SWR1c), a member of the Inositol requiring 80 (INO80) family of remodelers (Nguyen et al. 2013). Homologs of the 14 yeast and 11 human SWR1 subunits have been identified in Arabidopsis (March-Díaz and Reyes, 2009; Meagher et al., 2009), indicating that the SWR1 complex (SWR1c) exists also in plants. It has been shown that Arabidopsis mutants for genes encoding key subunits of the SWR1c (i.e. *PIE, ARP6* and *SUF*) are not able to efficiently incorporate H2A.Z into nucleosomes. Indeed, March-Díaz and coworkers (2008) shown mutants in those genes with phenotype similar to the H2A.Z double mutant *hta9 hta11*. Mutants in the ACTIN-RELATED PROTEIN 6 (*ARP6*), one of the subunits of the SWR1c, has been widely used to study the effects of H2A.Z depletion from chromatin (Choi et al., 2013; Smith et al., 2010; Bieluszewski et al., 2015; Zilberman et al., 2008; Rosa et al., 2013). In Arabidopsis, ARP6 and H2A.Z have been shown to control gene expression underlying development and environmental responses (March-Díaz and Reyes, 2009; Meagher et al., 2009). Plants defective in *ARP6* exhibit global reduction in size, curly leaves, altered inflorescence and flower morphology, and early flowering (Choi et al., 2005; Deal et al., 2005; Deal et al., 2007; Jarillo and Pineiro, 2015). Moreover, in *Atarp6* mutant Rosa et al. (2013) reported short and misshaped siliques with a seed set reduced of ≈50% due to impaired male and female gametophyte development. Defects in gametogenesis are frequently observed in mutants impaired in meiosis

(Li et al., 2004; Siaud et al., 2004; Samach et al., 2011). Indeed, Qin et al. (2014) observed defects in prophase I of female meiosis. Recently, Sura et al. (2017) observed a delayed germination for *hta9 hta11* and *arp6* mutants suggesting that nucleosome H2A.Z deficiency affect seed germination. Moreover, the authors reported that stress conditions such as saline stress make the seed germination worse. Kumar and Wigge (2010) revealed that H2A.Z is of great importance in regulating responses to heat and cold stress. Using a forward genetic screen approach, nucleosomes containing the H2A.Z variant were found to be essential for temperature perception. Transcriptome analysis of *Atarp6* plants displayed a constitutive up-regulation of genes induced by warm temperature (27°C), when the plants were grown at 12°C (Kumar and Wigge, 2010). A ChIP profile of H2A.Z on the *HSP70* gene showed eviction of H2A.Z during exposure to high temperatures at transcriptional start sites. Lack of H2A.Z allows RNA Polymerase (POL II) to initiate transcription. Therefore, failure of H2A.Z incorporation leads to a constitutively high expression of genes induced by heat.

2. Aim of PhD thesis

The aim of the present thesis is the identification and the functional characterization of two epiregulators in tomato, Histone Deacetylase 19 (HDA19) and Actin Related Protein 6 (ARP6). The role of SlHDA19 was investigated during the development and the ripening of tomato fruit. ARP6 was studied in response to abiotic stresses and in male meiosis during recombination.

The strategy was based on a reverse genetic approach mediated by artificial micro RNA (amiRNA) silencing. The research activity presented in this thesis was carried out at CNR-Institute of Biosciences and Bioresources, Portici in collaboration with prof. Jim Giovannoni at the Boyce Thompson Institute for Plant Research (BTI), NY, USA and the Department of Agriculture of University of Naples "Federico II".

3. Materials and methods

3.1 Plant Materials and Growth Conditions

Tomato *(Solanum lycopersicum)* material include amiHDA19 (herein *hda19-2*, *hda19-5* and *hda19-6*), and amiARP6 (herein *arp6-11* and *arp6-14*) lines and cv. Ailsa Craig as wild type (herein WT). For transformation, *A. tumefaciens* ElectroMax LBA4404 (Invitrogen, Italy) was used in co-colture with tomato cotyledons according to McCormick (1991). Transformants were selected on kanamycin (100 mg L^{-1}). Plants were grown in a controlled greenhouse with a photoperiod regime of 16/8 h light/dark, at 27°C/19°C and 70% of relative humidity. The plants used for the heat stress treatment were grown in artificial climate incubator under standard condition (16/8 h light/dark at 26°C/22°C) before the experiment. All the analysis were performed on transgenic plants in T_1 and T_2 generation. Primary transformants (T_0) were used for the copy number determination. For ripening time course aimed at molecular and biochemical analyses, fruits which have been tagged at 1cm corresponding to 7 days post anthesis (DPA) were collected at Breaker (BR) stage and between 1 and 7 days before Breaker (Br +1 to Br +7).

3.2 Artificial MicroRNAs

The specific artificial microRNA to silence *SlHDA19* (Solyc09g091440) and *SlARP6* (Solyc05g018600) and the oligonucleotide sequences were designed using the WMD3 Web tool according to the procedures and criteria described by Schwab and colleagues (2010; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The predicted mature microRNA sequence were 5'-UAUUCAGUAUCCGGUGGGCGC-3' and 5'-UAAAUAGAGUACUGCCGCCCG-3' for *SlHDA19* and *SlARP6*, respectively. Primers used in the construction of amiRNAs are listed in the table 2. The cloning of amiHDA19 and amiARP6 was performed using the miR319a precursor-containing plasmid pRS300 as a template (Schwab et al., 2010). Primers A and B were modified to allow the cloning of the final PCR product with Gateway technology into pK2GW7 binary vector (http://gateway.psb.ugent.be) using pDONR/ZEO (Invitrogen) as donor.

Table 2. List of primers used to clone amiHDA19 and amiARP6.

Primer	Sequence 5'-3'
ARP6_I_miR-s	gaTAAATAGAGTACTGCCGCCCGtctctcttttgtattcc
ARP6_II_miR*a	gaCGGGCGGCAGTACTCTATTTAtcaaagagaatcaatga
ARP6_III_miR-s	gaCGAGCGGCAGTACACTATTTTtcacaggtcgtgatatg
ARP6_IV_miR*a	gaAAAATAGTGTACTGCCGCTCGtctacatatatattcct
HDA19_I_miR-s	gaTATTCAGTATCCGGTGGGCGCtctctcttttgtattcc
HDA19_II_miR*a	gaGCGCCCACCGGATACTGAATAtcaaagagaatcaatga
HDA19_III_miR-s	gaGCACCCACCGGATTCTGAATTtcacaggtcgtgatatg
HDA19_IV_miR*a	gaAATTCAGAATCCGGTGGGTGCtctacatatatattcct
AttB1-amiRNA-Fw (A)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCCAAACACACGCTCGGA
AttB2-amiRNA-rev (B)	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCATGGCGATGCCTTAA

3.3 Expression Analysis

Total RNA was extracted from leaves of 4-week-old seedlings using the RNeasy Plant Mini Kit (Qiagen, Germany) and treated with DNAase I (Life Technologies, Italy) according to manufacturer's protocols. Quantitative and qualitative concentration measurements were performed using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). To obtain complementary DNA, SuperScript III Two-Step RT-PCR and Oligo (dT) 12-18 (Invitrogen) were used following the manufacturer's instructions. Gene-specific primers designed using Real time qPCR assay entry software (IDT company), are listed in Table 3. Real-time RT-PCR was carried out using the Applied Biosystem 7900 HT with SYBR green master mix (Life Technologies, Italy). Ubiquitin and Actin genes were used as reference.

Table 3. List of primers used for Real time RT-PCR experiments.

Primer	Gene ID	Sequence 5'-3'
HDA19-fw	Solyc09g091440	TGGGATGCTGATTCTGACAC
HDA19-rev		CGATTCTACTTCTCTTAGGTGCTC
ARP6-fw	Solyc05g018600	TCGAGAACACTGACAATTCCG
ARP6-rev		TGATTCAGTCCCAAGTCAGC
SlHSP90-fw	Solyc03g007890	TTCTTGGTGACAAGGTCGAA
SlHSP90-rev		ATCAGGATTAATTTCCATCGTCT
SlHsfA1-fw	Solyc08g005170	ACTTCTCCAGCTTTGTTCGG
SlHsfA1-rev		TCCATGAGCAGGTTTACGTC

SlHsfA2-fw	Solyc08g062960	TTCCACCACATTGTTGCCTA
SlHsfA2-rev		GCAAGCACCAGATCCTTGTT
SlHSP70-fw	Solyc04g011440	GGAAGTGGACTAAGCTCCACA
SlHSP70-rev		CGAAGGATATTTCTACATACACAAA
SlHSPMT-fw	Solyc08g078700	GCGGTGGAGGAGAACACGCT
SlHSPMT-rev		TCTCCGCCTTGATTCCATCCA
SlUBI-fw	Solyc07g064130	GGACGGACGTACTCTAGCTGAT
SlUBI-rev		AGCTTTCGACCTCAAGGGTA
SlACT-fw	Solyc11g005330	AGGTATTGTGTTGGACTCTGGTGAT
SlACT-rev		ACGGAGAATGGCATGTGGA

3.4 Cytology

Chromosomes number was determined on root tips harvested from seeds germinated on Petri dishes lined with filter paper, using a protocol performed by Chen *et al.* (2015) with some modifications. Briefly, root tips of 1-2 cm were pre-treated with 0.002 M 8-hydroxyquinoline for 4 h at room temperature in dark to arrest cells in metaphase, rinsed in distilled water and fixed in Carnoy's fixative (3:1 ethanol/acetic acid) (v/v) for 48 h. The samples were subsequently stored at -20°C for at least 48 h, or, alternatively, until use. Ten root tips for each sample were analysed. After two washes in distilled water, tips were incubated in a solution of 4% cellulase (Sigma) and 2% pectinase (Sigma) at 37°C for 30 minutes, rinsed in cold distilled water and re-fixed with cold freshly prepared 3:1 fixative solution. Before slides preparation tips were stained with aceto-carmine and then squashed in 45% acetic acid. Chromosomes were observed and imaged by a Leica DM R microscope equipped with a DFC 425 C camera (Leica Microsystems, Germany). Pollen viability was assessed by Alexander's staining (Alexander, 1969). For embryo development analysis, seeds were excised from different-stage fruits (10, 15, 20, and 25 DPA) previously fixed in ethanol:acetic acid (3:1) (v/v). They were cleared with a chloral hydrate solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water) and examined by differential interference contrast (DIC) microscopy (Leica DM6, Leica Microsystems, Germany). For microsporogenesis analysis young flower buds (0.1-0.2 mm long) were fixed in Carnoy's fixative (3:1 ethanol/acetic acid) (v/v) for at least 48 h. Meiosis was investigated using the spreading technique described by De Storme and Geelen (2013). Briefly, fixed floral buds rinsed twice in ddH2O and twice in 10 mM citrate buffer (pH 4.5) were digested in 0.3% (w/v) enzyme mixture consisting of cellulase (Sigma)

and pectolyase (Sigma) for 3 h at 37°C. After digestion, buds were rinsed in ddH2O. Each whole bud was then transferred to a slide where meiocytes were released from anthers by a needle. Fifteen µL of acetic acid (60% v/v) were added and slides were heated at 45°C for 30 s. The meiocytes were then fixed with Carnoy's fixative. The slides were air dried and stained using 4'-6-diamidino-2 phenylindole (DAPI, 10µg/mL). Slides were analyzed by a fluorescent microscope (Leitz, Aristoplan).

3.5 Copy Number Analysis

Transgene copy number was determined using Standard Addition Quantitative Real-time PCR (SAQ-PCR) as described by Huang et al. (2013). Tomato Phytoene Desaturase (PDS) (Solyc03g123760), a single copy gene (Corona et al., 1996), was selected as the internal reference gene, and neomycin phosphotransferase gene (NPT II) as the integrated target gene in this study.

3.6 Germination seed assay and salt stress treatment

Germinability tests were conducted on fully developed T2 seeds. Different batches of seeds were used throughout the study but the same batch was used within each experiment. The seeds of WT and amiRNA lines were collected at same period and stored at the same condition. Seeds were surface-sterilized by shaking in 70% (v/v) ethanol for 1 minute followed by wash in 20% commercial bleach (5.6% sodium hypochloride), followed by three rinses with sterile water. The seeds were then sown on media containing half-strenght of Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) and 0.8% (w/v) agar. For salt stress treatments, seeds were sown in the presence of NaCl (60 mM and 150 mM). Germination was scored daily in terms of first macroscopic appearance of root tips and of fully expanded cotyledons. To score growth of shoots and roots their lengths were measured on 14-days-old seedlings. Data were collected from three biological replicates.

3.7 Ethylene Measurement

Ethylene was measured by sealing whole fruits in airtight jars for at least 3 h at 22 °C, after which a 1-mL sample of the headspace was taken and injected on to an Agilent 6850 II gas chromatograph equipped with a flame ionization detector. Samples were compared with a standard of known concentration, normalized for fruit mass and time.

3.8 Carotenoid Extraction and Analysis

Carotenoids were extracted according to the method described by McQuinn et al. (2017). The HPLC was performed as described by Vrebalov et al. (2009).

3.9 Heat stress treatment

Heat stress (HS) treatment was conducted on four-week-old seedlings incubated at 39°C/26°C day/night cycle in artificial climate incubator for 7 days. Leaf samples from stress treated seedling were collected at 0 (control), 2, 8, and 72 h and at 7th day of treatment. All plant samples were frozen in liquid nitrogen and stored at -80°C for expression analysis.

3.10 Photochemical Efficiency, Electrolyte Leakage, and Relative Water Content

Injury to tomato plants was examined by measuring chlorophyll fluorescence and electrolyte leakage as described by Wu et al. (2012). Photochemical efficiency of leaves, as determined by chlorophyll fluorescence ratios (Fv/Fm), was monitored from the adaxial side of the leaf using a Fluorcam 800MF (Photon Systems Instruments, Czech Republic). Relative Water Content of tomato leaves was conducted according to the protocol of Sura et al. (2017). All the measurements were taken during and after (0, 2, 8, and 72 h and 7 days) the heat treatment.

3.11 Trichostatin A (TSA) treatment

Injections for TSA treatment were conducted according to Zhong et al. (2013) with some modifications. Briefly, the columella of tomato fruits at 10 DPA was needle-injected once from the pedicel with 100 µL of 15 µM TSA water solution. The control fruits were injected with 100 µL of water (Mock).

3.12 Statistical analysis

Data are shown as mean ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's test as post-hoc test. Different letters mean statistical different values.

4. Results

4.1 Functional characterization of tomato *HDA19* gene by loss-of-function strategy

4.1.1 In Silico Analysis of SlHDA19 expression in tomato fruit

SlHDA19 was reported to be highly expressed in the pericarp of tomato fruit at early fruit stage and at breaker up to the red ripe stage (Aiese Cigliano et al., 2013). To update the expression profile of *SlHDA19* in tomato fruit we interrogate the Tomato Expression Atlas (http://tea.sgn.cornell.edu) which reports transcriptome data from laser-capture micro-dissected tissues in M82 (Fernandez-Pozo N et al., 2017; Pattison RJ et al., 2015).

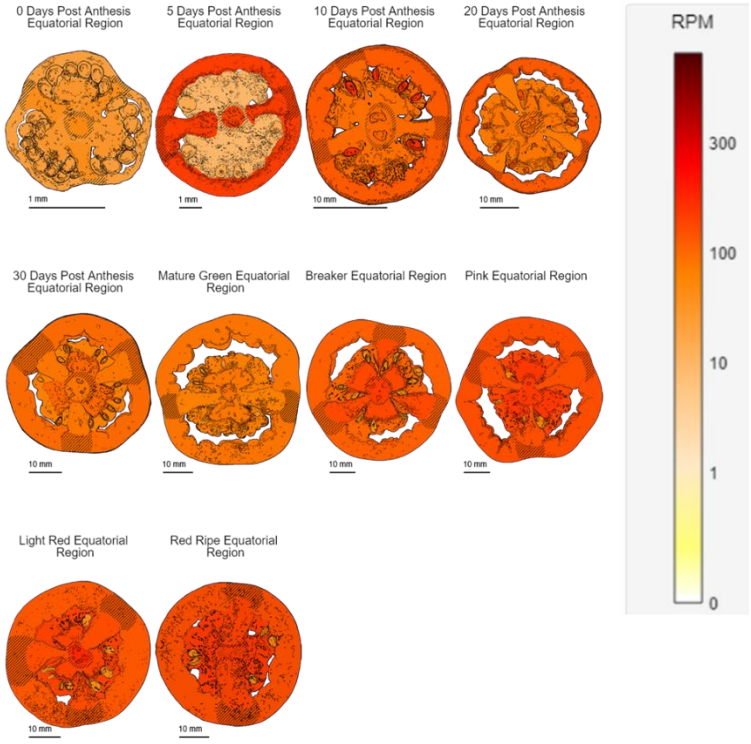

Figure 6. SlHDA19 expression pattern in tomato fruit (M82) at different developmental stages. Source: Tomato Expression Atlas (Fernandez-Pozo et al., 2017; Pattison et al., 2015 – http://tea.sgn.cornell.edu)

SlHDA19 shows a peak of expression in fruit at 5 and 10 days after anthesis, within the pericarp and the seeds, respectively. In pericarp tissue, the *SlHDA19* transcriptional activity remain constant from 20 days post anthesis to the mature green stage and it starts to rise again until the fruit is

completely ripe. On the contrary, the seeds shown a downregulation of *SlHDA19* up to red ripe stage (Fig. 6). We extract additional information out of a database reporting RNA-seq data from the tomato *cv.* Ailsa Craig, at four developmental stages corresponding to Immature Green (IG), Mature Green (MG), Breaker (Br) and 10 days after breaker (Br + 10) (private database from Prof. J.J. Giovannoni). *SlHDA19* is not highly expressed, in absolute terms, throughout the fruit ripening but it gradually increases in the expression from IG stage to Br +10 stage (Fig. 7).

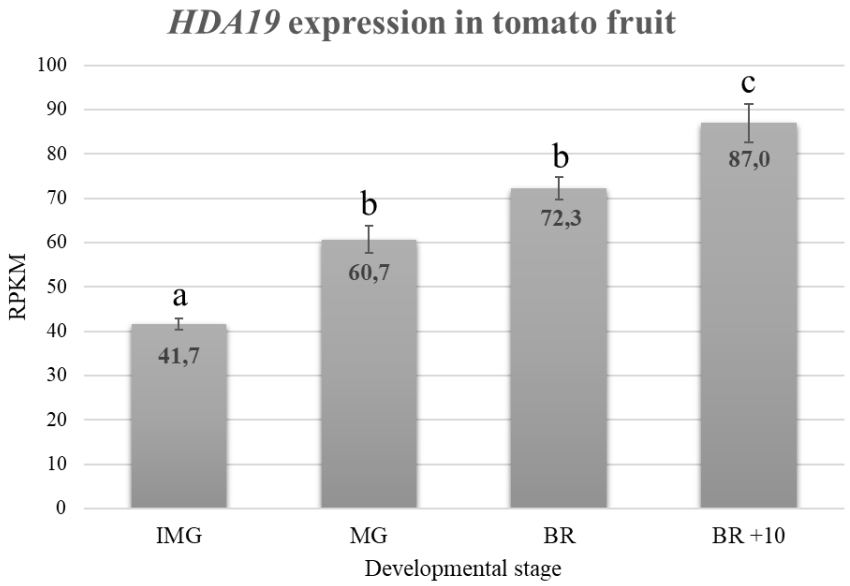

Figure 7. Expression profile of SlHDA19 in tomato fruit at four stages (cv. Ailsa Craig). Expression values are measured as reads per kilobase of exon model per million mapped reads (RPKM). Different letters mean statistical different values. P≤0.05. IG=Immature Green; MG=Mature Green; Br= Breaker. RNA-seq data are from private database of Prof. J.J. Giovannoni

4.1.2 Production of HDA19 Knockdown Tomato Lines

On the basis of in silico analysis, HDA19 was select to assess its biological function. RNA interference mediated by artificial miRNA (amiRNAi) was performed to silence *HDA19*. We designed an amiRNA with WMD3 tool (http://wmd3.weigelworld.org/) that was cloned into the expression vector pK2GW7 (pK2GW7_amiHDA19). After genetic transformation, nine To independent lines were selected by kanamycin resistance. Transgene integration was further

confirmed by PCR of the kanamycin resistance gene from plant genomic DNA. At maturity, the majority of the T_0 plants showed a reduced fruit size compared with control plants, and a reduction in seed number, as well. Three lines that shown a strong down-regulation of *SlHDA19* (*hda19-2*, *hda19-5* and *hda19-6*) were selected for further characterization. As shown in Fig. 8, *hda19-6* exhibits the highest downregulation of *SlHDA19*, 66% less than the WT, while *hda19-2* and *hda19-5* showed 44% and 39% of downregulation, respectively. To assess the transgene copy number, a Standard Addition Quantitative (SAQ)-PCR led us to detect a single T-DNA insertion in both *hda19-2* and *hda19-5*, and two insertions in *hda19-6*. To ascertain whether the regeneration of tomato seedling affected the chromosome number, ploidy analysis on root tips from *hda19-2*, *hda19-5* and *hda19-6* mitotic cells were performed. All these lines showed the diploid chromosome number of *S. lycopersicum* (2n=2*x*=24). To assess the inheritance of the transgene, T_1 progenies from the three *hda19* lines (T_0) were obtained. Substantial *HDA19* down-regulation was observed in each *hda19* progeny (Fig. 9).

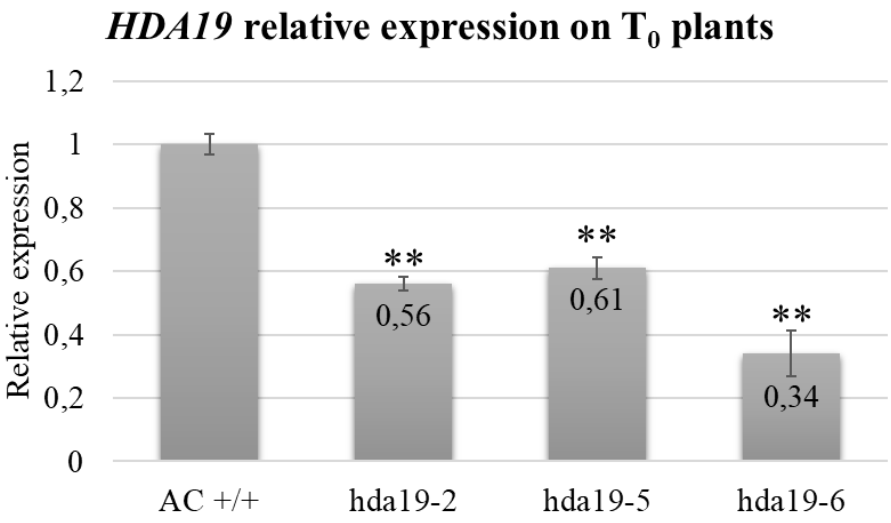

Figure 8. Expression analysis of HDA19 in hda19-2, hda19-5 and hda19-6 leaf respect to the WT (AC +/+). The expression values are the average of three replicates, the standard error is reported as black vertical bar. **P≤0.01 *P≤0.05.

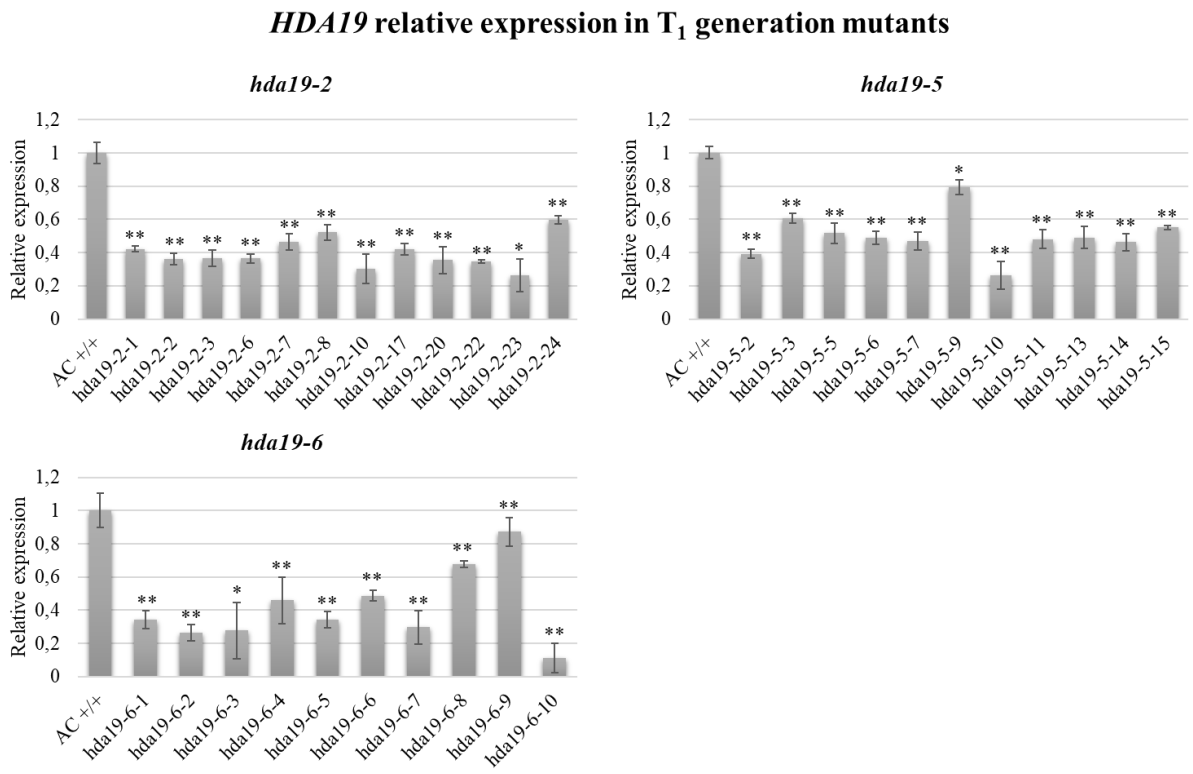

Figure 9. Expression analysis of HDA19 in T_1 progeny of hda19-2, had19-5, and hda19-6 leaf. The expression values are the average of three replicates, the standard error is reported as black vertical bar. **P≤0.01 *P≤0.05.

4.1.3 Phenotype of *HDA19* amiRNA Lines

To assess whether *HDA19* has a role in tomato development, *hda19-2*, *hda19-5* and *hda19-6* mutants (T_1) have been analyzed for different aspects related to plant growth and reproduction. The analyzed traits such as plant height, internode length, stem diameter, number of leaves and flowers, were not significantly different between WT and all *hda19* lines. Most characters associated to the flower phenotype (i.e. size and number of petals or sepals) were also not altered. Fruit size was consistently smaller than WT and there was no difference in locule number and pericarp thickness (Fig. 10). The fruit weight was reduced of 23%, 34% and 39% in *hda19-2*, *hda19-5* and *hda19-6*, respectively.

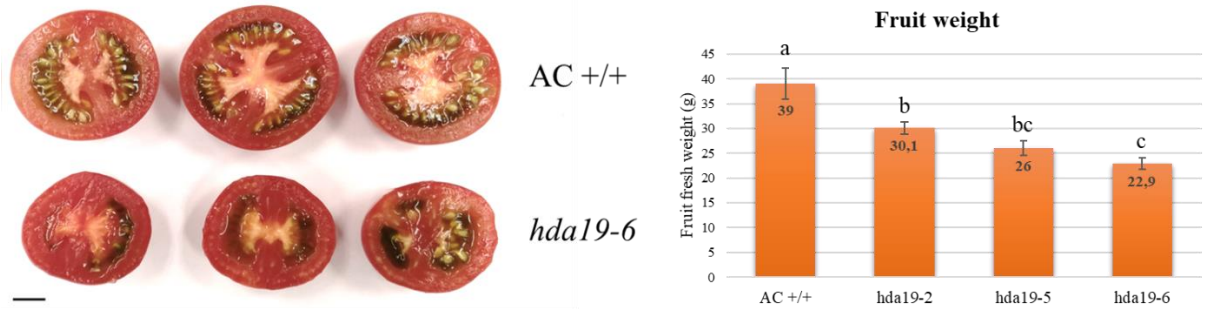

Figure 10. Cross section of fruits (A) and fruit weight (B) of WT and Slhda19 lines. Between 30 and 50 fruits were collected at Br +3 stage and immediately weighted. Different letters mean statistical different values P≤0.01

During the process of fruit development, fruits from *hda19* lines exhibited earlier and more pigment accumulation at the onset of ripening (Breaker +2) compared with WT (Fig. 11) suggesting alteration in carotenoid content and/or composition.

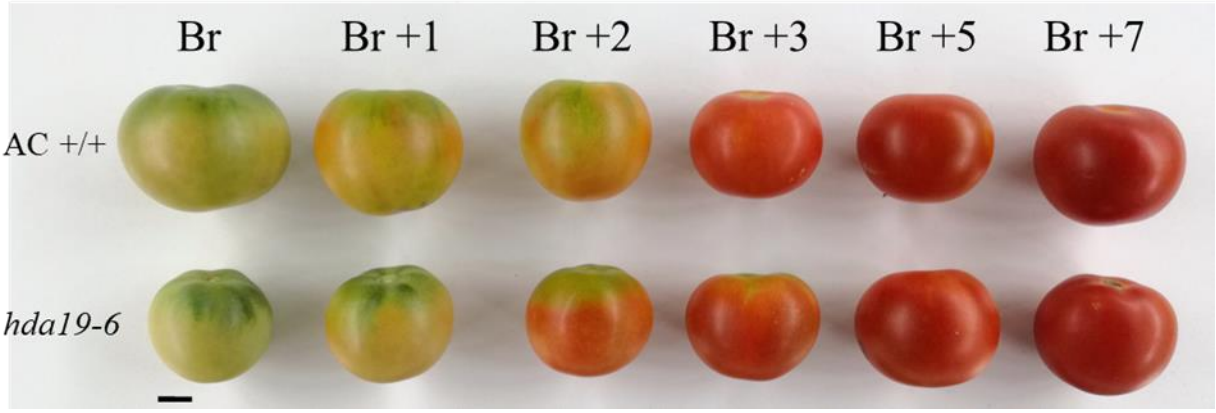

Figure 11. Ripening fruits of WT and slhda19-6. Br= Breaker. Black bar is 1 cm.

When the seeds from *Slhda19* fruits were examined, we found that the seed development was strongly affected (Fig. 12). Indeed, the number of fully developed seeds per fruit was significantly reduced of 82% in both *hda19-2* and *hda19-6* (n=21 seeds), and of 72 % in *hda19-5* (n=33 seeds) respect to the WT (n=120 seeds) (Fig. 13). However, the number of total seeds (developed and undeveloped) was counted and it did not differ between *hda19* and the WT. To assess the seed viability, both seed types, developed and undeveloped, isolated from *hda19* fruits were sown in

vitro to check their germination ability. While germination rate of developed seed was not affected, *hda19* undeveloped seeds did not germinate at all.

It is noteworthy that *hda19-6* showed the most severe phenotype in agreement with the weakest expression of *SlHDA19* among the three suppressed lines under study.

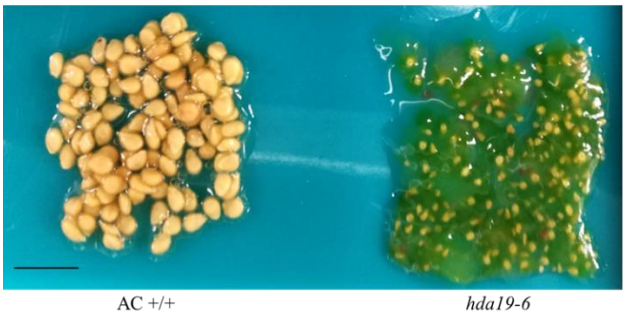

Figure 12. Undeveloped seeds from slhda19-6 fruits and a normal seeds from WT.

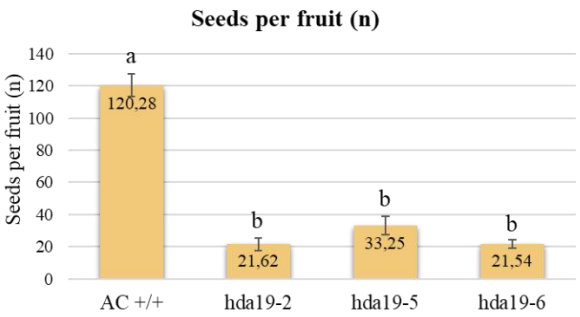

Figure 13. Number of fully developed seeds in each fruit in slhda19 lines and wild type. Different letters mean statistical different values. P≤0.01.

4.1.4 *SlHDA19* Impacts Ethylene Production

Given that the *SlHDA19* gene is upregulated at the onset of fruit ripening, we measured ethylene production as the primary regulatory parameter associated with fruit development and ripening. Ethylene production of *hda19-2* and *hda19-6* lines showed a higher induction of approximately 1.5 fold starting from 3 days post breaker stage and it remained at higher levels through 7 days post breaker stage with a similar pattern as compared with control fruits (Fig. 14). At the end of the time course (Br + 7), *hda19-2* and *hda19-6* exhibit, respectively, about 90% and 40% more ethylene than the WT. The enhancement in ethylene production rate at different time points suggests that *SlHDA19* is a negative regulator of ethylene biosynthesis in maturing fruits.

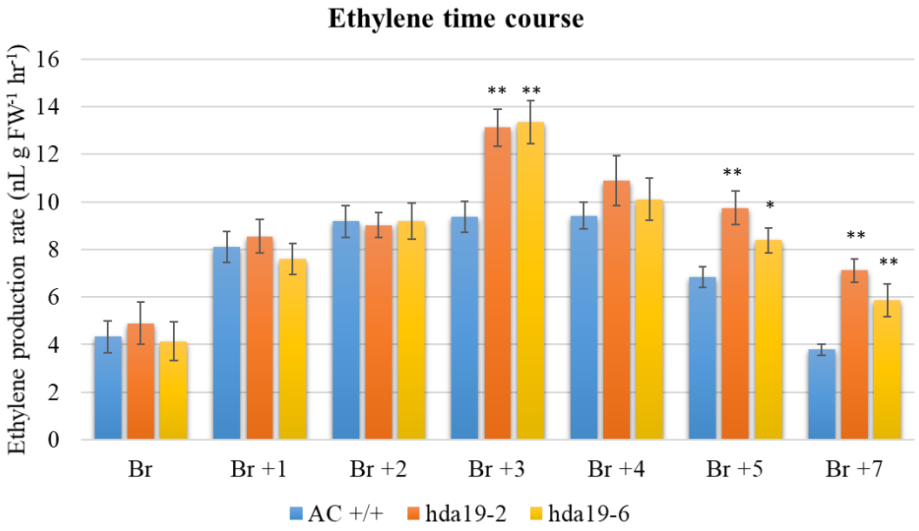

Figure 14. Production of ethylene in control and Slhda19 lines. Fruits of different ripening stages (Br, Br+1, Br+2, Br+3, Br+4, Br+5, Br+7) were sealed in airtight vials and 1-ml of gas was sampled from the headspace after 3 h. **P≤0.01. *P≤0.05

4.1.5 *hda19* lines exhibit an early accumulation of carotenoids in the pericarp during the ripening

To characterize the carotenoid accumulation profiles of *hda19-6* fruits we performed carotenoid analysis via HPLC. *Hda19*-6 revealed distinctly different carotenoid profiles respect to WT fruits (Fig. 15). In particular, major carotenoids such as lycopene, *β*-carotene, lutein and phytoene were accumulated at Br +2 days at a higher amount in *hda19-6* than in WT fruits. Consequently, *hda19-6* fruits had a 3.4 fold greater content of total carotenoids at Br +2 stage. At Br +5 days, the lycopene was about 2-fold less in *hda19-6* than in WT accounting for the total carotenoids reduction observed at this stage in *hda19-6* (Fig. 16). At Br +7 days, β-carotene amount increased about 1.6 fold in *hda19-6* whereas phytoene is slightly reduced compared with WT fruits. At the same stage (Br +7) both *hda19*-6 and WT accumulate the same amount of lycopene and lutein. However, the total amount of carotenoids is not significantly different at Br +7 between *hda19-6* and WT. In summary, *SlHDA19* repression is associated to a significant increase of total carotenoids at the onset of ripening thereby conferring the earlier pigmentation gained by *hda19-6* and to a *β*-carotene accumulation that, however, is not enough to confer a visible difference in fruit color at maturity.

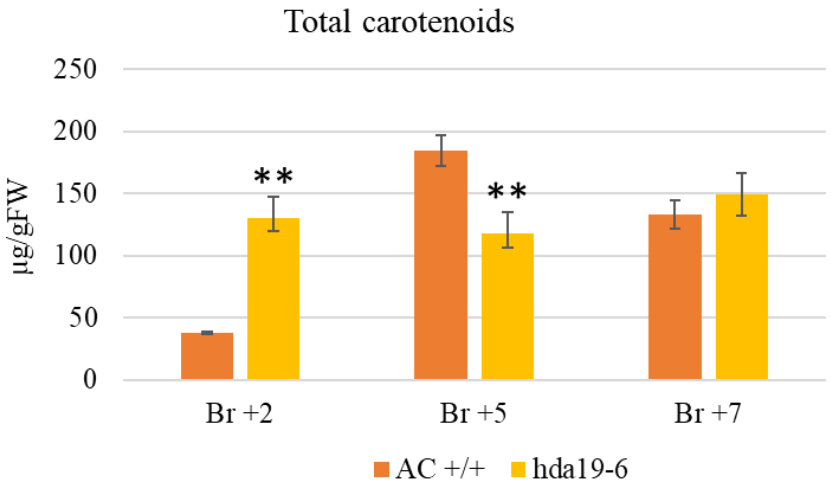

Figure 15. Total carotenoids in WT and hda19-6 fruits at different ripening stages (days after breaker). The carotenoid content (µg/gFW) is average of three biological replicates. The standard error is reported as black vertical bars. Asterisks mark statistical significant differences between hda19-6 and the control as verified by t-test (p<0.01). Br = breaker

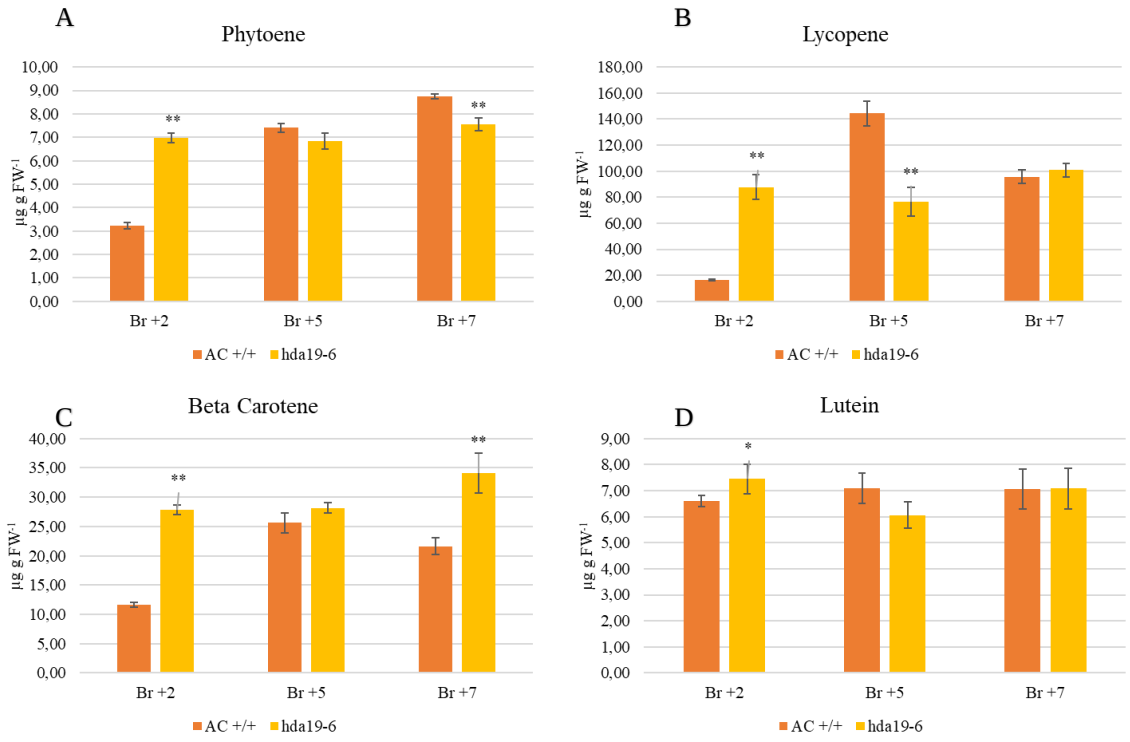

Figure 16. Major carotenoids, Phytoene (A), Lycopene (B), β-carotene (C) and Lutein (D) in WT and hda19-6 fruits at different ripening stages (days after breaker). The content of each carotenoid (µg/gFW) is average of three biological replicates. The standard error is reported as black vertical

bars. Asterisks mark statistical significant differences between hda19-6 and the control as verified by t-test (**P≤0.01; *P≤0.05). Br = breaker

4.1.6 *SlHDA19* is required for embryo development

In silico analysis performed in this work evidenced that *HDA19* was significantly up-regulated during the early development of seeds. Moreover, as above reported, when we evaluated the seed morphology, all the mutants (*hda19-2*, *hda19-5* and *hda19-6*) showed a strong reduction in the number of fully developed seeds. In order to identify the role of *HDA19* in seed development and consequently the cause underlying the undeveloped seeds, we analyzed clarified fruits at different developing stage by microscopy.

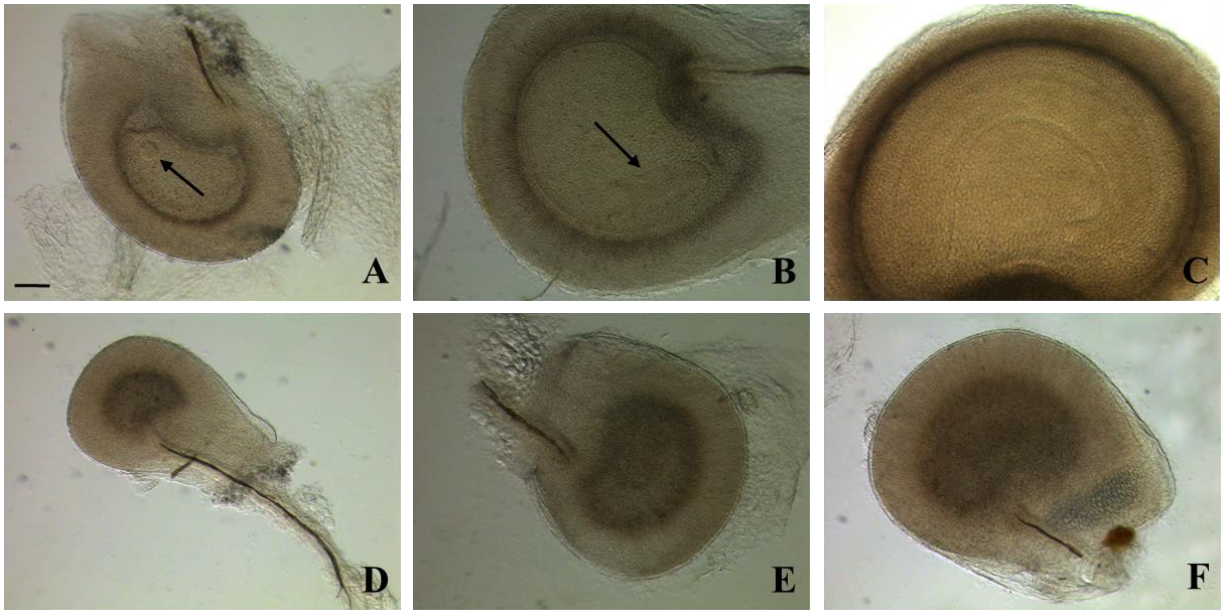

Figure 17. Embryogenesis of WT (A,B,C) and hda19 (D,E,F). globular embryo stage (indicated by arrow) at 10 DPA (A); Early torpedo stage (indicated by arrow) at 15 DPA (B); Coiled embryo stage at 20 DPA (C); globular embryo stage of hda19 at 10, 15 and 20 DPA (D,E,F). Black bar is 100 µm.

By this way, defects affecting embryo development were observed in *hda19* (Fig. 17). In particular, *hda19* mutant embryos were arrested at early globular stage before the onset of embryonic and seed maturation phase (Fig. 17E). For that reason, seeds remained smaller than WT (Fig. 17F) and unable to germinate.

Given that *HDA19* had high expression in flower buds (Aiese Cigliano et al., 2013), *hda19* plants were analyzed for pollen viability. Pollen viability as well as pollen size in *hda19* lines did not

differ from that in the WT. To further demonstrate that pollen functionality was not affected by *HDA19* silencing, we hand-pollinated emasculated WT flowers with *hda19* pollen. We obtained an average of 79.5 seeds per fruit with few or no undeveloped seeds. Conversely, when WT pollen was used to pollinate *hda19* we collected an average of 20.5 fully developed seeds per fruit. Collectively, these findings highlight that *HDA19* down regulation impairs partial the embryo development.

4.1.7 Trichostatin A treatment phenocopies *HDA19* down-expression

To obtain additional evidences that the developmental defects described in *hda19* lines were induced by an increase in histone acetylation, we treated WT fruits with Trichostatin A (TSA), an inhibitor of histone deacetylases (Perrella et al., 2010). The observation of treated fruits revealed that TSA treatment was able to phenocopy the fruit defects of *hda19* mutants. Indeed, the TSA injection on fruit at 10 DPA resulted in the production of smaller fruits with few or no seeds. No fruit defect was detected in untreated or water-treated control (Mock) (Fig. 18). Thus, these results indicate that the effect of TSA treatment during fruit development is substantially the same as down-expression of *SlHDA19*.

Figure 18. Cross-section of fruits of WT after the treatment with histone deacetylase inhibitor Trichostatin A (TSA, 15 µM) as compared with untreated (AC+/+) or water treated mock-control fruits. Black bar is 1 cm.

4.2 Functional characterization of tomato *ARP6* gene by loss-of-function strategy

4.2.1 Production of ARP6 knockdown tomato lines

To downregulate *ARP6* the same strategy adopted for *SlHDA19* was performed, i.e. RNA interference mediated by artificial miRNA (amiRNA). A specific *ARP6* amiRNA construct was designed with WMD3 tool (http://wmd3.weigelworld.org/) and cloned into the plant expression vector pK2GW7 (pK2GW7-amiARP6). After *A. tumefaciens* mediated transformation, seventeen T_0 plants deriving from independent transformation events were selected for kanamycin resistance. Transgene integration was further confirmed by PCR of the kanamycin resistance gene from plant genomic DNA. Candidate transgenic plants were evaluated for *ARP6* relative expression through quantitative RT-PCR approach. Two lines, *arp6-11* and *arp6-14*, showed the highest downregulation values (65% and 85%) compared to the WT (Fig. 19) and were used for further investigation.

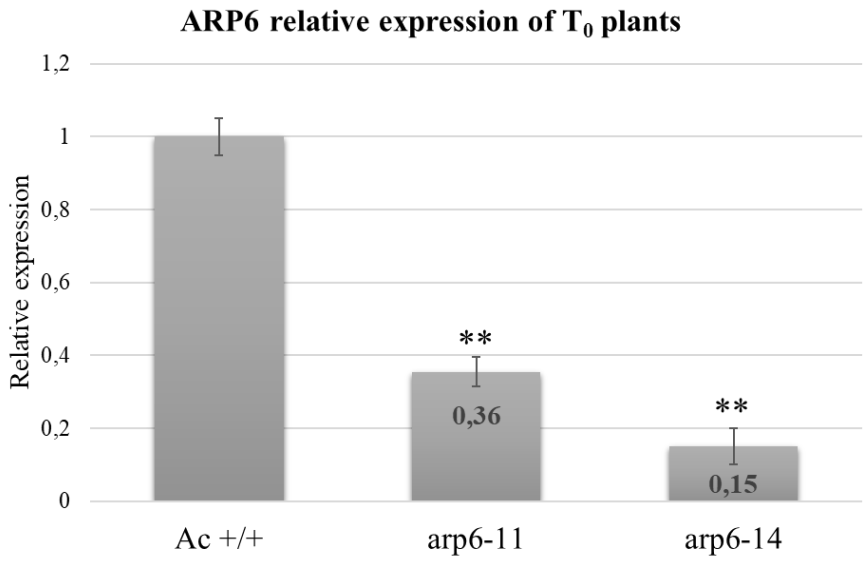

Figure 19. Expression analysis of ARP6 in arp6-11 and arp6-14 leaf respect to the WT (AC +/+). The expression values are the average of three replicates, the standard error is reported as black vertical bar. **P≤0.01

The transgene copy number was assessed using the Standard Addition Quantitative (SAQ)-PCR technique (Huang et al., 2013) that evidenced a double T-DNA insertion in both *arp6-11* and *arp6-14*. To check whether the regeneration of tomato seedling affected the chromosome number, ploidy analysis was performed on mitotic cells from root tips of both *arp6-11* and *arp6-14*. Both lines showed to be regularly diploid (2n=2*x*=24).

4.2.2 ARP6 influences vegetative and reproductive traits

Since *ARP6* has been reported in Arabidopsis to have a pleiotropic effect on vegetative and reproductive traits , we analyzed both of them, as reported in Table 4, in T_1 generation of tomato *arp6-11* and *arp6-14* (10 plants for each).

Table 4. List of traits analyzed in arp6-11, arp6-14 and WT (AC+/+). Values are means ± SE. Different letters mean statistical different values. P≤0.05.

Parameter	AC +/+	*arp6-11*	*arp6-14*
Height at 1st inflorescence (cm)	110,8±1,9 **a**	103,7±1,1 **b**	96,1±1,5 **b**
Leaves to 1st inflorescence (n)	9,1±0,4 **a**	11,3±0,3 **b**	10,7±0,0 **b**
Steam at 1st inflorescence (mm)	11,3±0,1 **a**	9,1±0,5 **b**	9,6±0,2 **b**
Internode length (cm)	7,3±0,3 **a**	5,8±0,3 **b**	5,5±0,5 **b**
Flowers in the first two inflorescences (n)	10,3±1,2	8,3±0,7	7,7±1,3
Sepals number (n)	6±0,0	6,0±0,2	5,9±0.1
Petals number (n)	6±0,0	6,0±0,3	5,9±0,1
Stamens number (n)	6±0,0	5,9±0,3	5,9±0,1
Fruit weight (g)	30,1±1,6	34,4±3,1	32±1,6
Days from anthesis to breaker (d)	37±0,2	37,3±0,6	36,2±0,1
Fruit Brix (°)	5,6±0,1	5,8±0,1	5,3±0,2

Both *arp6* transgenic lines exhibited a reduced plant height associate with shorter internode and thinner stems respect to the WT (Fig. 20). More, leaves exhibited a smaller size in *arp6* compared to the WT (Fig. 20).

AC +/+ *arp6-11* *arp6-14*

Figure 20. Plant development in arp6-11, arp6-14 and WT (AC+/+) at standard growth conditions.

Four weeks-old *arp6* plants were lighter in color than WT. Accordingly, spectrophotometric analysis on leaf tissue extract showed ≈20% less total chlorophyll in both mutant lines (Fig. 21A) and total carotenoids were reduced by 18% and 40% in *arp6-11* and *arp6-14*, respectively (Fig. 21B) compared to WT.

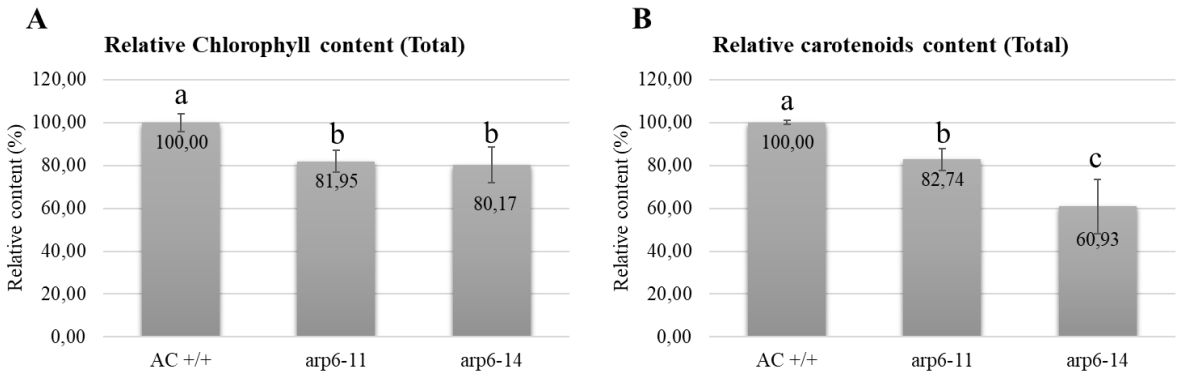

Figure 21. Total chlorophyll (A) and carotenoids content (B) in arp6-11, arp6-14 and wild type (AC+/+). Black bars are standard errors. Different letters mean statistical different values. P≤0.05.

The onset of fruit ripening and fruit traits such as weight and Brix° were not different between *arp6* and WT (Table 4). *Slarp6* seeds were consistently smaller than WT (Fig. 22A) and the seed weight was significantly reduced by 56% and 44% in *arp6-11* and *arp6-14*, respectively (Fig. 22B).

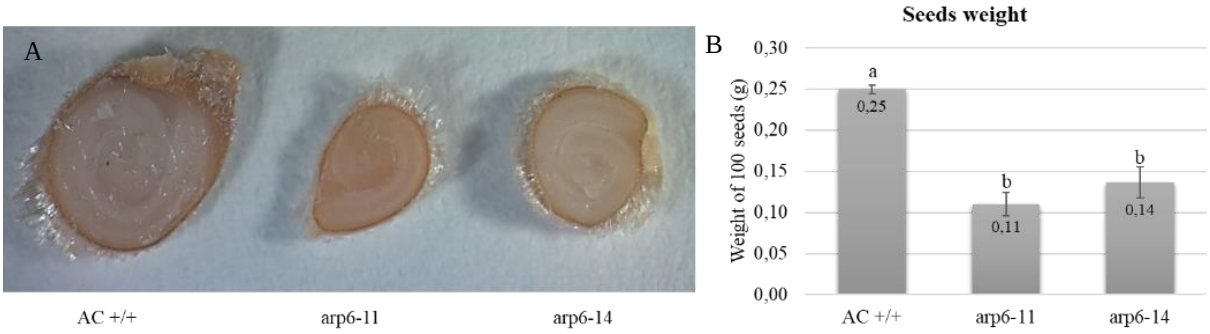

Figure 22. Transversal section of seeds in arp6-11, arp6-14 and WT (AC +/+) (A). Weight of 100 seeds in arp6-11, arp6-14 and WT (B). Black bars are standard errors. Different letters mean statistical different values. P≤0.01.

Flowering time measured counting the number of leaves before the first inflorescence resulted later in *arp6* plants than WT (11 leaves vs 9) (Table 4). The flower morphology in terms of number and size of sepals, petals, and stamens was the same in *arp6* as the WT (Table 4). Microsporogenesis analysis of flower buds from *arp6-14* T_0 plants showed that *arp6-14* cross-over (CO) frequency in diakinesis/metaphase did not differ from WT. Likewise pollen viability as well as pollen size in *arp6* lines did not differ from that in the WT.

Since *arp6* seeds were smaller than WT, we analyzed the *in vitro* seed germination ability recording the root tip emergence and cotyledon expansion every day for two weeks. We observed that ≈40% of WT seeds started to germinate at the second day after sowing while both *arp6* lines started only at the third day after sowing. At this time point, germination rate for the WT was about 80% while *arp6-11* and *arp6-14* root tip emergence was less than 20%. The delayed germination of *arp6* seeds respect to WT appeared significantly evident up to the sixth day after sowing (Fig. 23A). We found that WT cotyledons started to open on the fourth day after sowing while *arp6-11* and *arp6-14* started only 2 days later. At this point, there was 4-fold difference in cotyledons expansion between WT and *arp6* (Fig. 23B). At the end of the time course (14 days), the germination rate as well as the cotyledon expansion rate were close to 100% for both WT and *arp6*. However, we pointed out a reduction in length of roots, shoots and cotyledons in 14-days old seedlings (Fig. 23C-D). In particular, roots were 38% and 25% shorter than WT in *arp6-11* and *arp6-14*, respectively. Shoot length was reduced by 22% and 11% in *arp6-11* and *arp6-14* while cotyledons were 23% shorter than WT in both transgenic lines. Therefore, these data indicate that the downregulation of *SlARP6* affects tomato vegetative growth in adult plant and at seedling stage as well.

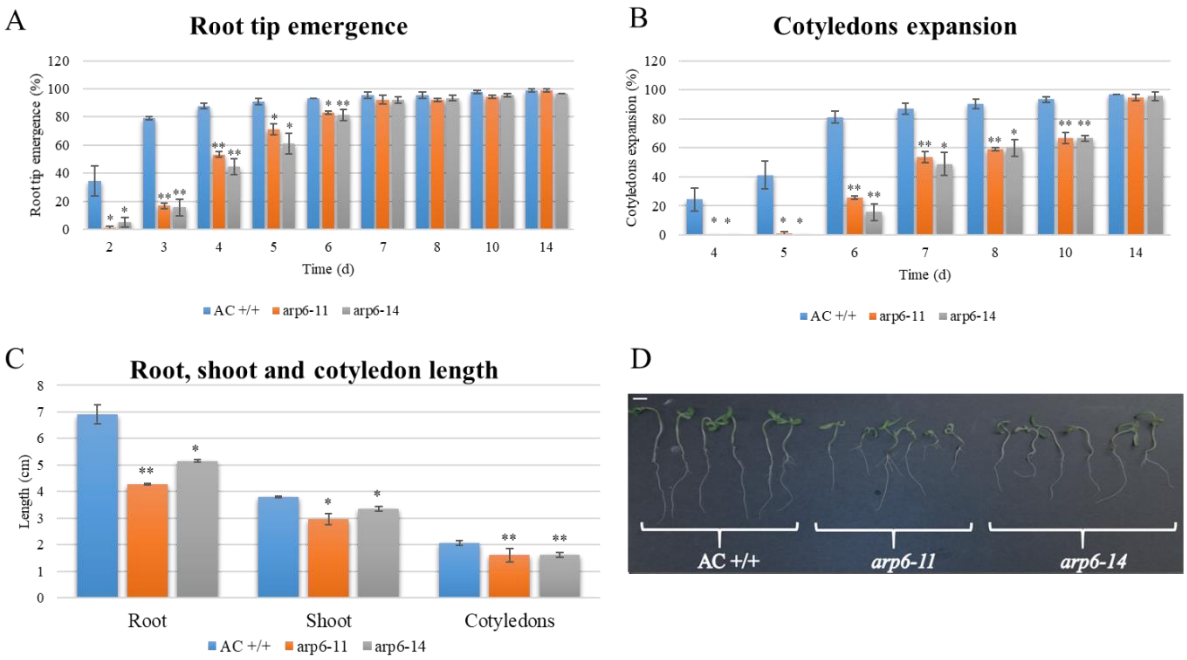

Figure 23. Root tip emergence (A), cotyledon expansion (B) and size of organs in 14-days old seedling (C,D) in arp6-11, arp6-14 and WT (AC +/+). Black bars represent standard errors. *P<0.05, **P<0.01.

4.2.3 ARP6 mediates tomato response to salt stress

In Arabidopsis, *ARP6* is involved in response to several abiotic stresses (Sura et al. 2017; March-Díaz et al., 2007). Since salt stress is considered as one of the most impacting factors on tomato production, we sought to investigate whether *ARP6* downregulated mutants present a different response to salt stress compared to WT. In NaCl supplemented medium (corresponding to 60 mM and 150 mM concentrations), we shown that the difference for germination rate, in terms of root tip emergence and cotyledons expansion, between *arp6* and WT increases (Fig. 24). In the stronger allele *arp6-14* the root tip emergence rate is reduced compared to the WT up to 14 and 10 days after sowing at 60 and 150 mM NaCl concentration, respectively (Fig. 24A-C). In particular, *arp6-14* when growth on 60 mM NaCl medium exhibits 23% less germination even 14 days after sowing. In *arp6-14* line cotyledons expansion rate is significant reduced up to 14 days after sowing at 60 and at 150 mM NaCl as well (Fig. 24B-D).

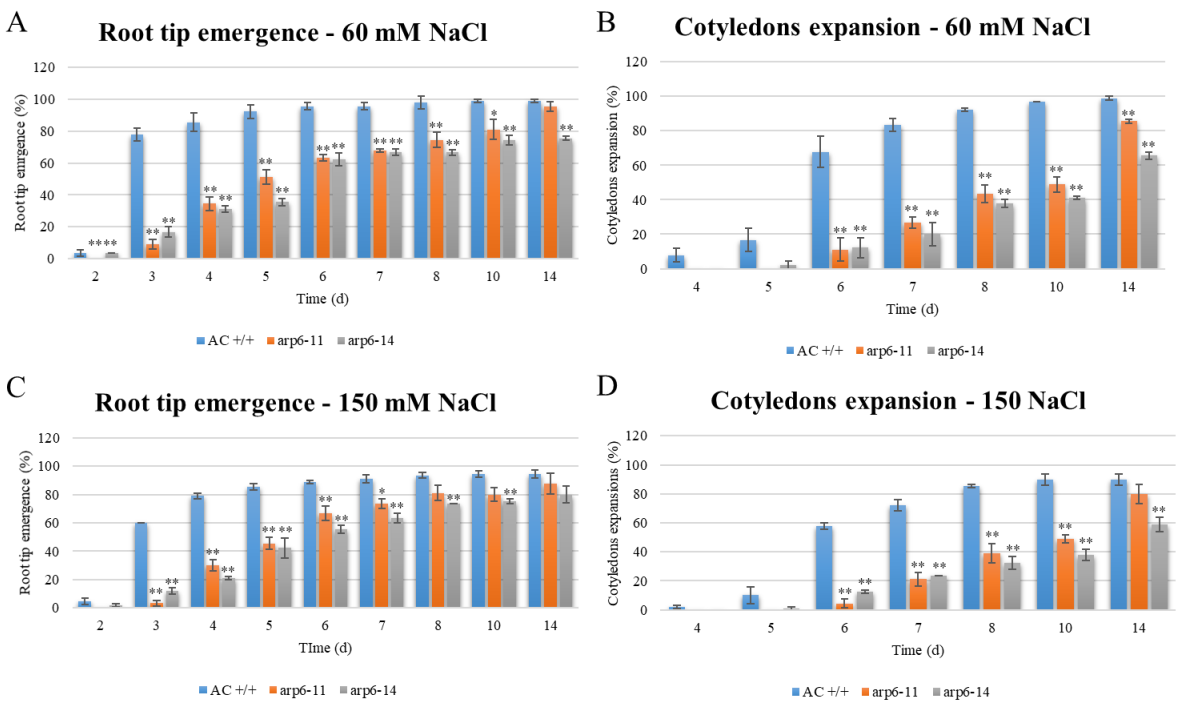

Figure 24. Root tip emergence and cotyledons expansion in presence 60 mM NaCl (A, B) and 150 mM NaCl (C, D) in WT and arp6 mutants. Black bars are standard errors. *P≤0.05;P≤0.01.**

Mutant seedling had shorter roots, shoots and cotyledons then WT at stressing conditions (Fig. 25).

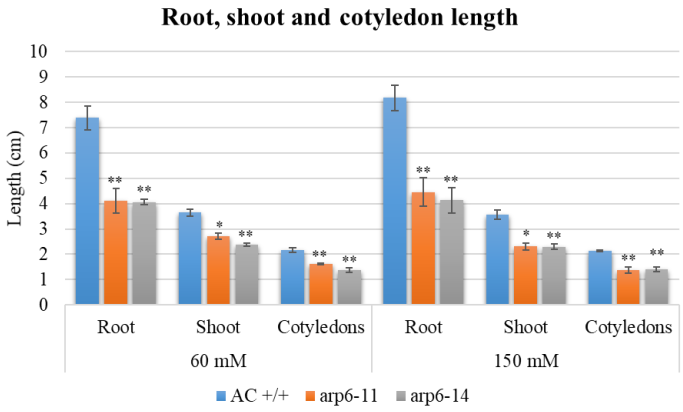

Figure 25. Roots, shoots and cotyledons length in arp6 mutants and in the WT under stressing conditions. Black bar are standard errors. *P<0.05, **P<0.01.

4.2.4 ARP6 is involved in heat stress regulation in tomato

Plants of Arabidopsis deficient in ARP6 phenocopy warm grown plants (Kumar and Wigge, 2010). Indeed, H2A.Z-containing nucleosomes provide thermosensory information to coordinate the heat response. To investigate whether SlARP6 has a similar function in tomato we performed heat stress experiments on 4 weeks-old seedlings of both *arp6* lines (Fig. 26A).

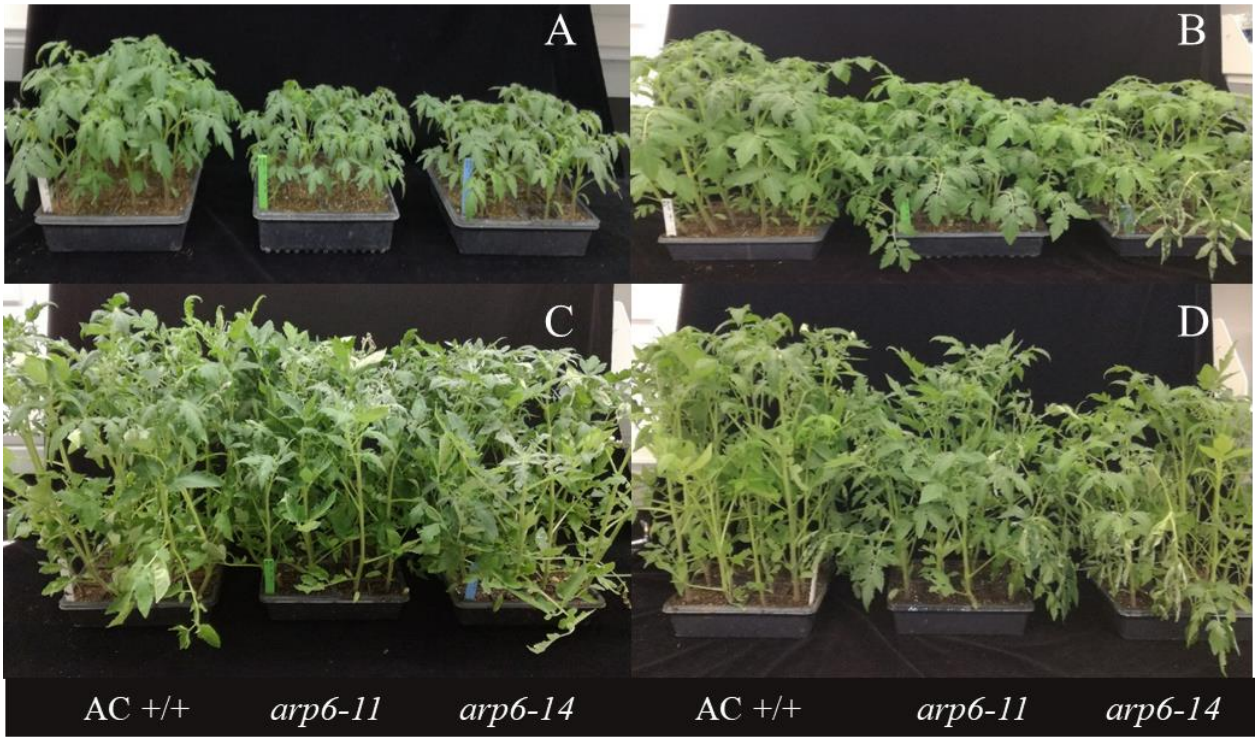

Figure 26. Four weeks-old plants (A). The same plants after 8 hours (B), 3 days (C) and 7 days (D) of exposition to heat stress.

As above reported, the growth habitus of *arp6* plants was reduced compared to the WT at control temperature (26°C/19°C day/night). These plants did not show any other visible sign of heat stress (Fig. 26A). We performed our analysis under heat stress conditions at 39°C/26°C day/night at three different time points (8 hours, 3 and 7 days of heat stress). *arp6* tomato plants appeared less tolerant to heat compared to the WT showing more withered leaves at all the time point of treatment (Fig. 26B-D). Heat stress sensitivity was assessed using relative water content (RWC), photosystem II efficiency (fv/fm) and electrolyte leakage analysis.

RWC is a measure of plant water status in terms of the physiological consequence of cellular water deficit and it is relative to the maximal water holding capacity at full turgidity. RWC was lower in both *arp6* plants than WT at control temperature and after 7 days of heat treatment (Fig. 27A).

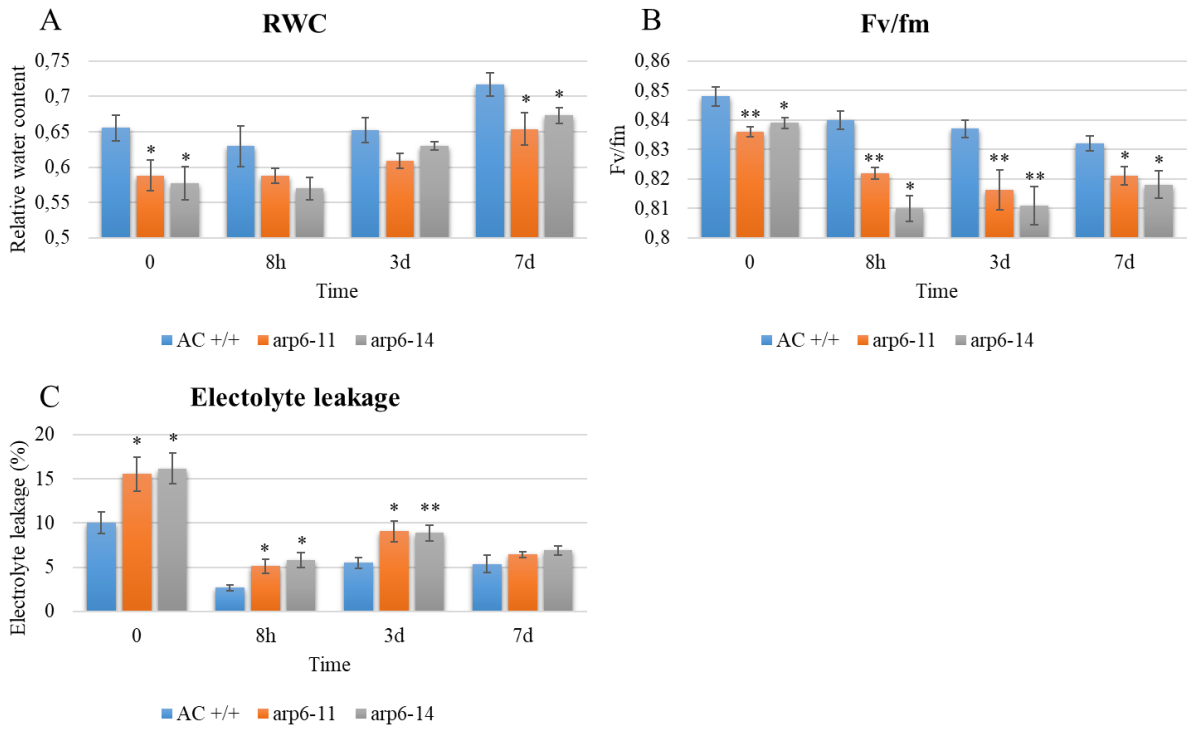

Figure 27. Relative water content (RWC) (A), fv/fm ratio (B) and electrolyte leakage (C) in arp6 and WT at 0 (26 °C), 8h (8 hours heat stress), 3d (3 days heat stress) and 7d (7 days heat stress). Black bars are standard errors. *P<0.05, **P<0.01.

The chlorophyll fluorescence Fv/Fm ratio, indicating the maximum quantum efficiency of Photosystem II, was reduced at all the time points including no stress condition when compared to WT (Fig. 27B). The loss of electrolytes, a parameter correlated negatively to the integrity of the plasma membrane, was higher in *arp6* plants than in the WT in non-treatment and treatment conditions except at 7 days (Fig. 27C). Our analysis suggested that *arp6* had a constitutive heat stress sensitivity, which increased even more under stress conditions.

The induction of heat shock proteins (HSPs) is one of the predominant response to temperature stress. HSPs perform important physiological functions as molecular chaperones for protein

quality control (Wu et al. 2014). Given that *arp6* lines were less tolerant to heat stress, we hypothesized a possible interplay between ARP6 and HSPs. The expression levels of some main HSPs were measured in the leaves of *arp6* lines and wild type from untreated and heat treated plants by relative qRT-PCR (Fig. 28).

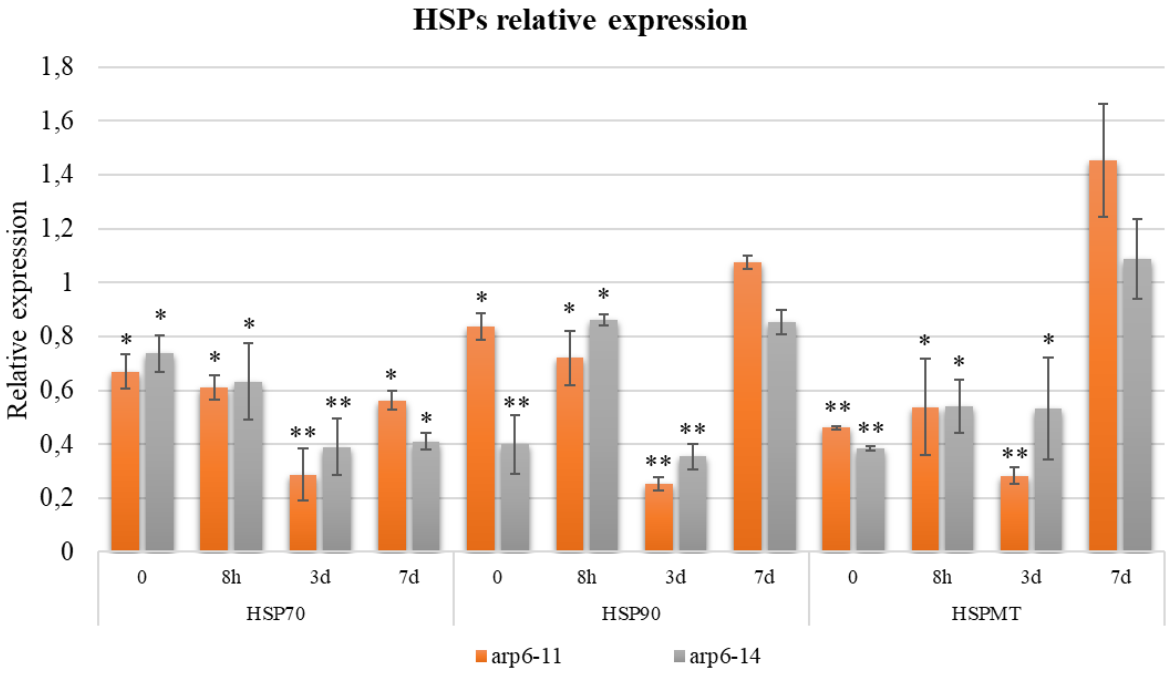

Figure 28. Relative expression of HSP70, HSP90 and HSPMT in arp6 and WT at 0 (26 °C), 8h (8 hours heat stress), 3d (3 days heat stress) and 7d (7 days heat stress). Black bars are standard errors. *P≤0.05;**P≤0.01.

The results displayed that *HSP70*, *HSP90* and *HSPMT* (mitochondrial small heat shock protein) were remarkably downregulated in both *arp6* lines compared with wild type under normal temperature, and at 8 hours and 3 days of heat stress (Fig. 28). *HSP70* were still downregulated after 7 days of heat stress. HSPs downregulation reached the highest level at 3 days of stress (Fig. 28).

Heat shock transcription factors (HSFs) play an important role in plant heat stress responses and thermo-tolerance since, as components of signal transduction, they regulate the expression of HSPs (Wu et al. 2012). We examined the transcript levels of Heat Stress Transcription Factors 1 (*Hsfa1*) and Heat Stress Transcription Factors 2 *(Hsfa2*) by relative qRT-PCR analysis. In tomato, Hsfa1 has been defined as a master regulator of heat stress response (Mishra et al., 2002), whereas Hsfa2

has an important role in thermotolerance (Kotak et al., 2007). As shown in Fig. 29, the expression of both *Hsfa1* and *Hsfa2* genes were significantly reduced in *arp6* lines in all detected time point of heat stress treatment. In *arp6-11*, the *Hsfa2* gene was slightly upregulated compared to the wild type at control condition (Fig. 29). Both HSFs reached the lowest expression level after 7 days of heat stress.

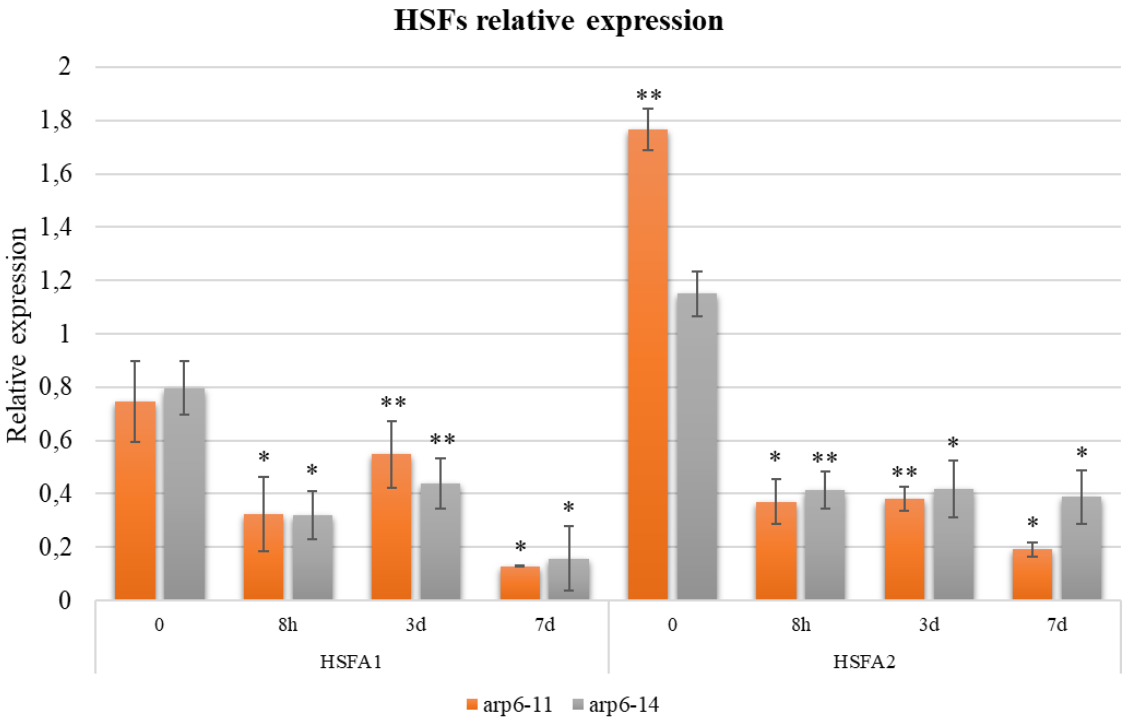

Figure 29. Relative expression of Hsfa1 and Hsfa2 in arp6 and WT at 0 (26 °C), 8h (8 hours heat stress), 3d (3 days heat stress) and 7d (7 days heat stress). Black bars are standard errors. *P≤0.05;**P≤0.01.

These data suggest that down-regulation of *SlARP6* affects the expression of HSPs and HSFs accounting for the reduced thermotolerance of *arp6* tomato plants.

5. Discussions

5.1 Functional characterization of tomato *HDA19*

In the last few years, several studies in *Arabidopsis* and other plants, including tomato have demonstrated the relevance of epigenetic mechanisms in the regulations of plant developmental processes (Gallusci et al., 2016). These discoveries have created new inroads into understanding of the primary control mechanisms that coordinate and modulate ripening phenotypes. For many years, great effort has been devoted to the study of DNA methylation and its requirement in controlling fruit development and particularly ripening. However, very few publications are available in the literature that address a biological role for other epigenetic factor such as HPTMs, in fleshy fruit development.

The function of the putative histone deacetylase *HDA19* was analyzed by means of *in silico* analysis and mutation inducing silencing. One of the first in *silico* analysis of tomato HDACs, performed by Aiese Cigliano and colleagues (2013) identified the class RPD3/HDA1 of HDACs as one of the most expressed during the fruit development and ripening. In particular, *SlHDA19* was highly expressed during the early development at 1 cm stage, at breaker and red ripe stages. In addition, *SlHDA19* expression reached a considerable level in flower buds suggesting a possible role in flower development and thus during the gametogenesis, as previously shown in *A. thaliana* for another member of RPD3/HDA1 deacetylases class HDA7 (Aiese Cigliano et al., 2013*)*. A comprehensive examination within the Tomato Expression Atlas of cv. M82 (Tomato Expression Atlas, http://tea.solgenomics.net; Fernandez-Pozo et al., 2017; Pattison et al., 2015) led us to observed *HDA19* expression in more stages and more tissues respect to previous studies. As expected, *SlHDA19* resulted expressed during fruit development and ripening, in particular the highest expression was at 5 and 10 days post anthesis and from breaking point to red ripe stage. *HDA19* is upregulated in pericarp tissue and even more in the seeds pointing out a possible involvement of *SlHDA19* in embryo development. Further, transcriptomic data derived from RNA-seq experiments on tomato cv. Ailsa Craig confirm the consistent increase in *SlHDA19* expression from IG to RR stages, reinforcing the idea that *HDA19* is required for the transition from development to ripening phase and its requirement is independent of the genotype analyzed. Taken

together transcriptomic profiles in different varieties and stages pointed the way to definitely identify *SlHDA19* as a good candidate to exert a role in fruit development and ripening.

To silence specifically *HDA19* expression, amiRNA approach was used. Indeed, amiRNAs were showed to efficiently silence multiple genes as well as single targets gene in tomato (Mitter et al., 2017). As previously stated, the nine primary regenerated amiRNA mutant plants shown variation in phenotypes strength, which could be linked to the different level of severity of *HDA19* down regulation. This kind of variability was also observed in Arabidopsis amiRNA lines by Schwab and colleagues (2006) as well as by others (Alvarez et al. 2006; Aiese Cigliano et al., 2013) which report strong, intermediate and weak phenotypes in transgenic plants overexpressing both natural and artificial miRNAs. Moreover, the authors linked the intensity of the phenotypes with the expression levels of the miRNAs, and thus of their target genes, which is in agreement with our results. Indeed, among the suppressed lines *hda19-6* showed the most severe phenotype associated to the weakest expression of *SlHDA19*. Frequently, it is possible to obtain from tomato transformation regenerated plants showing variated chromosomal number (Deverno, 1995). Often these plants are tetraploids, characterized by larger flowers and seeds and lower seed set than standard diploids, or sterile aneuploidy (Vasil, 1986). In order to avoid examining chromosomal variants and consequently invalidate the phenotypic characterization, we checked the chromosome set of each candidate plant under our study. We confirm the diploid genetic background of *hda19* transgenic mutants and the absence of traits commonly associated to chromosomal variation in tomato. HDA19 is considered a global regulator of gene expression in Arabidopsis (Fong et al. 2006). Indeed its down regulation leads to induction and repression of 10.7% and 7.8% of the transcriptome in leaves and flowers buds, respectively (Tian et al., 2005). In Arabidopsis knockout of *HDA19* obtained thought RNAi and T-DNA insertion affects several developmental process such as early senescence, vigor, serration of leaves, aerial rosette formation, flower lacking sepals and petals and gaining extra stamens (Tian and Chen, 2001; Tian et al., 2003; Zhou et al., 2005). When traits related to plant growth and flower morphology were investigate, no different phenotypes were observed in tomato *hda19*. The lack of vegetative and flower phenotype in the examined lines, at least regarding the morphological parameter used in our study such as plant height, internode length, stem diameter, number of leaves and flowers, size and number of petals or sepals could be due to different causes. Firstly, the presence of the residual expression levels (knockdown) can be enough to perform gene function (Krysan et al., 1999). Secondly, in the case

of functionally redundant genes, knockout alleles may not show a phenotype (Sridha and Wu, 2006) which is possibly our case because *HDA19* is member of multiple gene families. Finally, it is possible that *HDA19* in tomato is not contributing to the defects observed in Arabidopsis. Indeed, as an example, in Arabidopsis *HDA19* form a multi protein complex with the transcription factor APETALA 2 negatively regulating multiple floral organ identity genes (Krogan et al., 2012). In tomato, differently of what has been observed in Arabidopsis, the knockout mutations of AP2A, the close homolog of Arabidopsis AP2, do not affect floral organ development (Karlova et al., 2011).

If the *hda19* mutants showed flower indistinguishable from that of the WT, these developed into ripe fruit smaller than normal. Final fruit size results from the number of cells within the ovary before fertilization, the number of seeds, the number of cell divisions that occur in the developing fruit after fertilization, and the extent of cell expansion (Gillaspy et al., 1993). The involvement of all these factors in determining final fruit size clearly indicates the complexity of this phenomenon. As part of their phenotypes, the *hda19* lines exhibit reproductive alterations in terms of a strong reduction in the number of fully developed seeds. The reduced number of functional seeds arising from mutation in *HDA19* may explain the reduction in fruit size and their reduced fruit weight as well. Tian and co-workers (2003) already described an effect on both silique length and seed set in Arabidopsis *hda19* plants. Moreover, these authors in a loss of function *athda19* mutant have evidenced abortive seed development. When we investigated the cause of seed failure, we observed in *hda19* a noticeable embryos arrest during early embryogenesis. Wild-type tomato embryos follow a predictable pattern of cell divisions, going through a series of stages named after the shape of the embryo: globular, heart, young torpedo, torpedo and late torpedo, fully developed embryo and mature (Hocher et al., 1992). In Arabidopsis, where the embryogenesis pattern was deeply investigated, these stages encompass two major phases of development. The first part of embryogenesis, until the heart stage, is devoted to patterning, setting up the embryonic axes, meristems, and tissue types (Jenik et al., 2007). The heart-to-late-heart stage transition marks the onset of embryonic maturation, first evidenced by the appearance of chlorophyll auto fluorescence in the epidermis of the hypocotyl, signaling the beginning of proplastid maturation to chloroplasts (Mansfield and Briarty, 1991). Indeed, the embryos turn green in color and start accumulating seed storage products at the early torpedo stage (seed maturation phase). When we staged the *hda19* embryos by referring to the wild-type embryos in fruit of same age, *hda19* embryos were arrested

at early globular stage before the onset of embryonic and seed maturation phase. Studies concerning the relationships between *HDA19* and early embryogenesis have been reported in Arabidopsis by Long and co-workers (2006), where they demonstrated that *HDA19* is involved in the fate of the embryonic polarity during the transition stage, between the globular and the heart shape, of embryogenesis. Their data point to a transcriptional repression mechanism operated by HDA19 that guarantee the correct polarity during the first stage of Arabidopsis embryogenesis. It is worth noting that in Arabidopsis HDA19 interact with the corepressor proteins HIGH-LEVEL EXPRESSION OF SUGAR_INDUCIBLE GENES2-LIKE1 (HSL1) (Zhou et al., 2013), for repressing the seed maturation programme in seedlings. Although not demonstrated, it could not be excluded that *HDA19* has a role in the repression of seed maturation in the first stages of embryogenesis. Indeed, Zhou and colleagues (2013) were not able to recover double homozygous mutants for Arabidopsis *AtHDA19* and *HSL1*, and in silique isolated from the double heterozygous plants several aborted seeds were visible carrying embryo not properly developed. The authors, taken together these evidences suggested that *HDA19* and *HSL1* play a vital role during embryogenesis. Furthermore, *HDA19* together with another histone deacetylases *HDA6* were also proved to have an important role for the proper timing of embryogenesis. Indeed, *HDA19* and *HDA6* prevent the expression of embryonic traits after seed germination repressing several embryogenesis-related genes. A double RNA interference line with both *HDA6* and *HDA19* knocked down displayed growth arrest after germination and the formation of embryo-like structures on the true leaves of 6-week-old plants (Tanaka et al., 2008). A role of *HDA19* at early stage of embryogenesis is also deducible by the observations published by Long and colleagues (2006) of mRNA accumulation of *HDA19* in all cells of early heart-stage embryos and a HDA19-GUS fusion protein localized to the nuclei of cell at pre-globular phase. When we investigated the male side of *Slhda19* reproduction, we found regular pollen fertility in terms of pollen viability and size, which is in agreement with results published by Tanaka and colleagues (2008) in Arabidopsis. Besides the abovementioned embryo defect, fruit ripening is clearly affected in *hda19* mutants. We showed that *Slhda19* lines exhibited earlier and more pigment accumulation at the onset of ripening compared with wild type. Carotenoids, particularly lycopene and β-carotene, represent the primary components of ripe fruit pigmentation in tomato. The increase content of carotenoid pigments, mainly of the lycopene component, characterized by an increase of 30% in *hda19* fruits respect to wild type, accounted for the notable red color of pericarp tissues observed

in the silenced lines at Br +2. We detected a higher amount of β-carotene, which is usually associated to a darker orange fruit, during the red ripe Br +7 but an unchanged level of lycopene could hide the orange phenotype. Indeed, no difference of color intensity of fruit pericarps in *hda19* versus wild type is perceivable at fully ripe stage. Since it is well known that carotenoids biosynthesis is regulated by ethylene (Giovannoni, 2004), the elevated ethylene production observed in *hda19* fruits, during the progression of ripening, could explain the high accumulation of carotenoids in these genotypes. Even if further molecular analyses are required, our biochemical data suggest that the suppressed expression of SlHDA19 might promote the expression of ethylene biosynthesis genes. This subsequently elevates the ethylene biosynthesis leading to the induction of ethylene regulated phytoene synthase gene (PSY1) that is a major regulator of metabolic flux toward downstream carotenoids. It is worth noting that Zhou and colleagues (2005) showed that HDA19 can be induced by ethylene and that change in HDA19 expression levels could affect ethylene-regulated gene expression in Arabidopsis defense response.

5.2 Functional characterization of tomato *ARP6*

In this work, we describe for the first time in tomato the role of the chromatin remodeler *ARP6* during plant development and in response to saline and heat stresses. In Arabidopsis, it has been reported that *arp6* mutant has a significantly reduced H2A.Z content in the nucleosomes. the Indeed, ARP6 is a crucial subunit of the SWR1 chromatin remodeling complex necessary for H2A.Z deposition (Choi et al., 2005; Deal et al., 2005; Deal et al., 2007, March-Díaz et al., 2008; Zhang et al., 2015; Sura et al., 2017). For this reason, mutations in *ARP6* gene have been largely used to ascertain the biological function of the histone variant H2A.Z.

In this thesis, we report the characterization of tomato *arp6* mutants for different traits. Phenotypical analysis revealed several defects affecting vegetative development, including a reduced plant height, thinner stems with shorter internodes, and smaller leaves. Similar phenotype was observed in Arabidopsis *arp6* mutant (Deal et al., 2005; Deal et al., 2007). Reduced size of 14-days old *arp6* seedlings point out a role for *ARP6* in the early phases of plant development. Interestingly, the delayed transition between vegetative and reproductive phase in *arp6* suggests a role for *ARP6* in controlling genes relevant for flowering. However, this result is conflicting with

other studies, which have shown an early flowering in *A. thaliana arp6* mutants (Martin-Trillo et al., 2006; Choi et al. 2005). Seed size as well as germination time are also influenced by ARP6 but seed set appear to be normal in *arp6*. Flower morphology, microsporogenesis and pollen viability were apparently regular in *arp6* tomato plants thereby suggesting that *ARP6* has not a role in tomato flower organogenesis and male gametogenesis. This is in contrast with the results reported in Arabidopsis (Deal et al., 2005; Choi et al., 2005; Deal et al., 2007; Rosa et al. 2013). Development and ripening resulted not affected by the down-regulation of *ARP6*, as well. This is consistent with the fact that *ARP6* is weakly expressed during fruit development and ripening according to Tomato Expression Atlas database (http://tea.solgenomics.net).

Previous studies reported that histone modifications along with DNA methylation can be correlated with gene expression in response to abiotic stresses, such as water deficit, high-salinity, and temperature shift (Kim et al., 2008; Luo et al., 2012). In addition, histone variant H2A.Z and the subunits of chromatin remodeling complexes such as *ARP6* or *PIE1* have a role in transcription control of responsive genes in plants reaction to different environmental stresses (March-Díaz et al., 2008; Kumar and Wigge, 2010; Coleman-Derr and Zilberman, 2012; Choi et al., 2016; Sura et al., 2017). Given that, our goal was to ascertain how and whether tomato *arp6* mutant react to high temperature and soil salinity. These are two common and crucial abiotic stresses considered as the most impacting factors on crop production worldwide.

As abovementioned, seed germination was delayed in *arp6* under control conditions but the presence of salt prolonged further the germination time. Moreover, the control was less severely affected by the salt suggesting that *ARP6* is involved in the response to salinity stress. This phenotype is consistent with what observed by Sura et al. (2017) in Arabidopsis. Furthermore, the authors observed that *arp6* phenotype was even stronger than H2A.Z double mutant *hta9 hta11*, since the third H2A.Z-encoding gene, *HTA8*, remains fully functional in this line (March-Díaz et al., 2008).

ARP6 is known to be involved in the response to temperature through incorporation of histone variant H2A.Z into nucleosomes (Kumar and Wigge, 2010; Deal and Henikoff, 2010). For instance, in Arabidopsis *Atarp6* mutants showed a warm-temperature phenotype including hypocotyl and petiole elongation, leaf hyponasty, and early flowering, even when grown at 22°C or below. Our results showed that *arp6* tomato plants were more sensitive to high temperature than

wild type showing more visible signs of heat stress (i.e. leaf drooping). Relative water content (RWC) was reduced in both *arp6* lines undergone heat stress for seven days indicating a higher cellular water deficiency under stress. Likewise, quantum efficiency of photosystem II, assessed by Fv/Fm ratio, was slightly but significantly reduced under heat conditions. Conversely, electrolyte leakage was increased in both *arp6* lines suggesting a less heat tolerance in terms of plasma membrane integrity and enhanced disruption of cell membranes. Interestingly, RWC, photosystem efficiency and electrolyte leakage were misregulated even under control conditions when compared with wild type, indicating that *ARP6* is likely to be necessary to maintain plant temperature-related homeostasis. However, at a normal temperature no visible sign of heat stress was detectable in *arp6* mutants.

Response to heat stress is primarily regulated by heat shock transcription factors (HSFs) such as *HsfA1a* that serve as central coordinator of downstream TFs and other signaling components (Haak et al. 2017) and *Hsfa2,* which acts as coactivator of *HsfA1a,* is one of the major HSFs accumulating in response to elevated temperatures (Liu et al., 2015; Fragkostefanakis et al. 2016). In addition, heat shock proteins (HSPs) are part of the adaptive strategy for heat stress response and are transcriptionally induced by the heat shock factor (HSF)-class transcription factors (TFs) upon activation by heat stress (Jacob et al., 2017). In this work, expression analysis by qRT-PCR indicate that the relative expression of three of the most important HSPs in tomato, *HSP70, HSP90* and *HSPMT* were all downregulated under stress conditions especially after three days of heat stress. After seven days only one HSPs, *HSP70,* remained downregulated while *HSP90* and *HSPMT* were not significant different from wild type. We checked also the two master regulators in heat response *Hsfa1* and *Hsfa2*. Interestingly, both HSFs were significantly downregulated in *arp6* undergone heat stress, particularly after seven days. Under control conditions, these two HSFs were not different from wild type except *Hsfa2,* which was slightly upregulated in *arp6-11* line. The constitutive warm program of *arp6* tomato plants is consistent with the phenotype observed by Kumar and Vigge (2010) in Arabidopsis *Atarp6* displaying a high-temperature phenotype under normal temperature. Collectively, our data indicate a clear involvement of *ARP6* in affecting molecular and physiological response to heat stress in tomato. Since the downregulation of ARP6 likely affects the efficient incorporation of H2A.Z into nucleosomes our results indirectly demonstrate that H2A.Z is involved in the response to temperature shift in tomato.

6. Conclusions

Tomato is the most extensively investigated *Solanaceae* species and it is considered a model system for fleshy fruit development and ripening. Recently, increasing evidence has indicated that the regulatory network of tomato fruit development and ripening include not only hormonal and genetic regulation but also epigenetic modulations. Among the latter, substantial advances have been achieved in understanding DNA methylation, which plays a critical role as an important ripening regulatory component. However, the extent and the role of DNA methylation in fleshy fruits is by far more ahead than that relating to histone PTMs and histone variants, at least in the tomato plant. Given that, a better and more extended understanding of epigenome dynamics associated with the fruit development and maturing has the potential to provide novel strategies for generating sources of variation for crop improvement. In this context, the aim of this work was the identification and the functional characterization of two epiregulators, Histone Deacetylase 19 (*HDA19*) and Actin Related Protein 6 (*ARP6*), by using the tomato as model plant. *HDA19* was investigated during fruit development and ripening processes while *ARP6* was studied in tomato vegetative growth, in response to abiotic stresses and in male meiosis during recombination. The research activity presented in this thesis was carried out at CNR-Institute of Biosciences and Bioresources, Portici in collaboration with prof. Jim Giovannoni at the Boyce Thompson Institute for Plant Research (BTI), NY, USA and the Department of Agriculture of University of Naples "Federico II".

In conclusion our results can be summarized as follow:

- *HDA19* impacts on fruit development by affecting fruit weight and size. It also negatively influences ethylene biosynthesis and carotenoids accumulation during the ripening process. More, *hda19* displayed reduced fully developed seeds. Undeveloped seeds stopped to growth at globular embryo stage allowing us to conclude that *HDA19* is necessary for regular embryo development
- *ARP6* affects germination time, early seedlings growth and plant development. It is also involved in the response to saline stress during germination and early phase of seedlings development. Further, *ARP6* regulates the response to heat stress during vegetative growth by modulating HSPs and HSFs. *ARP6* does not seem to affect recombination during male meiosis.

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