Functional Analyses of AtCHR12 and AtCHR23

Plant Growth Responses upon Over-expression of Chromatin Remodeling ATPase Genes

Adam Folta

PROPOSITIONS

1. *AtCHR12* and *AtCHR23* over-expression during Arabidopsis embryo development causes reduced germination and early flowering.
   (This thesis)

2. *AtCHR12* over-expression disturbs H3K27me3 removal.
   (This thesis)

3. The conclusion by Wang *et al.* that increased fecundity is caused by *EPSPS* is not supported by their studies, as only a single transgenic line was studied.
   (Wang, W. *et al.* (2014) New Phytol 202, 679-688)

4. The conclusion by Liang *et al.* that Arabidopsis can recognize rhizobial Nod factors in a similar way as legumes has no experimental basis.
   (Liang, Y. *et al.* (2013) Science 341, 1384-1387)

5. Discussion skills should be an obligatory part of academic training.

6. Flexible working hours increase personal performance.

Propositions belonging to the thesis entitled

Functional Analysis of AtCHR12 and AtCHR23: Plant Growth Responses upon Over-Expression of Chromatin Remodeling ATPase Genes

Adam Folta
Wageningen, 19 November 2015

FUNCTIONAL ANALYSES OF ATCHR12 AND ATCHR23

PLANT GROWTH RESPONSES UPON OVER-EXPRESSION OF CHROMATIN REMODELING ATPASE GENES

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ADAM FOLTA

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CONTENTS

OUTLINE OF THE THESIS

In contrast to animals, plants are sessile organisms that have to cope with changing environmental conditions, to which they adjust their growth and developmental transitions. Therefore plants have evolved multiple mechanisms to regulate their growth and development in response to these environmental changes. When plants are exposed to stress conditions, a rather general response is a transient reduction of growth. To optimize their growth, plants have to integrate external cues with internal developmental programs. In this integration, regulation of gene expression by chromatin remodeling plays a major role.

Insight in mechanisms involved in regulation of growth is of great importance for agriculture, since the reduced growth of crops, in response to environmental stress, often leads to reduced yield. Therefore we have studied the underlying mechanisms affecting the plant growth in model plant *Arabidopsis thaliana*, and translated this knowledge to an important vegetable crop tomato (*Solanum lycopersicum*).

It has been shown, that altering the expression of *AtCHR12*, an Snf2-subfamily ATPase, which is in the core of an ATP-dependent chromatin remodeling complex, affects the plant growth in response to adverse environment (Mlynarova *et al.*, 2007). To understand better the mechanisms involved in this growth regulation, we have extended these studies to the close paralog of AtCHR12 in Arabidopsis, AtCHR23. Further, we have studied the possible application of this type of genes in crops by modifying the expression of the single ortholog of tomato.

Chapter 1 introduces the importance of growth regulation in plants in response to adverse conditions. Further, an overview is given on ATP-dependent chromatin remodeling as one of the important players involved in stress response, as well as in various developmental switches during the plant life cycle. With respect to chromatin remodeling, the introduction focuses on the roles of Snf2-subfamily ATPases, which are the core subunits of SWI/SNF chromatin remodeling complexes.

Chapter 2 describes the effect of *AtCHR23* over-expression on plant growth. In contrast to AtCHR12, over-expression of *AtCHR23* reduces vegetative growth. After application of mild abiotic stress, the growth reduction is enhanced. This indicates that AtCHR23 is involved in growth reduction during environmental stress. In addition, over-expression increases variability of growth in populations of genetically identical plants. This is associated with increased expression variability of a subset of genes that associate with environmental stress. This indicates that accurately controlled expression of *AtCHR23* is required for stability or robustness of growth, as well as the transcriptional activity of certain genes.

In addition to these studies on growth regulation, we have studied the effect of *AtCHR12* and *AtCHR23* over-expression on plant developmental transitions. Chapter 3 describes the role of AtCHR12 and AtCHR23 in regulation of the switch from dormancy to germination of seeds. Over-expression of *AtCHR12* or *AtCHR23* results in reduced frequency

of seed germination, which is more pronounced at stress conditions. The reduced germination upon over-expression coincided with reduced degradation of mRNAs of seed maturation genes. This shows that AtCHR12 and AtCHR23 are constituents of the regulatory framework that controls embryo maturation with clear effects on seed germination.

Chapter 4 explores the impact of *AtCHR12* over-expression on the developmental transition from vegetative growth to flowering. The *AtCHR12* over-expressing mutants are early flowering due to reduced expression of the main flowering repressor, *FLC*. The reduced *FLC* expression associates with increased levels of the repressive H3K27me3 histone mark on the *FLC* locus. Interestingly, the miss-regulation of *FLC* occurs already during embryo development, when *FLC* reprogramming takes place. This indicates that the underlying mechanism of flowering time control by *AtCHR12* over-expression is considerably different from another Snf2-subfamily ATPases. In contrast to AtCHR12, BRAHMA was shown to regulate flowering time via photoperiod pathway, while SYD affects flowering time by repressing *FT* expression.

Taken together the results indicate that similarly to AtCHR12, AtCHR23 has a role in growth regulation, however it is active at different developmental stages. In addition we have shown that both ATPases are involved in seed maturation during embryo development, and that over-expression of either of the ATPases affects the embryo maturation with consequences on the plant developmental transitions.

The regulation of growth is important for proper crop management, therefore we explored the role the Snf2-subfamily ATPase in regulation of growth in crop specie tomato. We studied the effect of over-expression of the only tomato ortholog of AtCHR12 and AtCHR23 on tomato growth. In Chapter 5 the identification and cloning of the tomato ortholog is described and the possible application in agriculture or horticulture is discussed. The over-expression of the ortholog reduces both vegetative and reproductive growth, indicating that the tomato ortholog possesses the function of both AtCHR12 and AtCHR23. Although, the tomato transgenic lines do not show difference in growth upon abiotic stress, the results indicate that modulation of the expression of chromatin remodeling ATPases could be used in novel strategies to control plant growth.

Chapter 6 discusses the most important results of this thesis with respect to the published research, the implications of this work on plant science and outlines the perspectives for future research. Focus is given to the role of the chromatin remodelers on plant growth and on plant developmental transitions, as well as to the interplay between chromatin remodeling processes.

CHAPTER 1

GENERAL INTRODUCTION

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PLANT GROWTH AND ENVIRONMENT

Plants, as sessile organisms, have to deal with changing environmental conditions, which affect multiple aspects of their life cycle. To ensure successful reproduction, plants go through two main developmental transitions, which work as "safe-points". The first developmental transition is germination, the switch from dormant seed to growing seedling. Seeds can stay dormant for a long period of time until the conditions are favorable to ensure successful germination and seedling development. The second developmental transition is the switch from vegetative to reproductive stage. At optimal conditions the vegetative growth can be very short, however, at unfavorable conditions plants remain longer in the vegetative stage until the conditions ensure formation of mature embryos and seeds.

To cope with unfavorable conditions, plants adapt with a wide range of responses at the molecular, cellular and whole-plant level. One of the general responses of plants to adverse conditions is a partial or complete arrest of growth, what can be achieved at any time when the environmental conditions are unfavorable (Xiong *et al.*, 2001). Generally, growth resumes after the environmental limitations are overcome (Rohde *et al.*, 1999). Another mechanism in response to adverse environments is growth plasticity, which provides plants the ability to continuously adapt their growth and development to the local environment (Nicotra *et al.*, 2010, Palmer *et al.*, 2012). Growth plasticity ensures the best possible strategy to complete the plant life cycle and propagate, however, it results in substantial variability of growth. For example, plants in different types of soils with different concentrations of nutrients will manifest alterations of leaf size and thickness (Sultan, 2000).

In addition to environmental conditions, plant growth is affected by internal factors, such as phytohormones (Srivastava, 2002). For successful growth, plants evolved mechanisms to integrate the external cues with internal developmental programs. In these mechanisms, regulation of gene expression plays an important role.

The modulation of gene expression in response to environment is mediated by transcription factors and regulation of chromatin organization. Transcription factors are proteins, which bind to DNA and stimulate or repress transcription of certain genes. Such transcription factors are for example DELLA's, which play a key role in temporal growth arrest. They repress growth of *Arabidopsis thaliana* by integrating the signals of adverse environment with phytohormone signals, and promote plant survival (Achard *et al.*, 2006). Transcription factors often cooperate with chromatin remodelers, which are involved in dynamic change of chromatin structure resulting in altered accessibility of genetic information. Also chromatin remodelers were shown to be involved in temporal growth arrest in response to abiotic stress (Mlynarova *et al.*, 2007).

The focus of this thesis is on the role of chromatin remodeling in growth control in response to environment, and during development. Therefore in the next subchapter chromatin and chromatin remodeling is introduced in more detail.

CHROMATIN AND MECHANISMS OF CHROMATIN REMODELING

Genomic information of a eukaryotic organism is stored in nuclear DNA, which forms complexes with histones and other proteins. The DNA-protein complex is called chromatin and it is organized at multiple levels. At the first level of compaction, genomic DNA is wrapped around histone octamers. These are formed from a H3-H4 tetramer, to which two H2A-H2B heterodimers are bound. Around the octamers approximately 147 bp of DNA is wrapped, forming the basic units of eukaryotic chromatin – nucleosomes. Individual nucleosomes are linked together by 10 to 50 bp of linker DNA. The first level of compaction creates what is known as the 10-nm fiber, or 'beads on a string'. With the help of H1 histones and other proteins like HP1, this fiber can be compacted to a 30-nm fiber and to an even higher order of compaction (Fisher *et al.*, 2011).

Originally it was thought that chromatin is just an organizational structure for compaction of the DNA inside the nucleus. However, it has now become clear that chromatin structure plays a prominent role in gene regulation. It has a highly dynamic structure, involving cell-, tissue- and stage-specific changes in chromatin organization and accessibility (Rosa *et al.*, 2013).

The dynamic changes in chromatin structure, known as chromatin remodeling, are required to enable access of regulatory proteins and transcriptional machinery to DNA. There are two main processes involved in chromatin remodeling: covalent DNA and histone modifications, which are recognized by other DNA binding proteins, and chromatin remodeling performed by ATP-dependent chromatin remodeling complexes that does not involve covalent modifications (Figure 1.1) (Fisher *et al.*, 2011).

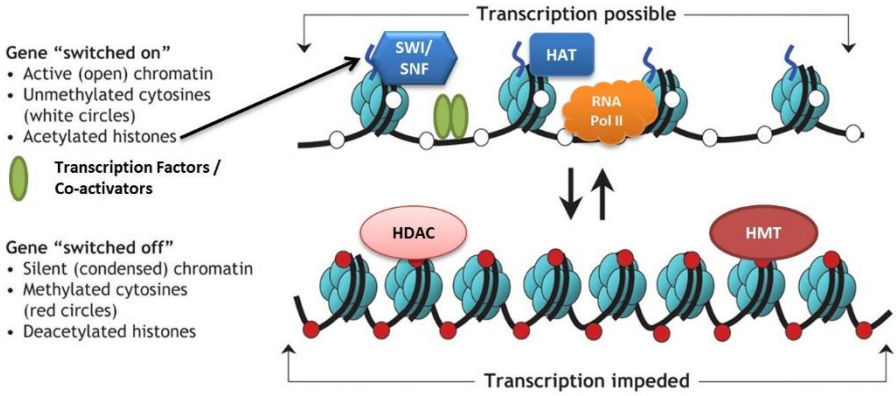

Figure 1.1: Chromatin remodeling in regulation of transcription. Histone acetyltransferases (HAT) and ATP-dependent chromatin remodeling (SWI/SNF) generally make chromatin more accessible for transcription factors or co-activators, and enable transcription. Histone deacetylases (HDAC) and histone methyltransferases (HMT) are generally involved in compaction of chromatin. Taken from Wikipedia (https://en.wikipedia.org/wiki/Chromatin_remodeling).

Methylation of DNA occurs at specific cytosine residues and is generally associated with transcriptionally inactive regions (Schubeler, 2015, Zemach *et al.*, 2007). Post-translational modification of histone proteins occurs at specific residues of protruding N-terminal tails. The most common and best studied histone modifications are acetylation and methylation. However, other modifications were described and new ones are still being discovered (Bannister *et al.*, 2011, Kouzarides, 2007).

The level of acetylation of lysines on histone tails is controlled by two groups of enzymes: histone acetyltransferases and histone deacetylases. Histone acetylation is generally associated with active gene expression. Histone methylation mainly occurs on lysine and arginine residues of histone tails and it is controlled by histone methyltransferases and histone demethylases (Figure 1.1). The effect of histone methylation depends on which lysine or arginine residue is methylated, and whether it is mono-, di- or tri-methylated (Bannister *et al.*, 2011, Pfluger *et al.*, 2007). Generally, methylation of lysine 4 on histone 3 (H3K4me1/2/3) is associated with activation of gene expression, while di- or tri-methylation of lysine 9 or 27 on histone 3 (H3K9me2/3, H3K27me2/3) is found in repressed genes.

Another important chromatin remodeling process is ATP-dependent chromatin remodeling. ATP-dependent chromatin remodeling modifies the chromatin structure by altering the histone-DNA interaction, which can lead to unwrapping, mobilization, reconstruction or ejection of histone octamers. ATP-dependent chromatin remodeling is in general associated with transcriptional activation, but it was found to function also in transcriptional repression (Varga-Weisz, 2001). ATP-dependent chromatin remodeling is performed by large multiprotein complexes with an ATPase as one of its components. The ATPase uses energy from ATP hydrolysis for the action of the complex (He *et al.*, 2012). The ATPases are members of large SNF2 family, which can be divided into 24 subfamilies. These are structurally and functionally conserved from yeast to animals and plants (Clapier *et al.*, 2009). However, not all subfamilies occur in every species or kingdom (Bargsten *et al.*, 2013). Based on the type of ATPase and the subunit composition, the ATP-dependent chromatin remodeling complexes can be classified into 4 groups: SWI/SNF, ISWI, INO80/SWR1 and CHD (Clapier *et al.*, 2009, Flaus *et al.*, 2011). Different chromatin remodeling complexes have unique biochemical activities. SWI/SNF chromatin remodeling complexes are involved in nucleosome sliding and ejection, whereas ISWI and CHD have a function in nucleosome spacing in chromatin assembly (Clapier *et al.*, 2009). INO80/SWR1 has a role in H2A – H2A.Z histone variant exchange. The presence of H2A.Z correlates with low DNA methylation and it was suggested to play a role in preventing gene silencing (March-Diaz *et al.*, 2009).

DIVERSE ROLES OF SNF2 ATPASES

The first ATP-dependent chromatin remodeling ATPase was identified in two independent genetic screens for *Saccharomyces cerevisiae* mutants exhibiting defective mating type switching (SWI) and sucrose non-fermenting (SNF) phenotypes (Neigeborn *et al.*, 1984, Stern *et al.*, 1984). Further studies, including also *Candida albicans* and *Tetrahymena thermophila*, revealed that SWI/SNF complexes are essential for viability (Kwon *et al.*, 2007).

The first ATP-dependent chromatin remodeling mutants in a multicellular organism were identified in *Drosophila* (Tamkun *et al.*, 1992). Since then, multiple chromatin remodeling ATPases belonging to the SNF2 family have been identified in yeast, *Drosophila* and human. A lot of research has been done to identify the underlying mechanisms of ATP-dependent chromatin remodeling, as well as the roles of the complexes (Flaus *et al.*, 2011, Narlikar *et al.*, 2013). In addition to regulation of gene expression, ATP-dependent chromatin remodeling complexes are involved in alternative splicing (Batsche *et al.*, 2006), DNA replication (Vincent *et al.*, 2008) or DNA double strand break repair (Lans *et al.*, 2012), but they are also important for proper chromosome segregation during mitosis (Huang *et al.*, 2004). These studies have especially been performed with yeasts and cell lines, because in metazoans such studies are hampered due to embryo lethality of null mutants (Kwon *et al.*, 2006).

Orthologs of SNF2 ATPases were found also in plants. They are best studied in the model plant *Arabidopsis thaliana*, which has 41 SNF2 ATPases distributed over 18 subfamilies (Bargsten *et al.*, 2013). Genetic approaches showed that they have diverse functions in plant growth and development, recently reviewed by Han (Han *et al.*, 2015). They have been shown to be involved in response and adaptation to stress, in embryo development and germination, in flower patterning and repression of flowering, in phytohormone signaling, and in immune responses.

The studies in Arabidopsis suggest that SNF2 ATPases may be interesting targets for improving agriculturally important crops. The SNF2 family genes have been identified in 33 annotated plant genomes (Bargsten *et al.*, 2013). However, more detailed analyses were performed only in rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*), both indicating similar roles of SNF2 ATPases in crop species as in Arabidopsis. The rice SNF2 orthologs were shown to be involved in response to drought, salt or cold stresses, and in phytohormone signaling (Guo *et al.*, 2014). In tomato, the expression analyses focused on DRD1- and Snf2-subfamilies involved in stress responses (Bargsten *et al.*, 2013). However, the function of any of the tomato ATPases has not been studied. A role of ATP-dependent chromatin remodeling in response to stress was also observed in pea (*Pisum sativum*) and maize (*Zea mays*) (Casati *et al.*, 2008).

The focus of this thesis is on the role of Snf2-subfamily ATPases, therefore this subfamily is introduced in more details in the next section.

SNF2-SUBFAMILY ATPASES IN PLANT GROWTH AND DEVELOPMENT

In Arabidopsis, the Snf2-subfamily has four members – BRAHMA (BRM), SPLAYED (SYD), AtCHR12 and AtCHR23 (Bargsten *et al.*, 2013). The latter two were also named MINU1 and MINU2 (Sang *et al.*, 2012). However, in this thesis the original names AtCHR12 and AtCHR23 will be used. BRAHMA (BRM) and SPLAYED (SYD) are large proteins, while the other two paralogs AtCHR12 and AtCHR23 are significantly smaller (Figure 1.2). All four have the same domains in their N-terminal sequence, including an HSA and QLQ domains (Gentry *et al.*, 2014) important for DNA-protein and protein-protein interactions, respectively (Bargsten *et al.*, 2013) (Figure 1.2). However, only BRM has a C-terminal bromodomain, which in yeast was found to target remodeling complexes to hyperacetylated lysines (Chatterjee *et al.*, 2011, Farrona *et al.*, 2004, Gentry *et al.*, 2014). On the other hand, SYD contains a large unstructured C-terminal part, which was suggested to control SYD accumulation and/or activity (Su *et al.*, 2006). The two smaller proteins AtCHR12 and AtCHR23 both carry an unfolded region at the C-terminal end and AtCHR12 contains also unfolded regions at the N-terminal end (Bargsten *et al.*, 2013) (Figure 1.2).

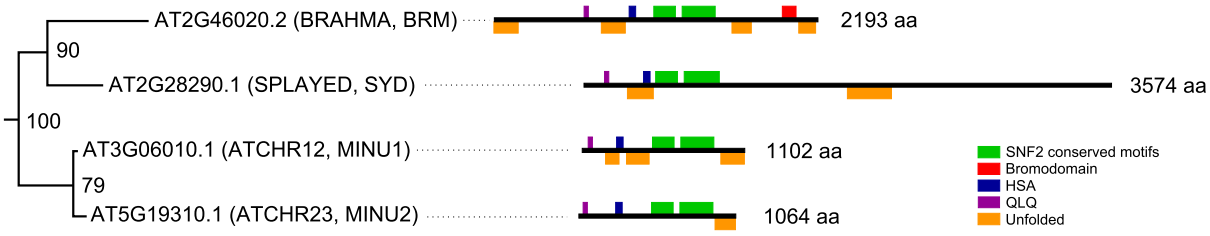

Figure 1.2: Schematic representation of the protein domains in Snf2-subfamily ATPases in Arabidopsis. The left side shows an unrooted tree of Snf2-subfamily with confidence values. On the right side are indicated domains in the proteins and the protein size in amino acids. Modified from (Bargsten *et al.*, 2013, Gentry *et al.*, 2014).

From the four Arabidopsis Snf2-subfamily ATPases, BRM and SYD have been investigated in more detail. It has been shown that they have some specific and some redundant functions. Both *brm* and *syd* single knockout mutants show severe pleiotropic developmental defects, yet they are viable. However, the double knockout is embryo lethal (Bezhani *et al.*, 2007). Both single mutants are slow growing and dwarfed with reduced apical dominance and have defects in cotyledon separation, patterning (leaf polarity, flower morphogenesis) and embryo development (Farrona *et al.*, 2004, Hurtado *et al.*, 2006, Kwon *et al.*, 2006, Su *et al.*, 2006, Wagner *et al.*, 2002). In addition, *brm* has a unique root growth defect, is male sterile and is involved in flowering time control (Farrona *et al.*, 2011). BRM has also been shown to be involved in phytohormone signaling (Archacki *et al.*, 2013, Han *et al.*, 2012). On the other hand, *syd* mutants exhibit defects in stem cell maintenance, because of misregulation of the SYD target *WUSCHEL*, a master regulator of stem cell

maintenance in Arabidopsis (Kwon *et al.*, 2005). Further, SYD was shown to be involved in biotic stress responses by regulating expression of genes downstream of ethylene and jasmonate signaling (Walley *et al.*, 2008). The unique and shared functions indicate that BRM and SYD have some common and unique targets (Bezhani *et al.*, 2007). So these two Snf2-subfamily ATPases exhibit partial functional diversification, but have retained some of their ancestral roles (Kwon *et al.*, 2007).

The role of the AtCHR12 and AtCHR23 ATPases is studied to a less extent. At optimal growth conditions, single knockouts of *AtCHR12* or *AtCHR23* do not show any phenotypical difference compared to wild-type. However, the double knockout mutant is embryo lethal (Sang *et al.*, 2012). The embryo lethality occurs at an early stage of embryo development (heart stage and early torpedo stage), suggesting a redundant function in embryo and seed development (Cong-Cong *et al.*, 2012, Sang *et al.*, 2012). It has been suggested that, similarly as for BRM and SYD, also AtCHR12 and AtCHR23 have some shared and unique functions. AtCHR12 has been shown to play a role in temporary growth arrest in response to mild abiotic stress (Mlynarova *et al.*, 2007). Over-expression of *AtCHR12* resulted in growth arrest of primary buds, as well as in reduced growth of the primary stems in response to drought, heat or salt stress. On the other hand, the *atchr12* knockout mutants show less growth arrest than wild-type under stress (Mlynarova *et al.*, 2007).

The role of AtCHR12 in growth regulation in response to abiotic stress has been studied by Mlynarova et al. (Mlynarova *et al.*, 2007). In this thesis, these studies are extended to its paralog, AtCHR23. In addition, the role of AtCHR12 and AtCHR23 was studied during developmental transitions, where integration of environmental signals with intrinsic developmental programs plays an important role, specifically in germination and flowering time control. The mechanisms regulating growth in response to adverse environments affect parameters important for crop agronomy, such as growth, yield and quality of crops. Therefore the role of such chromatin remodelers was studied in the major vegetable crop, tomato.

CHAPTER 2

OVER-EXPRESSION OF ARABIDOPSIS *ATCHR23* CHROMATIN REMODELING ATPASE RESULTS IN INCREASED VARIABILITY OF GROWTH AND GENE EXPRESSION

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ABSTRACT

Plants are sessile organisms that deal with their -sometimes adverse- environment in well-regulated ways. Chromatin remodeling involving SWI/SNF2-type ATPases is thought to be an important epigenetic mechanism for the regulation of gene expression in different developmental programs and for integrating these programs with the response to environmental signals. In this study, we report on the role of chromatin remodeling in Arabidopsis with respect to the variability of growth and gene expression in relationship to environmental conditions. Already modest (2-fold) over-expression of the *AtCHR23* ATPase gene in Arabidopsis results in overall reduced growth compared to the wild-type. Detailed analyses show that in the root, the reduction of growth is due to reduced cell elongation. The reduced-growth phenotype requires sufficient light and is magnified by applying deliberate abiotic (salt, osmotic) stress. In contrast, the knockout mutation of *AtCHR23* does not lead to such visible phenotypic effects. In addition, we show that over-expression of *AtCHR23* increases the variability of growth in populations of genetically identical plants. These data indicate that accurate and controlled expression of *AtCHR23* contributes to the stability or robustness of growth. Detailed RNAseq analyses demonstrate that upon *AtCHR23* over-expression also the variation of gene expression is increased in a subset of genes that associate with environmental stress. The larger variation of gene expression is confirmed in individual plants with the help of independent qRT-PCR analysis. Over-expression of *AtCHR23* gives Arabidopsis a phenotype that is markedly different from the growth arrest phenotype observed upon over-expression of *AtCHR12*, the paralog of *AtCHR23*, in response to abiotic stress. This demonstrates functional sub-specialization of highly similar ATPases in Arabidopsis. Over-expression of *AtCHR23* increases the variability of growth among genetically identical individuals in a way that is consistent with increased variability of expression of a distinct subset of genes that associate with environmental stress. We propose that ATCHR23-mediated chromatin remodeling is a potential component of a buffer system in plants that protects against environmentally-induced phenotypic and transcriptional variation.

Keywords
Arabidopsis, Chromatin remodeling, Growth, Gene expression, Variability, Robustness

INTRODUCTION

Plants have evolved finely orchestrated mechanisms to regulate their growth in response to the environment as a programmed part of their sessile life style. These mechanisms help them to cope with the (possibly adverse) environment at any period of their existence. Notably developing seedlings are vulnerable to short-term adverse environments (Lianes *et al.*, 2005, Rajjou *et al.*, 2012). As a result, plants display substantial variability of growth, a phenomenon also known as growth plasticity (Nicotra *et al.*, 2010). Such plasticity allows plants to optimize their growth and development according to the prevailing environmental conditions, ensuring the best possible strategy to complete their life cycle and propagate. Growth plasticity is potentially important for agronomic use as it affects yield and quality in unfavorable environments. Plasticity for a trait as growth is largely organized at the molecular level in which epigenetic mechanisms play a critical role (Nicotra *et al.*, 2010). Chromatin remodeling is part of the epigenetic machinery, next to DNA methylation, histone modification and small RNA-based mechanisms (Mirouze *et al.*, 2011), that is an integral part of overall plant development and is associated with plant responses to biotic (Walley *et al.*, 2008) and abiotic stress (Kim *et al.*, 2010).

We have shown previously that the SWI/SNF2-type ATPase encoded by *AtCHR12* is involved in the regulation of growth of *Arabidopsis thaliana* upon perceiving abiotic stress, such as drought or higher temperature (Mlynarova *et al.*, 2007). Arabidopsis plants over-expressing *AtCHR12* showed growth arrest of normally active primary buds, as well as reduced growth of the primary stem when stressed. Without stress, they were indistinguishable from the wild-type. The growth arrest response depended on the severity of the stress applied. Another SWI/SNF2-type ATPase, *SPLAYED* (*SYD),* was shown to be required for resistance against the necrotrophic pathogen *Botrytis cinerea* (Walley *et al.*, 2008), whereas a knockout of the *AtDRD1* ATPase gene showed increased susceptibility to fungal pathogen *Plectosphaerella cucumerina* (Lopez *et al.*, 2011). The SWI/SNF2-type ATPases are believed to mediate the complex interplay between chromatin remodeling and the enzymes involved in DNA and histone modification. This underlines the importance of ATP-dependent chromatin remodeling in responses of plants to environmental stress.

In addition, such chromatin modifications play a regulatory role during development (Feng *et al.*, 2010) in establishing epigenetic states with expression patterns that are tightly regulated in time and space. In animals, such epigenetic states are determined early during the development, while in plants epigenetic mechanisms also operate after embryonic development (Jarillo *et al.*, 2009). Several chromatin remodeling ATPase genes have a role in plant development. The CHD3-subfamily ATPase *PICKLE* (*PKL*) selectively regulates a suite of genes during embryogenesis, seed germination and root development (Aichinger *et al.*, 2011, Ogas *et al.*, 1999, Perruc *et al.*, 2007). Recently, this gene was identified as negative regulator of photomorphogenesis (Jing *et al.*, 2013). Out of four genes of the SWI/SNF2-

subfamily of Arabidopsis ATPases (Flaus *et al.*, 2006), *SYD* and *BRM* are involved in various, partially overlapping, developmental processes, such as root and floral development or seed maturation (Bezhani *et al.*, 2007, Farrona *et al.*, 2004, Tang *et al.*, 2008). The other two members of this subfamily, *AtCHR12* and *AtCHR23*, have roles in embryo and endosperm development. A nearly lethal *atchr12 atchr23* double mutant containing weak allele displayed a variety of severe pleiotropic morphological defects, including poor maintenance of shoot and root meristems (Sang *et al.*, 2012). Such ATPase-mediated chromatin modification establishes a level of gene regulation that is likely to integrate developmental programs with the response to environmental signals.

It is thought that epigenetic modifications help to establish a buffer against environmental perturbations (Lempe *et al.*, 2013) that results in the phenotypic robustness of the organism. Both in *Drosophila* (Sollars *et al.*, 2003) and in yeast (Lehner *et al.*, 2006, Levy *et al.*, 2008, Tirosh *et al.*, 2010) the deletion of chromatin regulator genes markedly increased the variability of the phenotype studied, indicating that proper chromatin modification may counteract genetic, environmental and/or stochastic perturbations (Landry *et al.*, 2010, Lehner, 2010).

We here report on the marked impact of over-expression of the *AtCHR23* gene on the phenotype of Arabidopsis in terms of growth, reaction to adverse environments and genome-wide expression levels. *AtCHR23* is a paralog of *AtCHR12* (Bargsten *et al.*, 2013) of which the effects of over-expression were presented earlier (Mlynarova *et al.*, 2007). Over-expression of *AtCHR23* results in reduced growth compared to wild-type Arabidopsis, but phenotypic details between *AtCHR12* and *AtCHR23* over-expression are notably different, showing sub-specialization of these two paralogs. The effect of *AtCHR23* over-expression is notably quantitative both in terms of growth phenotype as in terms of gene expression. The over-expression of *AtCHR23* increases the variability of growth and expression variability of subsets of genes in populations of identical plants. It emphasizes the important role of chromatin modification in the control of gene expression in plants. Based on these results, we propose that accurate and controlled expression of *AtCHR23* is required for the stability or robustness of growth. We propose that AtCHR23-mediated chromatin remodeling could be part of a buffer system in plants that protects against environmentally-induced phenotypic and transcriptional variation (Lempe *et al.*, 2013).

RESULTS

Construction Arabidopsis mutants with altered *AtCHR23* expression

To generate transgenic Arabidopsis lines over-expressing the *AtCHR23* gene a construct containing 35S CaMV promoter and genomic sequence of *AtCHR23* (including 5'-UTR) from the accession Columbia (Figure 2.S1) was used for transformation of wild-type

Arabidopsis (Col-0). Two single-copy transgenic lines were identified and analyzed in detail: *AtCHR23*-4ov and *AtCHR23*-5ov. In addition, transgenic lines over-expressing cDNA copy of *AtCHR23* fused in-frame to the *GFP* gene under the 35S CaMV promoter in front (Figure 2.S1) were generated. Two separate single-copy transgenic lines were identified and analyzed: G_*AtCHR23*-1ov and G_*AtCHR23*-3ov. A third type of over-expressing transgenic line was generated by transformation with the cDNA copy of *AtCHR23* including 5'-UTR fused in frame to *GFP* driven by the native *AtCHR23*-promoter (Figure 2.S1). For comparison, two loss-of-function T-DNA insertion lines affecting *AtCHR23* expression were obtained from the Arabidopsis Stock Center. Both knockout lines showed no expression of full length *AtCHR23* transcript. The data presented in this paper are from SALK_057856 that in the remainder of this paper will be designated as *atchr23*. The other insertion line gave similar results (data not shown).

Over-expression of *AtCHR23* reduces the growth of roots and increases phenotypic variation

The growth dynamics of seedlings of the knockout (*atchr23*) and over-expressing lines of *AtCHR23* was analyzed with the help of a root elongation assay using vertical agar plates described previously (Mlynarova *et al.*, 2007). Stratified seeds of wild-type and mutant plants germinated at approximately the same time and frequency. The lengths of the primary root and hypocotyl, as well as other phenotypic characteristics, were measured repeatedly during development in different environmental conditions. To prevent possibly confounding influences of the environment experienced by the previous generation (Elwell *et al.*, 2011), all comparisons were made using seeds from parental plants (both for the wild-type and for the mutants) grown at the same time and in the same environment. Assays were based on at least 40 roots per condition, with at most 16 roots (8 mutant; 8 wild-type) per agar plate and five agar plates per assay.

Clearly visible differences between different lines were observed, notably with respect to the length of the root (Figure 2.1A). The differences in root length depended on the environmental conditions applied. When grown at 23 °C under long-day conditions, roots of the two *AtCHR23*-ov mutants were considerably shorter than those of Columbia wild-type (Figure 2.1A,B). Data is summarized in Table 2.1. The average length of the roots of 8-day-old wild-type seedlings was 40.7 mm, whereas of *AtCHR23*-4ov seedlings it was 31.9 mm (21.6% reduction) and of *AtCHR23*-5ov 34.6 mm (14.9% reduction). Also up-regulation of *AtCHR23* with a cDNA copy of the gene (two G_*AtCHR23*-ov lines) resulted in seedlings with roots 14 and 22.7% shorter than wild-type, whereas the transgenic line with the native promoter showed 11% shorter roots (Figure 2.1C; Table 2.1). In such assays, the variation in the root length was considerable, with coefficients of variation (CV) ranging from 0.161 to 0.164 for over-expressing lines, whereas for wild-type it was 0.052 (Table 2.1). The variation

of over-expressing mutants was significantly higher than in the wild-type (Levene's test; Table 2.1). These data show that upon over-expression of *AtCHR23*, roots become not only significantly shorter, but also more variable and less uniform. In contrast, the knockout mutant *atchr23* develops roots that are only slightly longer than those of the wild-type (Figure 2.1B). In populations of 40 seedlings, this difference was not statistically significant. These root growth differences between the various *AtCHR23* mutants and the wild-type were consistently observed in several seed stocks that were produced in various growing conditions, greenhouse or growing chambers. Moreover, similar differences and variability patterns in root length were observed in seedlings grown at 18 °C and 25 °C (data not shown).

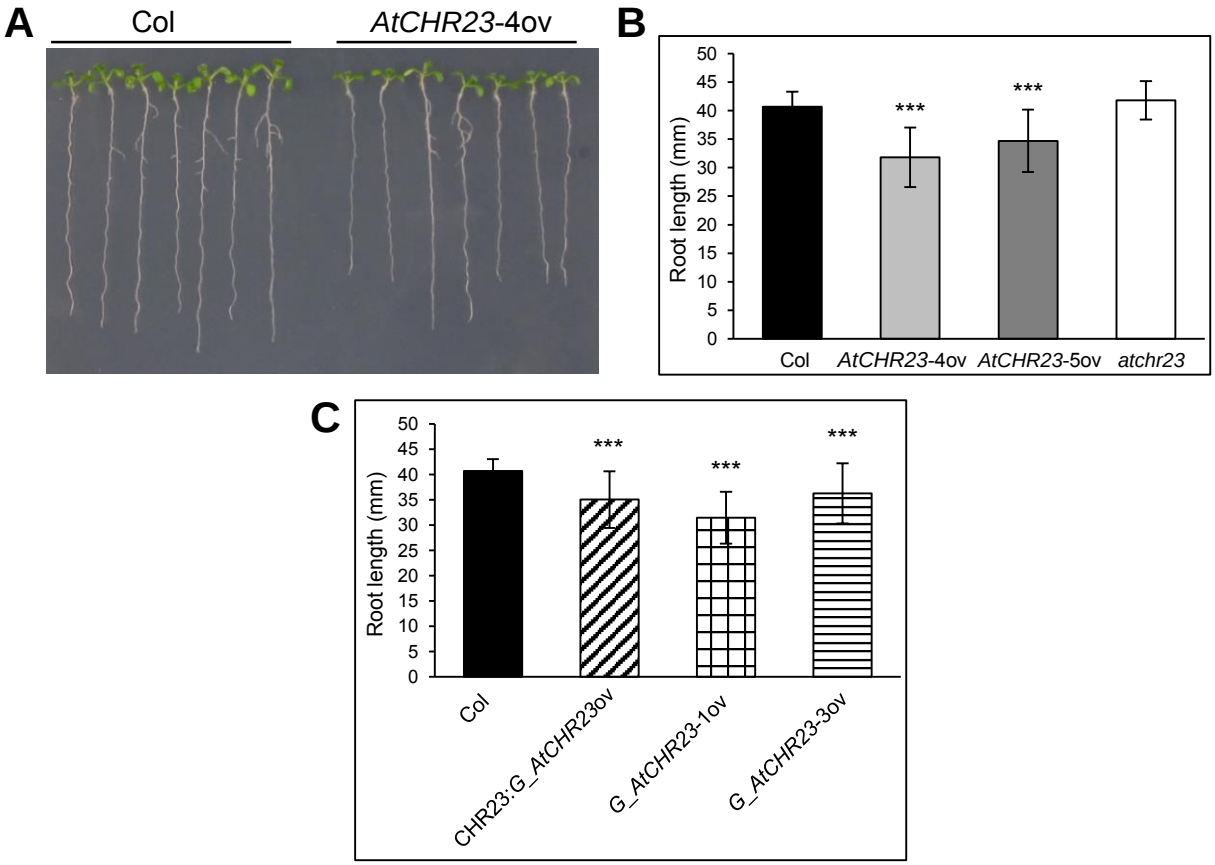

Figure 2.1: Over-expression of *AtCHR23* results in reduced root growth. (A) Seedlings grown for eight days at 23 °C, long-day (LD). **(B)** Mean (± standard deviation, s.d.) length of the primary root of Columbia wild-type (Col), knockout (*atchr23*) and two lines over-expressing the genomic copy of *AtCHR23*. **(C)** Mean (± s.d.) length of the primary root of Col wild-type and lines over-expressing the cDNA copy of *AtCHR23*. For each line, 40 seedlings were measured. Asterisks indicate significant differences from the wild-type: ***, $P < 0.001$.

Table 2.1: Root length reduction and *AtCHR23* mRNA up-regulation in transgenic Arabidopsis lines with modified *AtCHR23* expression

Plant line	Root length (mm)[a]	CV[b]	VAR[c]	P(VAR)[d]	Reduction in root length (%)[e]	Fold up-regulation *AtCHR23*[f]
Columbia – WT	40.53	0.052	4.76	na	na	na
AtCHR23-4ov	31.89	0.164	27.63	***	21.6	30
AtCHR23-5ov	34.65	0.161	31.32	***	14.9	40
atchr23	41.81	0.080	12.26	*	nd	na
G_*AtCHR23*-1ov	35.04	0.161	31.46	***	14.0	15
G_*AtCHR23*-3ov	31.46	0.163	26.49	***	22.7	13
CHR23:G_*AtCHR23*ov	36.26	0.164	35.47	**	11.0	2

[a] Mean root length; [b] coefficient of variation calculated as ratio of the standard deviation to the mean; [c] variance in root length; [d] significance of variance relative to WT as determined by Levene's test, *, $P < 0.05$; **, $P < 0.01$; ***; $P < 0.001$; [e] reduction in root length relative to WT; [f] fold up-regulation of *AtCHR23* relative to WT. WT, wild-type; na, not applicable; nd, not detected.

The variability in the phenotypic assays was assessed in more detail by analysis of the frequency distributions of the length data (Figure 2.2). The frequency distribution of the root lengths shows that the distribution is shifted to shorter roots when *AtCHR23* is over-expressed (Figure 2.2A), but still quite a number of individual seedlings have roots as long as the wild-type (Figure 2.2A, middle two panels). Also for the distribution of the hypocotyl length, the variation is larger in populations of over-expressing seedlings than in the wild-type (Figure 2.2B, middle two panels). In view of all experimental efforts to standardize the environment in the phenotypic assays, we think the variation between individuals of over-expressing lines is likely to have a molecular and/or functional basis.

To associate the growth arrest phenotypes with the level of *AtCHR23* mRNA, the amount of *AtCHR23* mRNA was determined in pools of (eight) seedlings with the help of qRT-PCR. The quantitative results are summarized in Table 2.1. A two-fold increase in *AtCHR23* mRNA (compared to wild-type) is observed in CHR23:G_*AtCHR23*ov. This is apparently sufficient for the growth arrest phenotype to become detectable. Higher levels of mRNA tend to make the phenotype more pronounced, without however a clear correlation between the level of up-regulation and the length of the root. Such an association indicates a complex interplay of interactions between steady-state mRNA levels and the penetrance of the root length phenotype. The lack of correlation between root length and the level of *AtCHR23* expression was also confirmed in individual seedlings of wild-type and mutant (10 seedlings of each) (data not shown).

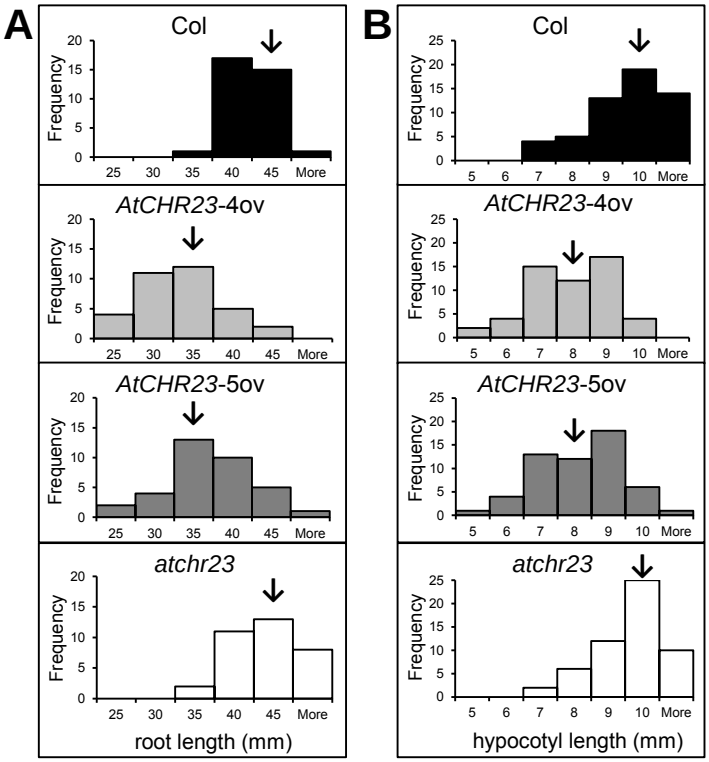

Figure 2.2: Frequency distribution of root (A) and hypocotyl (B) length. Seedlings (40 for each panel) were grown on agar plates for eight days at 23 °C **(A)** or 28 °C **(B)** in long-day conditions. In each panel, the arrow indicates the median length.

The reduction in root growth is due to reduced cell elongation

To determine whether the reduction of root length is due to reduced cell division or reduced cell elongation, we analyzed the size of the meristematic and elongation zone of 6-day-old seedlings. *AtCHR23*-4ov roots exhibited a normal cellular patterning compared to the wild-type (Figure 2.3A). For meristem we measured both the length of the meristematic zone and the number of meristematic cortex cells. None of them differ between wild-type and mutant roots (Figure 2.3B). To further assess the role of cell division, we also used the cell G2-M phase cycle marker pCYCB1;1:CYCB1;1-GUS (Colon-Carmona *et al.*, 1999). No clear difference in the pattern (Figure 2.S2) and number of GUS-positive cells was observed between the wild-type and the over-expressing mutant (data not shown). This is consistent with meristem size of wild-type and mutant (Figure 2.3B). On the other hand, the mutant showed a significantly shortened (16.8%) elongation zone relative to the wild-type as well as reduced length (23.1%) of the fully elongated cells (Figure 2.3C). Taken together, these results indicate that the major effect of *AtCHR23* up-regulation in the root is the reduction of cell elongation.

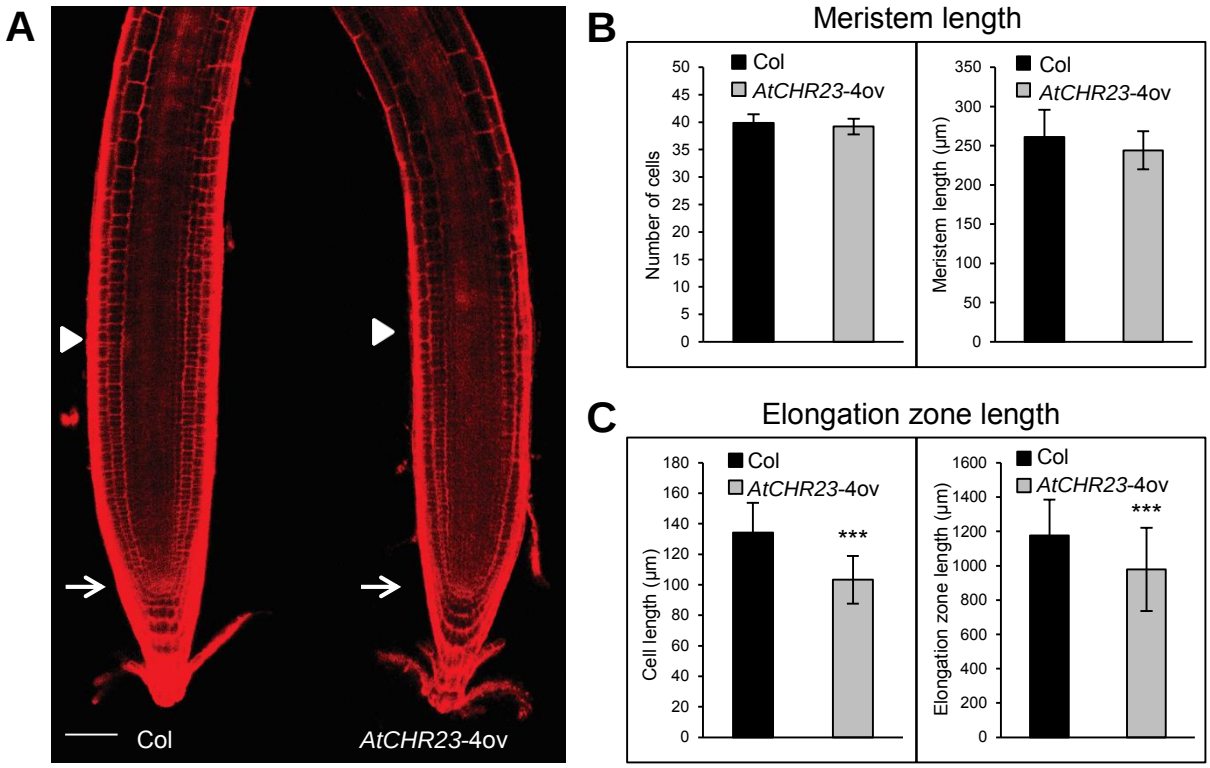

Figure 2.3: *AtCHR23* over-expression affects cell elongation. **(A)** Confocal images of 6-day-old Col wild-type and *AtCHR23*-4ov mutant roots grown at 23 °C in long day conditions stained with propidium iodide. Arrows indicate the quiescent center, arrowheads indicate the boundary between the proximal meristem and elongation zone of the root. Scale bar: 50 µm. **(B)** Number of cells (± s.d.) counted in meristem (left) and mean (± s.d.) meristem length (right) in Col wild-type and *AtCHR23*-4ov mutant. **(C)** Mean (± s.d.) length of fully elongated cells in elongation zone (left) and mean (± s.d.) length of the elongation zone (right) in Col wild-type and *AtCHR23*-4ov mutant. Asterisks indicate significant differences from the wild type: ***, $P < 0.001$.

Over-expression of *AtCHR23* results in smaller seedlings and smaller plantlets

Analyses of two *AtCHR23*-ov lines demonstrate that over-expression of *AtCHR23* also resulted in overall reduced seedling and plant growth (Figure 2.4). Over-expressing lines showed reduced growth of the cotyledon (Figure 2.4A) and hypocotyl (Figure 2.4B). The mean cotyledon area was reduced from 4.67 mm^2 in the wild-type to 3.35 mm^2 in *AtCHR23*-4ov (28.3% reduction) and to 3.83 mm^2 in *AtCHR23*-5ov (18% reduction). The length of the hypocotyls was determined from seedlings grown at 25 °C or 28 °C. The latter temperature is known to induce considerable hypocotyl elongation (Gray *et al.*, 1998). The average hypocotyl length of 25 °C-grown 8-day-old seedlings of over-expressing lines was reduced to 1.97 mm (about 20% reduction) compared to 2.42 mm of the wild-type, while the length of the hypocotyl of the knockout did not differ significantly from the wild-type. Such differences become more obvious at 28 °C (Figure 2.4B). Both temperatures show that up-regulation of *AtCHR23* leads to a significant overall reduction in the growth of seedlings.

The increased growth variability of mutant cotyledon and hypocotyl was not significant (Levene's test; Table 2.S1).

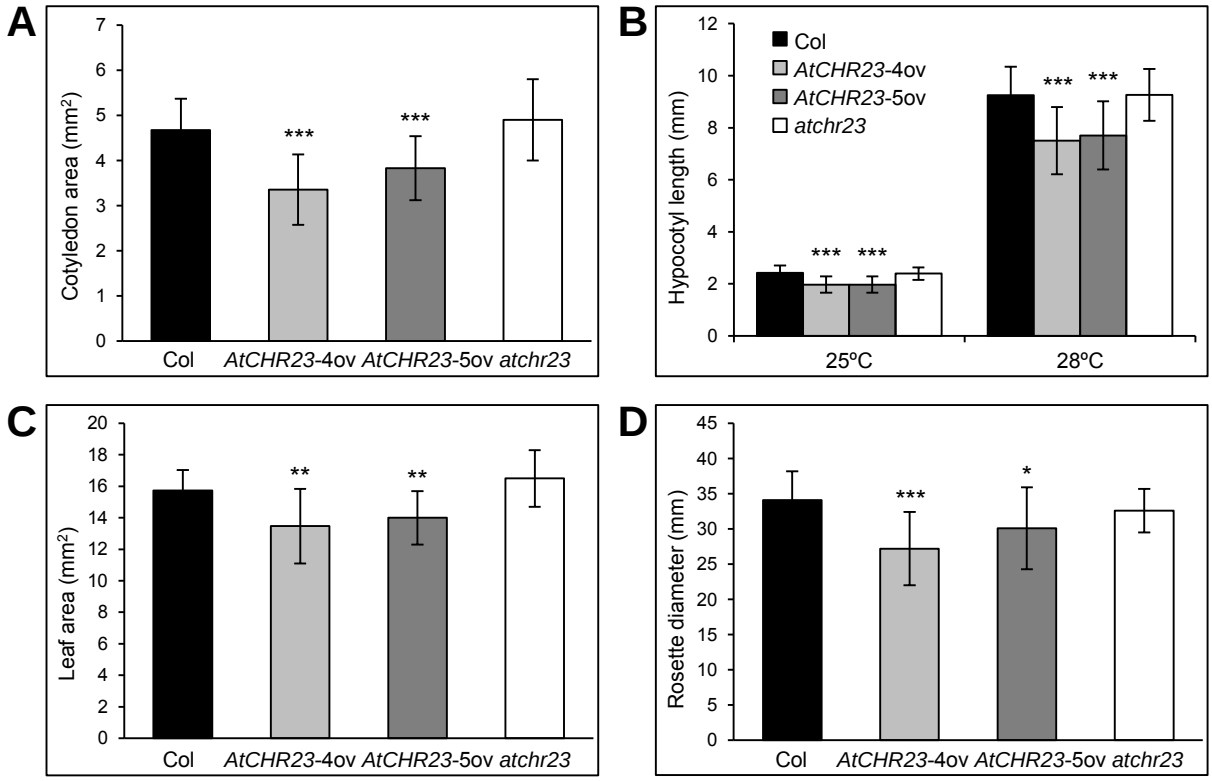

Figure 2.4: Over-expression of *AtCHR23* leads to overall reduced seedling and plant growth. **(A)** Mean (± s.d.) cotyledon area of 8-day-old wild-type (Col) and mutant seedlings grown at 25 ºC in long day conditions. **(B)** Mean (± s.d.) of hypocotyl length of wild-type (Col) and mutant plants grown for 8 days at 25 ºC or 28 ºC in long-day conditions. **(C)** Mean (± s.d.) leaf area of first rosette leaf of 15-day-old soil grown wild-type (Col) and mutant plants in long- day conditions. **(D)** Mean (± s.d.) rosette diameter of 4-week-old wild-type (Col) and mutant plants grown as in **(C)**. For each line, 40 seedlings or 15 plants were measured. Asterisks indicate significant differences from the wild type: **, $P < 0.01$; ***, $P < 0.001$.

To determine if and how the effects on plant size due to *AtCHR23* over-expression generate phenotypic changes further in development, two parameters for vegetative growth were measured in soil-grown plants: the leaf area and the diameter of the rosette. Both parameters were determined from digital images of 15 soil-grown plants. The average surface area of the first rosette leaf of the wild-type was 15.7 mm^2. This was reduced to 13.5 mm^2 in *AtCHR23*-4ov and to 14.0 mm^2 in *AtCHR23*-5ov, so over-expressing lines have up to 15% smaller leaves than the wild-type (Figure 2.4C). The knockout line had slightly larger leaves (5%), but again this difference was not statistically significant in the experimental set-up chosen. Similar growth differences were observed for the third rosette leaf (data not shown). Leaves of over-expressing mutants also showed significantly increased growth variability relative to wild-type (Levene's test; Table 2.S1). Furthermore, the average rosette diameter of 4-week-old over-expressing mutants was reduced in size (Figure 2.4D). While the wild-type

rosette diameter was 34.1 mm, it was 27.2 mm in *AtCHR23*-4ov and 30.1 mm in *AtCHR23*-5ov. Compared to the wild-type it represents 20% and 12% reduction in the size of the rosette in the mutants, respectively. It shows that also during vegetative development plants over-expressing *AtCHR23* tend to stay smaller than the wild-type.

Light conditions determine the growth characteristics of over-expressing lines

As light is a crucial environmental factor affecting plant growth (Jiao *et al.*, 2007), we evaluated the growth dynamics of the various *AtCHR23* expression variants under different light regimes. In continuous light, all *AtCHR23* mutants confirm the pattern of root length as presented above for long-day conditions. Over-expressing lines have a significantly reduced root length relative to the wild-type, whereas the knockout tends to have (in this case indeed significantly) longer roots (Figure 2.5A). In the dark, however, none of the lines significantly differed in root length from that of wild-type (Figure 2.5B). In the dark, root growth is known to be significantly reduced (Kircher *et al.*, 2012, Yazdanbakhsh *et al.*, 2010), while the hypocotyl is known to elongate (etiolate) more than in the light (Gendreau *et al.*, 1997).

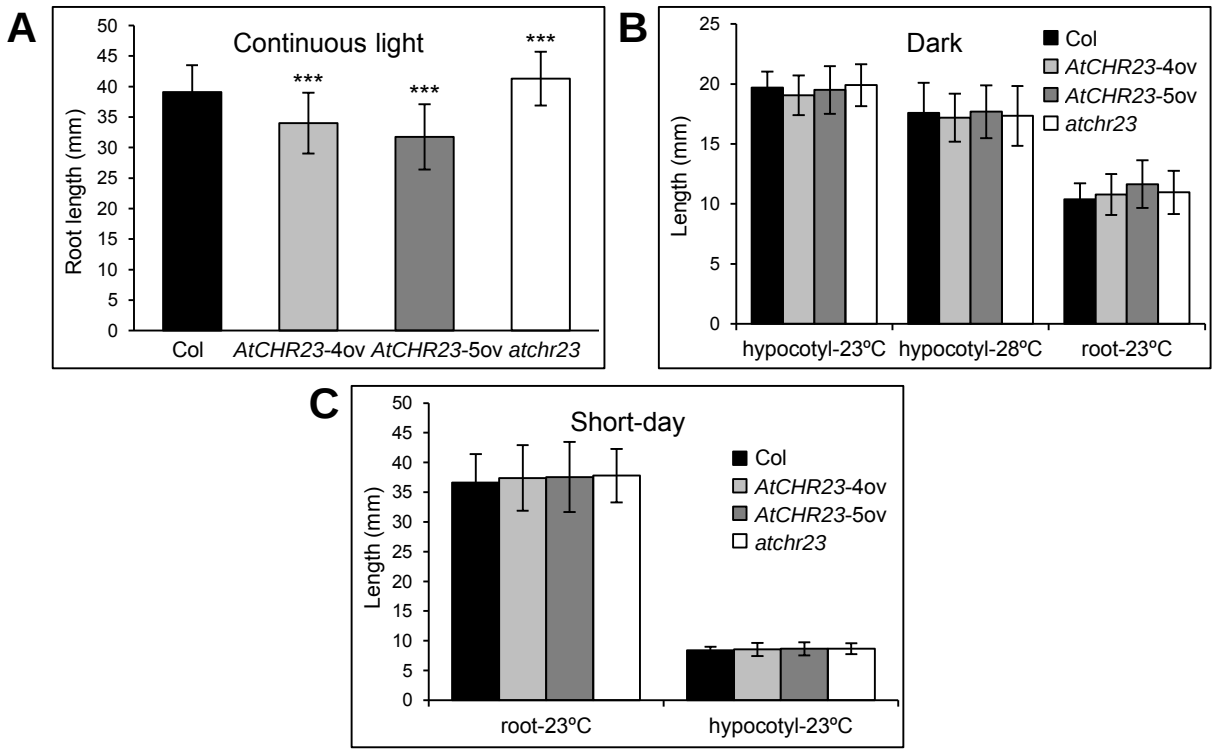

Figure 2.5: *AtCHR23* over-expression only affects root length in sufficient light. **(A)** Mean (± s.d.) root length of wild-type (Col) and mutant seedlings grown for 10 days at 23 °C in continuous light. **(B)** Mean (± s.d.) root and hypocotyl length of 10-day-old wild-type (Col) and mutant seedlings grown at the indicated temperature in the dark. **(C)** Mean (± s.d.) root and hypocotyl length of 10-day-old wild-type (Col) and mutant seedlings grown at 23 °C in short-day conditions. For each line 40 seedlings were measured. Asterisks indicate significant differences from the wild type: ***, $P < 0.001$.

Establishing further reductions in root length in such an environment is therefore less reliable. However, also the length of the hypocotyl of seedlings grown in the dark at either 23 °C or 28 °C (Figure 2.5B) was not different from the wild-type. Also at short day conditions (10 days at 8 h light/16 h dark at 23 °C; Figure 2.5C), the length of neither roots nor hypocotyls of mutants could be distinguished from the wild-type. One possible cause for the lack of the phenotype in dark and short-day could be the instabilities of *AtCHR23* mRNA over-expression. However, quantitative expression analysis of *AtCHR23* in dark and short-day grown seedlings confirmed the same level of up-regulation relative to wild-type as in long-day (data not shown). The lack of phenotype in dark and short-day grown mutants cannot be therefore explained by reduced levels of *AtCHR23* over-expression. These results show that light markedly influences the impact of modified *AtCHR23* expression on the growth dynamics of Arabidopsis seedlings: sufficient (amounts of) light is required to establish the *AtCHR23*-mediated growth phenotype.

Abiotic stress magnifies the impact of *AtCHR23* over-expression

The impact of modified *AtCHR23* expression is also apparent in environmental stress. Seedlings were assayed under abiotic stress conditions on agar plates containing 75 mM NaCl (salt stress; Figure 2.6A) or 200 mM mannitol (osmotic stress; Figure 2.6C). Both stresses had, as expected, a clear negative impact on root growth. The average length of the roots of wild-type seedlings in an environment with salt stress was 30.92 mm (Figure 2.6B) and in osmotic stress 32.51 mm (Figure 2.6D), whereas without such stress the length was 40.7 mm (see Table 2.1 and Figure 2.1). This shows that salt stress reduces the root length of the wild-type by 24% and osmotic stress by 20%. The over-expressing mutants *AtCHR23*-4ov and *AtCHR23*-5ov respond to salt by 32% and 36% reduction of root length, respectively (Figure 2.6B). In osmotic stress, this reduction was 29% and 31%, respectively (Figure 2.6D). Similar results were obtained with the lines over-expressing *AtCHR23* cDNA copy (Figure 2.S3). In contrast, the knockout line *atchr23* has slightly longer roots than the wild-type, but only in osmotic stress (average length 33.9 mm; Figure 2.6D). These data indicate that the *AtCHR23* over-expressing lines respond to stress conditions by stronger growth arrest of the root length than the wild-type. A non-parametric factor analysis showed highly significant ($P < 0.001$) effects of both genotype and stress treatment on root length, and significant ($P < 0.01$) effects of genotype X treatment interaction on root length, in all mutant lines except for knockout line at osmotic stress (Table 2.S2). The same is observed in further vegetative development. After applying salt stress by watering two-week-old plants with 100 mM NaCl twice in 3 days, the rosette diameter of soil-grown plants (Figure 2.6E) was measured. The rosette diameter of wild-type without stress was 34.1 mm^2 whereas after stress, it was 30.34 mm^2, which is a reduction of 11%. The *AtCHR23*-4ov plants respond to salt stress by two-fold higher (22%) reduction of the rosette diameter: from 30.1 mm^2 to 23.49 mm^2 (Figure 2.4D, 2.6F). The

non-parametric factor analysis showed highly significant ($P < 0.001$) effects of both genotype and treatment on rosette diameter, however the effect of genotype X treatment interaction was not significant (Table 2.S2). It shows that abiotic stress magnifies the effect of *AtCHR23* over-expression on the seedlings growth and that the effect extends beyond the seedling stage.

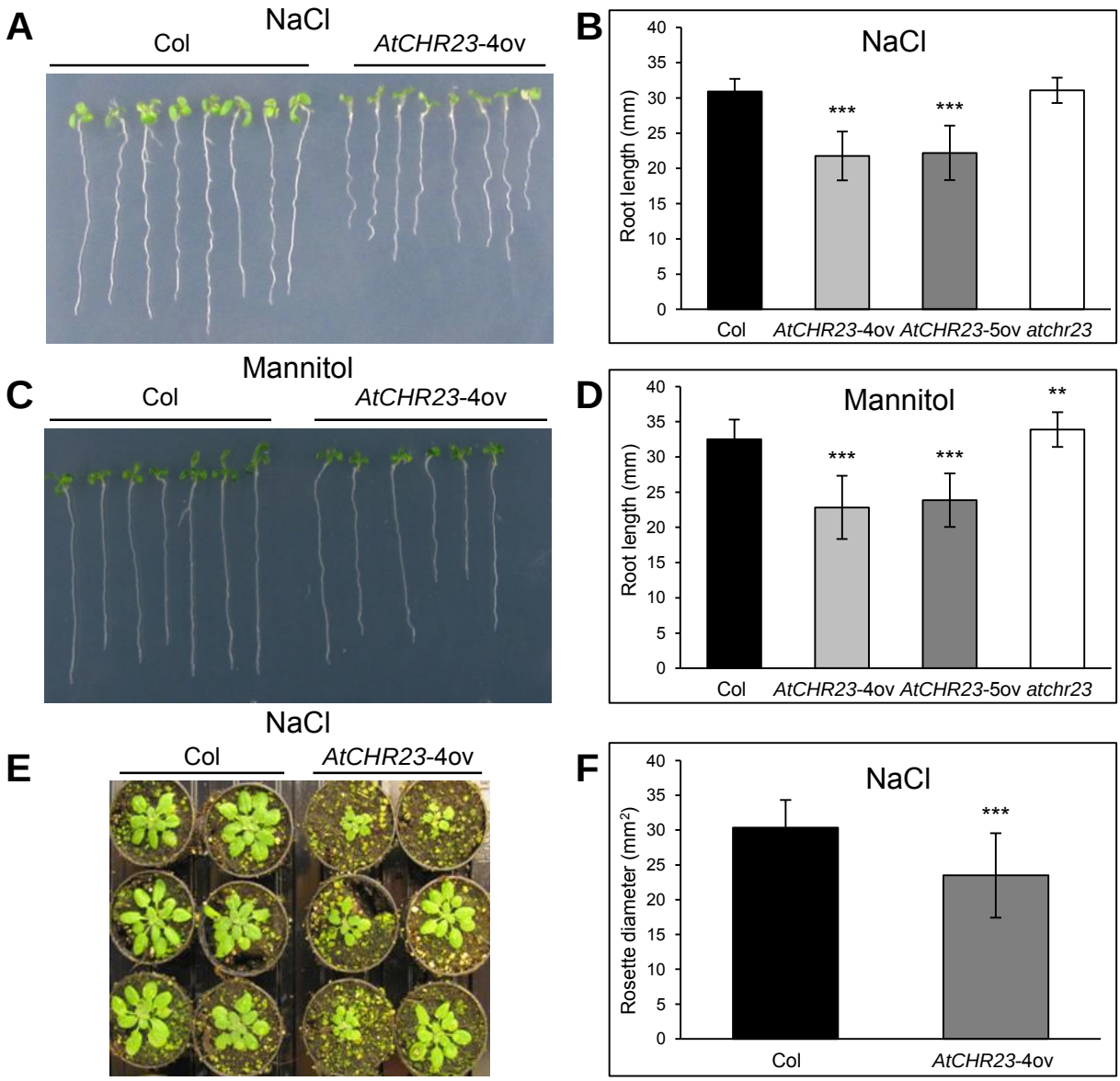

Figure 2.6: Abiotic stress emphasizes the reduction of growth in case of *AtCHR23* over-expression. **(A)** Photograph of 8-day-old seedlings grown at 23 °C in long-day conditions on medium supplemented with 75 mM NaCl. **(B)** Mean (± s.d.) length of the primary roots of 8-day-old seedlings grown on 75 mM NaCl. **(C)** Photograph of 8-day-old seedlings grown at 23 °C in long-day conditions on medium supplemented with 200 mM mannitol. **(D)** Mean (± s.d.) length of the primary roots of 8-day-old seedlings grown on 200 mM mannitol. **(E)** Photograph of 4-week-old wild-type and *AtCHR23*-4ov plants two weeks after application of salt stress. **(F)** Mean (± s.d.) rosette diameter of 4-week-old plants two weeks after application of salt stress. For each assay and line, 40 seedlings or 15 plants were measured. Asterisks indicate significant differences from the wild type: **, $P < 0.01$; ***, $P < 0.001$.

Genome-wide RNAseq analysis demonstrates increased variability of gene expression upon *AtCHR23* over-expression

The growth phenotype conferred by *AtCHR23* over-expression was evaluated by RNA sequencing. Two biological replicates of pooled eight-day-old seedlings of *AtCHR23*-4ov and the wild-type (Columbia) grown at 23 °C in long-day (with the reduced growth phenotype) and short-day (without the reduced growth phenotype) photoperiods were evaluated. For each of the eight samples, more than 60 million reads were generated. Given the experimental set-up, expression differences associated with the reduced growth phenotype were expected between the over-expressing lines in long-day conditions relative to all other samples.

Differential expression analysis using DESeq (Anders *et al.*, 2010) or cuffdiff (Trapnell *et al.*, 2010) resulted in lists of potentially differentially expressed (DE) genes. However, in additional biological replicates many of these could not be confirmed. From 96 genes identified by DESeq as potentially DE in long-day mutant (File 2.S1), 24 genes were analyzed by qRT-PCR and 7 were confirmed as differentially expressed (33.3% of tested genes). We concluded that identified DE genes cannot be biologically validated. Further analyses therefore focused on the apparent variation in gene expression. Comparison of the expression values expressed as summed fragments per kilobase of transcript (exon model) per million mapped reads (FPKM) of replicates R1 and R2 for each sample showed the Pearson's correlation coefficients above 0.99 (Figure 2.7), except for the only sample in which the growth phenotype was present: *AtCHR23* over-expression in long-day conditions. In this case the data are much more disperse from the line of best fit and the Pearson's correlation coefficient is just above 0.97 (Figure 2.7). In order to assess the larger between-replicate expression variability in mutant long-day, we calculated for all genes the absolute differences between the $\log_2$(FPKM + 1) expression level in the two replicates. The larger expression difference shown by the top 1% of the genes in wild-type (195 genes) was taken as cut-off for variability and used to select the number (and identity) of the genes in all other samples that showed variability higher than specified cut-off. This threshold was equivalent to an expression difference of about 1.5 fold on the normal scale. In the scatter plots of genome-wide gene expression, these genes are depicted in red (Figure 2.7).

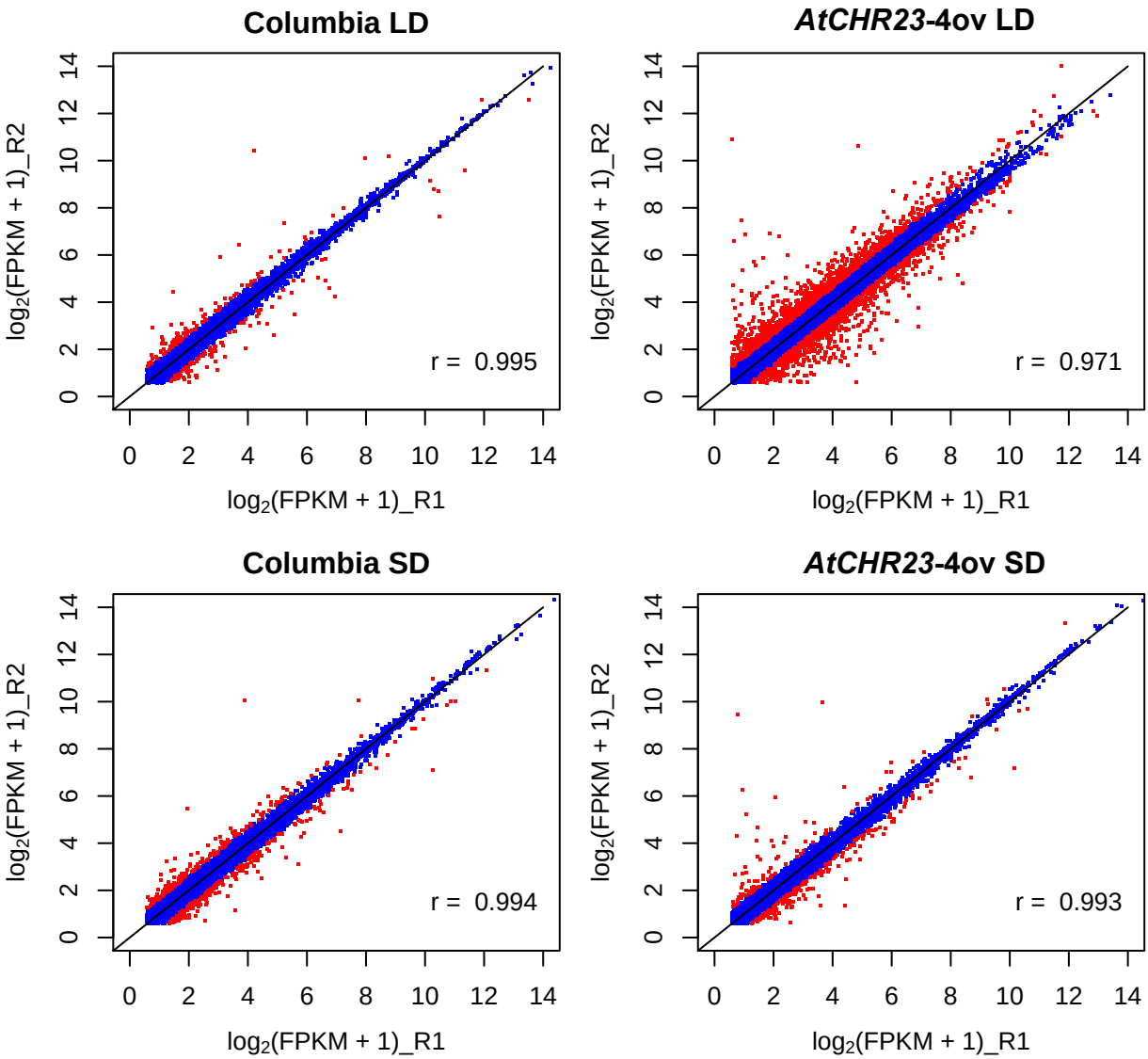

Figure 2.7: Scatter plots of gene expression expressed as $\log_2$(FPKM + 1) show more pronounced variability in long-day grown over-expressing mutant. Expression was determined from RNAseq reads for the wild-type (Columbia) and mutant (*AtCHR23*-4ov), with biological replicates indicated with R. Each dot represents a gene. Genes displaying a variability of expression above the cut-off specified (see text) are shown in red. In the bottom of each graph the pair-wise Pearson's correlation of all genes depicted is shown. LD, long-day; SD, short-day; R1, biological replicate 1; R2, biological replicate 2.

In long-day conditions, the *AtCHR23* over-expressing mutant has no less than 2007 genes with larger variation (Figure 2.8A). Of these, 68 genes were also variable in wild-type (Figure 2.8; File 2.S2). This shows that *AtCHR23* over-expression increases the expression variability of a considerable subgroup of genes compared to the wild-type. In contrast, in short-day conditions, 381 genes were identified as variable in the wild-type, whereas 276 genes were identified in the mutant line, of which 82 were shared (Figure 2.8B; File 2.S2). The larger subgroup of variable genes is therefore associated with the higher over-expression of *AtCHR23* observed in long-day conditions. This may point to a causal relationship between *AtCHR23* over-expression and increased variability of gene expression. The 68 long-day

variable genes shared between the wild-type and the mutant are less correlated between the two replicates of *AtCHR23* over-expressing mutant (R^2 = 0.038) relative to the wild-type (R^2 = 0.625) (Figure 2.9). It indicates that the expression of genes which are already noisy in natural conditions (the wild-type) become even noisier when *AtCHR23* is over-expressed.

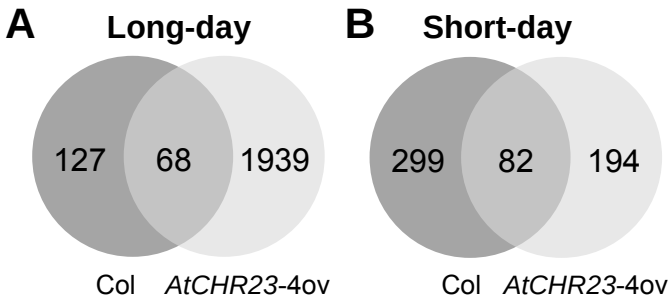

Figure 2.8: Numbers of genes with high expression variability. The number of plant-line specific and shared genes that are identified as variable are given for the wild-type (Col) and over-expressing mutant (*AtCHR23*-4ov) in long-day **(A)** and short-day **(B)** conditions.

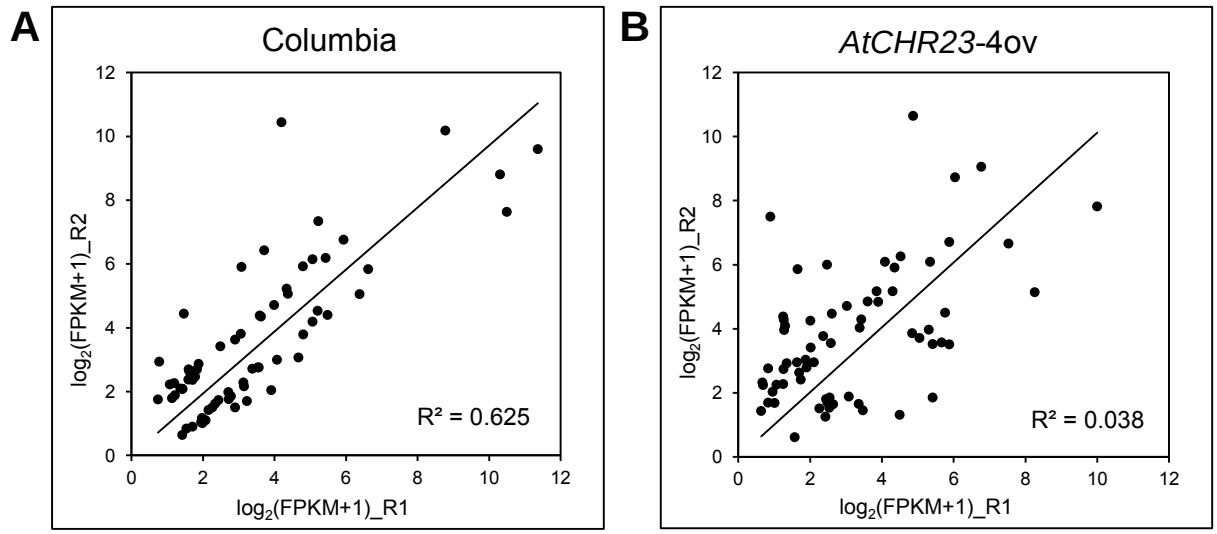

Figure 2.9: Over-expression of *AtCHR23* pronounces the variation in genes that are already variable in the wild-type. The panels show scatter plots and the linear regression line of the gene expression expressed as $\log_2$(FPKM + 1) of the 68 genes identified as variable of the two biological replicates of **(A)** the wild-type (Columbia) and **(B)** the *AtCHR23*-4ov over-expressing line, both grown in long-day conditions. The coefficient of determination (R^2) is shown in the panel. R1, biological replicate 1; R2, biological replicate 2.

To evaluate the function of the genes with higher variation in gene expression when *AtCHR23* is over-expressed, gene ontology (GO) analysis was performed. For this, the subset of 298 genes (from the 2007) was selected that had at least 3-fold expression difference between the two biological replicates. Genes were classified using the Classification SuperViewer (Provart *et al.*, 2003) as being over- or under-represented. The main results are summarized in Figure 2.S4. Biological Process subcategories that were over-represented include responses to stress, stress stimuli and developmental processes, in addition to other biological processes. This is in good agreement with the phenotypic observations presented above.

AtCHR23 over-expression enlarges differences in gene expression among individuals for selected subsets of genes

To address the impact of variation on gene expression in individual seedlings, eight genes were selected for additional analyses. Four genes were randomly selected from the list of *AtCHR23*-4ov variable genes at long-day conditions. In addition four genes were randomly selected that were identified as not variable (including *AtCHR23*/*At5g19310*). Details of these genes are given in Table 2.S3. The expression of these eight genes was analyzed by quantitative RT-PCR in six individual seedlings of over-expressing mutants and the wild-type grown at long-day conditions. Box plots summarizing these data show considerably more variation in expression among individual seedlings of the various mutants compared to the wild-type for the four variable genes (Figure 2.10A). In contrast, none of the genes selected for lack of variation showed such a large expression variability between individual seedlings in any of line tested (Figure 2.10B). In individual seedlings different from seedlings analyzed in Figure 2.10 additional three variable (*At1g04220*, *At3g22640*, *At3g12580*) and three not variable genes (*At5g02490*, *At5g10140*, *At2g01422*) were analyzed. For all of them, except for *At3g12580*, the variability as detected by RNAseq was confirmed. Although based on limited number of individuals these data show that the increased variation of gene expression of distinct subset of genes is also apparent in individual seedlings. This emphasizes the importance of studying the expression pattern in individual plants. For direct biological proof of increased expression variation in *AtCHR23* over-expressing mutant more genes should be tested, preferably by extensive RNAseq analysis of a larger number of individual seedlings.

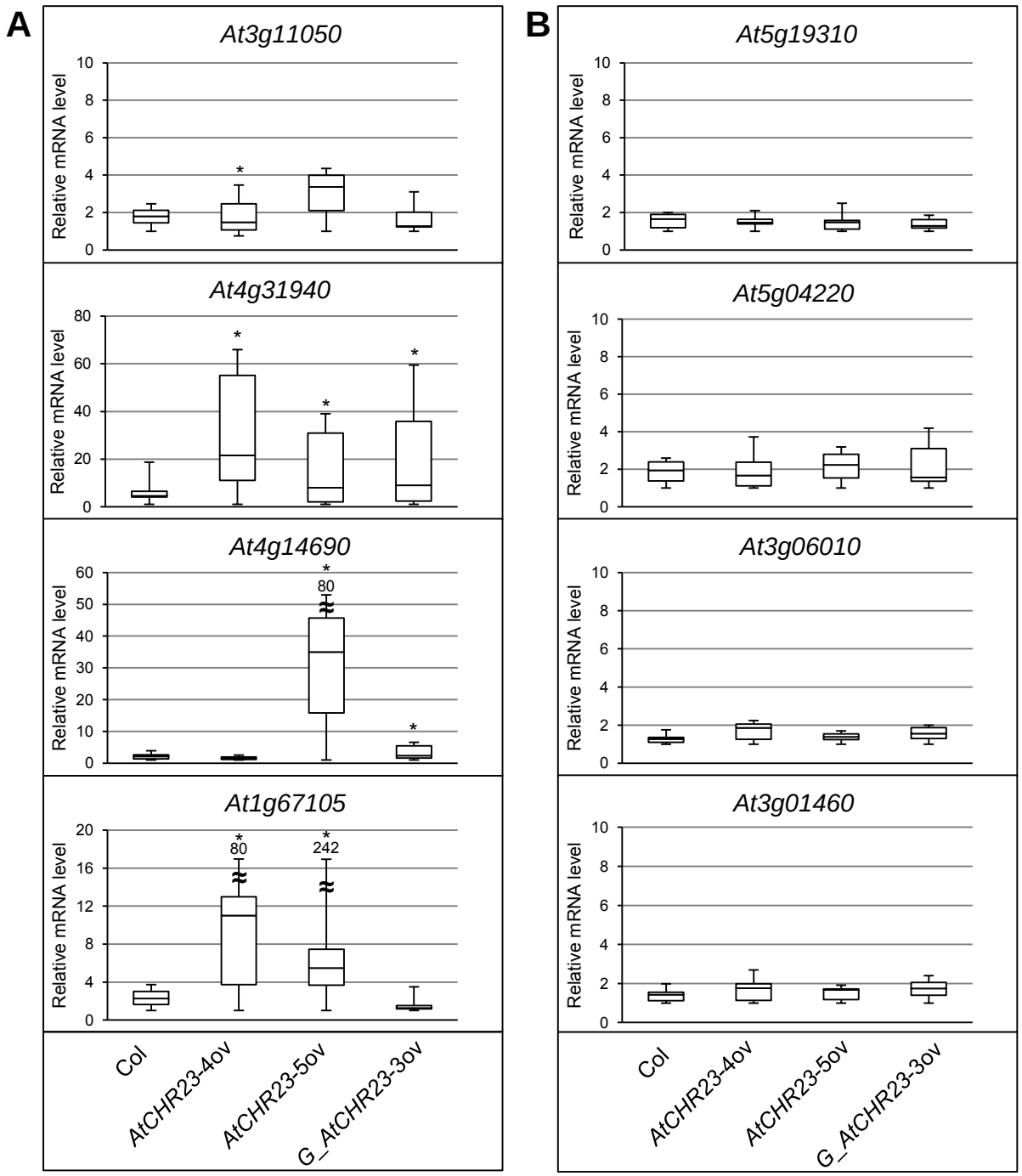

Figure 2.10: Box plots of the relative mRNA levels of selected genes in individual seedlings. Relative mRNA levels were determined in six individual seedlings, each of wild-type (Col) and three *AtCHR23* over-expressing lines, by quantitative RT-PCR using gene specific primers for eight different genes. Four of these genes **(A)** were characterized by high variability between the replicates of the over-expressing lines and four genes **(B)** did not show such variability. After normalization to the *UBC* reference gene, the expression value for each gene and each individual seedling was scaled relative to the lowest expression value (set to 1) for that gene in the line. In each box plot, the box area represents the lower and the upper percentiles, the horizontal line within the box indicates the median value and the horizontal dashes at the top and the bottom of the box show the minimum and maximum values observed. Asterisk indicate significantly different variances (Levene's test) from the wild type at $P < 0.05$.

DISCUSSION

Previously, we have presented detailed analyses of the over-expression of *AtCHR12* (Mlynarova *et al.*, 2007). *AtCHR12* is a paralog of *AtCHR23*: the two genes are very similar in (protein) sequence and are thought to be result of recent gene duplication in Arabidopsis (Bargsten *et al.*, 2013). Yet, their expression patterns are different in spatial and quantitative terms (Duarte *et al.*, 2006) and the results presented here show that over-expression of these two genes have different impact on the growth parameters of Arabidopsis. Over-expression of *AtCHR12* had not the phenotypic effects here documented for *AtCHR23* over-expression. Under normal environmental conditions, the phenotype of the *AtCHR12* mutant cannot be distinguished from the wild-type. However, exposing the *AtCHR12* over-expressing mutant to mild stress conditions led to growth arrest of normally active primary buds and reduced growth of the primary stem. It demonstrated that chromatin remodeling-associated growth arrest is priming the plants for growth arrest upon actual stress after the transition to reproductive development (Mlynarova *et al.*, 2007).

AtCHR23 over-expression affects the growth of seedlings and the vegetative rosette. As the two ATPases affect plant growth in different ways at different stages of development, these two genes present a clear and not-so-common example in Arabidopsis of sub-functionalization of very similar genes after gene duplication (Duarte *et al.*, 2006). In the genome of crops as potato and tomato, only a single gene instead of two paralogs is present (Bargsten *et al.*, 2013). This single ortholog is supposed to combine the function of both *AtCHR12* and *AtCHR23* in the environmental growth response of such crops. If confirmed in future experiments it could open a new possibility to improve the environmental growth response of agriculturally relevant crops.

Over-expression rather than loss-of-function is required to observe the growth phenotype

The *atchr23* loss-of-function mutant did not show any visible phenotype, neither did the equivalent *atchr12* loss-of-function mutant. The *atchr12 atchr23* double knockouts fail to initiate root and shoot meristems and are embryo lethal (own observations; Sang *et al.*, 2012). A barely viable double mutant containing weak knockout alleles showed severe defects in the maintenance of stem cells, extremely delayed development, bushy appearance, floral organ aberrations and substantially shortened roots (Sang *et al.*, 2012). The short roots of such double knockouts had significantly reduced cell division and other defects in meristem organization (Sang *et al.*, 2012). It suggests that in embryo and endosperm development, these two paralogous genes seem to be redundant (Sang *et al.*, 2012). In contrast to the phenotypic impact of the weak double knockout, *AtCHR23* over-expression is not associated with any morphological effect on the root meristem (Figure 2.3A). Over-

expression correlates with a shorter root elongation zone and a reduced hypocotyl and cotyledon growth. The post-embryonic growth of these organs is mostly the result of controlled cell elongation and does not involve significant cell divisions (Gendreau *et al.*, 1997, Stoynova-Bakalova *et al.*, 2004). Therefore, *AtCHR23* over-expression exerts (most) impact on cell expansion rather than on cell division. The phenotype of the weak double knockout was accompanied by the changes in the expression of several meristem marker genes (*WOX5, SHR, SCR*) and several cell cycle genes (Sang *et al.*, 2012). None of these genes was identified as differentially expressed and/or variable in the RNAseq analysis of the *AtCHR23* over-expression phenotype here presented.

Over-expression accomplished by either the native or the much stronger CaMV 35S promoter resulted in the same growth phenotype. A relatively small fold up-regulation of about 2 (Table 2.1; line CHR23:G_*AtCHR23*ov) is apparently sufficient and no clear correlation between fold up-regulation and root length reduction is evident (Table 2.1). This lack of dose dependency of up-regulation indicates that other limiting factors are likely to be involved. SWI/SNF2 ATPases function in the context of protein complexes (Mohrmann *et al.*, 2005) and one of the participants of such a complex may become limiting. The larger amount of AtCHR23 may be in competition with such a factor, or drains it from such complexes. If so, the over-expression phenotype would represent an on/off case and allow for more fine-grained analysis of the detailed role of AtCHR23 in growth regulation than the analyses of knockouts (Prelich, 2012). Such severe effects from small folds of up-regulation are rather difficult to study, but especially in a complex phenotype as vegetative development, such subtle effects may be more the rule than the exception (Birchler *et al.*, 2012). Several other examples are known where relatively small fold over-expression results in clear phenotypic effects (Chen *et al.*, 2002, Delgado *et al.*, 2010).

ATCHR23 function needs long-day light condition

The growth phenotype of *AtCHR23* over-expression is only apparent in (sufficient) light. Its function or the function of (one of its) direct partners must therefore be photoperiod-dependent. Photoperiodicity is one of the most significant and complex of interactions between plants and their environment. It is the major stimulus that plants use to detect seasons (Davis, 2002). A well-known response to the photoperiod is flowering, but it also affects seed germination, leaf formation rate, leaf size and dry matter production (Adams *et al.*, 2005). The lack of a clear phenotype in dark and short-day condition could result from dark-induced proteolytic degradation of either AtCHR23 or AtCHR23 targets. An example of such a regulation is the transcriptional regulator CONSTANS (CO) that promotes flowering of *Arabidopsis thaliana* under long summer days, but not under short winter days (Valverde, 2011). More detailed analyses of AtCHR23 will be required to assess whether protein stability plays a role in the regulation of and/or by *AtCHR23*. Conversely, the putative target genes of AtCHR23 may be light regulated and the AtCHR23 remodeling

complex contributes to the fine-tuning of this regulation. In view of the reaction of the various plant lines to environmental stress (Figure 2.6), light or light duration may become perceived as stress.

Over-expression of *AtCHR23* increases variability of growth and gene expression

The length of the root (or the hypocotyl) in populations of seedlings behaves as a typical quantitative trait: it has a frequency distribution around a population average of in this case genetically identical plants. Over-expression of *AtCHR23* shifts the distribution to an average of shorter roots (Figure 2.2A) and hypocotyls (Figure 2.2B). Such a continuous distribution indicates a polygenic trait rather than a phenotype controlled by a single locus and/or the involvement of the environment in which epigenetic processes are believed to play an important role (El-Lithy *et al.*, 2004, Richards, 2009). The frequency distributions tend to overlap and when considered on an individual basis, some over-expressing seedlings are individuals with growth comparable with individual wild-type seedlings (Figure 2.1A, Figure 2.2). In the individual case, over-expression of *AtCHR23* may not necessary result in reduced growth. This indicates that over-expression does not have inhibitory effect on growth *per se*. Upon over-expression, the mutant seedlings show a more broad distribution of growth parameters than the wild-type (Figure 2.2). Therefore, *AtCHR23* over-expression increases the within-population variability of growth, best expressed as coefficient of variation (CV; Table 2.1) of growth.

In long-day conditions, *AtCHR23* over-expression associates with increased variability of gene expression between biological replicates. It is tempting to assume a causal relationship between these two associated phenomena. Although the larger variation could originate from environmental factors and/or the biological material used (seed batches), extreme care was taken to exclude interference of such experimental factors. Light intensity, temperature, humidity were carefully controlled and monitored; wild-type and mutant seedlings were without exception grown at the same time and the same conditions; RNA samples were isolated simultaneously and RNA handling procedures were synchronized as much as possible. Moreover, the variability of the same over-expressing line in short-day conditions was similar to that of the wild-type.

The apparent effect of *AtCHR23* over-expression on increasing expression variability is not unknown in other biological systems. Inter-individual differences in gene expression is observed in many organisms, including human, mice, fish and yeast (Fay *et al.*, 2004, Li *et al.*, 2010, Oleksiak *et al.*, 2002). Also in plants, considerable variability of gene expression can occur between genetically identical individuals in identical environments. Gene expression can differ seemingly randomly in amplitude, frequency and timing between genetically identical cells. Such stochasticity of gene expression is nongenetic or epigenetic in nature and thought to be an intrinsic property of gene expression itself: stochastic noise (Raj *et al.*, 2008). The 68 genes identified as variable in both the wild-type and the over-expressing mutant

could represent intrinsically variable or noisy genes in the tissues and conditions examined. Because *AtCHR23* over-expression makes intrinsically variable genes more variable, the AtCHR23 remodeling complex could be involved in tuning the noise levels of variable genes. Stochastic noise can be beneficial, *e.g.* for survival in fluctuating stressful environment (Chalancon *et al.*, 2012, Raser *et al.*, 2004), but in general it is considered to decrease fitness or interfere with development (Raj *et al.*, 2008, Wang *et al.*, 2011).

Chromatin-related events are thought to be a component of the regulation of the stochastic noise in gene expression. For example, in yeast, deletion of individual components of chromatin remodeling complexes such as SWI/SNF increased the expression fluctuation from the PHO5 promoter significantly (Raser *et al.*, 2004) and variable genes are distinctly regulated by chromatin modifiers (Choi *et al.*, 2008). In most biological systems known today, the intrinsic variability of expression has to be controlled or buffered (Raj *et al.*, 2010) to ensure optimal development and growth. The ability to buffer variations generated by molecular noise, or environmental fluctuations is termed robustness (Lempe *et al.*, 2013). It is suggested that the expression of genes with an essential role in development or differentiation is highly robust (Macneil *et al.*, 2011), whereas expression of stress-responsive genes tends to be much more variable between cells and individuals (Blake *et al.*, 2006, Raj *et al.*, 2008). The latter suggestion is in agreement with the significant enrichment for stress and stress stimuli responsive genes in the GO analyses of genes that are highly variable between the two replicates of the *AtCHR23* over-expressing line grown in long-day conditions (Figure 2.S4).

The putative perils of pooling

The best way of RNAseq analysis is still being discussed (McCarthy *et al.*, 2012, Trapnell *et al.*, 2013) and may depend on both biological and statistical issues, such as sampling, pooling, pooling design, the distribution of (biological or environmental) variation and others (Kendziorski *et al.*, 2005, Kliebenstein, 2012). Accounting for biological variation in gene expression is important for reliable and biologically relevant differential expression analysis (Oshlack *et al.*, 2010). Large variation of the expression of subset of genes between the individual seedlings in pools (Figure 2.10) could for example result in poor reproducibility between data from different pools.

We have here presented an RNAseq data analysis that is focusing on the variability of gene expression as the topic-of-interest. Independent validation by qRT-PCR showed the validity of the approach developed, although more advanced statistics may distill more understanding from the RNAseq data here presented. This data is based on pools of genetically identical seedlings that however may show highly variable gene expression. Such between-individual variation in gene expression did not yet get too much attention, but the depth of the new sequencing technologies could provide approaches to circumvent this

limitation (Whitehead *et al.*, 2006). In fact, also the analysis of a single whole seedling concerns a pool of various tissues in different developmental stages that may have differences in gene expression. In the future, large-scale single cell transcriptomics may resolve such complexities (Fritzsch *et al.*, 2012, Tang *et al.*, 2011).

We have shown that in transgenic Arabidopsis, the over-expression of the SWI/SNF2-type ATPase *AtCHR23* increases the variability of growth and the variability of expression of a distinct subset of genes in populations of genetically identical plants. These results suggest that accurate and controlled expression of *AtCHR23* contributes to more stable or robust gene expression that results in a more uniform growth phenotype. Based on the phenotypic and expression data here presented we propose that the AtCHR23 remodeling complexes could be a component of a buffering system of gene expression in plants. If that system of buffering is disrupted by over-expression of *AtCHR23*, downstream genes become more variable and compromise the expression of other genes in ways that result in the reduced growth phenotype here documented. Phenotypic robustness influences all parameters important for plant growth, yield and quality. The findings presented here will help to better understand and use chromatin remodeling genes as exponents of a potential buffer of phenotypic and transcriptional variation, particularly in conditions of changing environments.

MATHERIALS AND METHODS

Construction of T-DNA plasmids for transformation

To generate plants that over-express *AtCHR23* the genomic copy of the gene sequence (including all 11 introns) was obtained by PCR from Arabidopsis Col-0 wild-type. Three sets of primers were used with the Phusion™ DNA polymerase (see Table 2.S4 for details). All three PCR fragments were cloned into pJET (Fermentas) and verified by sequencing. The genomic copy of *AtCHR23* was assembled by ligation of appropriate restriction fragments of three PCR sequences into pENTR4 (Invitrogen). The resulting plasmid carries the whole gene including 127 nucleotides of 5'UTR. This was recombined in an LR Gateway (Invitrogen) reaction with pB2GW7 (http://gateway.psb.ugent.be/). The resulting binary vector 35S::*AtCHR23* (Figure 2.S1) was introduced into *Agrobacterium tumefaciens* C58C1 (pMP9) and used for Arabidopsis transformation. Transgenic lines were selected based on PPT resistance (5 μg ml^{-1} phosphinothricin-DL) and screened for the level of transgene expression. Such lines were designated *AtCHR23*-ov. In addition, two GFP-tagged constructs carrying a cDNA copy of *AtCHR23* driven either by the CaMV 35S or the endogenous promoter were prepared (Figure 2.S1). The cDNA copy of *AtCHR23* was prepared from RNA with the SuperScript® III First-Strand Synthesis System employing the oligo$(dT)_{20}$ primer

(Invitrogen) and PCR amplification using the CHR23_F4 and CHR23_R4 primers (Table 2.S3). The full length cDNA sequence was recombined by Gateway BP clonase into the pDONR221 entry vector (Invitrogen). The resulting plasmid was next recombined in an LR Gateway reaction into the destination vector pK7FWG2 (http://gateway.psb.ugent.be/). The resulting binary vector 35S::*GFP-AtCHR23* was used for Arabidopsis transformation. Transgenic lines were selected based on kanamycin resistance (50 μg ml^{-1}) and the level of transgene expression. Such lines were designated G_*AtCHR23*-ov. The promoter sequence (918 bases) of *AtCHR23* including the 5 'UTR was isolated by PCR using the primers pCHR23_F and pCHR23_R (Table 2.S4) and cloned into the pENTR4 entry vector. The desired promoter sequence was selected with appropriate restriction enzymes and cloned into a derivative of pENTR4 carrying the *GFP* gene. The resulting clone was, together with *AtCHR23* cDNA entry clone described above, assembled in a multi-step LR Gateway reaction into the modified destination vector pBGW (http://gateway.psb.ugent.be/). The resulting binary vector pCHR23::*GFP-AtCHR23* was transformed to Arabidopsis. Transgenic plants were selected based on PPT resistance (5 μg ml^{-1} phosphinothricin-DL) and the line used for further analysis was designated CHR23:G_*AtCHR23*ov.

Plant material and growth conditions

All transgenic Arabidopsis plants over-expressing *AtCHR23* were generated by transformation of wild-type *Arabidopsis thaliana* Col-0 using the floral dip method (Clough *et al.*, 1998). For analysis, homozygous F3 plants were used. The loss-of-function mutant lines of *AtCHR23* (*At5g19310*) SALK_057856 and SALK_139883 were obtained from the Arabidopsis Stock Center (Salk Laboratory, Institute of Genomics Analysis, USA; generated by J.R. Ecker (Alonso *et al.*, 2003)). SALK_057856 carries the T-DNA insertion in the first exon and SALK_139883 carries T-DNA in the fifth exon of *AtCHR23*. The zygosity of both SALK lines was determined on 30 μg ml^{-1} kanamycin plates. For both knockouts no full length cDNA product was detected (data not shown). The marker line pCYCB1;1:CYCB1;1-GUS (Colon-Carmona *et al.*, 1999) in Col-0 was obtained from M. Koornneef (Cologne/Wageningen). In all cases seeds were stratified for 3 days at 4 °C in the dark before sowing or analysis to synchronize germination. Seedlings were grown vertically in fully controlled growing chambers lit by Philips TD 32 W/84HF lamps at either 23 °C, 25 °C or 28 °C in long-day (LD; 16 h light/8 h dark) or short-day (SD; 8 h light/16 h dark) photoperiods. Light conditions were adjusted according to the experimental set-up. Plants were grown in standard potting soil in 16 h light/8 h dark (long-day conditions) at 21 ± 2 °C in either a growth room lit by Philips-Master 36 W/830 lamps or in a controlled greenhouse with supplemental light provided by four Son-T (Philips Greenpower, 400 W) lamps when required.

Analysis of growth parameters

Root elongation assays were performed as described (Mlynarova *et al.*, 2007) on seedlings grown vertically on 0.5 x MS agar plates. For salt or osmotic stress treatments, seedlings were grown on plates supplemented with 75 mM NaCl or 200 mM mannitol, respectively. Seedlings were photographed (Canon SX120) after 8–10 days of growth and the root length was measured from the root tip to the base of the hypocotyl using ImageJ (http://rsb.info.nih.gov/ij). Detached hypocotyls, cotyledons and leaves were flattened on double-sided tape and also photographed for analysis with ImageJ. For the analysis of vegetative rosette growth, plants were photographed 4 weeks after germination, just before the transition to flowering. The diameter of the rosette was estimated using ImageJ after enclosing the entire rosette in a rectangular selection. The size of the meristem and elongation zone was determined in 6-day-old seedlings grown vertically on 0.5 x MS agar plates. The meristematic zone was measured as the length from the quiescent center till the transition zone and as the number of cells in cortex file between the quiescent center and the first cell of the transition zone. In the elongation zone was analyzed the length as the distance from the transition zone till the beginning of the differentiation zone and the size of the fully elongated cell. Images were obtained with Leica microscopes (Leica Microsystems) and were used in ImageJ for length determination. For most of the growth parameters, at least two to three replicates were performed. For measurements 15–20 roots were used. GUS patterns were observed as described previously (Mlynarova *et al.*, 2007) with Nikon Optiphot-2 microscope.

Statistical analysis

For statistical analysis, normality of data was evaluated with Shapiro-Wilk test (Henderson, 2006) and homogeneity of variances was tested with the Levene's test (Lim *et al.*, 1996) (http://www.stat.ufl.edu/~winner/sta6166.html). The significance of the difference between the means of wild-type and mutants in the same growth condition were calculated by non-parametric Mann–Whitney U test. For comparison of different growth conditions a non-parametric adjusted rank transform test (Leys *et al.*, 2010) was used. In charts and tables, asterisks *, **, and ***, respectively, indicate significance at the 0.05, 0.01 and 0.001 of confidence levels.

Analysis of gene expression by qRT-PCR and RNAseq

Seedlings were grown on agar plates in the same set-up and conditions as used for growth measurements (see above). Total RNA was isolated from the pools of eight intact 8-day-old seedlings using the E.Z.N.A.™ Plant RNA Mini Kit (Omega Bio-Tek, Inc., USA), followed by on column DNase treatment (Qiagen, RNase-free DNase Set). One microgram

of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). Ten times diluted cDNA was used for quantitative RT-PCR using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA) in an iCycler thermal cycler. Reactions were performed in triplicate. The *UBC* gene (*At5g25760*) was used as reference (Czechowski *et al.*, 2005). Sequences of primers used are given in Table 2.S3.

For RNA sequencing, total RNA was isolated as above from eight 8-day-old seedlings of either mutant (*AtCHR23*-4ov) or the wild-type, grown at 23 °C in either long-day (with the reduced growth phenotype) or short-day (without the reduced growth phenotype) photoperiods. In all cases, two biological replicates were included from two different seed stocks, either one year old (biological replicate 1) or half a year old (biological replicate 2). With four different conditions each with two biological replicates a total of 8 samples were analyzed. All eight RNAseq library preparations were performed according to manufacturer's recommendations (Illumina Truseq RNA sample Preparation Low Throughput protocol). The eight samples were multiplexed in one Hiseq 2000 lane (WUR sequencing facility) and sequenced in 100 bases paired-end reads with an insert size of approximately 300 bases. After demultiplexing, for each of the eight samples, more than 60 million reads were generated.

RNASeq bioinformatics

The quality of reads was assessed with FastQC (obtained from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter and quality trimming was performed with the CLCbio Genomics workbench (v. 5.5.1) using default settings. Reads were mapped against the *Arabidopsis thaliana* genome (v. TAIR10) using TopHat (v. 2.0.5; Trapnell *et al.*, 2009) with as default parameter settings: –-no-mixed, –-no-discordant, –M, –g 1, –-min-intron-length 50, –-max-intron-length 11000. Differential expression was analyzed with DEseq (v1.10.1; Anders *et al.*, 2010) and with cuffdiff in the cufflinks package (v. 2.0.2; Trapnell *et al.*, 2010) using setting options –u and –b without quality trimming. Gene expression levels were determined by calculating the FPKM (Fragment per Kilobase of transcript (exon model) per Million mapped reads) values. To analyze the variation in expression, the expression levels between two replicates for each sample and conditions were compared for all genes with FPKM > 0.5 in both replicates. The absolute difference of the $\log_2$ transformed FPKM values [$\log_2$ (FPKM + 1); approximately equivalent to fold change on the normal scale] was calculated and the top 1% of the genes of the wild-type plants grown in long-day conditions was used to define a cut-off for all other conditions to determine the number (and identity) of genes with a difference (*i.e.* variation) larger than this cut-off. Gene ontology (GO) analysis was performed with the Classification SuperViewer tool (Provart *et al.*, 2003) obtained from the Bio-Array Resource (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). Genes were functionally classified according to the three main GO categories: biological process, molecular function, and cellular component.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

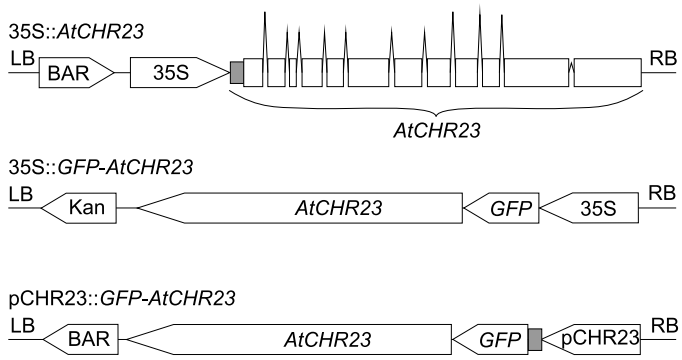

Figure 2.S1: Schematic layout of T-DNA regions of plasmids used to generate transgenic Arabidopsis over-expressing *AtCHR23* gene. 35S, CaMV 35S promoter; pCHR23, *AtCHR23* promoter; *GFP*, green fluorescent protein gene; Kan, kanamycin resistance gene; BAR, barnase herbicide resistance gene; RB, LB, right and left T-DNA borders. Grey shaded box indicate the presence of 5'UTR.

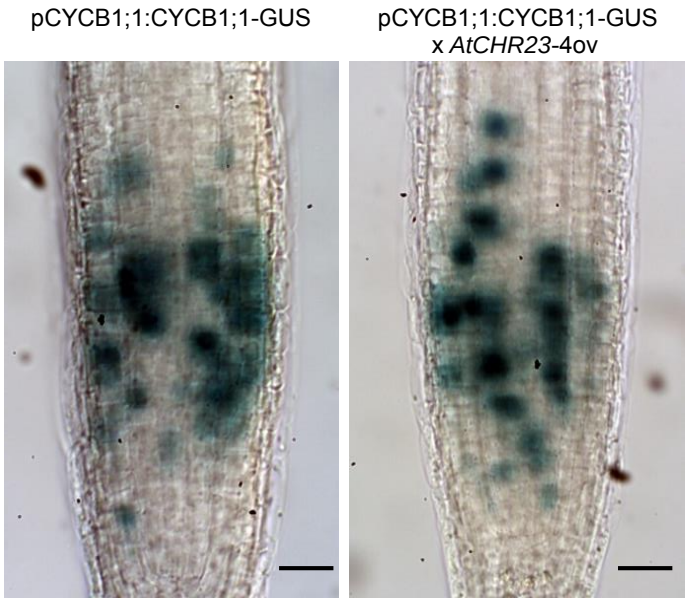

Figure 2.S2: Photograph of whole-mount, GUS-stained 4-day-old roots of CYCB1;1:CYCB1;1-GUS in wild-type (left) and in *AtCHR23*-4ov homozygous for both transgenes. *AtCHR23*-4ov was crossed with the transgenic line pCYCB1;1:CYCB1;1-GUS that contains the *GUS* reporter fused to the mitotic destruction sequence (D-box) and the cyclin *CYCB1;1* promoter. In this reporter line, *GUS* is expressed upon entry into the G2 phase of cell cycle via the *CYCB1;1* promoter and its protein product is degraded upon exit from the metaphase via the D-box. Bars: 20 μm.

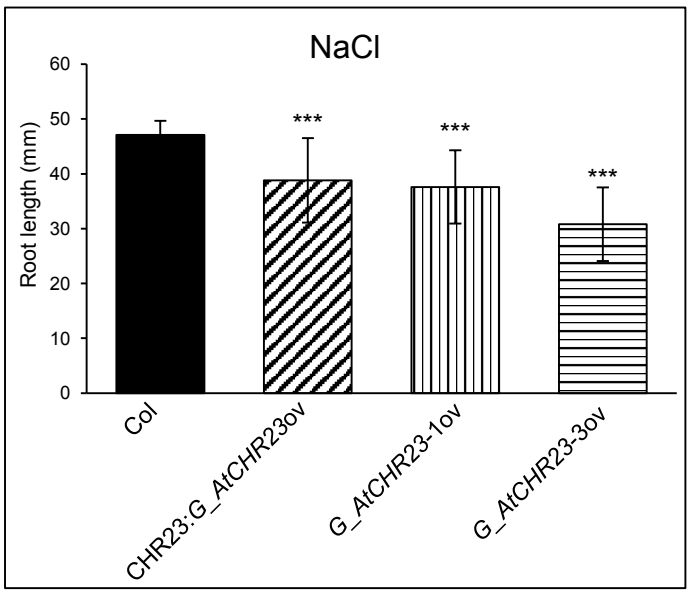

Figure 2.S3: The negative impact of *AtCHR23* cDNA over-expression on growth is enhanced by salt stress. Mean (± s.d.) length of the primary roots of 10-day-old wild-type (Col) and mutant seedlings grown on 75 mM NaCl. For each line 40 seedlings were measured. Asterisks indicate significant differences from the wild type: ***, $P < 0.001$.

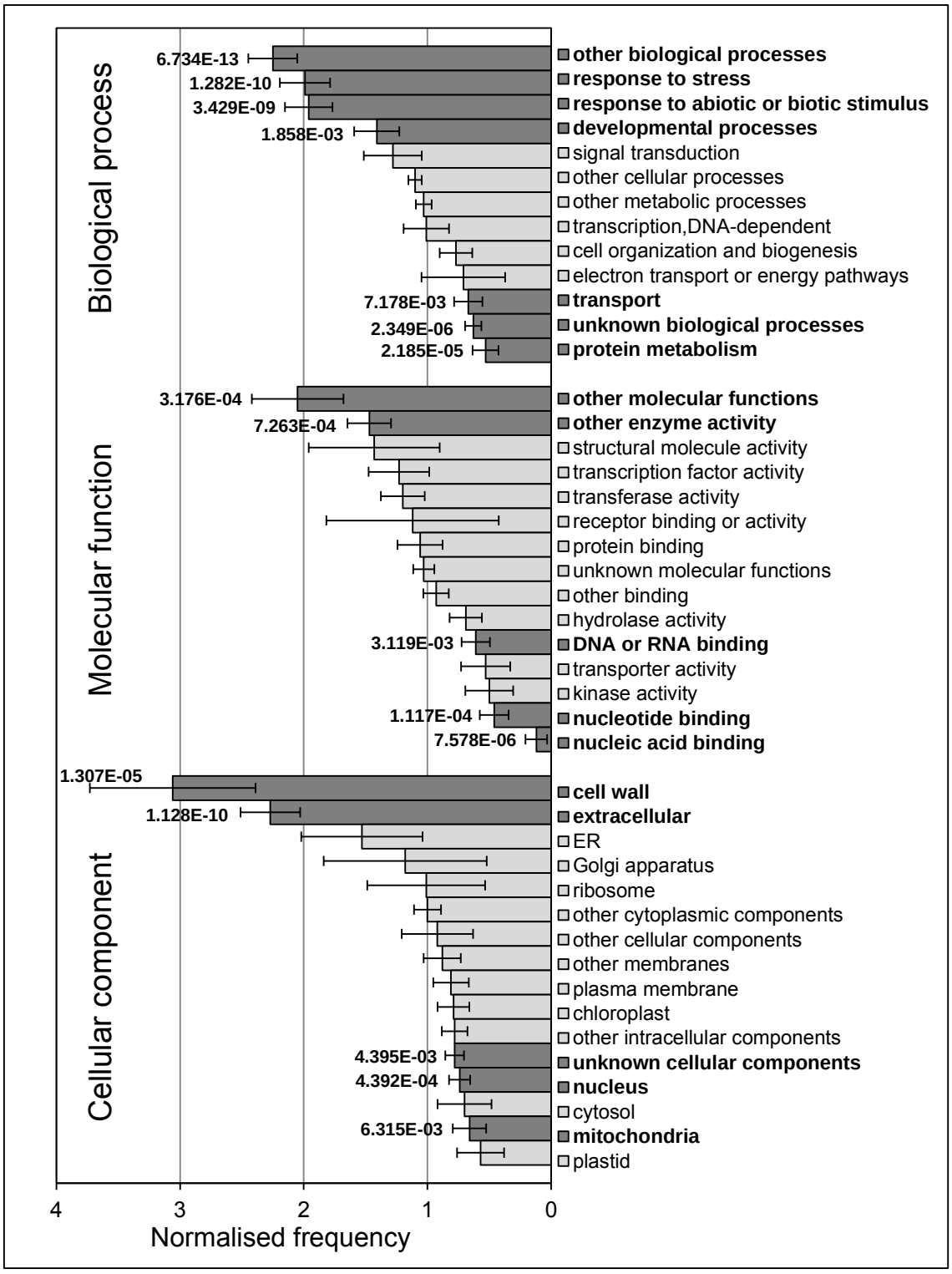

Figure 2.S4: Gene ontology (GO) analysis of the genes showing high variability in expression between the two replicates of *AtCHR23* over-expressing mutant grown in long-day conditions. The 298 genes showing at least 3-fold expression difference between the two replicates were classified with Classification SuperViewer. Normalised frequency of GO categories ± bootstrap s.d. is presented. Categories with a normalized frequency greater than 1 are over-represented and lower than 1 are under-represented. The over- or under-representation of categories highlighted in dark grey and bold are statistically significant at $P < 0.01$; the *P*-value is indicated next to the s.d.

Table 2.S1: Effect of modified *AtCHR23* expression on growth traits.

Trait	Line	Mean[a]	CV[b]	VAR[c]	P(VAR)[d]
Cotyledon area (mm^2)	Columbia - WT	4.675	0.183	0.730	na
	AtCHR23-4ov	**3.357**	0.232	0.605	ns
	AtCHR23-5ov	**3.830**	0.206	0.624	ns
	atchr23	4.925	0.195	0.938	ns
Hypocotyl 25°C (mm)	Columbia - WT	0.242	0.117	7.963E-04	na
	AtCHR23-4ov	**0.198**	0.159	9.875E-04	ns
	AtCHR23-5ov	**0.197**	0.147	8.344E-04	ns
	atchr23	0.239	0.132	9.992E-04	ns
Hypocotyl 28°C (mm)	Columbia - WT	0.933	0.121	1.269E-02	na
	AtCHR23-4ov	**0.754**	0.172	1.686E-02	ns
	AtCHR23-5ov	**0.771**	0.171	1.734E-02	ns
	atchr23	0.926	0.117	1.171E-02	ns
Root length continuous light (mm)	Columbia - WT	38.370	0.118	20.460	na
	AtCHR23-4ov	**34.213**	0.145	24.780	ns
	AtCHR23-5ov	**31.750**	0.173	30.238	ns
	atchr23	**41.333**	0.106	19.376	ns
Root length: salt stress (mm)	Columbia - WT	30.926	0.060	3.449	na
	AtCHR23-4ov	**21.763**	0.160	12.064	**
	AtCHR23-5ov	**22.222**	0.174	14.898	***
	atchr23	**31.076**	0.058	3.279	ns
Root length: mannitol (mm)	Columbia - WT	32.516	0.087	7.998	na
	AtCHR23-4ov	**22.831**	0.198	20.415	**
	AtCHR23-5ov	**23.871**	0.159	14.453	ns
	atchr23	**33.953**	0.073	6.135	ns
Leaf area (mm^2)	Columbia - WT	15.732	0.083	1.718	na
	AtCHR23-4ov	**13.479**	0.176	5.641	**
	AtCHR23-5ov	**14.049**	0.176	6.144	*
	atchr23	16.506	0.113	2.462	ns
Rosette diameter (cm)	Columbia - WT	3.400	0.120	0.167	na
	AtCHR23-4ov	**2.724**	0.165	0.201	ns
	AtCHR23-5ov	**3.055**	0.190	0.337	ns
	atchr23	3.256	0.097	0.100	ns
Rosette diameter: salt stress (cm)	Columbia - WT	3.030	13.58	0.169	na
	AtCHR23-5ov	**2.323**	25.77	0.358	ns

[a] Mean of growth parameter indicated in left, bold indicates significant difference relative to WT as determined by Mann-Whitney U test; [b] coefficient of variation calculated as ratio of the standard deviation to the mean; [c] variance in growth parameter indicated in left; [d] significance of variance relative to WT as determined by Levene's test, ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***; $P < 0.001$. WT, wild-type; na, not applicable.

Table 2.S2: Results of non-parametric adjusted rank transform test.

		Salt stress			Mannitol stress		
		G	**T**	**G x T**	**G**	**T**	**G x T**
Root length	*AtCHR23*-4ov	***	***	**	***	***	***
	AtCHR23-5ov	***	***	**	***	***	**
	atchr23	***	***	**	***	***	ns
Rosette diameter	*AtCHR23*-5ov	***	***	ns	na	na	na

Significance of the sources of variation are Genotype (G), Treatment (T) and Genotype x Treatment interaction (G x T).
**, $P < 0.01$; ***, $P < 0.001$; ns, not significant; na, not applicable

Table 2.S3: Definition of genes tested by quantitative RT-PCR in individual seedlings.
Names and descriptions from TAIR10 of the 14 genes tested for expression variability in 6 individual seedlings.

AGI number	Gene name	Function
At3g11050	FER2, FERRITIN 2	response to oxidative stress, abscisic acid stimulus, cellular iron ion homeostasis and iron ion transport
At4g31940	CYP82C4, CYTOCHROME P450	early Fe deficiency response
At4g14690	ELIP2, EARLY LIGHT-INDUCIBLE PROTEIN 2	biogenesis of all chlorophyll-binding complexes
At1g67105		other RNA
At5g19310	ATCHR23, CHROMATIN REMODELING 23	homeotic gene regulator
At5g04220	ATSYTC, SYNAPTOTAGMIN 3	unknown
At3g06010	ATCHR12, CHROMATIN REMODELING 12	temporary growth arrest in Arabidopsis upon perceiving environmental stress
At3g01460	ATMBD9, METHYL-CPG-BINDING DOMAIN 9	modification of the FLC chromatin acetylation state
At1g04220	KCS2, 3-KETOACYL-COA SYNTHASE 2	involved in the biosynthesis of VLCFA (very long chain fatty acids)
At3g22640	PAP85	nutrient reservoir activity
At3g12580	ATHSP70, ARABIDOPSIS HEAT SHOCK PROTEIN 70	ATP binding
At5g02490	ATHSP70-2, ARABIDOPSIS HEAT SHOCK PROTEIN 70-2	protein binding
At5g10140	AGL25, AGAMOUS-LIKE 25, FLC, FLOWERING LOCUS C, FLF, FLOWERING LOCUS F, RSB6, REDUCED STEM BRANCHING 6	MADS-box protein encoded by FLOWERING LOCUS C - transcription factor that functions as a repressor of floral transition and contributes to temperature compensation of the circadian clock
At2g01422		other RNA

Table 2.S4: List of primers used in the study.

Primer name	Sequence 5' > 3'	Used for
CHR23_F1	CCCGTCTCGTTTATCTTTCG	Gene cloning
CHR23_R1	GGCATCTTTCTGACGCTGG	
CHR23_F2	CACCCGAGCTGAAAAACTAA	
CHR23_R2	GCTTGTATGACTTTCGCATC	
CHR23_F3	GATCGTGCTCATCGGATAG	
CHR23_R3	TCAGTTTCGTTTACTTCCTTTT	
CHR23_F4	GGGGCAACTTTGTACAAAAAAGTTGGCATGGTGAAGCAGCTACAAG	
CHR23_R4	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTTTCGTTTACTTCCTTTTGAG	
pCHR23_F	CACCGCTTCGATAAAAAGAGTCAAAG	Promoter cloning
pCHR23_R	GGCGGGAGTTTCTAATTAGA	
qCHR23_F	CTAGGAACTGGCTACCGGA	qRT-PCR
qCHR23_R	AGCGACCATAGTTCTTGCAGA	
At1g67105_F	CATCTTCGTCACCTCCGATT	
At1g67105_R	TCAGTGCGATGGGTAGACTG	
At3g01460_F	ATGGTTTCCCTGAGCAAAAGGGTAG	
At3g01460_R	ACTGCATCGGACATCCATTCTTAGC	
At3g06010_F	TTCCACTGCACAAGACAGAAG	
At3g06010_R	TCTTGCTCTTGCATCAGACG	
At3g11050_F	TCGAACCTTTTGAGGAGGTG	
At3g11050_R	AATCGTCGGAGAACTTGTGG	
At4g14690_F	CGCCATGGAGTTATCAAAGG	
At4g14690_R	CCTTTTGACTTTGCCTCTGC	
At4g31940_F	GCAACCATCGAGCTTCTTTC	
At4g31940_R	CTGGTTTTGTACCGCCATTC	
At5g04220_F	AGATGTCGAGGGCAAGAAGA	
At5g04220_R	GAAAGTGAAAGCCGGTCCCT	

Supplementary files. Available upon request or at http://www.biomedcentral.com/ website:

- **File 2.S1.** List of potentially DE genes in long-day mutant seedlings identified by DESeq.
- **File 2.S2.** List of genes variable between replicates of RNASeq analysis.

CHAPTER 3

REDUCED SEED GERMINATION IN ARABIDOPSIS OVER-EXPRESSING SWI/SNF2 ATPASE GENES

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ABSTRACT

In the life of flowering plants, seed germination is a critical step to ensure survival into the next generation. Generally the seed prior to germination has been in a dormant state with a low rate of metabolism. In the transition from a dormant seed to a germinating seed, various epigenetic mechanisms play a regulatory role. Here, we demonstrate that the over-expression of chromatin remodeling ATPase genes (*AtCHR12* or *AtCHR23)* reduced the frequency of seed germination in *Arabidopsis thaliana* up to 30% relative to the wild-type seeds. On other hand, single loss-of-function mutations of the two genes did not affect seed germination. The reduction of germination in over-expressing mutants was more pronounced in stress conditions (salt or high temperature), showing the impact of the environment. Reduced germinations upon over-expression coincided with increased transcript levels of seed maturation genes and with reduced degradation of their mRNAs stored in dry seeds. Our results indicate that repression of *AtCHR12/AtCHR23* genes in germinating wild-type Arabidopsis seeds is required for full germination. This establishes a functional link between chromatin modifiers and regulatory networks towards seed maturation and germination.

Keywords
Arabidopsis, Chromatin remodeling, Germination, Seed maturation genes

INTRODUCTION

In the life cycle of flowering plants, seed germination is a critical step to ensure survival into the next generation. It is also a major parameter of seed quality in agronomy and breeding. Germination involves the resumption of active growth by the embryo. It results in the rupture of the seed coat and emergence of new photosynthetically active plant. Generally the seed has been in a relatively inactive, so-called dormant, state with a low rate of metabolism prior to germination. Germination is therefore the result of many cellular and metabolic events that are coordinated by complex regulatory networks (Bewley, 1997). Germination is affected by environmental factors such as temperature, availability of water, oxygen and light, as well as by intrinsic factors such as dormancy (Bove *et al.*, 2002). Dormancy is here defined as the failure of an intact viable seed to complete germination under favorable conditions (Bewley, 1997). Most seed-specific traits, such as dormancy, desiccation tolerance and reserve accumulation, are acquired during a developmental phase called seed maturation (Vicente-Carbajosa *et al.*, 2005). The environmental conditions during seed maturation strongly affect seed quality and germination characteristics, in combination with the genetic make-up of the parents.

In the transition from a dormant seed to a germinating seed, various epigenetic mechanisms play a regulatory role (van Zanten *et al.*, 2013). Here, we demonstrate the impact of over-expression of chromatin remodeling ATPase genes on seed germination. Chromatin remodeling ATPases rearrange chromatin and change the accessibility of the associated DNA for the transcriptional machinery. Such chromatin remodeling is an important control mechanism of transcription during development and is also involved in the reactions of organisms towards their environment (Luo *et al.*, 2012, Mlynarova *et al.*, 2007). Previously we have shown that over-expression of two paralogous *Arabidopsis thaliana* SWI/SNF-class ATPases, *AtCHR12* and *AtCHR23,* reduce the growth of Arabidopsis in response to adverse environmental conditions. Mild abiotic stress led to increased growth arrest of normally active primary buds and reduced growth of the primary stem compared to wild-type plants in case of *AtCHR12* over-expression (Mlynarova *et al.*, 2007) and reduced growth of seedlings and vegetative rosette in case of *AtCHR23* over-expression (Folta *et al.*, 2014). On other hand, a knockout mutants of both genes show no visible phenotype different from wild-type plants (Folta *et al.*, 2014, Mlynarova *et al.*, 2007).

In this study, it is shown that by elevating the expression level of either *AtCHR12* or *AtCHR23* genes, germination is reduced by 20-30% compared to wild-type seeds. In the presence of abiotic stress, over-expression results in more reduction of germination than in wild-type Arabidopsis. RNA profiling of 48 hours germinating seeds revealed that the reduced germination of mutants coincided with increased RNA levels of several seed maturation-related genes. Seed maturation genes, such as genes for seed storage proteins (SSPs), lipid accumulation and late-embryogenesis-related processes, are highly expressed

during seed maturation phase and their expression has to be repressed during germination. Further transcript analysis showed that over-expression of chromatin remodeling ATPases impairs the degradation of RNAs for maturation-related genes stored in dry seeds during early germination. These results identify *AtCHR12* and *AtCHR23* as constituents of the regulatory framework that controls the seed maturation program with clear effect on seed germination.

RESULTS

Reduced germination upon over-expression of chromatin remodeling ATPases

Over-expression of *AtCHR12* and/or *AtCHR23* genes reduces the growth of Arabidopsis plants during reproductive and vegetative development. To evaluate the impact of the over-expression of these two chromatin remodeling ATPase genes on seed germination, the germination of after-ripened seeds from the over-expressing mutants with seeds from the wild-type plants was compared. Seeds of AN *AtCHR12*ov activation-tagged mutant showed a significantly lower proportion of germinating seeds compared to the corresponding wild-type of ecotype Ws (Figure 3.1). After 3 days of germination, the proportion of Ws seeds that had germinated was 0.89, while it was only 0.59 in *AtCHR12*ov (reduction 33%). After 4 days, the reduction in the germination proportion of mutant seeds was 20% (Figure 3.1C). After longer germination time (up to 8 days) similar differences in germination were observed (data not shown), indicating that over-expression of chromatin remodeling genes has no effect on the germination rate.

Transgenic lines over-expressing the *AtCHR12* gene under the control of the 35S CaMV promoter showed similarly reduced relative germination proportions, both in ecotype Columbia (Col; data not shown) and in Ws (Figure 3.S1).

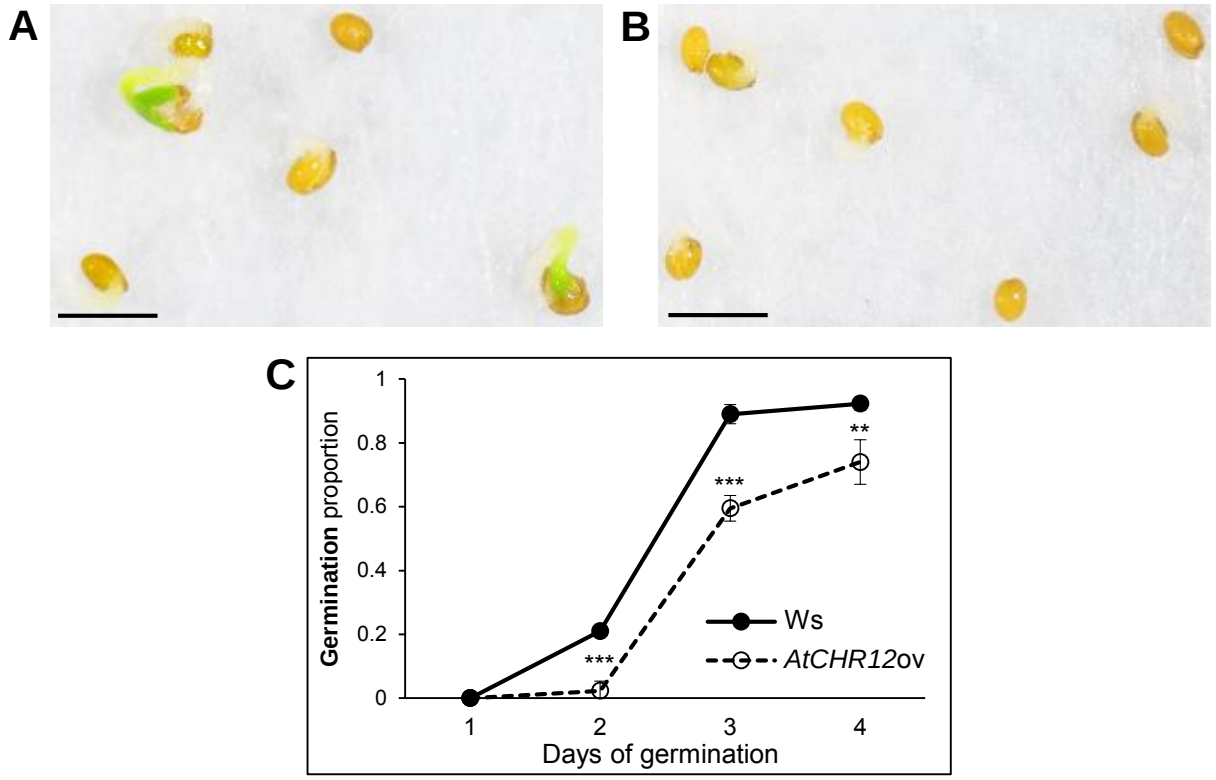

Figure 3.1: Over-expression of the chromatin remodeling gene *AtCHR12* decreases the proportion of germinating seeds. Seeds of the wild-type Ws **(A)** and the *AtCHR12*ov mutant **(B)** were germinated on water for 3 days. **(C)** Time-course of germination of *AtCHR12*ov and corresponding Ws wild-type seeds. Bars: 1 mm. The plot shows the mean proportion of viable seeds that germinated (± standard deviation, s.d.) for five independent germination tests (n = 80). Asterisks indicate significance levels for differences between mutant and corresponding wild-type seeds at the same time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Also over-expression of *AtCHR23* shoved such results. The proportion of *AtCHR23*-4ov seeds that had germinated after 2 days was 0.61 compared to 0.84 in the corresponding Col wild-type (Figure 3.2). This represents 27.4% reduction of germination frequency. After 3 days, *AtCHR23*-4ov had 22% lower proportion of germinated seeds than the wild-type (Figure 3.2C). *AtCHR23*-5ov has a similar phenotype of reduced germination (Figure 3.S1). Same results were obtained with several independent seed batches. The reduced germination was not due to seed mortality because seeds from all mutant lines germinated with the same frequency (> 90%) as wild-types after cold stratification at 4°C for 3 days (data not shown). The knockout lines of both *AtCHR12* and *AtCHR23* (both in ecotype Columbia) did not show any significant difference in germination frequency compared to the wild-type (data not shown). Similar differences in germination frequencies between wild-type and over-expressing mutants were observed in seeds after-ripened for up to 6 month (data not shown). These data establish that it is the over-expression of either *AtCHR12* or *AtCHR23* that results in decreased seed germination frequencies and the difference in germinability is likely to be related to dormancy.

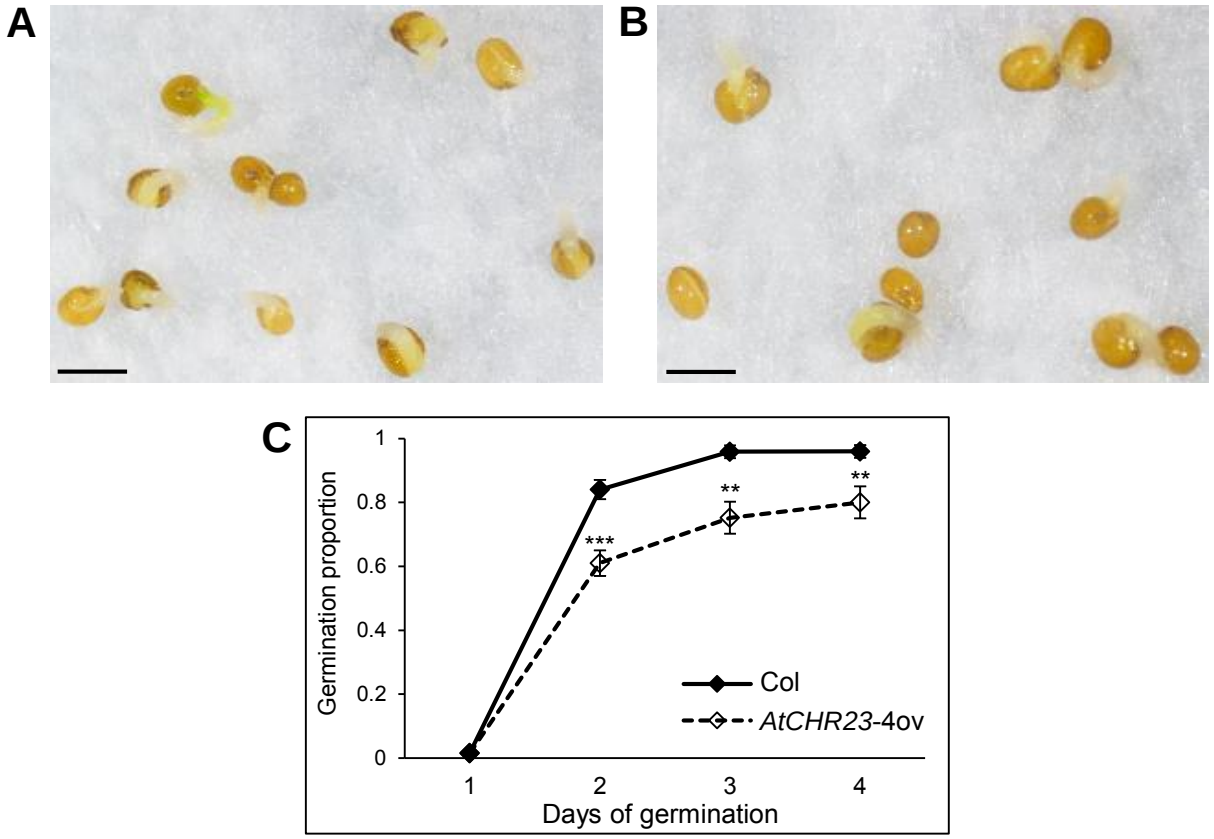

Figure 3.2: Over-expression of the chromatin remodeling gene *AtCHR23* decreases the proportion of germinating seeds. Seeds of the wild-type Col **(A)** and *AtCHR23*-4ov mutant **(B)** seeds germinated on water for 2 days. **(C)** Time-course of germination of *AtCHR23*-4ov and corresponding Col wild-type seeds. Bars: 1 mm. The plot shows the mean proportion of viable seeds that germinated (± s.d.) for five independent germination tests (n = 80). Asterisks indicate significance levels for differences between mutant and corresponding wild-type seeds at the same time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Abiotic stress enhances the reduced germination phenotype of *AtCHR12/AtCHR23* over-expression

In order to establish the relationship between *AtCHR12* and *AtCHR23* over-expression and germination in stressful conditions, the germination frequency in the presence of salt (100 mM NaCl) or elevated temperature (28°C) was determined. Compared to germination in the no-stress environment, both stresses reduced the proportion of germinating seeds as expected in adverse conditions (Table 3.1). However, seeds of over-expressing lines showed considerably more reduction of germination proportion than seeds of the wild-type. After 4 days in salt conditions, the germination frequency of *AtCHR12*ov seeds was reduced to 55.4% of the germination in control condition, while in Ws wild-type the reduction was 60.9% (Table 3.1). The germination frequency of *AtCHR23*-4ov and *AtCHR23*-5ov seeds in salt conditions was reduced to 63.8% and 39.2% of control germination, respectively. The reduction was 81.3% in Col wild-type (Table 3.1). Over-expressing mutants also showed more

sensitivity to elevated temperature than wild-type (Table 3.1). The proportion of *AtCHR12*ov seeds germinating at 28°C was just 64.9% of germination in control condition, whereas in Ws it was 82.6% of the control. The germination frequency of two *AtCHR23* over-expressing mutants at elevated temperature was reduced to about 45% of the germination at control condition, while in Col wild-type it was to 70.8% (Table 3.1). The phytohormone ABA is known to regulate seed germination in response to environmental stresses (Rodriguez-Gacio Mdel *et al.*, 2009). Also in ABA-treated seeds, mutants showed more reduced germination relative to control condition than wild-type seeds (Table 3.1). This demonstrates that the impact of over-expression of chromatin remodeling genes on the reduction of seed germination is more pronounced in adverse environmental conditions.

Table 3.1: Environmental stress, either salt (NaCl) or elevated temperature, as well as ABA pronounce the reduction of seed germination upon over-expression of chromatin remodeling genes.

Line	Control	NaCl 100 mM		Temperature 28°C		ABA 0.5 µM	
	Proportion[a]	Proportion[a]	Reduction (%)[b]	Proportion[a]	Reduction (%)[b]	Proportion[c]	Reduction (%)[b]
Ws	0.92 ± 0.02	0.56 ± 0.02	60.9	0.76 ± 0.04	82.6	0.48 ± 0.12	52.4
*AtCHR12*ov	0.74 ± 0.07	0.41 ± 0.06	55.4	0.48 ± 0.04	64.9	0.24 ± 0.05	32.8
Col	0.96 ± 0.02	0.78 ± 0.05	81.3	0.68 ± 0.04	70.8	0.43 ± 0.09	44.4
AtCHR23-4ov	0.80 ± 0.05	0.51 ± 0.05	63.8	0.36 ± 0.06	45.0	0.23 ± 0.06	29.2
AtCHR23-5ov	0.79 ± 0.07	0.31 ± 0.06	39.2	0.36 ± 0.06	45.6	0.28 ± 0.07	35.0

[a] Mean (± s.d.) proportion of germinated seeds after 4 days of germination. [b] Reduction (%) in germination proportion relative to control germination of the same line. [c] Mean (± s.d.) proportion of germinated seeds after 6 days of germination.

Reduced germination associates with increased RNA levels of seed maturation genes

To get more insight into the transcriptional events affected by the over-expression of chromatin remodeling genes during the seed germination, an Agilent transcript profiling analysis was performed on RNA isolated from seeds of *AtCHR12*ov and wild-type (Ws). For comparison, RNA was isolated from dry seeds and from seeds germinating for 48 hours without cold stratification. The 48 h time point marks the end of phase II of germination when seeds either enter stage III to complete germination via visible radicle protrusion, or remain dormant (Bewley, 1997). Genes that were expressed with at least 2-fold difference in both biological replicates were considered differentially expressed (DE). In dry seeds, no significant differences between wild-type and mutant in the levels of stored RNAs (Nakabayashi *et al.*, 2005) was observed (data not shown). The level of the *AtCHR12* transcript in dry seeds of the over-expressing line was only 1.2-fold higher than in Ws, reflecting the lower activity of 35S CaMV promoter to the end of seed development (data not shown).

In germinating seeds, 68 DE genes were identified, of which 53 were up-regulated and 15 were down-regulated in the mutant (File 3.S1). Among the up-regulated genes, 13 genes are specifically associated with the seed maturation phase (Table 3.2). These include genes coding for several seed storage proteins (SSPs), embryo specific proteins (*AtS1-AtS3*) and the late embryogenesis protein *M10* that has been linked to seed dormancy and/or late embryogenesis (Nuccio *et al.*, 1999, Toorop *et al.*, 2005). Among the SSPs genes were four genes for 2S albumins (*At2S1* to *At2S4*), three for 12S globulins (*CRA1*, *CRB* and *CRC*) and one for the cupin family storage protein *At1g03890* (Krebbers *et al.*, 1988). In addition, a higher transcript level of *DOG1* (*DELAY OF GERMINATION 1*) was observed. *DOG1 is* associated with natural variation in dormancy in *Arabidopsis*, but its precise biochemical function is yet unknown (Bentsink *et al.*, 2006). None of the major transcriptional factors (*LEC1*, *LEC2*, *FUS3* and *ABI3*) that regulate the expression of SSPs and other maturation genes (Verdier *et al.*, 2008) was up-regulated in the germinating seeds of the over-expressing mutant (data not shown).

Table 3.2: Maturation-related genes with increased RNA levels relative to wild-type (Sw) in 48-h germinating seeds of *AtCHR12ov* based on microarray analysis.

Locus	Fold increase[a]		Gene description
	R1	R2	
At4g27140[b]	5.0	3.3	2S seed storage *At2S1*
At4g27150	4.9	2.4	2S seed storage *At2S2*
At4g27160[b]	6.9	3.0	2S seed storage *At2S3*
At4g27170	5.2	2.3	2S seed storage *At2S4*
At5g44120[b]	2.9	2.4	12S seed storage *CRA1*
At1g03880	4.0	2.1	12S storage *CRB*
At4g28530	3.7	2.8	12S storage *CRC*
At4g26740	3.4	2.2	Embryo-specific *AtS1*
At5g55240[b]	2.9	2.5	Embryo-specific *AtS2*
At5g07190	4.9	2.1	Embryo-specific *AtS3*
At2g41280[b]	3.6	2.4	Embryogenesis *M10*
At1g03890	6.0	2.4	Cupin 1
At5g45830[b]	3.9	2.2	*DOG1*

[a] Fold increase of RNA level in the mutant relative to the wild-type. [b] Gene for which the increased RNA level was validated by qRT-PCR (see Figure 3.3). R1, biological replicate 1; R2, biological replicate 2.

For six genes listed in Table 3.2, the array results were validated by quantitative RT-PCR (qRT-PCR). In addition to the plant material used for RNA profiling (*AtCHR12* and Ws), the analysis included dry and 48-hour germinating seeds of *AtCHR23*-4ov and Col wild-type. The qRT-PCR results confirmed the microarray profiling results: in 48-hour-germinating seeds the RNA levels of three SSP genes (*At2S1*, *At2S3*, *CRA1A*), two embryo specific genes (*AtS2*, *M10*) and *DOG1* were 2-fold up to 12-fold higher in over-expressing mutants relative to their corresponding wild-type (Figure 3.3). In dry seeds, the RNA levels of the maturation genes did not differ between mutant and wild-type seeds (data not shown).

The qRT-PCR also confirmed the lack of up-regulation of the transcriptional factors (*LEC1*, *LEC2*, *FUS3* and *ABI3*) (data not shown).

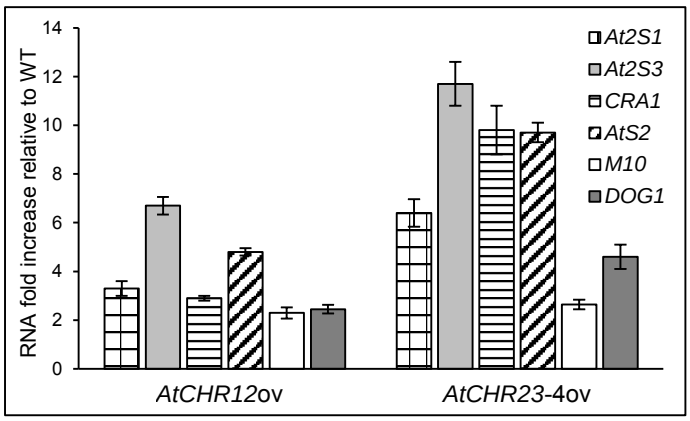

Figure 3.3: Analyses by qRT-PCR show increased RNA levels of seed maturation-related genes (see Table 3.2) in germinating seeds of over-expressing lines. Mean (± s.d.) fold increase in RNA level in over-expressing mutant seeds relative to the corresponding wild-type (Ws for *AtCHR12*ov, Col for *AtCHR23*-4ov) after 48 hours of germination. For each assay three replicates were used. WT, wild-type.

These data suggest that over-expression of chromatin remodeling ATPases is interfering with the repression of maturation genes, or with the degradation of their RNAs stored in dry seeds during early germination. To confirm it, the RNA levels of *At2S3* and *AtS2* genes in a time-course of germination with qRT-PCR were determined. In dry and 1-day germinating seeds, the transcript levels of these two genes in over-expressing mutants did not differ significantly from that of wild-type seeds (Figure 3.4A,B). However, after 2-days of germination, the RNA levels in the mutants were up to 12-fold higher than in corresponding wild-type. Up-regulated RNA levels were also found in the mutants after 3-days of germination, though less pronounced than at 2 days. In the course of germination, the RNA levels of the ATPase genes used for over-expression progressively decreased in wild-types seeds (Figure 3.3C,D). In the over-expressing mutants, a marked up-regulation relative to wild-type of *AtCHR12* (4-fold) and *AtCHR23* (8.5-fold) was observed after 2 days of germination (Figure 3.4C,D). The up-regulation of chromatin remodeling genes thus coincided with considerably increased RNA levels of maturation genes. In 3-day-germinated seeds the transcript levels of the maturation genes seem already on the decline whereas the up-regulation of chromatin remodeling genes is even stronger. There is apparently a narrow window of time for the chromatin remodeling to exert its effects on seed germination.

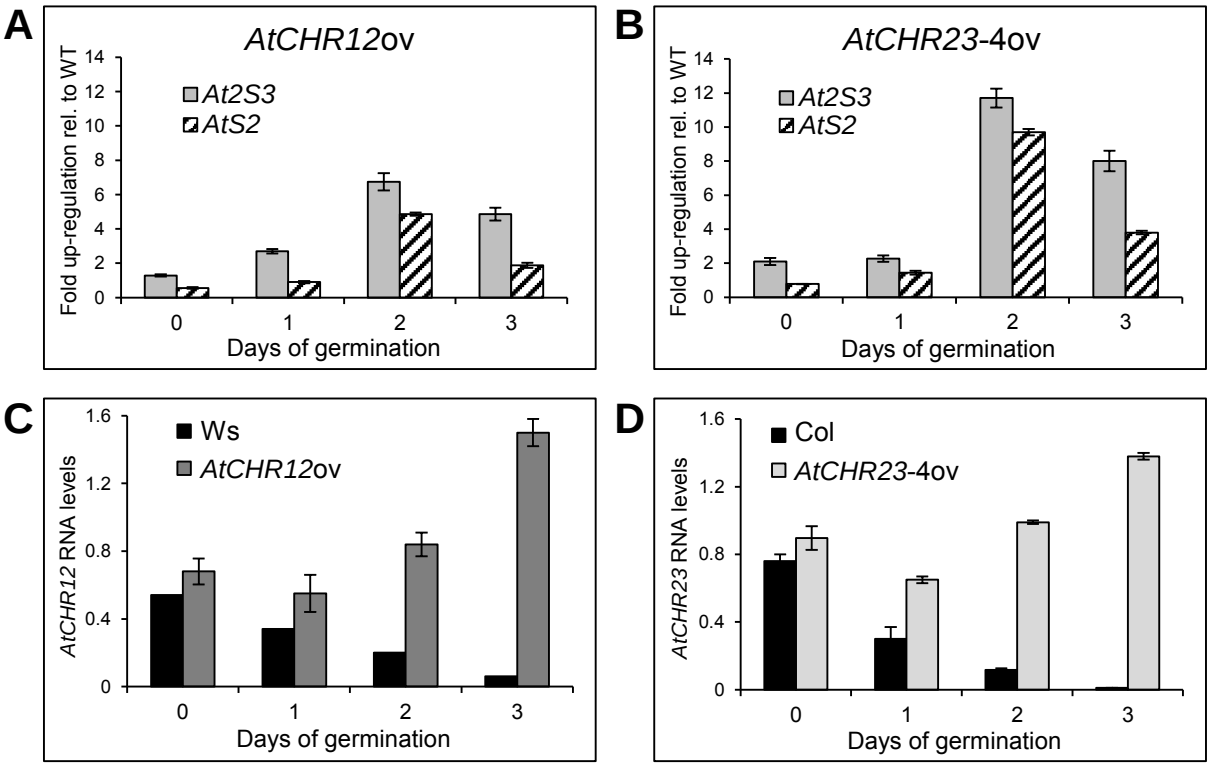

Figure 3.4: Analyses by qRT-PCR show that elevated levels of *At2S3* and *AtS2* transcripts in germinating mutant seeds coincide with marked up-regulation of chromatin remodeling genes. Day 0 represents dry seeds. **(A)** Mean (± s.d.) fold increase in RNA levels in seeds of the *AtCHR12*ov mutant relative to Ws wild-type seeds in the course of germination. **(B)** Mean (± s.d.) fold increase in RNA levels in seeds of the *AtCHR23*-4ov mutant relative to Col wild-type in the course of germination. **(C)** Mean (± s.d.) expression levels of *AtCHR12* in the course of germination. **(D)** Mean (± s.d.) expression levels of *AtCHR23* in the course of germination. In all panels, values are the mean of three replicates. WT, wild-type.

DISCUSSION

We here demonstrated that over-expression of either *AtCHR12* or *AtCHR23* SWI/SNF ATPase genes results in decreased seed germination in Arabidopsis. Environmental stress always reduces germination frequencies, but the response of the over-expressing mutants was more pronounced: compared to control condition the germination frequencies of the over-expressing mutants were more reduced in conditions of environmental stress than the germination frequencies of the wild-type. The reduced germination associated with increased RNA levels of several seed maturation genes.

The maturation phase starts when the embryo and endosperm morphogenesis and patterning are completed. It is characterized by storage compound accumulation, acquisition of desiccation tolerance, growth arrest and the entry into dormancy (Santos-Mendoza *et al.*, 2008). Maturation-related genes are highly expressed, but only during the late embryogenesis and their expression slows down towards the end of the stage of seed filling (Verdier *et al.*,

2008). Relatively high transcript levels are stored in dry matured seeds (Nakabayashi *et al.*, 2005), supposedly to allow fast action upon germination (Rajjou *et al.*, 2004). The levels of stored RNAs of maturation genes in dry seeds (Figure 3.4; day 0) are comparable. No detectable expression of maturation-related genes stored in dry seeds was observed in 2-week-old seedlings or mature plants of both wild-type and over-expressing mutants (data not shown). In vegetative tissues, maturation genes are thought to be repressed via chromatin modifications (van Zanten *et al.*, 2013). Given that in dry seeds the RNA levels of the maturation genes did not differ between mutant and wild-type, it was concluded that the over-expression of chromatin remodeling ATPases is likely to impair the degradation of stored mRNAs during the early steps of seed germination.

The importance of RNAs (and proteins) stored in dry mature seeds for germination was shown previously, indicating that the potential for germination is largely programmed during seed maturation (Rajjou *et al.*, 2004). Stored RNAs are specifically degraded during seed germination and such degradation seems to be required for new RNA synthesis (Li *et al.*, 2006). The inhibition of the degradation of stored RNAs in germinating seeds was shown to inhibit new RNA synthesis with negative impact on germination (Li *et al.*, 2006). The reduced germination of seeds of the *AtCHR12* and *AtCHR23* over-expressing mutants may therefore be the consequence of reduced degradation of stored RNAs from maturation-related genes.

Data extracted from public expression arrays (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) show that the expression of maturation genes affected in our RNA profiling analysis (Table 3.2) peaks at mid maturation phase (from curly cotyledons stage) with relatively high levels of RNA stored in dry seeds (Figure 3.S2A). Upon germination the transcript levels decline dramatically reflecting the degradation of their RNAs during this process (Figure 3.S2A). The expression of both *AtCHR12* and *AtCHR23* during embryogenesis increases from curled cotyledons on and peaks in dry seeds (Figure 3.S2B). Upon the first 24 hours of germination (imbibition), the expression of chromatin remodeling genes is repressed considerably (Figure 3.S2B). This points towards a role of the two ATPases in the seed maturation program (Vicente-Carbajosa *et al.*, 2005). When the repression of chromatin remodeling genes during germination was disrupted through over-expression by the 35S promoter, the degradation of stored RNAs encoding maturation-related genes was reduced. This was most prominent in 2-day germinating seeds where it coincided with markedly up-regulated *AtCHR12/AtCHR23* expression (Figure 3.4). These results identify *AtCHR12* and *AtCHR23* ATPases as constituents of the regulatory framework in control of the seed maturation program. The repression of *AtCHR12/AtCHR23* therefore results in full germination. How they affect the expression of maturation genes during late embryogenesis needs further investigation. A likely reason for the lack of differential expression of maturaion-related genes in dry mutant seeds is the 35S CaMV promoter used to over-express chromatin remodeling genes. Expression analysis of developing mutant seeds shows that till 17 days after flowering both transgenes are highly up-regulated relative to wild-

type: *AtCHR12* 10-fold and *AtCHR23* 30-fold (unpublished data). However in dry seeds (Figure 3.4C,D) the over-expression is not significant. This indicates a very low activity of the 35S promoter in dessicated and/or dry seeds. Upon the germination, activity of the 35S promoter is re-established (Figure 3.4C,D). We speculate that during germination the 35S promoter is activated, chromatin remodeling genes are up-regulated and consequently the maturation programme can be reset partially. It was shown previously that under stress conditions maturation program can be recapitulated during early stages of germination (Lopez-Molina *et al.*, 2001, Rajjou *et al.*, 2004).

The single loss-of-function mutation of gene *AtCHR12* or *AtCHR23* does not lead to a detectable phenotypic effect as a likely result of gene redundancy (Thomas, 1993). The same chromatin remodeling genes were shown previously to have mutually redundant roles in early embryogenesis (Sang *et al.*, 2012). The *atchr12 atchr23* double knockouts are embryo lethal and only double mutants containing weak knockout alleles give rise to a viable seedlings with severe defects in the maintenance of stem cells (Sang *et al.*, 2012). The data presented here indicate that AtCHR12/AtCHR23 ATPases act as regulatory components of the seed maturation program with clear effects on seed germinability. As the maturation process is affected by environmental conditions and also the impact of chromatin remodeling genes over-expression is environment-dependent (Table 3.1), these two ATPases could be involved in the molecular dialog between maturing/germinating seeds and their environment. Environmental conditions during seed maturation on a maternal plant include temperature, photoperiod, light quality, water stress and the position of the seed in the fruit or on the plant. All these conditions influence the germination of seeds (Donohue, 2009). Better understanding of the epigenetic components of seed formation and germination could be of practical interest for the production of high quality seeds less sensitive to unfavorable maternal environments.

MATERIALS AND METHODS

Plant material and growth conditions

The Arabidopsis mutant over-expressing *AtCHR12* (indicated as *AtCHR12*ov) was identified in activation tagged lines in the Wassilijewskaja (Ws) genetic background (Mlynarova *et al.*, 2007). To generate transgenic lines over-expressing this gene with the help of the 35S CaMV promoter (35S::CHR12-ov) in the Ws accession, the genomic copy of *AtCHR12* was cloned using appropriate Gateway technology. The knockout line for *AtCHR12* was SALK_105458 in the Col-0 background. Arabidopsis over-expressing *AtCHR23* was generated by transformation of wild-type Col-0 as described (Folta *et al.*, 2014). Two transformants were included in the analyses, indicated as *AtCHR23*-4ov and *AtCHR23*-5ov. The knockout line for this gene was SALK_057856. Both knockouts were obtained

from the Salk Institute of Genomics Analysis Laboratory, USA; in either case, no full length cDNA product was detected (data not shown). Plants were grown in a fully controlled growing chamber lit by Philips TD 32W/84HF lamps at 23°C in long-day (LD; 16h light/8h dark).

Seed germination

For germination, homozygous seeds of the F3 progeny were used. Fully matured seeds from 8 plants (both wild-type and mutant) grown side-by-side were harvested and dried for 1 month at room temperature before subsequent germination assays. In the remainder of this paper, such seeds are referred to as 'after-ripened' seeds. Seeds were sown without sterilization on 2 layers of filter paper soaked with 2 ml of distilled water in Petri dishes (approximately 80 seeds) and incubated in germination cabinet in long-day conditions (16 h light/8 h dark) at 21°C. Day zero refers to dry seeds. To apply salt stress, seeds were germinated on filter papers soaked with 100 mM NaCl. To apply temperature stress, seeds sown on water-soaked papers were incubated in the same long-day conditions at 28°C. Seed was counted as germinated after visible protrusion of the radicle tip. In case of cold stratification, seeds were sown as above and incubated for 3 days at 4°C in the dark prior to germination. All germination assays were performed in five replicates. Data were evaluated with the two-sample unequal variance t-test in Excel. In charts, asterisks indicate the significance level obtained: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Microarray analysis

Agilent transcript profiling analysis was performed on RNA isolated from seeds of *AtCHR12*ov and Ws wild-type plants that were either dry or germinating for 48 hours without cold stratification. For both stages, two biological replicates of pooled seeds from 8 plants were used. In addition, two technical replicates were performed with a dye swap for each biological replicate. Total RNA was isolated using the E.Z.N.A.™ Plant RNA Mini Kit (Omega Bio-Tek, Inc., USA) followed by on column DNase treatment (Qiagen, RNase-free DNase Set). Microarray analysis including RNA quality check, labelling, hybridization, scanning and data analysis was performed by ServiceXS, Leiden, The Netherlands (http://www.servicexs.com/). For Agilent transcript profiling analysis the Agilent Quick Amp Labeling Kit was used to synthesize Cy3 and Cy5 labeled cRNA. From each cRNA sample, 500 ng was used for labeling. The Agilent Arabidopsis 4x44K microarray (AMADID # 015059) was used for hybridization (Agilent Technologies; www.agilent.com). The Agilent protocols were followed for the hybridizations, washing and scanning of the chips. The XDR function was used to extend the dynamic range and the arrays were scanned twice with 10% PMT and 100% PMT laser power. Feature extraction software (Agilent, version 9.5) was used to generate the feature extraction data. For background subtraction, the option "no

background subtraction" and "spatial detrend" was used. Text files were imported into GeneSpring v10 (Agilent Technologies; www.agilent.com). A baseline transformation using the median of all arrays was performed. Data from dye-swap were combined. Statistical analyses (unpaired T-test; asymptonic p-value computation) were performed on the combined duplicates to identify differential expression between wild-type and mutant lines in the same conditions. Differentially expressed genes were analyzed using Rosetta Resolver software (Agilent Technologies; www.agilent.com) according to the manufacturer's instructions.

Quantitative RT-PCR

One microgram of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). Ten-fold diluted cDNA was used for quantitative PCR using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA) in an iCycler thermal cycler. *SAND (At2g28390)* was used as reference gene (Czechowski *et al.*, 2005). Sequences of primers used are given in Table 3.S1.

ACKNOWLEDGMENTS

We thank Dr. Leonie Bentsink (Laboratory of Plant Physiology, Wageningen University) for critical reading of the manuscript and helpful suggestions. AF was supported by The Netherlands Organization for Scientific Research (NWO), through its ALW/TTi Green Genetics program. This research was supported in part by the Centre for BioSystems Genomics (CBSG2012), The Netherlands, part of the Netherlands Genomics Initiative (NGI), and by RaakPRO BioCOMP coordinated by Hanze University of Applied Sciences Groningen. We acknowledge Prof. Dr. Ton Bisseling (Molecular Biology, WUR) for general support and encouragement.

SUPPLEMENTARY DATA

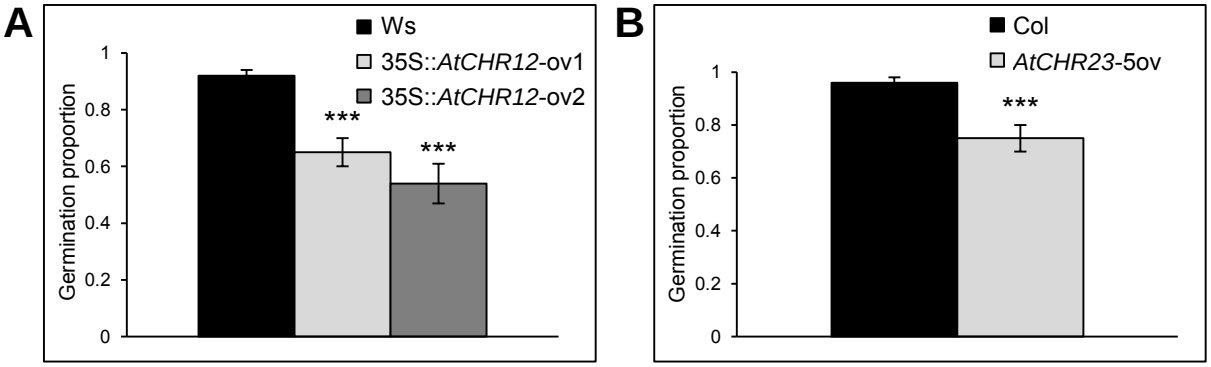

Figure 3.S1: Over-expression of *AtCHR12* and *AtCHR23* genes results in markedly reduced seed germination frequencies relative to the wild-type. **(A)** Proportion of germinated seeds in two independent transgenic lines over-expressing *AtCHR12* gene with the help of 35S CaMV promoter relative to the corresponding Wassilewskaja (Ws) wild-type. **(B)** Proportion of germinated seeds in *AtCHR23*-5ov transgenic line over-expressing *AtCHR23* gene with the help of 35S CaMV promoter relative to the corresponding Columbia (Col) wild-type. Bars represent the mean proportion of viable seeds that germinated (± s.d.) for 5 independent germination tests (n = 80) after 3 days of germination. Asterisks indicate significance levels for differences between mutant and corresponding wild-type seeds. ***, $P < 0.001$.

A

	globular		heart	torpedo	walking stick	curled cotyledons		green cotyledons		
stage	3	4	5	6	7	8	9	10	dry	24 HAI
At4g27140	5.06	470	3390	5007	5245	9158	11932	10839	2019	496
At4g27150	0.63	732	2746	3799	3806	6612	8447	7835	2104	491
At4g27160	3.05	995	3061	4229	4351	7763	9945	8912	2641	1051
At4g27170	2.23	489	2982	5575	6024	10071	13500	12100	2821	594
At5g44120	2.35	1921	3972	5278	5494	9571	12869	11851	1095	1378
At1g03880	4	830	4356	6812	7373	12241	15201	14120	1893	474
At4g28520	9.95	1611	4255	5930	6389	10864	14390	12979	1557	1485
At4g26740	4.1	124.5	993	2590	3109	6131	5304	4704	2413	102
At5g55240	10.76	19.65	120	715	836	2737	2620	3368	2521	115
At5g07190	11.1	280.13	1558	3856	4390	6493	7839	7413	246	19.32
At2g41280	0.53	0.51	0.51	0.78	75.38	3111	5797	5800	2221	254
At1g03890	4.23	31.53	853	3600	4277	6886	8568	8243	1987	28.7
At5g45830	2.83	11.03	51.6	339	1192	1705	3100	1918	227	1.4

B

stage	3	4	5	6	7	8	9	10	dry	24 HAI
AtCHR12	31.7	37.38	30.11	38.61	31.15	37.85	56.68	56.95	146.9	60.3
AtCHR23	51.35	52.48	46.3	51.96	39.81	80.36	89.21	92.2	133.4	41.51

Figure 3.S2: Heat map of expression for **(A)** the 13 maturation-related genes up-regulated in germinating mutant seeds and **(B)** *AtCHR12/AtCHR23* in the course of embryogenesis (from stage 3 to 10), dry seeds and upon 24 hours of imbibition (24h HAI).

Table 3.S1: List of primers used in this study.

Gene	Forward sequence 5' > 3'	Reverse sequence 5' > 3'	Used for
AtCHR12	TTCCACTGCACAAGACAGAAG	TCTTGCTCTTGCATCAGACG	qRT-PCR
ATCHR23	CTAGGAACTGGCTACCGGA	AGCGACCATAGTTCTTGCAGA	
AT2S1	CACCAACCAATGCAAGTCAG	GGGATGTTGAAGGGACAAAC	
AT2S3	GCAAAACATGGCTAACAAGCTCT	TGGCATCTCTGTCTTGGACCTTTTG	
CRA1A	TGACGTTCAGTTGGCTCAGCAG	GCAGATAGTCTCCTCAAGGCCG	
ATS2	TTACTCGCGTCGCTTATCTTGC	TTAGAGTCGCTTCCGTGC	
M10	CGTGGCTTTGCTCTTTTCTC	TTGCTTCAGTACTCGGCTTC	
DOG1	AGGCTCGTTTATGGTTTGTGTGG	GCACTTAAGTCGCTAAGTGATGC	
ACT8	CTCAGGTATTGCAGACCGTATGAG	CTGGACCTGCTTCATCATACTCTG	
SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	
LEC1	ACCAGCTCAGTCGTAGTAGCC	GTGAGACGGTAAGGTTTTACGCATGAT	
LEC2	CGATCTCATGGACCTCACCAC	CACCTGATCATCGGATGAAC	
FUS3	GCCAAACAACAATAGCAGAA	TTTCTTGCTTGTATAACGTAATTG	
ABI3	GGCAGGGATGGAAACCAGAAAAGA	GGCAAAACGATCCTTCCGAGGTTA	

Supplementary files. Available upon request or at http://onlinelibrary.wiley.com/ website:

- **File 3.S1.** Differentially expressed genes in *AtCHR12ov* relative to wild-type in 48 hours germinating seeds.

CHAPTER 4

OVER-EXPRESSION OF ARABIDOPSIS *ATCHR12* CHROMATIN REMODELING ATPASE INTERFERES WITH *FLC* REPROGRAMMING DURING EMBRYO DEVELOPMENT

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ABSTRACT

Transition into flowering is an important developmental switch which ensures survival of the next generation. Therefore multiple environmental and intrinsic factors are coordinated in a complex regulatory network. In this network, *FLC* is a key flowering regulator, which expression is well controlled during the plant life. One of the key processes in *FLC* regulation is the *FLC* reprogramming, when *FLC* expression gradually increases during embryo development. However, the molecular mechanisms of *FLC* reprogramming are not very well known, although several factors have been shown to be required for this process. Here we show that over-expression of *AtCHR12*, a SWI/SNF-type chromatin remodeling gene, interferes with the *FLC* reprogramming at later stages of embryo development leading to reduced *FLC* expression in mature embryos. The reduction of *FLC* expression correlates with increased levels of repressive H3K27me3 histone mark at the *FLC* locus, and is maintained during vegetative growth leading to early flowering. Our results indicate that the chromatin remodeling gene *AtCHR12* is able to control *FLC* expression through H3K27me3 modulation, and its biological role as well as the underlying mechanism is considerably different from that of another chromatin remodeling genes from the same subfamily, *BRAHMA* and *SPLAYED*.

Keywords
Arabidopsis, Chromatin remodeling, Flowering time, *FLC* reprogramming

INTRODUCTION

In plant life cycle, the transition from vegetative to reproductive growth is a major developmental switch that is critical for reproductive success. Therefore, plants evolved multiple genetic pathways that integrate environmental and endogenous signals to regulate the expression of floral pathway integrators *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*). When activated, the floral integrator genes act on their downstream targets to promote flowering (Boss *et al.*, 2004). In *Arabidopsis thaliana*, the floral integrator genes are activated depending on the light quality and duration, (ambient) temperature, hormone levels or plant age (Boss *et al.*, 2004). On the other hand, the central player in repressing the floral pathway integrators is a MADS-domain transcription factor encoding gene *FLOWERING LOCUS C* (*FLC*) (Yan *et al.*, 2010), which directly blocks transcription of floral integrators.

FLC is extensively regulated through epigenetic modifications and transcriptional control. The *FLC* regulators control the *FLC* expression either by affecting RNA-binding and -processing of *FLC* transcripts (members of autonomous pathway) or by altering the chromatin state of the *FLC* locus (members of autonomous pathway, RNAII polymerase associated (Paf1) and SWR1 complexes) (Yan *et al.*, 2010). The importance of the *FLC* chromatin state suggests that chromatin remodeling plays an important role in *FLC* regulation, both covalent change of *FLC* chromatin by histone modifications, and ATP-dependent chromatin remodeling. The covalent modifications of histones on the *FLC* locus are associated with several members of autonomous pathway and Paf1 complex, while SWR1 complex is an ATP-dependent chromatin remodeler from the SWI/SNF class involved in exchange of H2A histone variant for H2A.Z on the *FLC* locus (He, 2012). In addition to SWR1, also BRAHMA (BRM) and SPLAYED (SYD), Snf2-subfamily ATPases, were shown to be involved in flowering time control. BRM affects the flowering time via photoperiod pathway by reducing the expression of *CONSTANS* (*CO*) and *FT* (Farrona *et al.*, 2011). Modification of *SYD* expression correlates with changes in *FT* expression, however, the mechanism is not known (Su *et al.*, 2006).

The key role of *FLC* in transition to flowering indicates that the *FLC* expression has to be well regulated throughout the plant life. To ensure that the plant will flower at the correct time of the year, *FLC* expression is repressed at optimal environmental conditions enabling transition to flowering. However, the *FLC* gene has to be reset to an active transcriptional state in the next generation. This control of *FLC* expression prevents premature flowering of the new generation of plants. The *FLC* levels are reset during embryo development in a process called *FLC* reprogramming (Choi *et al.*, 2009, Crevillen *et al.*, 2014, Sheldon *et al.*, 2008). The reactivation can be divided into three phases: repression in gametogenesis, reactivation in early embryogenesis and maintenance in late embryogenesis (Choi *et al.*, 2009).

It was shown that different *FLC* regulators play a role in *FLC* reactivation and maintenance (Choi *et al.*, 2009). The activators of *FLC* expression *FRIGIDA* (*FRI*), Paf1 and SWR1 complexes are essential for both reactivation and maintenance (Choi *et al.*, 2009, Yun *et al.*, 2011). On the other hand, the main repressors of *FLC*, the members of autonomous pathway, play a role only during the maintenance phase (Choi *et al.*, 2009).

The *FLC* reprogramming is mainly important for the winter accessions, which require a prolonged period of cold (vernalization) to reduce *FLC* expression to allow flowering (Crevillen *et al.*, 2014, Kim *et al.*, 2014). In contrast, summer accessions do not require vernalization to flower (Ding *et al.*, 2013). However, differences in *FLC* expression still affect the flowering time (Shafiq *et al.*, 2014). Also in these accessions a similar *FLC* resetting was observed, although with lower final levels of *FLC* expression (Choi *et al.*, 2009). The work presented here is focused on summer accessions and the mechanisms and processes in *FLC* regulation specific to winter accessions (*FRIGIDA* regulation, vernalization) are not described and discussed in detail.

Here, we show that over-expression of Snf2-subfamily ATPase gene *AtCHR12* affects the timing of flowering by interfering with the *FLC* reprogramming. During embryo development, *AtCHR12* over-expressing lines showed reduction in *FLC* expression compared to wild-type. The reduced *FLC* expression is maintained during vegetative growth leading to early flowering of mutant lines in both short-day and long-day conditions. This early flowering phenotype is associated with increased levels of H3K27me3 histone modification. These data suggest that AtCHR12 might affect flowering time by increasing the level of H3K27me3 on the *FLC* locus during embryogenesis.

RESULTS

Over-expression of *AtCHR12* leads to early flowering due to reduced *FLC* expression

During the phenotypic analysis of *AtCHR12*ov mutant (Mlynarova *et al.*, 2007) we have observed a new role of *AtCHR12* chromatin remodeling gene in flowering time control. Arabidopsis (*Arabidopsis thaliana*) lines over-expressing *AtCHR12* under the constitutive 35S CaMV promoter were early flowering compared to its corresponding wild-type Wassilewskija (Ws). To confirm this phenotype and compare it with knock-out mutant line, we generated new transgenic Arabidopsis line expressing a genomic copy of *AtCHR12* with the native promoter and with a C-terminal GFP-tag in *atchr12* knockout background (Col, SALK_105458), which we named *AtCHR12*ov-GFP.

To quantify the flowering time, the number of leaves at the time of bolting was counted at both long-day (LD) and short-day (SD) conditions. Both over-expressing lines displayed clear early flowering phenotype with less rosette leaves at bolting time than the corresponding

wild-type (Figure 4.1). The early flowering phenotype was more distinctive at SD conditions. On the other hand, the knockout mutant did not show significant difference in flowering time compared to the wild-type at any of the two tested conditions.

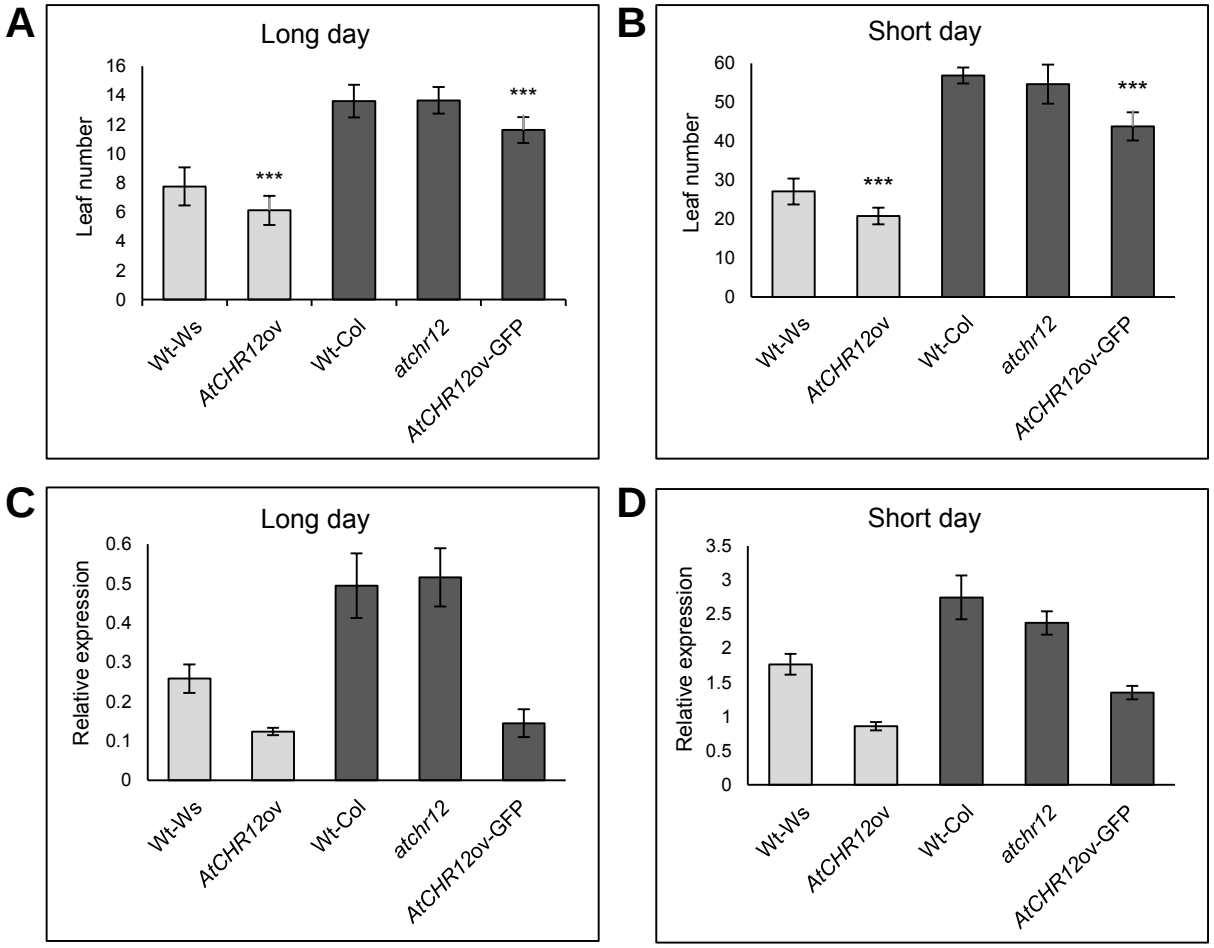

Figure 4.1: Over-expression of *AtCHR12* leads to early flowering. (A, B) Rosette leaf number at bolting of wild-type and *AtCHR12* mutants grown at LD **(A)** and SD **(B)** conditions. At least 15 plants were analyzed. The error bars represent standard deviation (s.d.). Statistically significant differences from corresponding wild-type were analyzed using Student's *t*-test (significance: ***, $P < 0.001$). **(C, D)** Relative expression of the main flowering repressor, *FLC* gene in 10-days-old seedlings of wild-type and *AtCHR12*ov mutants grown at LD **(C)** and SD **(D)** conditions. *UBC* was used as a reference gene. The error bars represent s.d. Light grey bars represent Arabidopsis lines in Ws background, dark grey bars represent Arabidopsis lines in Col background.

To determine the molecular basis of the early flowering phenotype of *AtCHR12* over-expressing mutants, we analyzed the expression of *FLC* gene, the main flowering repressor, as well as the expression of the flowering pathway integrators *FT* and *SOC1* genes. Quantitative RT-PCR analysis of 10 days old seedlings grown in LD and SD conditions showed that in both over-expressing lines *FLC* expression was significantly reduced compared to the wild-type (Figure 4.1). In agreement with reduced *FLC* expression we detected 2-3 fold

increased expression levels of the floral pathway integrators *SOC1* and *FT* leading to early transition into flowering (Figure 4.S1). The *atchr12* mutant did not show a significant change of *FLC* expression at LD nor at SD conditions (Figure 4.1). These data show that reduced expression of *FLC* is the most likely cause of early flowering phenotype of *AtCHR12* over-expressing mutants.

The chromatin state of *FLC* is altered in *AtCHR12*ov mutants

To investigate the mechanism of *FLC* down-regulation in over-expressing lines, first we measured the expression of the major *FLC* regulators.

We analyzed 10 days-old seedlings (SD) for expression levels of the most important *FLC* regulators. For analysis, 14 activators and 13 repressors were selected, including all components of the autonomous pathway (Table 4.S1). The expression levels of *FLC* activators were not significantly reduced in the mutant lines compared to wild-type, nor was the expression of *FLC* repressors significantly increased in these mutants (Figure 4.S2). The results indicate that *AtCHR12* over-expression does not affect the expression of *FLC* regulators in a way that could explain low *FLC* levels in mutants. However, we cannot exclude that *AtCHR12* over-expression does affect the expression of some other *FLC* regulators, which were not tested or not yet identified and studied.

The unaffected expression of *FLC* regulators suggests that *AtCHR12* over-expression could affect the *FLC* expression through changes in *FLC* chromatin state. Two main histone marks influence the *FLC* expression. Trimethylation of lysine 4 on H3 (H3K4me3) is associated with active transcription, and trimethylation of lysine 27 on H3 (H3K27me3) is associated with *FLC* repression (Crevillen *et al.*, 2011). To analyze the change of histone modifications on the *FLC* locus, we performed chromatin immunoprecipitation assays (ChIP) with antibodies specific to H3K4me3 and H3K27me3 on 10-days-old seedlings of *AtCHR12*ov and Ws wild-type. While the level of H3K4me3 on the *FLC* locus was not significantly different between wild-type and mutant (Figure 4.2B), the level of H3K27me3 was about 2 fold increased in several regions of the *FLC* locus in *AtCHR12*ov line (Figure 4.2C). The increased level of H3K27me3 was detected at the promoter region, first intron and at the beginning of the first exon (regions A, B, C, D, E), which is part of the so-called nucleation region important for *FLC* expression (Berry *et al.*, 2015). The level of H3K27me3 at the end of the *FLC* gene (regions F, G, H) was not significantly different from Ws wild-type. The increased level of H3K27me3 at nucleation region was observed also in *AtCHR12*ov-GFP line compared to Col wild-type, suggesting that the increased levels of repressive H3K27me3 histone mark at the 5'-end of the *FLC* locus in *AtCHR12*ov lines cause the reduction of *FLC* expression.

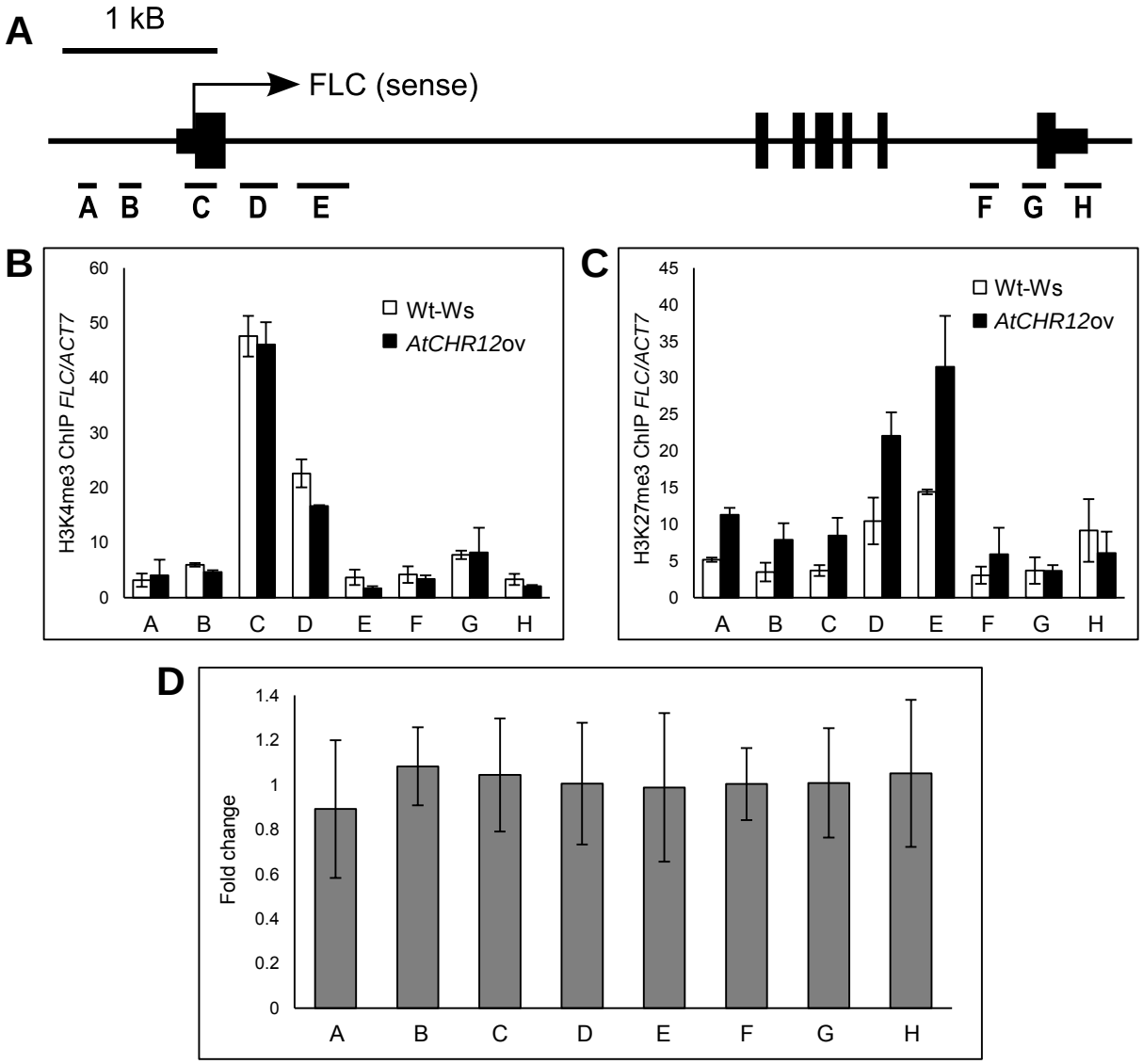

Figure 4.2: ChIP analysis of the *FLC* locus. (A) Schematic representation of the *FLC* locus and the regions examined by qPCR after ChIP. Transcription start sites are indicated by arrows, black, higher boxes represent exons, black lower boxes untranslated regions and black lines introns and intergenic regions. **(B, C)** Analysis of H3K4me3 **(B)** and H3K27me3 **(C)** levels using ChIP followed by qPCR, on 10-days-old seedlings of *AtCHR12*ov and Ws wild-type. Error bars represent s.d. from 3 replicates. **(D)** Relative fold change of AtCHR12 binding on the *FLC* locus analyzed by ChIP-qPCR with anti-GFP antibodies on 10-days-old seedlings of *AtCHR12*ov-GFP and Col wild-type. The fold change is normalized to Col wild-type (set to 1). Error bars represent s.d. from two biological replicates.

The data indicate that AtCHR12 chromatin remodeling complex could be involved in direct regulation of *FLC* by modifying the chromatin structure. To examine, if AtCHR12 directly binds to the *FLC* locus, we performed ChIP with anti-GFP antibodies on 10-days-old seedlings of *AtCHR12*ov-GFP and Col wild-type. However, no direct binding of AtCHR12 to the *FLC* locus was observed (Figure 4.2D), suggesting that AtCHR12 functions via different mechanism or at different developmental stage.

AtCHR12 over-expression interferes with *FLC* reprogramming

Throughout Arabidopsis vegetative growth the *FLC* expression is silenced. However, during embryogenesis the *FLC* expression is restored to high levels in accessions both requiring and not requiring vernalization (Choi *et al.*, 2009, Sheldon *et al.*, 2008). Previously, we have shown that the promoter of *AtCHR12* is active during embryo development (Leeggangers *et al.*, 2015, Mlynarova *et al.*, 2007), which led to the hypothesis that over-expression of *AtCHR12* could affect the *FLC* reactivation already during embryo development.

Therefore we measured the *FLC* levels in wild-type and *AtCHR12* over-expressing mutants during embryo development. *FLC* expression was determined in whole siliques collected at 4, 6, 8, 10 and 12 days after pollination (DAP) in *AtCHR12*ov and *AtCHR12*ov-GFP lines, and compared to the *FLC* expression in siliques of the same stage of the corresponding wild-type.

In the first phase of *FLC* reprogramming (*FLC* reactivation) till 8 DAP, over-expressing mutants showed a similar increase in *FLC* expression as the corresponding wild-types. However, during the later stages of embryo development from 10 DAP, both *AtCHR12*ov and *AtCHR12*ov-GFP lines showed reduced *FLC* expression compared to wild-type. While in Ws wild-type the *FLC* expression during maintenance phase (10-12 DAP) stayed at a high level, in *AtCHR12*ov the *FLC* expression level decreased (Figure 4.3A). In Col wild-type, the *FLC* expression was increasing till the last measured time point (12 DAP), while in *AtCHR12*ov-GFP the *FLC* expression remained more or less at the level as at 8 DAP (Figure 4.3B). At the last measured point of embryo development (12 DAP), the *FLC* expression in *AtCHR12*ov was reduced to about 15% of Ws wild-type level, and in *AtCHR12*ov-GFP to 50% of Col wild-type level. The timing of the observed effect of *AtCHR12* over-expression on *FLC* reprogramming during embryo development correlates with the highest levels of *AtCHR12* promoter activity shown earlier (Mlynarova *et al.*, 2007).

Taken together the results show that the over-expression of *AtCHR12* in Arabidopsis interferes with the second phase of *FLC* reprogramming during embryo development leading to reduced levels of *FLC* at late stages of embryo development. We assume that *AtCHR12* over-expression affects the removal and/or deposition of H3K27me3 histone mark on the *FLC* locus during embryo development resulting in lower *FLC* expression in mature embryos. The reduced *FLC* expression is maintained during vegetative growth causing early flowering of *AtCHR12* over-expressing mutants.

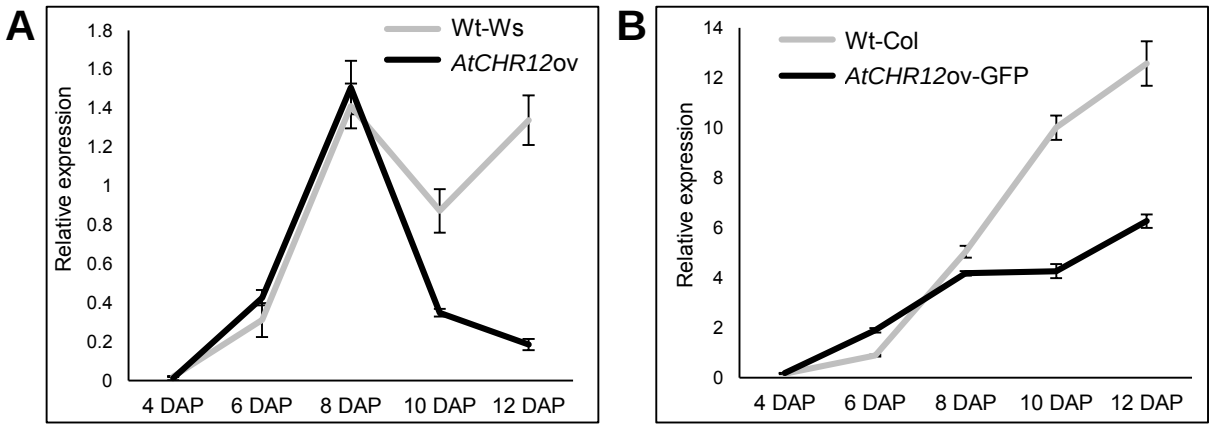

Figure 4.3: *FLC* expression in *AtCHR12*ov mutant lines and corresponding wild-types during embryogenesis in siliques at 4, 6, 8, 10 and 12 DAP. **(A)** *FLC* expression in *AtCHR12*ov and Ws wild-type. Error bars represent s.d. **(B)** *FLC* expression in *AtCHR12*ov-GFP and Col wild-type. Error bars represent s.d.

DISCUSSION

Regulation of the *FLC* expression, and hence flowering time control, is a well-studied topic and a lot of *FLC* regulators have been identified. However, most of the regulators are studied after germination and not too much is known about the role of *FLC* regulators during *FLC* reprogramming. We have here shown that over-expression of SWI/SNF-type chromatin remodeling gene *AtCHR12* interferes with the second phase of *FLC* reprogramming during embryo development. We assume that *AtCHR12* over-expression reduces the removal or increases deposition of H3K27me3 histone mark on the *FLC* locus during embryo development leading to reduced *FLC* expression in mature embryos and seeds. The reduced *FLC* expression is maintained during vegetative growth and results in early flowering of *AtCHR12*ov mutant compared to wild-type.

We have shown previously that AtCHR12 ATPase act as a regulatory component of seed maturation program (Leeggangers *et al.*, 2015). The mutants over-expressing *AtCHR12* showed reduced frequency of seed germination. Reduced germination was associated with increased transcript levels of several seed maturation genes with reduced degradation of their mRNAs stored in dry seeds. This coincides with the maintenance phase of *FLC* reactivation.

As shown previously, the two phases of *FLC* reprogramming are regulated by different *FLC* regulators (Choi *et al.*, 2009). Members of autonomous pathway are involved only in the maintenance of *FLC* expression, suggesting that *AtCHR12* over-expression may affect the *FLC* expression via members of autonomous pathway. The expression of autonomous pathway genes was not different from wild-type in transgenic seedlings, however, we cannot exclude that *AtCHR12* over-expression increases their expression during embryo development.

AtCHR12 over-expression led to a reduced level of H3K27me3 on the *FLC* locus. AtCHR12 ATPase might be therefore involved in regulation of *FLC* locus in concert with some members of autonomous pathway that are in control of H3K27me3.

REF6, also known as Jumonji domain-containing protein 12, is an H3K27me3-specific demethylase and in *ref6* mutants hundreds of genes are hypermethylated, including *FLC* (Kim *et al.*, 2014, Ko *et al.*, 2010, Lu *et al.*, 2011, Noh *et al.*, 2004). *REF6* is expressed during embryo development, but its role in *FLC* reprogramming was not studied in detail (Choi *et al.*, 2009). In addition to *ref6*, mutants in two other autonomous pathway genes, *fld* and *fca*, showed reduced H3K27me3 levels at *FLC* locus (Yu *et al.*, 2010). However, the reduction of H3K27me3 levels in these mutants was accompanied by increased H3K4me3 levels (Yu *et al.*, 2010), and the *FLC* reprogramming was shown to be independent of FLD (Choi *et al.*, 2009). This indicates that *AtCHR12* over-expression does not affect *FLC* reprogramming via *FLD*.

If AtCHR12 has a role in direct regulation of the *FLC* locus, a direct binding of AtCHR12 would be expected. However, we have not observed such binding in 10-days-old seedlings. The reason could be that AtCHR12 chromatin remodeling complex associates with the *FLC* locus only temporary at a certain developmental stage. We have observed the effect of *AtCHR12* over-expression on the *FLC* locus during embryo development, when also its expression peaks. It is possible that the binding can be detectable only at that stage. To establish, if it is true, it would require to analyze the AtCHR12 binding on *FLC* locus during embryo maturation. However, such analysis is technically difficult and due to time limitations it was not performed.

The effect of *AtCHR12* over-expression on *FLC* reprogramming at later stages of embryo development could be due to activity of promoters used. Both 35S CaMV promoter and *AtCHR12* promoter show the highest activity during later stages of embryo development (Mlynarova *et al.*, 2007, Völker *et al.*, 2001), therefore *AtCHR12* over-expression in embryos appeared at 7 – 8 DAP. Although at least 5 fold over-expression of *AtCHR12* was measured at all stages in both over-expressing lines (data not shown), the samples were a mixture of tissues of siliques and embryos and the actual mRNA level in extracts could be diluted.

The *AtCHR12* over-expression could interfere also with the function of Paf1 or SWR1 complexes, which are essential for both phases of *FLC* reprogramming (Choi *et al.*, 2009). However, Paf1 and SWR1 complexes are involved in H3K4me3 deposition and in H2A.Z histone variant exchange (He, 2012, March-Diaz *et al.*, 2009), which was not changed or studied in *AtCHR12*ov lines, respectively.

During *FLC* reprogramming, the *FLC* expression increases, suggesting reduction of the level of repressive histone mark H3K27me3 during embryo development (Crevillen *et al.*, 2014). The results suggest that in *AtCHR12*ov lines the H3K27m3 level on *FLC* locus is affected either by increased deposition or reduced removal. The H3K27me3 mark is deposited on the *FLC* locus by Polycomb Repressive Complex 2 (PRC2) (He *et al.*, 2013). However, PRC2 is not active during embryo development (Xiao *et al.*, 2015), which suggests

that the *AtCHR12* over-expression does not affect the *FLC* expression via PRC2. The removal of H3K27me3 histone mark is performed by H3K27me3-specific demethylases. In addition to REF6 mentioned above, a closely related demethylase ELF6 was shown to be involved in *FLC* reprogramming (Crevillen *et al.*, 2014). Mutation in *ELF6* impaired the *FLC* reactivation during embryo development resulting in reduced *FLC* expression in mature embryos and increased H3K27me3 levels on the *FLC* locus. Although, our results do not clearly indicate, which of the processes is affected by *AtCHR12* over-expression, the reduced removal by ELF6 may be the most probable mechanism, since it was shown to be involved in *FLC* reprogramming.

In addition to AtCHR12, also other Snf2-subfamily ATPases affect flowering time, however, via different pathways. The early flowering of *brm* mutants associates with increased expression of CO and *FT*, suggesting a role of BRM in the photoperiod pathway (Farrona *et al.*, 2011). In contrast, the *AtCHR12* over-expressing lines were early flowering in both LD and SD conditions, suggesting photoperiod-independent regulation. Another Snf2-subfamily ATPase SYD affects the flowering time through modulation of *FT* expression (Su *et al.*, 2006). However, neither BRM nor SYD were shown to be involved in *FLC* reprogramming. On the other hand, we have indications that over-expression of *AtCHR23*, an *AtCHR12* paralog, leads to early flowering by reducing the *FLC* expression (data not shown). Since *AtCHR23* is expressed during embryo development (Leeggangers *et al.*, 2015), its role in flowering time control could explain the lack of phenotype of *atchr12* knockout mutant due to redundant functions of these two ATPases (Folta *et al.*, 2014, Sang *et al.*, 2012).

Based on the results described in this manuscript and other published data we suggest that AtCHR12 ATPase might play a role in *FLC* reprogramming during embryo development. Over-expression of *AtCHR12* affects the H3K27me3 deposition most probably by reduced removal by H3K27me3-specific demethylases. However, the results indicate, that AtCHR12 affects the flowering time by different mechanism than other Snf2-subfamily ATPases.

MATERIALS AND METHODS

Construction of T-DNA plasmids and transformation

A genomic copy of *AtCHR12* (*At3g06010*) including its promoter (1253 bp promoter, 194 bp 5'-UTR, 4849 bp gene) was isolated from genomic DNA of Arabidopsis (*Arabidopsis thaliana*) accession Colombia by PCR and cloned into a pENTR Gateway plasmid (Invitrogen) using *E. coli* CopyCutter cells (Epicentre) and verified by sequencing. The correct plasmid was used for multi-step LR Gateway reaction with plasmids pEN-L4-MCS-R1 and pEN-R2-F-L3 containing multi-cloning site and C-terminal GFP-tag, respectively, into destination vector pB7m34GW (https://gateway.psb.ugent.be/). The resulting binary vector

pAtCHR12::AtCHR12-GFP was introduced into *Agrobacterium tumefaciens* C58C1 by freeze-thaw method (Weigel *et al.*, 2006) and used for transformation of Arabidopsis Columbia (Col-0) *AtCHR12* knockout line *atchr12* by floral dip (Clough *et al.*, 1998). Transgenic lines were selected based on PPT resistance (7.5 µg/ml phosphinothricin-DL) and screened for the level of transgene expression. The line was designated *AtCHR12*ov-GFP.

Plant material and growth conditions

To investigate the role of *AtCHR12* in flowering time control, two Arabidopsis (*Arabidopsis thaliana*) lines over-expressing a genomic copy of *AtCHR12* were used. In addition to *AtCHR12*ov-GFP (see above), a previously described *AtCHR12*ov line in Wassilewskija (Ws) background was used (Mlynarova *et al.*, 2007). *AtCHR12*ov is an activation tagged mutant of *AtCHR12* with four tandem copies of the 35S enhancer sequence integrated about 1 kb upstream of the transcription initiation site. The *AtCHR12* expression was 15 – 20-fold up-regulated in *AtCHR12*ov line compared to Ws wild-type, and 3 – 7-fold up-regulated in *AtCHR12*ov-GFP line compared to Col wild-type. Two *AtCHR12*ov-GFP lines were used for the phenotypical analyses with similar results, therefore the results of only one line are shown in the manuscript. For comparison, loss-of-function knockout mutant *atchr12* was used (Mlynarova *et al.*, 2007). The knockout mutant did not show any *AtCHR12* expression.

For seedling analysis, Arabidopsis seeds were surface sterilized, placed on 0.5x MS plates and cold stratified for 3 days. The plates were placed vertically in fully controlled growth chambers lid by Philips TD 32 W/84 HF lamps at 23 °C in long-day (LD, 16 h light/8 h dark) or short-day (SD, 8 h light/16 h dark) conditions. Arabidopsis plants were grown in standard potting soil in growth room lid by Philips-Master 36W/830 lamps at 21 ± 2 °C in LD or SD conditions. For embryo analysis, the flowers were marked every two days and later the siliques were collected at 4, 6, 8, 10 and 12 DAP and used for further analysis.

RNA isolation and expression analysis

Total RNA was isolated from 10-days-old seedlings or siliques collected at certain stage as described above, using E.Z.N.A.™ Plant RNA Mini Kit (Omega Bio-Tek, Inc., USA), followed by on column DNase treatment (Qiagen, RNase-free DNase Set). One microgram of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). Ten times diluted cDNA was used for quantitative RT-PCR using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA) in an CFX Connect™ Real-Time System thermal cycler (Bio-Rad Laboratories, Inc., USA). Reactions were performed in triplicate. The *UBC* gene (*At5g25760*) was used as reference (Czechowski *et al.*, 2005). Sequences of primers used are given in Supplementary table S1.

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed with 10-days-old seedlings or siliques older than 10 DAP. The ChIPs with antibodies against H3K4me3 or H3K27me3 histone marks (both Cell Signalling Technology®, USA) were performed as described by Kaufmann et *al.* (Kaufmann *et al.*, 2010), and ChIP experiments directed to GFP-tagged proteins were performed as described by Smaczniak et *al.* (Smaczniak *et al.*, 2012). The immunoprecipitated DNA and 100-fold diluted input DNA were analyzed by qPCR with primers listed in Supplementary table S1. The qPCR results were re-calculated as a fold change of the mutant line compared to the corresponding wild-type. All ChIP experiments were performed at least two times, in graphs is depicted the average with its standard deviation.

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SUPPLEMENTARY DATA

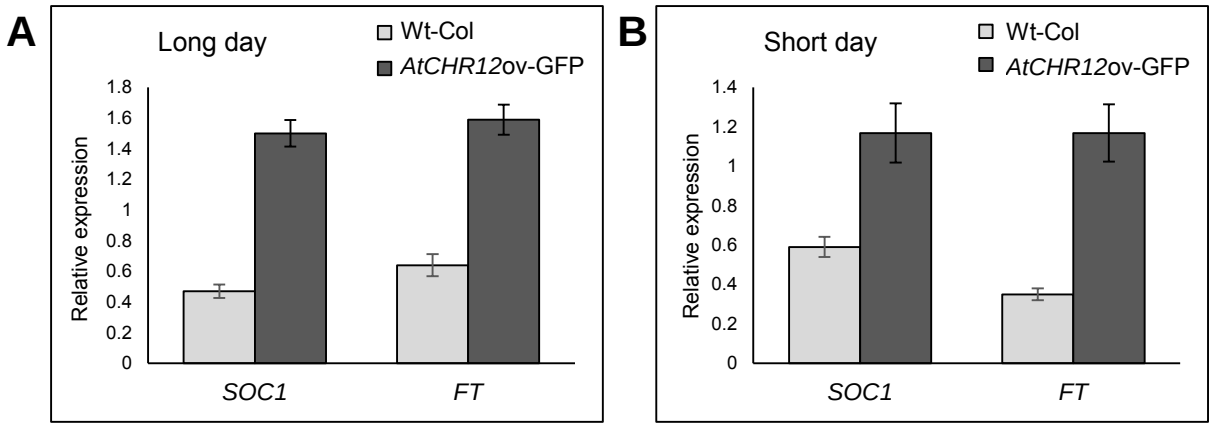

Figure 4.S1: Expression of *SOC1* and *FT* in 10 days old seedlings of *AtCHR12*ov-GFP mutant line at LD (**A**) and SD (**B**) conditions. The error bars represent s.d.

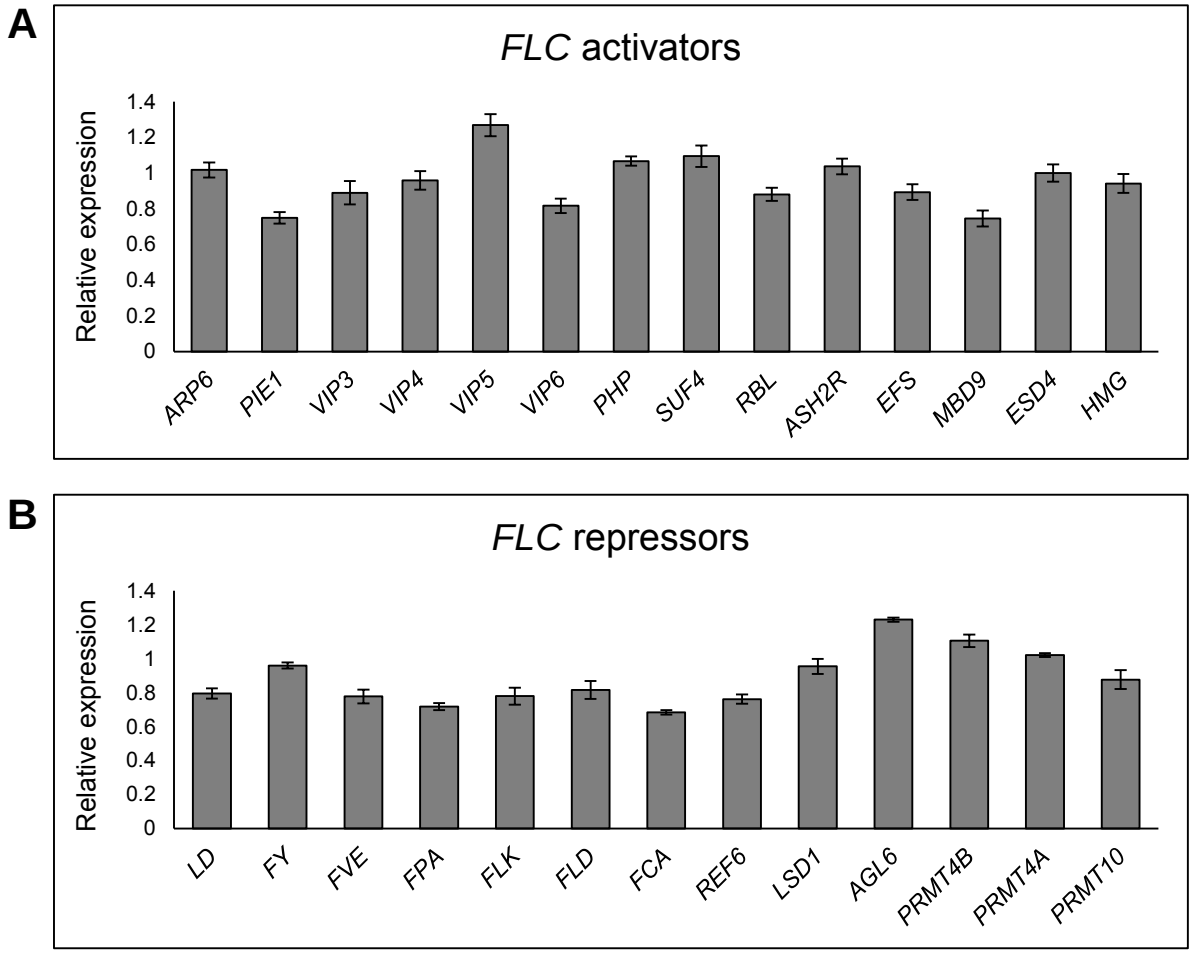

Figure 4.S2: Expression of the main *FLC* activators (**A**) and repressors (**B**) in 10 days old seedlings of *AtCHR12*ov line. The expression is normalized to the Ws wild-type (set to 1). The error bars represent s.d.

Table 4.S1: Description of various *FLC* regulators.

	Gene	Function	Reference
Activators	*ARP6*	H2A.Z histone variant deposition	(He, 2009)
	PIE1		
	VIP3	H3K4 methylation	(He, 2009)
	VIP4		
	VIP5		
	VIP6		
	PHP	H3K4 methylation	(Yu *et al.*, 2010)
	SUF4	H3K4 methylation	(Kim *et al.*, 2006)
	RBL	H3K4 methylation	(Jiang *et al.*, 2011)
	ASH2R		
	EFS	H3K4 and H3K36 methylation	(He, 2009)
	MBD9	H3 and H4 acetylation	(Yaish *et al.*, 2009)
	ESD4	SUMO protease	(Henderson *et al.*, 2004)
	HMG	Component of FACT transcription factor	(Lolas *et al.*, 2010)
Repressors	*LD*	Transcriptional regulator	(Kim *et al.*, 2006)
	FY	RNA processing factor	(Henderson *et al.*, 2004)
	FLK		
	FPA	RNA chromatin silencing	(He, 2009)
	FCA		
	FVE	Histone deacetylation	(He, 2009)
	FLD	H3K4 demethylation	(He, 2009)
	REF6	H3K27me3 demethylation	(Lu *et al.*, 2011)
	LSD1	H3K4 demethylation	(He, 2009)
	AGL6	MADS-box transcription factor	(Yoo *et al.*, 2011)
	PRMT4B	H3R3 dimethylation	(He, 2009)
	PRMT4A		
	PRMT10		

Table 4.S2: List of primers used in this study.

Gene	Forward sequence 5' > 3'	Reverse sequence 5' > 3'	Used for
AtCHR12	TTCCACTGCACAAGACAGAAG	TCTTGCTCTTGCATCAGACG	
FLC	TCTCCTCCGGCGATAACCT	GCATGCTGTTTCCCATATCGAT	
SOC1	CTTCTAAACGTAAACTCTTGGGAGAAG	CCTCGATTGAGCATGTTCCTATG	
FT	CAACCCTCACCTCCGAGAATAT	TGCCAAAGGTTGTTCCAGTTGT	
UBC	TTAGAGATGCAGGCATCAAGAGCGC	CATATTTCTCCTGTCTTGAAATGAA	
ARP6	CCAGCCTGCAGATTTAGGGATGA	CATCAAAGTGATCTGGGACAAGTGG	
PIE1	ACGTGGCAATGATGATTCCCTGA	AGCATTTGCAGCATTCCTGTAACG	
VIP3	CCGTTTGGGCAGCGACGTGGGTT	CAAGATCCAGCTCGTCCGGTCGCCA	
VIP4	GCCTTTTGATGCCAAAACAT	CCACCTTACAAACCGAGCAT	
VIP5	CCGACGATTTCTCCGTCTGATCTC	CCTCACGTCTCCTCGATGATGG	
VIP6	TGCCCCTGATAATGTTCCTGCTC	CGCTTGCCGTGCTTTATCTAGTTG	
PHP	CGTGTTGTTGCGGTTTTTGTATTGG	GGTCTTGATGCCGCTTATTCTTGCT	
SUF4	TGCAAGGAATTCCACCCCATGT	GGAGGACCCGGATAATACATTGGTC	
RBL	GAAGATGAATTTGATTTGATACCTG	TGTCTCACCCATTTCTTCTGC	
ASH2R	GGAAGGGTACAAGGSSGGTG	AACGATATTTCACTGCCTGGT	
EFS	AACTGCCGTACTGAAAAGTGGATGG	TCAGAGGATCTCCCCCAATATACCC	qRT-PCR
MBD9	ATGGTTTCCCTGAGCAAAAGGGTAG	ACTGCATCGGACATCCATTCTTAGC	
ESD4	GCTCTGGCAAAATACATGGGTGG	GTATGGCATGTGTTCCTGGCT	
HMG	TTGAGGATGCGGTCGTCACATT	TGTGTGGTTGGTTTGACTTTGGAAG	
LD	TGATTGGCATGTACCACCAGGAAT	GTCCATTTCCCTATCCCATGGTTCT	
FY	GATGCCTGGATCAATGGGAATG	TGCTGCTGTTGGAAAGGGTTGT	
FLK	AACTGCCGTGCAGCTTATTCAGAAC	TGTTGCGTATGGGTTATAGCCTTGC	
FPA	TGGGTGTCGATGAGAGGTCAT	CAATTGACGACCTCAGGCAGT	
FCA	GAACTGGACAGCAGCAAGGCTGTTG	TAGGGTGCCTATGCGTTCTCTCTCC	
FVE	ACAAACCCTGTCACGAAGGTTGAA	GCGGTAAGCTTCCTACGATCAAACA	
FLD	TGACGCAGTGACTCGTGTTCTTCAT	AAGCCTTCCATCTCCCACACTTTCT	
REF6	CCGGAATACCGTGTTGCAGGTTAGT	ATTCGGGAGTGGCAATGTTAGATGC	
LSD1	GCTGCGGAAAGATTCGAGACATTG	GATGATCCGACCGCAACATATGAGT	
AGL6	GGATTGCAACACCGAACCCTTTTTA	TGGCATCGTGGTCTAATCAACAGAG	
PRMT4B	CGCTGACCGAATCACAGTCATCA	TGCCATGTGAATCCTTCCGACA	
PRMT4A	CACCAGCTCAGTCGCAAGACATACA	AAGGGTTGGTGCTAAACCGTAGGAA	
PRMT10	CGGCTCCAGTTGACAAAGAAGTTG	CGGTGCCCACGTCTAAAACAGTC	
A	GCTGATACAAGCATTTCACCAAA	CTTAAATGTCCACACATATGGCAAT	
B	TGTAGGCACGACTTTGGTAACACC	GCAGAAAGAACCTCCACTCTACATC	
C	CGACTTGAACCCAAACCTGA	GGATGCGTCACAGAGAACAG	
D	ATCTCTTGTTGTTTCTCGGTTCTG	AACAAATCGTGAATGACATGC	
E	TCATTGGATCTCTCGGATTTG	GAATCGCAATCGATAACCAGA	ChIP-qPCR
F	CAGGTTATGGTCTGGTTCAGTCT	CACTCGGATCCAAACCTAACC	
G	TGGTTGTTATTTGGTGGTGTG	ATCTCCATCTCAGCTTCTGCTC	
H	GCTGATAAGGGCGAGCGTTTG	GTAGGCTTCTTCACTGTGAAGC	
ACT7	CGTTTCGCTTTCCTTAGTGTTAGCT	AGCGAACGGATCTAGAGACTCACCTTG	

CHAPTER 5

COMPACT TOMATO SEEDLINGS AND PLANTS UPON OVER-EXPRESSION OF A TOMATO CHROMATIN REMODELING ATPASE GENE

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ABSTRACT

Control of plant growth is an important aspect of crop productivity and yield in agriculture. Over-expression of the *AtCHR12/AtCHR23* genes in *Arabidopsis thaliana* reduced growth habit without other morphological changes. These two genes encode Snf2 chromatin remodeling ATPases. Here, we translate this approach to the horticultural crop tomato (*Solanum lycopersicum*). We identified and cloned the single tomato ortholog of the two Arabidopsis Snf2 genes, designated *SlCHR1*. Transgenic tomato plants (cv. Micro Tom) that constitutively over-express the coding sequence of *SlCHR1* show reduced growth in all developmental stages of tomato. This confirms that *SlCHR1* combines the functions of both Arabidopsis genes in tomato. Compared to the wild-type, the transgenic seedlings of tomato have significantly shorter roots, hypocotyls and reduced cotyledon size. Transgenic plants have a much more compact growth habit with markedly reduced plant height, severely compacted reproductive structures with smaller flowers and smaller fruits. The results indicate that either GMO or non-GMO-based approaches to modulate the expression of chromatin remodeling ATPase genes could develop into methods to control plant growth, for example to replace the use of chemical growth retardants. This approach is likely to be applicable and attractive for any crop for which growth habit reduction has added value.

Keywords
Chromatin remodeling, Growth, Plant habit, SWI/SNF2, Tomato

INTRODUCTION

An important aspect of crop productivity and yield in agriculture is plant growth (Del Moral *et al.*, 1985, Ozalkan *et al.*, 2010). Control of plant growth is therefore an important feature of proper crop management. Plant growth is affected by both internal genetic factors and external environmental conditions. Plants evolved finely orchestrated mechanisms to regulate growth either in response to short-term adverse environments or as programmed part of their life cycle (Claeys *et al.*, 2013). A growing body of evidence indicates that epigenetic modifications provide mechanisms that help plants to integrate intrinsic and environmental signals (Gutzat *et al.*, 2012, Sahu *et al.*, 2013, Seffer *et al.*, 2013). In such epigenetic modifications, chromatin remodeling plays a major role. Chromatin remodeling is based on the activity of multi-protein enzymes that are conserved from yeast to man. These enzymes alter the accessibility of chromatin to the transcriptional machinery (Kennison, 1995, Vignali *et al.*, 2000), particularly in case of inducible or increased gene expression (Narlikar *et al.*, 2002, Sudarsanam *et al.*, 2000, Tsukiyama, 2002). Prominent chromatin remodeling moieties are ATPase-dependent chromatin remodeling complexes such as SWI/SNF, which utilize ATP hydrolysis to generate the energy to restructure chromatin.

Previously, we have shown a functional relationship between the expression of the *Arabidopsis thaliana* *AtCHR12* and *AtCHR23* genes and the regulation of plant growth. These two genes are paralogs encoding chromatin remodeling ATPases of the SWI/SNF2-type. Over-expression of *AtCHR12* resulted in the growth arrest of primary buds, as well as reduced growth of the primary stem (Mlynarova *et al.*, 2007). Over-expression of *AtCHR23* led to reduced growth of seedlings and vegetative rosette compared to the wild-type (Folta *et al.*, 2014). Upon applying abiotic stress, over-expressing mutants were reduced in overall growth significantly more than wild-type Arabidopsis. Except for this reduction in growth, the over-expressing plants showed no other morphological changes. Another Arabidopsis Snf2-type chromatin remodeler BRAHMA (BRM) was shown to affect growth regulation. A loss-of-function mutant of this gene shows reduced growth and is early flowering (Tang *et al.*, 2008). BRM promotes vegetative growth by suppression of PcG activities at the SHORT VEGETATIVE PHASE locus (Li *et al.*, 2015).

Modulated expression of chromatin remodeling genes, either through genetic engineering or by genetic selection, could therefore present an innovative technology for the control of plant growth in crops. Although it requires that Arabidopsis growth regulation is a good model for growth regulation in crops.

To be able to investigate if comparable phenotypes are obtained in a crop upon over-expression of a SWI/SNF2 chromatin remodeling ATPase, we have analyzed all putative Snf2 family members in all currently available plant genomes (Bargsten *et al.*, 2013). In tomato (*Solanum lycopersicum*), this analysis identified one gene that is the putative ortholog of both *AtCHR12* and *AtCHR23*. The two Arabidopsis paralogs are likely the result of a gene

duplication specific to the Arabidopsis genus. In view of the evolutionary relationships, it was suggested that the one tomato ortholog would combine the functions of both its Arabidopsis counterparts (Bargsten *et al.*, 2013).

Tomato is a major vegetable crop with increasing popularity over the last decades. Commercial production of tomato, either field- or greenhouse-grown, makes use of transplanting pre-grown seedlings. The major benefit of the use of such tomato transplants is uniformity, earlier production and it results in increases in crop yield and quality (Hochmuth *et al.*, 2012). In addition, smaller plants with equal yield and/or smaller fruits with better taste are attractive breeding targets.

Here we show that over-expression of a single chromatin remodeling gene affects plant growth habit markedly. We have isolated and cloned the tomato ortholog of the Arabidopsis *AtCHR12* and *AtCHR23* genes. Transgenic tomato plants constitutively over-expressing this gene show reduced growth at all developmental stages. Compared to wild-type, the transgenic seedlings have significantly shorter roots, hypocotyls and reduced cotyledon size. The growth reduction also affects vegetative growth, resulting in smaller, more compact, tomato plants with severely compacted reproductive structures and smaller flowers. These results show that modulating the expression of chromatin remodeling ATPase genes could develop into novel methods to control plant growth habit that may prove attractive for agricultural or horticultural practice.

RESULTS

Characterization and isolation of the coding sequence of the tomato Snf2 ATPase gene *SlCHR1*

Detailed phylogenetic analyses have shown that the two *Arabidopsis thaliana* Snf2 ATPase in-paralogs *AtCHR12* (*At3g06010*) and *AtCHR23* (*At5g19310*), have only one tomato (*Solanum lycopersicum*) ortholog (Bargsten *et al.*, 2013). In the ITAG1 tomato genome release (10 March 2010), this gene was annotated as *SL100sc05189_42.1.1* (http://solgenomics.net/), whereas in the more recent ITAG2.4 annotation release (23 February 2014) two genes are predicted in the same genomic region: *Solyc01g79690.2.1* and *Solyc01g079700.2.1* (Figure 5.1). ITAG2.4 *Solyc01g079700.2.1* is identical to the first two exons and first intron of *SL100sc05189_42.1.1* apart from an additional 5'-UTR and 16 additional bases including stop codon at the 3'-end. ITAG 2.4 *Solyc01g79690.2.1* covers most of the ITAG1 *SL100sc05189_42.1.1* gene; it corresponds to ITAG1 *SL100sc05189_42.1.1* from its third exon, except for an additional exon at the 5'-end and 3'-UTR sequence (Figure 5.1). The ITAG2.4 *Solyc01g079700.2.1* gene does not contain any domain that is characteristic for chromatin remodeling ATPases (Bargsten *et al.*, 2013).

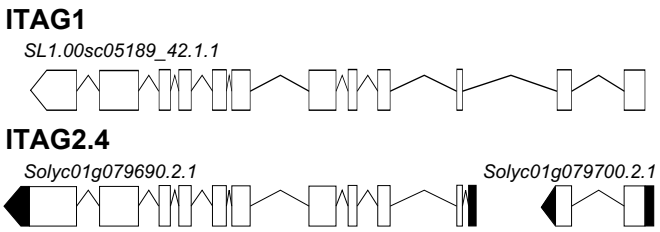

Figure 5.1: Layout of the structure of *SlCHR1* gene in two different tomato genome annotations, ITAG1 and ITAG2.4. Exons are illustrated as boxes, lines represent introns. White-filled boxes show exons common to both annotations, black boxes represent exons specific for the ITAG2.4 annotation.

To determine the correct configuration in the Heinz tomato genome, exploratory RT-PCR analyses were undertaken with several primer sets specific for each annotation (Figure 5.S1A). Total RNA was isolated from both leaves and flowers. No PCR product was obtained with primer sets specific for cDNA according to the ITAG2.4 annotation (Figure 5.S1B). In contrast, the expected PCR product was obtained with primer set specific for the ITAG1 annotation (Figure 5.S1B). Moreover, in publicly available RNA-seq libraries for *S. lycopersicum* (Sato *et al.*, 2012), no reads covering the 16 bases present at the 3'end of the *Solyc01g079700.2.1*, nor reads mapping to the first exon of *Solyc01g79690.2.1*, are present. In contrast, a number of reads are present that start in the second exon of *Solyc01g079700.2.1* and continue into the second exon of *Solyc01g79690.2.1.* (Figure 5.S2). These results show that the ITAG1 annotation is most close to the true situation in the tomato genome and we therefore based the isolation of the coding sequence on the ITAG1 annotation of *SL100sc05189_42.1.1.* In the remainder of this paper, we refer to this tomato gene as *SlCHR1* and to its coding sequence as c*SlCHR1.*

The coding sequence of *SlCHR1* was amplified by RT-PCR from total RNA isolated from leaves of *in vitro* grown tomato cv. Heinz 1706. The DNA sequence of c*SlCHR1* confirms the existence of a single (3321 bp) transcript, essentially matching the ITAG1 annotation, except for three changes on exon/intron boundaries that were correct in the ITAG2.4 annotation: a deletion of 57 b at position 1645, an insertion of one base at position 2763 and an insertion of 20 b at position 2871. The resulting sequence of the *SlCHR1* coding sequence and the derived protein sequence are given in Figure 5.S3. The distribution of protein domains and elements in this sequence was presented earlier (Bargsten *et al.*, 2013).

Generation and first characterization of c*SlCHR1* over-expressing transgenic lines for Arabidopsis and tomato

To test whether the over-expression of c*SlCHR1* would affect plant growth, we generated transgenic Arabidopsis and tomato lines over-expressing c*SlCHR1.* The binary plasmid 35S:c*SlCHR1*-GFP contains the full-length coding sequence of *SlCHR1* with a C-terminal GFP-tag put under the control of the constitutive 35S CaMV promoter (Figure

5.2A). The T-DNA was transferred to *Arabidopsis thaliana* Col-0 using the floral dip method (Clough *et al.*, 1998). Single-locus homozygous F3 lines were selected based on kanamycin segregation. Two such lines, At-c*SlCHR1*-ov1 and At-c*SlCHR1*-ov2, were selected for more detailed analyses.

Transgenic tomato lines were generated by transformation of the tomato cultivar Micro Tom (Carvalho *et al.*, 2011) using the same binary plasmid and regenerating transgenic shoots from cotyledons (Qiu *et al.*, 2007). Single-locus homozygous F3 lines were selected as for Arabidopsis. From 10 transgenic lines obtained, two lines, Sl-c*SlCHR1*-ov1 and Sl-c*SlCHR1*-ov2 were randomly selected for more detailed analysis.

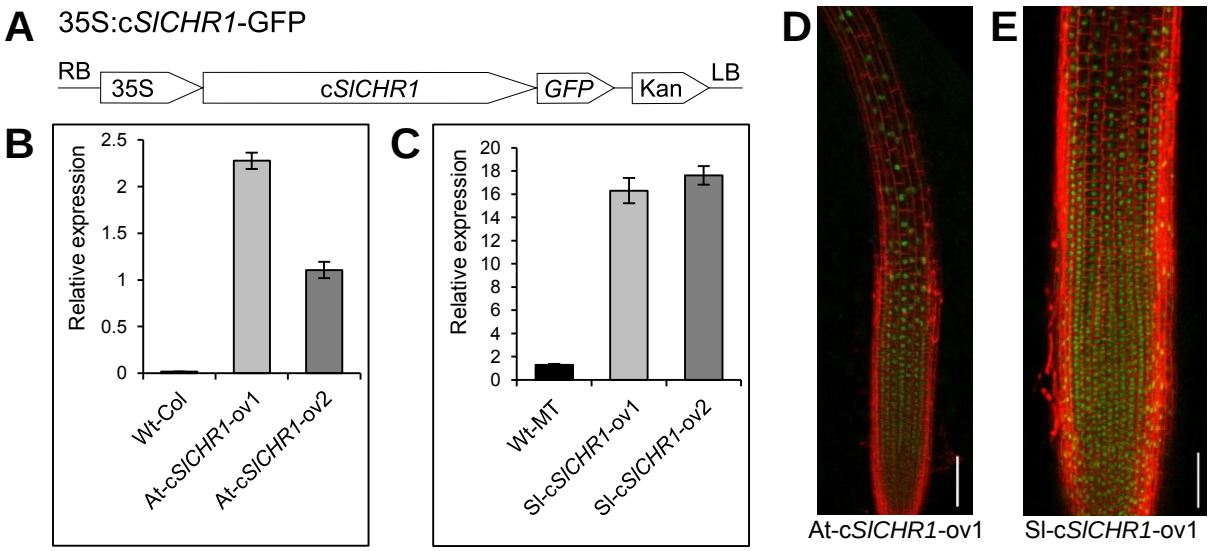

Figure 5.2: Expression and localization of c*SlCHR1* in transgenic lines. **(A)** Schematic layout of plasmid T-DNA region used to generate transgenic Arabidopsis and tomato lines over-expressing c*SlCHR1* gene. 35S, CaMV 35S promoter; *GFP*, green fluorescence protein gene; Kan, kanamycin resistance gene; RB, LB, right and left T-DNA borders. **(B)** Relative expression levels of *SlCHR1* mRNA in Arabidopsis At-c*SlCHR1*-ov1 and At-c*SlCHR1*-ov2 transgenic lines as measured by qRT-PCR. No expression was detected in Arabidopsis wild-type Col-0. The *UBC* gene was used as reference. The error bars represent standard deviation (s.d.). **(C)** Relative expression levels of *SlCHR1* mRNA in tomato Sl-c*SlCHR1*-ov1 and Sl-c*SlCHR1*-ov2 transgenic lines compare to the wild-type Micro Tom (MT). The *L33* gene was used as a reference. The error bars represent s.d. **(D, E)** Nuclear localization of cSlCHR1-GFP in roots of transgenic Arabidopsis line At-c*SlCHR1*-ov1 **(D)** and transgenic tomato line Sl-c*SlCHR1*-ov1 **(E)**. Confocal images of 6-day-old seedlings taken with Leica confocal microscope. Propidium iodide (1 μg/ml) was used to color the cell walls red. Bar = 100 μm.

Analysis of *SlCHR1* expression levels by qRT-PCR in Arabidopsis and tomato lines showed the intended over-expression in both plant genera (Figure 5.2B,C). In Arabidopsis, the two lines differ in expression level (Figure 5.2B). The expression relative to the reference gene *UBC* was about 2.3 for At-c*SlCHR1*-ov1 and 1.0 for At-c*SlCHR1*-ov2. Relative to the endogenous Snf2 genes *AtCHR12* and *AtCHR23*, expression of the transgene is about 8-10

fold higher (data not shown). Relative expression levels of the endogenous genes are not compromised (data not shown), so introduction of the tomato cDNA does not result in silencing of the endogenous Snf2 paralogs. In tomato, in both lines the *SlCHR1* gene was about 15-17 fold higher expressed, relative to either the reference gene *L33* or the endogenous gene *SlCHR1* (Figure 5.2C).

In both Arabidopsis (Figure 5.2D) and tomato (Figure 5.2E), the GFP-tagged SlCHR1 protein was localized in root nuclei, as well as in nuclei of hypocotyls and leaves (data not shown). These data confirm the expected nuclear localization of a chromatin remodeling ATPase (Sang *et al.*, 2012, Sarnowski *et al.*, 2002).

Over-expression of c*SlCHR1* in Arabidopsis does not affect growth and development

The growth of the two transgenic Arabidopsis lines was monitored during early seedling and vegetative development. Transgenic seedlings did not differ from the wild-type in the length of the hypocotyl or in cotyledon size when grown under optimal conditions (data not shown). To assess the impact of c*SlCHR1* over-expression on vegetative growth when exposed to environmental stress, the length of the primary root was compared between the wild-type and transgenic plants under salt stress (75 mM NaCl). Salt stress reduces the length of the root of the wild-type to about half and the reduction of the root length was similar in the two transgenic lines (Figure 5.3A). Also the diameter of the leaf rosette of soil-grown plants was compared between standard conditions and salt stress. The rosette diameters were determined from digital images of 4-week-old plants as described previously (Folta *et al.*, 2014). No differences in rosette diameter between wild-type and transgenic lines were observed (data not shown). To compare the phenotypic effects of c*SlCHR1* over-expression with the phenotypes obtained with *AtCHR12* (Mlynarova *et al.*, 2007), we measured the length of the primary stem of 40-day-old plants without (control) and with a heat stress treatment of 12 days as described previously. The results (Figure 5.3B) show that although the length of the primary stem of line At-c*SlCHR1*-ov1 was slightly reduced compared to wild-type in control conditions, the length of the primary stem of both transgenic lines upon stress was not different from the primary stem length of the wild-type (Figure 5.3B). These results show that over-expression of the tomato c*SlCHR1* gene does not affect seedling and vegetative growth in Arabidopsis as seen upon over-expression of the *AtCHR23* (Folta *et al.*, 2014) or the *AtCHR12* gene (Mlynarova *et al.*, 2007). Constitutive expression of the tomato c*SlCHR1* gene has no significant impact on vegetative growth and development of Arabidopsis plants and also does not seem to affect the response of Arabidopsis to adverse environmental conditions.

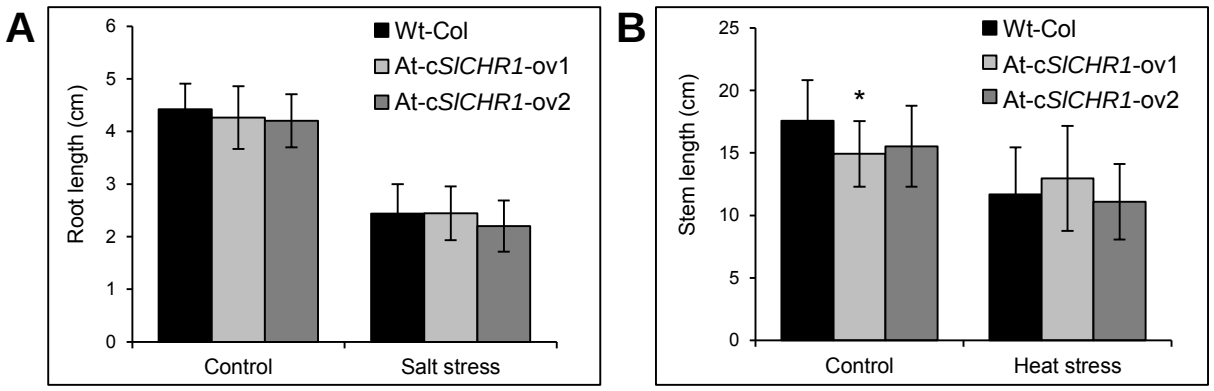

Figure 5.3: Over-expression of c*SlCHR1* does not affect the growth habit of Arabidopsis. **(A)** Mean length of the primary roots of 8-day-old seedlings grown in control and salt stress (75 mM NaCl) conditions. **(B)** Mean length of primary stem of 40-day-old control and heat-stressed plants. The heat stress (37 °C for 16 hours) was applied to 28-day-old plants. Control, non-treated plants, were grown and measured in parallel with stressed plants. The error bars represent s.d. For each condition, asterisks indicate significant differences from wild-type: *, $P < 0.05$. For each line, at least 32 seedlings and 13 plants were measured.

Over-expression of c*SlCHR1* in tomato results in considerably compacter growth

To quantify the effect of c*SlCHR1* over-expression on the growth of tomato seedlings, three parameters were measured: the cotyledon area, the length of the main root and the length of the hypocotyl. The two transgenic tomato lines over-expressing c*SlCHR1* showed significantly reduced growth compared to the wild-type in all parameters measured (Figure 5.4). The average length of the root of 7-day-old seedlings was reduced from 6.7 cm in wild-type to 5.1 cm and 5.7 cm in the Sl-c*SlCHR1*-ov1 and Sl-c*SlCHR1*-ov2 lines, respectively (Figure 5.4A). This is a reduction in growth of 23.9% and 14.9% relative to wild-type. A similar reduction was observed for the length of the hypocotyl (Figure 5.4B). While the average length of the wild-type hypocotyl was 2.1 cm, in Sl-c*SlCHR1*-ov1 and Sl-c*SlCHR1*-ov2 lines it was 1.6 cm (23.8% reduction) and 1.8 cm (14.3% reduction), respectively. The cotyledon area of 0.32 cm^2 in the wild-type was reduced to 0.24 cm^2 (25% reduction) in both transgenic lines (Figure 5.4C). Upon over-expression of c*SlCHR1*, tomato seedlings become markedly more compact than the wild-type. In contrast, transgenic tomato lines obtained via RNAi that had markedly reduced (about 50%) levels of *SlCHR1* expression revealed no differences in growth habit relative to the wild-type (data not shown).

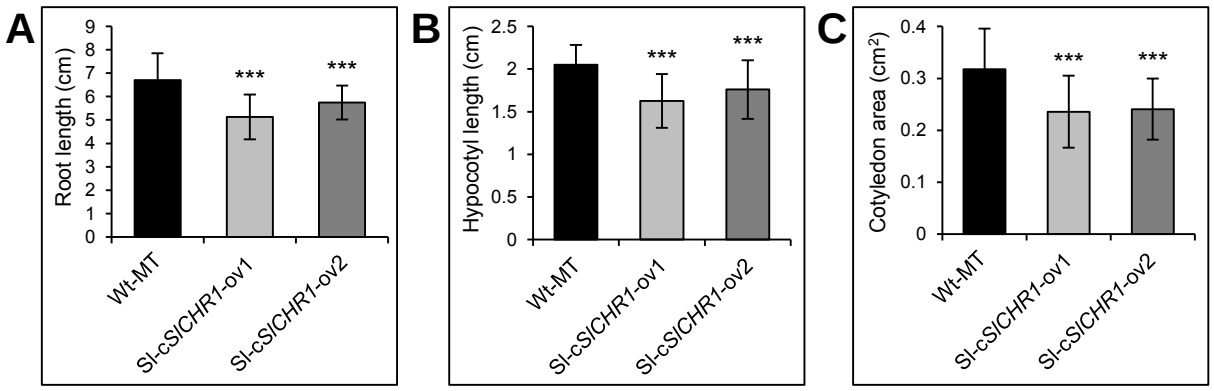

Figure 5.4: Over-expression of c*SlCHR1* in tomato results in reduced seedling growth. **(A)** Mean root length, **(B)** mean hypocotyl length, and **(C)** mean cotyledon area of 7-day-old seedlings of wild-type MT and two transgenic lines grown in normal environmental conditions. The error bars represent s.d. Asterisks indicate significant differences from wild-type: ***, $P < 0.001$. For each line, at least 15 seedlings were measured.

To evaluate how the over-expression of c*SlCHR1* and compactness of tomato seedlings translates to later stages of vegetative growth and development of tomato, height and diameter of 6-week-old greenhouse-grown plants were measured. Both height and diameter of the two transgenic lines were significantly reduced compared to the wild-type (Figure 5.5). An example of the height difference is shown in Figure 5.5A. The average height of wild-type plants was 17.8 cm. It was 12.8 cm (28.1% reduction) in Sl-c*SlCHR1*-ov1 and 13.4 cm (24.7% reduction) in Sl-c*SlCHR1*-ov2 (Figure 5.5B). The reduced height is due to shorter internodes at the same number of nodes (data not shown). The diameter of the wild-type plants was 24.2 cm. It was reduced to 14.8 cm (reduction 38.8%) in Sl-c*SlCHR1*-ov1 and to 18.3 cm (reduction 24.4%) in Sl-c*SlCHR1*-ov2 (Figure 5.5C).

Also the individual leaves of the two transgenic lines show a more compact phenotype compared to the wild-type (Figure 5.5D). The average length of the fourth leaf from the plant base was 12.5 cm in the wild-type, while in the Sl-c*SlCHR1*-ov1 and Sl-c*SlCHR1*-ov2 lines it was 9.6 cm (22.9% reduction) and 10.4 cm (16.4% reduction), respectively (Figure 5.5E). In addition, the top leaflet of the fourth compound leaf is smaller in the transgenic lines than in the wild-type. The average length of the wild-type top leaflet was 6.3 cm, it was 4.9 cm (22.2% reduction) in Sl-c*SlCHR1*-ov1 and 5.5 cm (12.7% reduction) in Sl-c*SlCHR1*-ov2 (Figure 5.5F). All data demonstrate that over-expression of c*SlCHR1* in tomato leads to overall markedly reduced vegetative growth, resulting in more compact seedlings and plants.

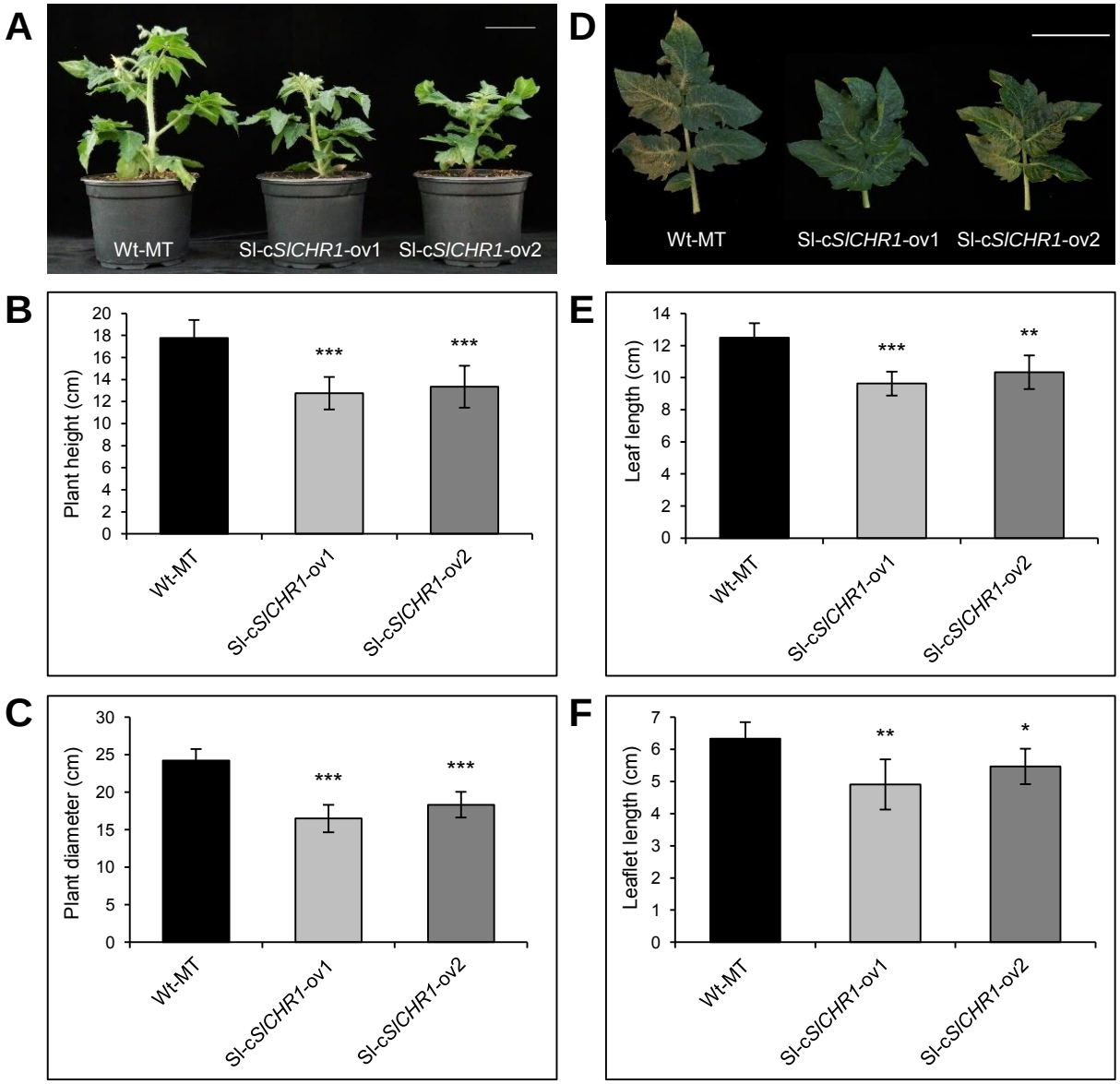

Figure 5.5: Tomato plants over-expressing c*SlCHR1* show reduced vegetative growth. **(A)** Phenotype of wild-type MT and two transgenic lines 6 weeks after sowing; bar = 5cm. **(B)** Plant height and **(C)** plant diameter of 6-week-old plants grown in normal environmental conditions. **(D)** Phenotype of the fourth leaf of wild-type MT and over-expressing lines 6 weeks after sowing; bar = 5 cm. **(E)** Mean length of the fourth leaf and **(F)** mean length of the terminal leaflet on the same leaf. The error bars represent s.d. Asterisks indicate significant differences from wild-type: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. For each line, at least 10 plants and 6 leaves were measured.

A prominent feature of the phenotype associated with c*SlCHR1* over-expression was associated with flowering and reproduction organs. Flowering of the transgenic lines was on average six days delayed compared with non-transgenic wild-type plants (Figure 5.S4). The reproductive structures of the two transgenic lines was severely compacted compared to the wild-type (Figure 5.6A). The average diameter of the wild-type reproductive structure was 16.2 cm. It was reduced to only 3.6 cm (77.8% reduction) in Sl-c*SlCHR1*-ov1 and to 5.2 cm (67.9%

reduction) in Sl-c*SlCHR1*-ov2 (Figure 5.6B). Closer examination of the inflorescence architecture revealed significantly shortened peduncles and pedicels in the transgenic lines.

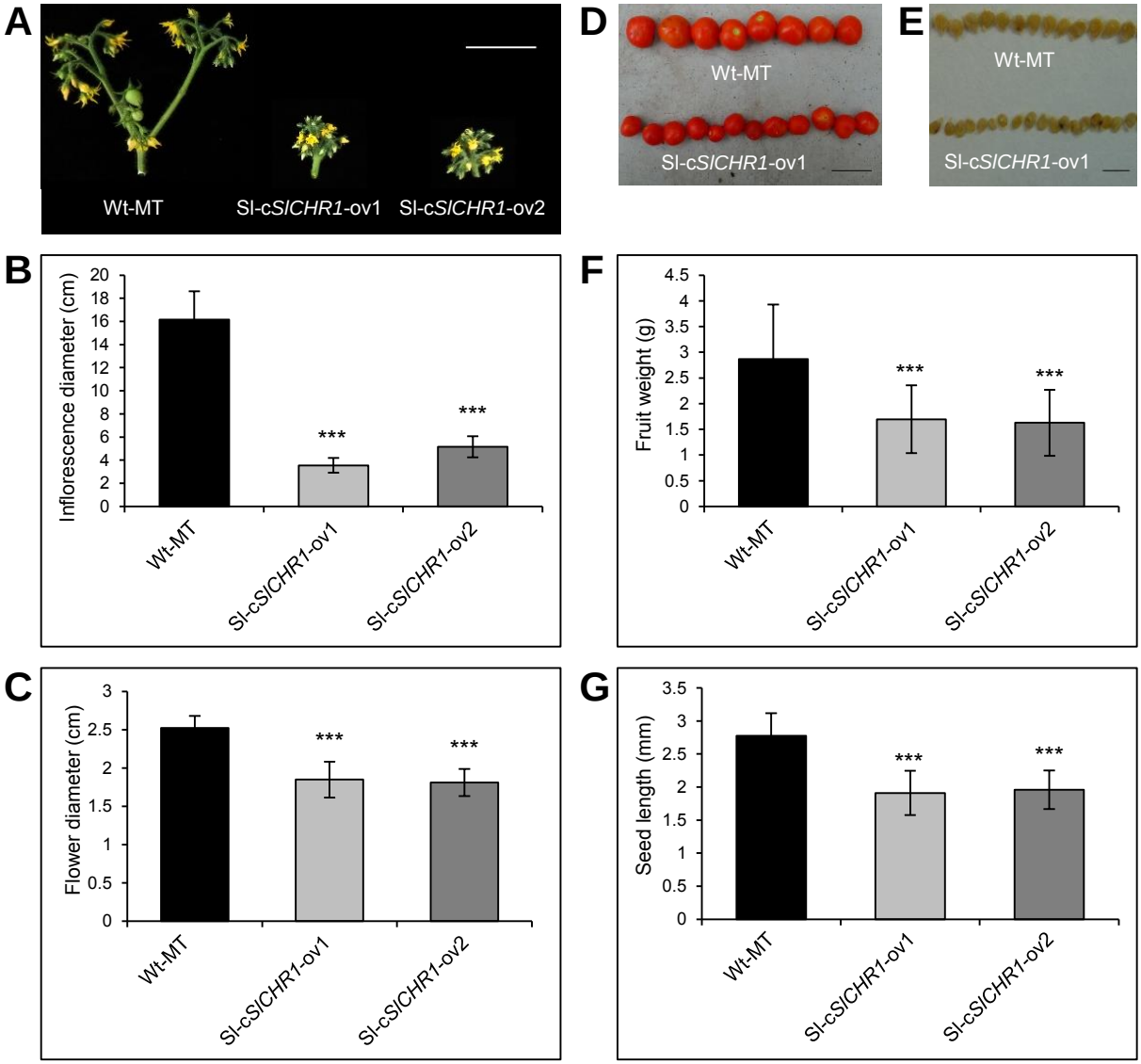

Figure 5.6: Over-expression of c*SlCHR1* in tomato results in compact reproductive structures and smaller flowers and fruits. **(A)** Phenotype of the reproductive structure of wild-type MT and over-expressing mutants; bar = 5 cm. **(B)** The mean diameter of the reproductive structures and of fully open flowers **(C)** in 6-week-old wild-type and two transgenic lines. **(D, E)** Phenotype of the fruit **(D)** and seed **(E)** of wild-type MT and Sl-c*SlCHR1*-ov1 line; bar = 2.5 cm for fruits **(D)** and 3 mm for seeds **(E)**. **(F)** Mean weight of the ripe fruit and the seed length **(G)** in wild-type and mutant. The error bars represent s.d. Asterisks indicate significant differences from wild-type: ***, $P < 0.001$. For each line, at least 10 reproductive structures, 15 flowers and 30 fruits and seeds were measured.

In addition, also the diameter of fully open individual flowers was significantly reduced in transgenic plants (Figure 5.6C). The diameter of wild-type flower was 2.5 cm, the transgenic lines have both a flower diameter of about 1.8 cm (28% reduction).

Although the transgenic lines set fruit that appeared to ripen normally, the fruits were considerably smaller in size (Figure 5.6D) and in weight (Figure 5.6F); the number of fruits per plants appeared smaller than for the corresponding wild-type tomato, but the compact nature of the transgenic plants did not allow proper quantification of the average number of fruits per plant. The number of seeds per fruit was smaller and also the size of the seeds themselves was reduced in length (Figure 5.6E,G).

The effect of c*SlCHR1* over-expression on growth parameters of plants grown under stress conditions

In Arabidopsis, over-expression of the Snf2 chromatin remodeling genes *AtCHR12* and *AtCHR23* particularly affected the growth under adverse environmental conditions (drought, heat, salt) (Folta *et al.*, 2014, Mlynarova *et al.*, 2007). To check if over-expression of c*SlCHR1* has similar effects in tomato, the c*SlCHR1* over-expressing transgenic tomato plants were subjected to drought and salt stress and compared to the wild-type. 2-week-old plants were subjected to drought stress by withholding water supply. After 2 weeks of water shortage, plant height was measured after another 2 weeks of growth without stress and compared with wild-type plants that had undergone the same treatment. The wild-type plants showed a reduction in height from 17.1 cm to 13.3 cm (reduction 22.2%). The height of Sl-c*SlCHR1*-ov1 was reduced from 13.8 cm to 11.8 cm (14.5% reduction) and of Sl-c*SlCHR1*-ov2 from 13.2 cm to 11.2 cm (15.1% reduction) (Figure 5.7). The two transgenic tomato lines seem to be a bit more resistant to water shortage (less growth reduction). While in control conditions the height of transgenic plants was significantly shorter than wild-type, after drought stress the difference was not significant ($P < 0.05$).

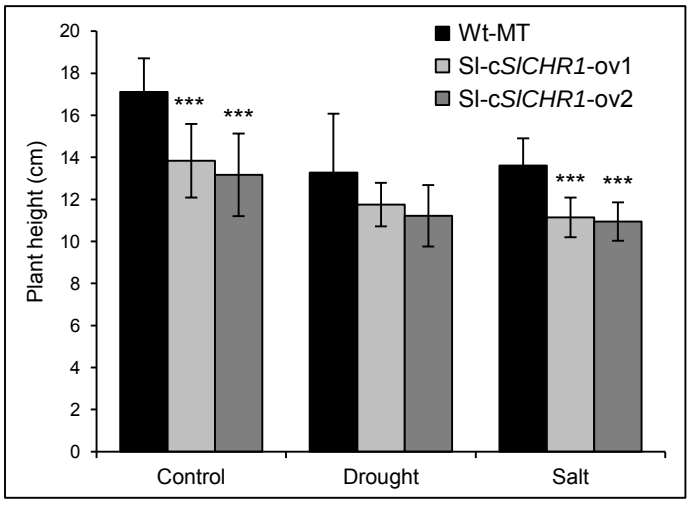

Figure 5.7: The height of wild-type and transgenic tomato plants after stress. Plants were grown for 2 weeks without stress followed by 2 weeks of drought or salt stress. The plant height was measured after another 2 weeks of growth without stress. Control, non-stressed, plants were grown and measured in parallel with stressed plants. The error bars represent s.d. For each condition, asterisks indicate significant differences from wild-type: ***, $P < 0.001$.

Growth in the presence of salt stress was analyzed by growing plants for 2 weeks under standard conditions followed by 2 weeks of watering with 150 mM NaCl. After another 2 weeks of growth without stress, plant height was measured. Salt stress reduced the height of

wild-type plants from 17.1 to 13.6 cm (20.5% reduction). The average height of salt-treated Sl-c*SlCHR1*-ov1 was reduced to 11.1 cm (19.6% reduction), and of Sl-c*SlCHR1*-ov2 it was 10.9 cm (17.4% reduction) (Figure 5.7). These data show that in case of salt stress, both wild-type and transgenic plants show the same reduction in growth relative to control conditions.

DISCUSSION

We here present the cloning of the coding sequence of a tomato (*S. lycopersicum*) chromatin remodeling Snf2-type ATPase gene (*SL100sc05189_42.1.1./Solyc01g079690*, here designated *SlCHR1*) and the first phenotypic characterization of plants upon over-expression in both Arabidopsis and tomato. To our knowledge, this is the first tomato chromatin remodeling gene analyzed this way. Two tomato genome annotations (ITAG1 and ITAG2.4) were contradictory with respect to the structure of this tomato gene. Such discrepancy between the two tomato genome annotations demonstrates the intrinsic difficulties for automated annotation in case of gene families and/or the presence of alternative transcripts (Fawal *et al.*, 2014). Detailed PCR analyses showed the earlier annotation (ITAG1) to be most close to the true genomic structure. This result emphasizes the importance of experimental confirmation and manual curation of automated gene prediction, especially in case of newly sequenced genomes. Based on phylogenetic analyses, *SlCHR1* is thought to combine in tomato the functions of its two Arabidopsis paralogs (Bargsten *et al.*, 2013), that upon over-expression will affect the growth habit of tomato. Constitutive over-expression of the coding sequence of *SlCHR1* indeed resulted in significant reduction of growth and development of tomato plants. Compared to the wild-type, transgenic tomato lines have smaller seedlings, much more compact vegetative growth habit, and severely compacted reproductive structures.

Over-expression of a tomato chromatin remodeling ATPase gene does not affect the growth habit of Arabidopsis

The finding that over-expression of c*SlCHR1* in Arabidopsis did not impact plant growth as expected based on the over-expression of either *AtCHR12* or *AtCHR23* was quite surprising. Transgenic Arabidopsis plants over-expressing *SlCHR1* could not be distinguished phenotypically from the wild-type, neither in standard growth conditions, nor in environmentally adverse conditions (Figure 5.3). It seems sufficiently unlikely that the lack of phenotype is due to too low expression levels. The relative level of over-expression accomplished seems high enough and since AtCHR12-GFP fusion protein gives the same phenotype in Arabidopsis as AtCHR12 (Folta, unpublished data), the small GFP tail is not likely to affect the chromatin remodelling function of the fusion protein.

One of the possible explanations for the lack of growth phenotype in Arabidopsis could be that the structure of the single tomato gene deviates to such an extent that it cannot take the functions of the two Arabidopsis genes. Both *AtCHR12* and *AtCHR23* carry at their C-terminal end an unfolded region that is not present in the tomato *SlCHR1* gene (Bargsten *et al.*, 2013). Subtle differences in domain architecture may change the function of orthologous proteins (Gabaldon *et al.*, 2013). Possibly the lack of the C-terminal unfolded region in SlCHR1 is crucial for the apparent lack of function in Arabidopsis. Such unfolded or disordered regions help or guide protein-protein or protein-DNA interactions (Uversky *et al.*, 2010, Uversky *et al.*, 2000). Disordered regions could potentially adopt different conformations that allow interactions with multiple binding partners (Grau *et al.*, 2011). SWI/SNF2 ATPases function in the context of protein complexes and the recruitment of one of the components of the remodeling complex in Arabidopsis may become affected. More detailed analyses are required to show whether this part of the Snf2 protein family has indeed such an influence on function. Alternatively, the two species may be evolutionary too far apart for proper gene function analysis. Arabidopsis and tomato belong to two different clades of the eudicots, the *Cruciferae* and the *Solanaceae*, respectively. However, Arabidopsis has been used to characterize the function of tomato genes (Fradin *et al.*, 2011, Li *et al.*, 2014, Li *et al.*, 2013).

Vice versa, Arabidopsis genes have been successfully used to modify tomato (Zhang *et al.*, 2004). When introduced into the solanaceous tobacco (*Nicotiana tabacum*), over-expression of the Arabidopsis *AtCHR12* gene did result in more compact plants (data not shown). These results indicate that the evolutionary distance is not necessarily a bottleneck for functional characterization. The lack of phenotype here obtained for Arabidopsis only implies that Arabidopsis cannot be used as model species for this type of growth-related genes, possibly because the detailed regulation of growth in the two species differ subtly. Arabidopsis can be considered a pioneer species used to encouter adverse environments (Chew *et al.*, 2011), whereas tomato has been subject to many years of selection and breeding for uniformity and stability of growth.

Over-expression of *SlCHR1* results in more compacted tomato seedlings and plants

Transgenic seedlings of the tomato cultivar Micro Tom over-expressing c*SlCHR1* driven by the near-constitutive CaMV 35S promoter were more compact than the untransformed controls. They showed up to 25% reduction of growth compared to the wild-type. Also during vegetative growth, the plants have more compact growth habit (Figure 5.4, 5.5). The most severe effect of *SlCHR1* over-expression was observed for the reproductive organs. The average diameter of the reproductive structures was reduced up to one fifth of the wild-type (Figure 5.6). Micro Tom is already one of the smallest tomato cultivars known (Marti *et al.*,

2006). It is remarkable that over-expression of a single gene can reduce plant habit so much further.

In Arabidopsis, the phenotype upon over-expression of *AtCHR12* could only be distinguished from the wild-type in case of mild stress conditions. It resulted in growth arrest of primary buds and reduced growth of the primary stem that recovered in the absence of the environmental stress. In mature plants, notably the growth after the transition to the reproductive development was affected (Mlynarova *et al.*, 2007). Over-expression of *AtCHR23* in Arabidopsis resulted in reduced growth of seedlings and more compacted vegetative rosette (Folta *et al.*, 2014). Tomato *SlCHR1* is considered to be the single ortholog of *AtCHR12* and *AtCHR23* and supposed to combine their functions (Bargsten *et al.*, 2013). This is indeed reflected in the phenotype obtained. Upon over-expression, tomato shows a compact vegetative growth habit (*AtCHR23* over-expression-like) and considerably smaller reproductive organs (*AtCHR12* over-expression-like). However, the compact growth habit is seen without the need for applying additional stress conditions. This suggests that in this respect the AtCHR23 function of the SLCHR1 protein overrides the AtCHR12 function. The concept of priming the plants for growth arrest upon actual environmental stress associated with *AtCHR12* over-expression (Mlynarova *et al.*, 2007) is either less important in Arabidopsis than in tomato, or is taken over by other protein or mechanisms.

The c*SlCHR1* over-expressing tomato lines do however differ markedly from the *AtCHR12/AtCHR23* over-expressing Arabidopsis lines with respect to their reaction to environmental stress. In Arabidopsis, environmental stress results in stronger growth reduction (Folta *et al.*, 2014, Mlynarova *et al.*, 2007), irrespective of the type of environmental stress applied (drought, heat, salt). In tomato, the growth reduction was not significantly different between over-expressing lines and the wild-type when subjected to salt stress. When subjected to drought stress, tomato over-expressing c*SlCHR1* showed even less growth reduction, hence more stress tolerance, than wild-type plants (Figure 5.7). We speculate that these differences may be related to the intrinsic differences between Arabidopsis and tomato, their natural habit and habitat as well as human selection in tomato breeding. The better performance of the transgenic tomato plants under drought stress may be a side effect of the reduced plant size and slower growth rate that result in decreased water evaporation (Blum, 2005). The *SlCHR1* over-expression phenotype may be related to hormone signaling. In Arabidopsis, chromatin remodeling plays a role in growth regulation and hormone signaling (Archacki *et al.*, 2013, Sarnowska *et al.*, 2013). However, over-expression of *AtCHR12/AtCHR23* in Arabidopsis was not associated with notable differences in expression of any of the known phytohormone-related genes (Folta *et al.*, 2014, Mlynarova *et al.*, 2007). More data are required to speculate about a relationship between chromatin remodeling, growth regulation and hormone signaling in tomato.

Potential applications of modulated expression of chromatin remodeling genes in crops

The markedly reduced growth habit of tomato as result of the over-expression of c*SlCHR1* could be exploited in several ways. The compact growth habit is advantageous for the production of field-growth tomatoes. It could reduce production costs because of diminished labor costs for stalking, tying and pruning. The latter account to up to 55% of the field-grown tomato production cost (Davis *et al.*, 1993, Kemble *et al.*, 1994). Alternatively, it could help develop cultivars with smaller, more cherry-like tomatoes from larger-fruit cultivars. Possibly the smaller tomatoes have a shape or taste that is more appreciated by consumer panels (Jones Jr, 2008, Rocha *et al.*, 2013).

In view of the current controversy about transgenic approaches, notably in Europe, these potential applications should and can be translated into non-GMO strategies based on breeding and selection (e.g. marker assisted selection, MAS). Promoter activity will be critical for the targeted modification of tomato growth through chromatin remodeling. Methods such as TILLING, EcoTILLING, or CRISPR/CAS (Barkley *et al.*, 2008, Belhaj *et al.*, 2013) can be used to induce or identify mutations in the *SlCHR1* promoter sequence to generate plant lines which produce higher levels of the chromatin remodeling protein SlCHR1. As it is only one particular promoter that must be targeted, such approaches will become more straightforward in the future.

When it becomes feasible to modulate the specificity of an endogenous promoter, new options for application arise. The use of an endogenous promoter redesigned to be specifically active in the seedling stage would allow targeted adjustments of tomato growth habit. Possibly a tunable transcriptional factor could provide the desired regulation for inducible, spatial or temporal expression (Liu *et al.*, 2013). This may give better control of the growth of tomato seedlings used as transplants. Commercially-grown tomatoes are generally produced from transplanted seedlings previously grown in greenhouses. Short, uniform and sturdy seedlings are required to enable the use of mechanical transplanting machinery. Seedlings can become tall and leggy prior to field establishment and good control of notably the height of tomato transplants is important. Nowadays, transplant growth rate is regulated in nurseries through nutrient and water management, as well as temperature control, clipping shoots and mechanical treatment (brushing) (Garner *et al.*, 1996), but nurseries have not always the desired flexibility. In industry, the use of plant growth retardants (PGRs) is explored (Choudhury *et al.*, 2013, Nickell *et al.*, 1982). PGRs are synthetic chemicals, which temporarily inhibit the elongation of stem and shoots, without irreversible blocking of vital metabolic and developmental processes. The use of PGRs, when used appropriately at the correct stage of development and in the required concentration, enables to get shorter, sturdier and possibly healthier transplants (Biles *et al.*, 2001). To date, only Sumagic (Valent Professional Products, USA) is registered for use of height control of tomato transplants in greenhouse production (Runkle *et al.*, 2012). The active compound of

this very potent growth retardant, Uniconazole, suppresses stem elongation by the inhibition of gibberellin acid biosynthesis (Zandstra *et al.*, 2007). However, the use of such a PGR in plant production is not without controversy or risk. Misapplication can result in phytotoxicity, delayed flowering and stunned growth (Whipker *et al.*, 2001). In addition, there is the possibility of undesired persistence in plant material or in the environment (Wu *et al.*, 2013). Therefore, alternative methods for temporary growth retardation of seedlings are still desired (Gargul *et al.*, 2015). Growth retardation of tomato seedlings based on over-expression of the *SlCHR1* gene in specifically the seedling stage could develop into a promising and environmentally friendlier alternative for the use of chemical plant growth retardants. In all applications, possibly compromised fruit yield will have to be assessed for economic feasibility and sustainability.

All crop species carry genes orthologous to *SlCHR1* (Bargsten *et al.*, 2013). Therefore, the use of chromatin remodeling genes to reduce plant height is likely to be applicable to and attractive for any crop for which height reduction could have added value. This applies to edible crops such as vegetables and herbs. In grasses such as wheat or barley, shorter-stemmed plants will be more resistant to wind and rain, therefore reducing the lodging losses before the harvest (Jones *et al.*, 2013). Growth control would also be beneficial for horticultural uses, such as the reduction of vegetative growth in turf, fruit trees, grapes and other woody plant species. Improvements in ornamental floral crops and bedding plants are also feasible (Chandler *et al.*, 2012). Short, compact ornamental plants look more balanced and are less likely to be damaged during shipping. Our data do indicate however that the phenotype conferred by this type of genes after inter-species transfer cannot be predicted easily and should be cautiously managed and/or interpreted. This way, modulation of the expression of chromatin remodeling genes could develop into a widely applicable approach to control the growth of plants for agronomic and commercial purposes.

MATERIALS AND METHODS

RNA isolation and RT-PCR analysis

Total RNA and RT-PCR analysis were performed as previously described (Bargsten *et al.*, 2013, Folta *et al.*, 2014). Quantitative RT-PCR was performed at least in triplicate with 2.5 µl of 10-times diluted cDNA using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). Genes *L33* (*Solyc01g007450.1.1*) (Schijlen *et al.*, 2007) and *UBC* (*At5g25760*) (Czechowski *et al.*, 2005) were used for normalization of tomato and Arabidopsis samples, respectively. Primers were designed with Primer3Plus (Untergasser *et al.*, 2007) and are listed in Table 5.S1.

Cloning of the coding sequence of the tomato *SlCHR1* gene

To obtain the cDNA sequence of the *SlCHR1* gene, RNA from leaves of tomato cultivar Heinz 1706 was isolated as described above. The cDNA was prepared from one microgram of total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen) with oligo$(dT)_{20}$ primers and 1µl of the first strand cDNA was used as a template for PCR with Phusion® High-Fidelity DNA Polymerase (Finnzymes, Finland) with the primer pair SlCHR1-F1 and SlCHR1-R1 (Table 5.S1). The conditions used for PCR reaction were 98 °C for 4 min; 35 cycles: 98 °C for 30 s, 61 °C for 30 s and 72 °C for 150 s; 72 °C for 7 min. The PCR product was cloned into pENTR™/D-TOPO® vector (Invitrogen Corporation, USA) and its integrity was verified by DNA sequencing. Next, the *SlCHR1* coding sequence was cloned by an LR Gateway (Life Technologies) recombination reaction into the destination vector pK7FWG2.0, obtained from VIB Gent, Belgium (Karimi *et al.*, 2002). This generated a fusion gene with a C-terminal GFP moiety driven by the (near-) constitutive CaMV 35S promoter (35S:c*SlCHR1*-GFP, Figure 5.2A). The final plasmid was transferred into *Agrobacterium tumefaciens* strain C58C1 with the freeze-thaw method (Weigel *et al.*, 2006).

Plant growth conditions

Arabidopsis thaliana seedlings and plants were grown in control (without stress) and stress conditions as previously described (Folta *et al.*, 2014, Mlynarova *et al.*, 2007). To analyze the growth of tomato (*Solanum lycopersicum*) seedlings, seeds of the cultivar Micro Tom were surface sterilized and grown on 0.5 x MS agar plates. For salt stress treatment, the agar plates were supplemented with 75 mM NaCl. Seedlings were grown vertically in fully controlled growing chambers lit by Philips TD 32W/84HF lamps at 25 °C in long day conditions (16 h light/8 h dark). The tomato cultivar Heinz used for RNA isolation was grown in the same conditions in pots containing 0.5 x MS agar. Tomato plants were grown in standard potting soil in a controlled greenhouse at 21 °C with supplemental light provided by four Son-T (Philips Greenpower, 400 W) lamps when required, in long day conditions (16 h light/8 h dark). To apply salt stress, 2-week-old greenhouse-grown plants were watered for two weeks with 150 mM NaCl. To apply drought stress, water supply of 2-week-old greenhouse-grown plants was stopped for 2 weeks. Wild-type and transgenic plants were grown and treated in parallel. In all cases, also untreated plants were grown in parallel.

Generation of transgenic plants

Transgenic Arabidopsis plants (ecotype Col-0) were obtained by the floral dip method (Clough *et al.*, 1998) using C58C1 agrobacteria bearing the 35S:c*SlCHR1*-GFP binary plasmid. To obtain transgenic tomato lines, cultivar Micro Tom was transformed with the

same binary plasmid using a method described previously (Qiu *et al.*, 2007) with minor modifications. Cotyledons of 10-day-old seedlings were used, when the first true leaves were only 2-3 mm long. During regeneration, 50 mg/l of vancomycin was used instead of carbenicillin, and in all media 0.5 g/l of MES (2-N-morpholinoethanesulfonic acid) was used to buffer the pH. Transgenic lines were selected based on kanamycin resistance and segregation. Homozygous F3 transgenic plants of both Arabidopsis and tomato were used.

Analysis of growth

Arabidopsis seedlings, vegetative and reproductive growth parameters were analyzed as described previously (Folta *et al.*, 2014, Mlynarova *et al.*, 2007). Tomato growth parameters, such as the length of the main root, the length of the hypocotyl and the area of the cotyledon were analyzed in a similar way. 7-day-old seedlings grown vertically as described above were photographed and the root and hypocotyl length were measured using ImageJ (http://imagej.nih.gov/ij/). The cotyledon area was determined from a photograph of flattened cotyledons in ImageJ. The growth of the tomato plants was determined on 6-week-old plants. Plant height was measured by a ruler from the stem base till the top of the plant. The length of the fourth leaf and the terminal leaflet on fourth leaf was determined from a photograph of flattened leaves using ImageJ. The leaf length was measured from the axil till the tip of terminal leaflet, the terminal leaflet length from the rachis till the tip of the leaflet. To analyze the growth during reproductive development, 6-week-old plants were photographed from the top and the reproductive structure diameter was measured after enclosing in a square section using ImageJ. The individual flowers were also flattened and photographed. Using ImageJ software, the flowers were enclosed in a square section and the diameter was determined. The fully ripe fruits were weighted on a laboratory weight and the seed length was determined from a photograph using ImageJ software. The significance of differences was determined with the Student's *t*-test assuming unequal variances in Excel.

GFP imaging and photography

The location of the cSlCHR1-GFP fusion protein in 6-day-old Arabidopsis and tomato seedlings grown vertically was determined with a Leica TCS SP2 confocal microscope (Leica Microsystems B.V., Rijswijk, The Netherlands) with a 16x objective. To visualize the cell walls, the tissue was incubated for 1 minute in a solution of 1 μg/ml of propidium iodide and washed in water before inspection. The photographs were obtained by Olympus SZ-30MR camera against a black background.

ACKNOWLEDGEMENTS

We acknowledge the Centre for BioSystems Genomics 2012 (CBSG2012) which was part of the Netherlands Genomics Initiative (NGI) for financial support. AF was supported by the Netherlands Organization for Scientific Research (NWO), through its ALW/TTi Green Genetics program. JPN was supported by the RaakPRO BioCOMP project coordinated by Hanze University of Applied Sciences Groningen.

SUPPLEMENTARY DATA

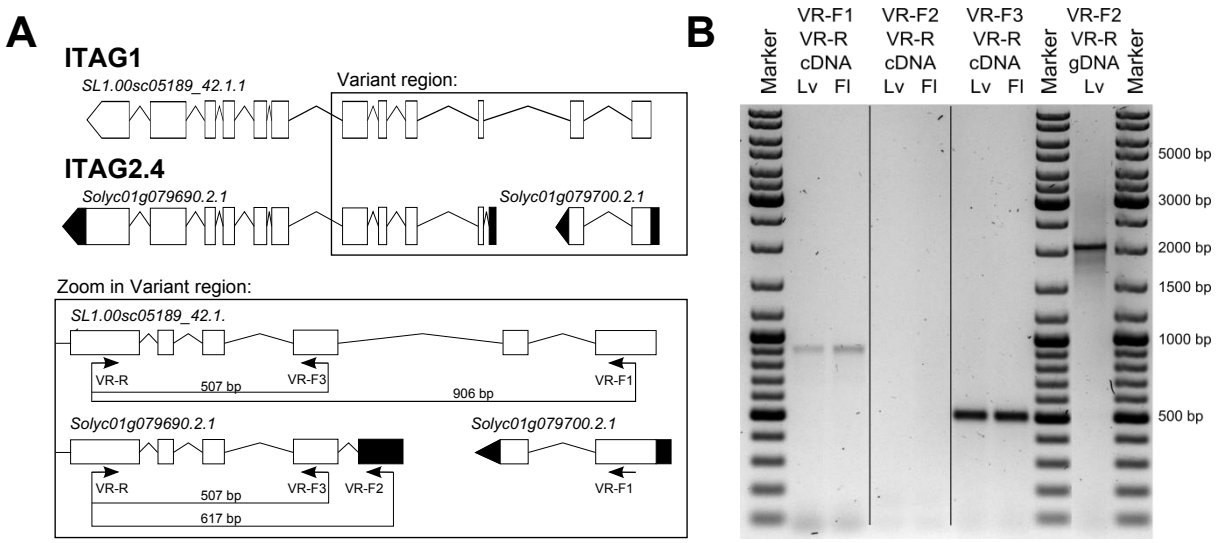

Figure 5.S1: RT-PCR analysis of the structure of *SlCHR1* in two tomato annotations. **(A)** Schematic layout of the *SlCHR1* gene in two tomato annotations with enlarged variant region. Arrows give the position of primers used in RT-PCR with the length (bp) of the expected RT-PCR product. White boxes represent exons, the black lines introns. In black boxes are indicated exon sequences present only in ITAG2.4 annotation. **(B)** Photograph of gel electrophoresis of RT-PCR analysis using primers VR-F1 and VR-R (specific to ITAG1), VR-F2 and VR-R (specific to ITAG2.4) and VR-F3 and VR-R primer pair common for both annotations. RNA isolated from leaves (Lv) and flowers (Fl) of tomato cv. Heinz 1706 was used for cDNA preparation. Genomic DNA from leaves (gDNA) was used as positive control for VR-F2 and VR-R primer pair with expected length of PCR product 2260 bp. Molecular weight marker used is GeneRuler™ DNA Ladder Mix (Thermo Scientific).

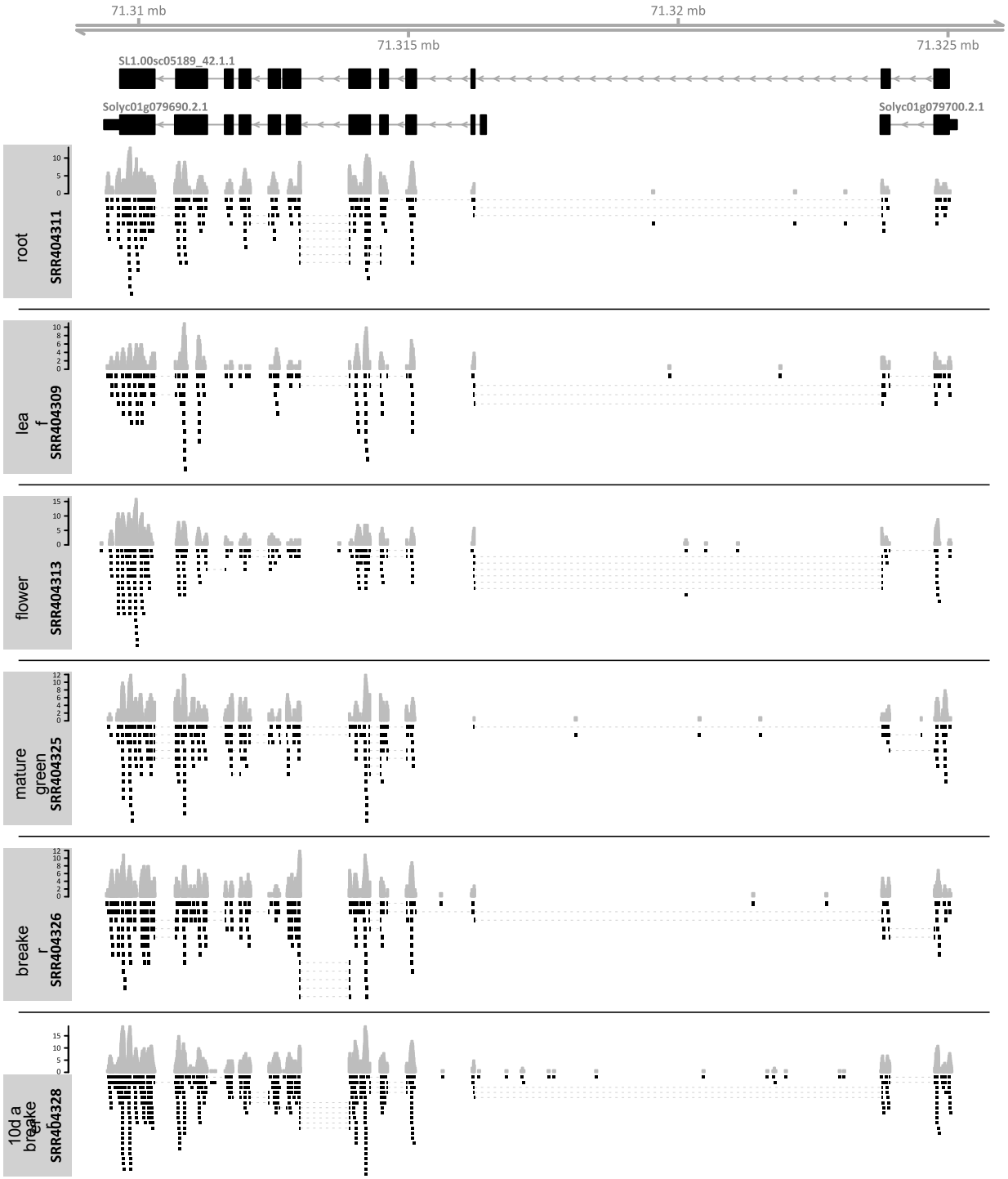

Figure 5.S2: Tomato RNA-seq libraries from different developmental stages (described on left) were used to map the RNA-seq reads to the genomic region of *SlCHR1* gene. On top, the black bars in the schematic layout of *SlCHR1* gene in ITAG1 and ITAG2.4 represent exons and arrowed lines introns. The reads over an intron region are connected by dashes. For each library, the RNA-seq coverage is represented as grey peaks above the RNA-seq reads.

```
   1 ATGGTGGCTCAGATAGAGACCAACGCCGCCGCTGACGGTGGTGGAGGAGGAGGAGGAGGAGGAATGGTTGGAGGTTCTTCCCCTCCCGTTGATGAATCGC 100
     M  V  A  Q  I  E  T  N  A  A  A  D  G  G  G  G  G  G  G  G  M  V  G  G  S  S  P  P  V  D  E  S  Q
 101 AGGAACAGTTAGAGAAGACGAAGACATTGATTTGTGCTCTCAATTTCCTCTCACGTAATCTTCCGATACCTCCTGACGTATTCGATGCTGTCTCTTCAAT 200
       E  Q  L  E  K  T  K  T  L  I  C  A  L  N  F  L  S  R  N  L  P  I  P  P  D  V  F  D  A  V  S  S  I
 201 TTACAATAGCGACGCCAACGATGTTGATGTCGGTGACGGAGATGCATCCCCTGCTGACGTGGACAGTTTGTCTGTGCAAAATGGTCCAGGAATGGGAAGT 300
       Y  N  S  D  A  N  D  V  D  V  G  D  G  D  A  S  P  A  D  V  D  S  L  S  V  Q  N  G  P  G  M  G  S
 301 TATGGAGACCTGATGGCAGATTTAGAGGAGTCGTTGTTGAGCCAACGGTCAAGTTATACATCAGGTTCTGGTTTGACAAAATTAAAGGAAGATCGTTTCC 400
     Y  G  D  L  M  A  D  L  E  E  S  L  L  S  Q  R  S  S  Y  T  S  G  S  G  L  T  K  L  K  E  D  R  F  R
 401 GAAGCCATATCCAGCATCGCCTAACTGAACTTGAAGACTTGCCAACCAGCAGAGGTGAGGACTTGCAATCGAAGTGCTTGCTTGAACTTTATGAATTAAA 500
       S  H  I  Q  H  R  L  T  E  L  E  D  L  P  T  S  R  G  E  D  L  Q  S  K  C  L  L  E  L  Y  E  L  K
 501 GTTAGCAGATTTACAACAAAAAGTTCGGTCTGAAGTGAGTTCTGAATATTGGCTTCGCCTGCATTGCGCTAATCCTGACAAGCAATTGTTTGACTGGGGG 600
       L  A  D  L  Q  Q  K  V  R  S  E  V  S  S  E  Y  W  L  R  L  H  C  A  N  P  D  K  Q  L  F  D  W  G
 601 ATGACACGATTGCGACGCCCTGTATATGGTATAGGAGATGCCTTTGCTGTTGAATCTGATGATCCATTGAGGAAGAAGCGTGATGCGCAGAGGTTGTCAA 700
     M  T  R  L  R  R  P  V  Y  G  I  G  D  A  F  A  V  E  S  D  D  P  L  R  K  K  R  D  A  Q  R  L  S  R
 701 GAATAGAGGAAGAGGAAAGAAACCGCGTGGAAACTACAAAAAGGAAGTTCTTTGCGGATGTACTCAATGCAGCACGTGAACTCCAATTACAAGTACAGGC 800
       I  E  E  E  E  R  N  R  V  E  T  T  K  R  K  F  F  A  D  V  L  N  A  A  R  E  L  Q  L  Q  V  Q  A
 801 TGTTCAGAAGCGGCGGAAACAGAGGAATGATGGTGTTCAGGCTTGGCATGGAAGGCAAAGGCAACGGGCTACTCGTGCTGAGAAACTGAGGTTACAAGCA 900
       V  Q  K  R  R  K  Q  R  N  D  G  V  Q  A  W  H  G  R  Q  R  Q  R  A  T  R  A  E  K  L  R  L  Q  A
 901 CTGAAAGCTGATGATCAAGAAGCTTACATGAAGATGGTGGAGGAAAGCAAGAACGAAAGGTTGACAATGCTTCTAGGGAAAACAAATGACCTCCTGGGTC 1000
     L  K  A  D  D  Q  E  A  Y  M  K  M  V  E  E  S  K  N  E  R  L  T  M  L  L  G  K  T  N  D  L  L  G  R
1001 GTTTGGGAGCTGCTGTTCAACGGCAGAAAGATGCTGATCATGATGGCCTTGAATCTTTGGAAGGTTCAGATGCTGAAATGGCTGCTACTAAGACTGATAC 1100
       L  G  A  A  V  Q  R  Q  K  D  A  D  H  D  G  L  E  S  L  E  G  S  D  A  E  M  A  A  T  K  T  D  T
1101 TCCTGGGCAGTCGCTTCCTGAGGAAGAAGAGGATGTAATTGATGATGAGTCCACTCATGATGTGAAGACTAATGACTTACTTGAAGGTCAAAGGAAGTAC 1200
       P  G  Q  S  L  P  E  E  E  E  D  V  I  D  D  E  S  T  H  D  V  K  T  N  D  L  L  E  G  Q  R  K  Y
1201 AATTCAGCTGTACATTCGATCCAGGAGAAGGTTACGGAGCAACCAGCTATGCTTCAGGGTGGAGAACTGAGATCTTACCAGATAGAAGGGCTTCAATGGA 1300
     N  S  A  V  H  S  I  Q  E  K  V  T  E  Q  P  A  M  L  Q  G  G  E  L  R  S  Y  Q  I  E  G  L  Q  W  M
1301 TGTTGTCTTTGTTTAATAACAACTTAAATGGAATTTTAGCGGATGAAATGGGACTCGGGAAAACGATCCAGACAATTGCTTTAATTGCTTACCTCTTGGA 1400
       L  S  L  F  N  N  N  L  N  G  I  L  A  D  E  M  G  L  G  K  T  I  Q  T  I  A  L  I  A  Y  L  L  E
1401 AAACAAGGGTGTACGTGGTCCCCACTTGATTGTGGCTCCAAAAGCTGTACTACCTAATTGGATTACGGAATTCTCAACATGGGCTCCTAGCATTGTTGCT 1500
       N  K  G  V  R  G  P  H  L  I  V  A  P  K  A  V  L  P  N  W  I  T  E  F  S  T  W  A  P  S  I  V  A
1501 ATTCTTTACGATGGTCGTTTGGAAGAAAGGAAGGCACTGAGGGAGGAGTTGACAGGAGAGGGGAGGTTCAGTGTGTTGATCACACATTATGATCTTATTA 1600
     I  L  Y  D  G  R  L  E  E  R  K  A  L  R  E  E  L  T  G  E  G  R  F  S  V  L  I  T  H  Y  D  L  I  M
1601 TGAGAGACAAAGCATTTCTGAAGAAAATTCATTGGCACTATCTGATAATTGATGAAGGCCACAGATTGAAAAATCACGAATGTGCTCTTGCACGGACACT 1700
       R  D  K  A  F  L  K  K  I  H  W  H  Y  L  I  I  D  E  G  H  R  L  K  N  H  E  C  A  L  A  R  T  L
1701 TGTGTCAGGTTATAGGATTCGACGAAGACTCCTATTGACAGGTACCCCAATCCAAAACAGCTTACAGGAGTTGTGGTCTCTTCTCAATTTTCTTCTTCCA 1800
       V  S  G  Y  R  I  R  R  R  L  L  L  T  G  T  P  I  Q  N  S  L  Q  E  L  W  S  L  L  N  F  L  L  P
1801 AACATCTTTAATTCAGTGGAGAATTTTGAGGAGTGGTTTAATGCCCCCTTCGCCGATAAGTGTGATGTCTCTCTAACTGATGAAGAGGAACTATTGATTA 1900
     N  I  F  N  S  V  E  N  F  E  E  W  F  N  A  P  F  A  D  K  C  D  V  S  L  T  D  E  E  E  L  L  I  I
1901 TTCGCCGGTTGCACCATGTGATAAGGCCATTTATATTGAGGAGGAAGAAGGATGAAGTGGAGAAATTTCTTCCTGGGAAAACACAAGTTGTTCTTAAATG 2000
       R  R  L  H  H  V  I  R  P  F  I  L  R  R  K  K  D  E  V  E  K  F  L  P  G  K  T  Q  V  V  L  K  C
2001 TGATATGTCTGCATGGCAAAAAGTATACTACCAGCAGGTCACAGATGTTGGGAGGGTTGGATTGGATTCTGGAACTGGGAGGTCTAAGAGTCTGCAGAAT 2100
       D  M  S  A  W  Q  K  V  Y  Y  Q  Q  V  T  D  V  G  R  V  G  L  D  S  G  T  G  R  S  K  S  L  Q  N
2101 CTCTCCATGCAACTTAGGAAATGTTGTAATCACCCTTACCTATTTGTGGCCGAGTATAATATTTATCGAAAGGAGGAGATAGTGAGAGCATCTGGAAAAT 2200
     L  S  M  Q  L  R  K  C  C  N  H  P  Y  L  F  V  A  E  Y  N  I  Y  R  K  E  E  I  V  R  A  S  G  K  F
2201 TTGAGCTTCTTGATCGTTTACTACCCAAACTTCGCAGAGCTGGGCACAGGGTCCTTCTCTTCTCACAAATGACCCGCCTCATGGACATTCTTGAAGTCTA 2300
       E  L  L  D  R  L  L  P  K  L  R  R  A  G  H  R  V  L  L  F  S  Q  M  T  R  L  M  D  I  L  E  V  Y
2301 TCTGCAGGTTCATGACTTCAAGTATCTTAGACTTGATGGCTCAACCAAAACAGAGGAGCGAGGGACTTTGCTAAAGCAATTTAATGCTCCTGACTCTCCT 2400
       L  Q  V  H  D  F  K  Y  L  R  L  D  G  S  T  K  T  E  E  R  G  T  L  L  K  Q  F  N  A  P  D  S  P
2401 TACTTTATGTTTCTTTTGAGTACTCGTGCTGGAGGACTCGGTCTAAATCTGCAAACAGCAGATACTGTGATAATTTTTGATAGCGACTGGAACCCTCAAA 2500
     Y  F  M  F  L  L  S  T  R  A  G  G  L  G  L  N  L  Q  T  A  D  T  V  I  I  F  D  S  D  W  N  P  Q  M
2501 TGGACCAGCAGGCAGAAGATAGGGCACATCGAATTGGGCAAAAAAaAGAAGTTAGGGTTTTTGTGTTGGTCAGTGTTGGATCAATTGAAGAGGTCATTTT 2600
       D  Q  Q  A  E  D  R  A  H  R  I  G  Q  K  K  E  V  R  V  F  V  L  V  S  V  G  S  I  E  E  V  I  L
2601 GGAGCGTGCAAAACAGAAGATGGGTATTGATGCTAAGGTCATCCAGGCTGGCTTGTTCAATACAACATCTACAGCTCAAGAAAGAAGGGATATGTTGGAA 2700
       E  R  A  K  Q  K  M  G  I  D  A  K  V  I  Q  A  G  L  F  N  T  T  S  T  A  Q  E  R  R  D  M  L  E
2701 GAGATCATGCGTAAAGGTACAAGCACTCTTGGAACAGATGTGCCAAGCGAAAGAGAGATTAATCGTCTTGCTGCCCGCTCCGATGAGGAATTTTGGCTGT 2800
     E  I  M  R  K  G  T  S  T  L  G  T  D  V  P  S  E  R  E  I  N  R  L  A  A  R  S  D  E  E  F  W  L  F
2801 TTGAGAAAATGGACGAGGAGAGAAGACAAAAAGAGCGTTACAGATCTCGTCTCATGGAAGATCATGAGGTACCAGATTGGGCTTATGCTACACCTGACTC 2900
       E  K  M  D  E  E  R  R  Q  K  E  R  Y  R  S  R  L  M  E  D  H  E  V  P  D  W  A  Y  A  T  P  D  S
2901 TAAGGAAAAGGGAAAAGGATTCTTATACGAAAGTGCTAATATCACTGGAAAGCGACGTAGAAAGGAAGTGGTTTATGCCGATACTTTAAGTGATGTACAG 3000
       K  E  K  G  K  G  F  L  Y  E  S  A  N  I  T  G  K  R  R  R  K  E  V  V  Y  A  D  T  L  S  D  V  Q
3001 TGGATGAAGGCTGTGGAGAACGGAGATGATTTCTTTACGCAATCAAGTAAAGGCAGGAACAGGGACCATCAGTCAGTCTCAAATGGTGAGTTGCCTAGTG 3100
     W  M  K  A  V  E  N  G  D  D  F  F  T  Q  S  S  K  G  R  N  R  D  H  Q  S  V  S  N  G  E  L  P  S  G
3101 GCAATGCTGATTCTGAGAGGACTGGACATGACTTGAAGCCCGATACTGTGTCTGTGGCGAGTGAGGCAACAAGTGAAGATACCTATGGAAGAACTCCCAA 3200
       N  A  D  S  E  R  T  G  H  D  L  K  P  D  T  V  S  V  A  S  E  A  T  S  E  D  T  Y  G  R  T  P  K
3201 AAGGTTCAAATCCGAGAGTGCAAGTTCCATGAGAAATGATTATCACGATTTGACTGGCCATAGTGTGGACGGTTTATCGTGGAAGGCCCACAGGAGGAGG 3300
       R  F  K  S  E  S  A  S  S  M  R  N  D  Y  H  D  L  T  G  H  S  V  D  G  L  S  W  K  A  H  R  R  R
3301 AGATCAAGCTTAATATCCTGA 3321
     R  S  S  L  I  S  *
```

Figure 5.S3: The coding sequence of *SlCHR1* gene (upper) and the derived protein sequence of SlCHR1 in one-letter amino acid abbreviations (lower). At the end of the lines is indicated length of cDNA sequence from the first base.

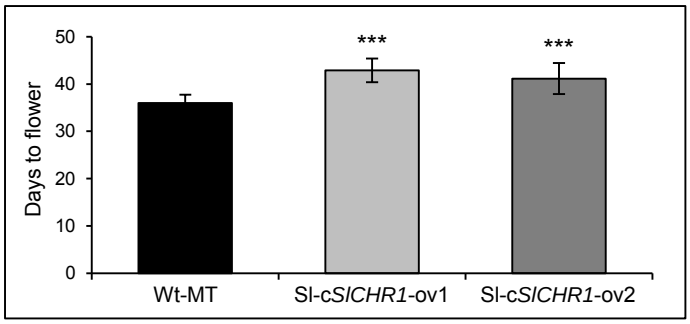

Figure 5.S4: Flowering time of wild-type and the two transgenic lines as the number of days from sowing till the first flower opens. ***, $P < 0.001$

Table 5.S1: List of primers used in this study.

Primer name	Sequence 5' > 3'	Used for
SlCHR1-F1	CACCATGGTGGCTCAGATAGAGACCAAC	c*SlCHR1* isolation
SlCHR1-R1	GGATATTAAGCTTGATCTCCTC	
SlCHR1-F2	ACTCAATGCAGCACGTGAAC	Expression analysis (qRT-PCR)
SlCHR1-R2	AGCCTGAACACCATCATTCC	
UBC-F	TTAGAGATGCAGGCATCAAGAGCGC	
UBC-R	CATATTTCTCCTGTCTTGAAATGAA	
L33-F	CGCACTATCGTTGCATTTGG	
L33-R	CAACGCCACTGTTTCCATGT	
VR-F1	GAGGAGGAGGAGGAGGAATG	Primers specific to Variant region (Figure 5.S1A)
VR-F2	GTTGTTGCTGTGTTGGTTAATTTT	
VR-F3	CCAGCAGAGGTGAGGACTTG	
VR-R	TCTTGCTTTCCTCCACCATC	

CHAPTER 6

GENERAL DISCUSSION

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The focus of this thesis is on ATP-dependent chromatin remodeling. The catalytic subunit of the ATP-dependent chromatin remodeling complex is an SNF2 ATPase. In Arabidopsis 41 genes encoding for SNF2 ATPases have been identified (Bargsten *et al.*, 2013). I studied the role of two Snf2-subfamily ATPases, AtCHR12 and AtCHR23, in the regulation of plant growth responses and in major developmental switches. These studies show that these two ATPases play a role in the regulation of growth at different developmental stages and in response to various types of environmental stresses (Mlynarova *et al.*, 2007, Chapter 2). In addition, the two ATPases play a role during embryo development and this affects germination (Chapter 3) as well as transition to flowering (Chapter 4). Genes with similar functions in crop species are potentially attractive candidates for future breeding. Therefore, I also studied the role of the tomato ortholog in growth regulation (Chapter 5).

AtCHR12 and AtCHR23 diverged but maintained functional redundancy

AtCHR12 and *AtCHR23* are the result of a recent duplication event within the *Brassicaceae* and has been identified in Arabidopsis, *Capsella rubella* and *Thellungiella halophila*. Other plant species have, in general, only one ortholog or in case more orthogs are present this is the result of an independent duplication (Bargsten *et al.*, 2013). In general, after gene duplication genes can go through sub-functionalization or one of the genes neo-functionalizes. SNF2 ATPases have multiple functions and AtCHR12 and AtCHR23 still share several functions. However, they partially diverged. Whether this is the result of neo- or sub-functionalization remains to be determined.

The diverged functions of AtCHR12 and AtCHR23 are apparent in the effect of *AtCHR12* or *AtCHR23* over-expression on growth. In Chapter 2 of this thesis it is shown that over-expression of *AtCHR23* leads to reduced vegetative growth. Upon application of mild abiotic stress (drought, salt), the *AtCHR23* over-expressing lines responded with stronger reduction of growth than wild-type plants. On the other hand, *AtCHR12* over-expressing mutants are indistinguishable from wild-type at standard conditions (Mlynarova *et al.*, 2007). Upon stress, the plants manifest growth arrest of the main stem of the inflorescence and normally active primary buds. Interestingly, the two ATPases affect growth at different developmental stages. *AtCHR12* over-expression results in reduction of growth during reproductive development, while *AtCHR23* over-expression affects only vegetative growth.

Although the effect of *AtCHR12* or *AtCHR23* over-expression on growth is different, the over-expressing mutants have also some similar phenotypes. In this thesis it is shown that over-expression of either of the genes affects embryo maturation with effects on important developmental transitions in the plant life cycle, germination and flowering time control. Over-expression of *AtCHR12* or *AtCHR23* reduced the frequency of seed germination compared to wild-type, and the reduction of germination was more pronounced under stress

(Chapter 3). In Chapter 4 of this thesis it is shown that over-expression of *AtCHR12* led to reduced *FLC* expression and early flowering. Similar results were observed for *AtCHR23* over-expressing lines (own observations).

In addition, redundant functions were shown by studies with knockout mutants (Sang *et al.*, 2012). Knockout mutation in *AtCHR12* or *AtCHR23* does not lead to a detectable phenotype. Even plants homozygous for a mutation in one gene and heterozygous for a mutation in the other gene were phenotypically indistinguishable from the wild-type (Sang *et al.*, 2012). However, the double knockout mutant is embryo lethal. These data indicate that although the function of AtCHR12 and AtCHR23 partially diverged, they maintained important redundant functions in plant growth and development.

AtCHR12 and AtCHR23 play a role in stem cell niche maintenance and cell elongation

atchr12 atchr23 double knockout mutants are embryo lethal. However, when weak alleles are used a double knockout, *atchr12-3 atchr23-1*, is viable. This double mutant is severely affected in growth and development (Sang *et al.*, 2012). It forms small seedlings and small bushy plants. This double mutant as well as *AtCHR23* over-expressing lines have reduced growth. However, while *atchr12-3 atchr23-1* showed severe reduction of growth, *AtCHR23* over-expressing lines showed not so extreme growth reduction.

The severe reduction of growth in seedlings of *atchr12-3 atchr23-1* was caused by dramatic defects in both shoot and root meristem. The reduction of root length was the result of a smaller root meristem due to defects in maintenance of the stem cell niche, as well as reduced cell expansion (Sang *et al.*, 2012).

In contrast, over-expression of *AtCHR12* or *AtCHR23* is not associated with reduced root meristem size (Mlynarova *et al.*, 2007, Chapter 2). The reduced root length of *AtCHR23* over-expressing seedlings correlates with a shorter elongation zone in roots, which is caused by smaller size of the cells. This indicates that *AtCHR23* over-expression has mainly an effect on cell elongation and not on maintenance of the stem cell niche. The *AtCHR23* over-expressing lines also have smaller hypocotyls and cotyledons, whose post-embryonic growth is mostly the result of cell elongation (Gendreau *et al.*, 1997, Stoynova-Bakalova *et al.*, 2004). This could mean that over-expression of *AtCHR23* has an effect on cell elongation in various organs.

Taken together, studies with the double mutant show that AtCHR12 and AtCHR23 are required for proper stem cell niche maintenance in root meristems (Sang *et al.*, 2012). It also suggests that AtCHR23 is not a limiting factor in maintenance of root meristem, because an increased level of AtCHR23 does not affect the size and/or patterning of the root meristem (Chapter 2). This is in contrast to for example PLETHORA2 (PLT2), a transcription factor required for stem cell niche maintenance. Increased PLT2 level causes

the formation of a larger meristem (Galinha *et al.*, 2007). In agreement with this, *AtCHR23* over-expression did not result in changes of expression level of key regulators like *WUSCHEL-related homeobox 5* (*WOX5*), *SHORT-ROOT* (*SHR*) or *SCARECROW* (*SCR*). In contrast, their expression was reduced in root meristems of the *atchr12-3 atchr23-1* double mutant (Sang *et al.*, 2012).

Both *atchr12-3 atchr23-1* and *AtCHR23* over-expressing mutants have a shorter elongation zone in roots (Chapter 2, Sang *et al.*, 2012). The results in Chapter 2 of this thesis suggest that proper and accurate expression of *AtCHR23* is required for stability and robustness of growth. This could relate to reduced levels of both AtCHR12 and AtCHR23, or increased levels of AtCHR23. The reduced or increased level of these ATPases might interfere with proper complex formation with the regulatory proteins and thereby affect the control of cell elongation.

AtCHR12 and AtCHR23 regulate root and shoot meristems by different pathways than BRM and SYD

It has also been studied whether the other two members of the Snf2-subfamily, SPLAYED (SYD) and BRAHMA (BRM), have a function in stem cell maintenance. *brm* mutants have defects in root stem cell niche maintenance as well as reduced elongation zone (Yang *et al.*, 2015). The *brm* mutants are affected in auxin distribution and impaired expression of *PLT1* and *PLT2,* which are transcription factors that play a key role in maintaining the stem cell niche. The affected auxin distribution is associated with reduced expression of *PIN-FORMED* (*PIN*) genes, which encode auxin efflux carriers. Further, BRM was shown to bind to several *PIN* genes. In contrast, AtCHR12 and AtCHR23 were shown to affect the root stem cell niche via the SHR/SCR related pathway (Sang *et al.*, 2012). The expression of *SHR* and *SCR* was reduced, while *WOX5* expression was increased and AtCHR23 binds to the *WOX5* promoter, suggesting a direct regulation. The data show that the Snf2-subfamily ATPases are important for both, maintenance of the stem cell niche and cell elongation. Interestingly, the data suggest that BRM and AtCHR12/AtCHR23 regulate the root stem cell niche via different pathways.

Further, both SYD and BRM are required for the proper maintenance of shoot apical meristem (Farrona *et al.*, 2004, Wagner *et al.*, 2002). However the molecular mechanism was only studied for SYD. SYD is recruited to the promoter of *WUSCHEL* (*WUS*), a transcription factor that acts as repressor of stem cell formation (Kwon *et al.*, 2005). Interestingly, *WUS* expression was also increased in the *atchr12-3 atchr23-1*, but AtCHR23 did not bind to the *WUS* promoter (Sang *et al.*, 2012). This suggests that SYD and AtCHR12/AtCHR23 also regulate the maintenance of shoot meristem, but by different mechanisms.

Snf2-subfamily ATPases are involved in response to abiotic stress

During their life, plants often have to deal with stress conditions, which could be permanent or temporary. Here the effects of temporary abiotic stress are discussed. When plants sense abiotic stress, the first phenotypic response is reduction of growth (Xiong *et al.*, 2001). In Arabidopsis two types of mutants with an altered response to stress have been identified. One type of mutants responds to stress with reduced sensitivity, which results in less reduction of growth than wild-type upon abiotic stress. The other type of mutants responds to abiotic stress with stronger reduction of growth than wild-type upon abiotic stress. There are advantages to both types of stress responses. The reduced sensitivity to abiotic stress may facilitate plants to maintain growth in places with stress. On the other hand, stronger reduction of growth or growth arrest allows plants to temporarily cope with stress and to continue growth when the stress is over. The latter requires that stronger growth retardation is a well-controlled physiological process and not the result of reduced fitness of the mutant.

The response of plants to stress conditions is associated with structural changes of chromatin (Probst *et al.*, 2015), where ATP-dependent chromatin remodeling plays a role. Previously, it was shown that over-expression of *AtCHR12* leads to growth arrest upon application of mild abiotic stress (Mlynarova *et al.*, 2007). When the temporary stress is over, the over-expressing plants resumed normal growth. The response of *atchr12* knockout mutant is less pronounced, but tends to go in the opposite direction (Mlynarova *et al.*, 2007). This indicates that AtCHR12-mediated growth arrest is an actively controlled response to abiotic stress.

In Chapter 2 of this thesis it is shown that also over-expression of *AtCHR23* leads to stronger reduction of growth upon abiotic stress compared to wild-type. The growth response of *atchr23* tended to go in the opposite direction, but was less pronounced and the difference was significant only at osmotic stress or continuous light. In contrast to *AtCHR12* over-expression, the reduced growth of *AtCHR23* over-expressing mutants was observed already at non-stress conditions, while only 10-20% of *AtCHR12* over-expressing plants shows growth arrest without stress (Mlynarova *et al.*, 2007). In addition, *AtCHR23* over-expression correlates also with increased variability of growth and gene expression levels between individuals/biological replicates (Chapter 2). The increased variability of gene expression levels at normal conditions might cause reduced fitness of the transgenic plants. This could lead to increased sensitivity to stress causing more severe reduction of growth. Because the ability to grow better than wild-type after relieve of stress was not analyzed, it is not possible to conclude if the reduction of growth upon stress in *AtCHR23* over-expressing lines is an actively regulated process or a consequence of reduced fitness.

Arabidopsis *brm* mutants also respond to abiotic stress with an increased growth reduction. Upon application of abscisic acid (ABA) the *brm* mutants have a stronger

reduction of primary root growth than wild-type (Han *et al.*, 2012). This hypersensitivity of *brm* to ABA is caused by increased expression of *ABA INSENSITIVE5* (*ABI5*), a transcription factor involved in ABA responses. *ABI5* might be regulated directly by BRM since BRM binds to the *ABI5* locus. The *brm* mutant has already at normal conditions severely reduced root growth due to defects in meristem and elongation zone (Yang *et al.*, 2015), which could lead to reduced fitness of the plant and could cause also stronger (non-specific) growth reduction upon stress. However, the increased ABA sensitivity in *brm* mutants led to increased tolerance to drought stress and higher survival rate, suggesting a controlled process.

In contrast to *brm*, the growth of a quadruple *della* mutant, which has four of the five *DELLA* genes mutated, is less inhibited in growth than wild-type upon application of ABA or salt stress (Achard *et al.*, 2006). DELLAs are transcription factors that repress plant growth in an ABA-dependent manner. In addition, the quadruple mutant has also reduced survival rate of seedlings compared to wild-type at continuous high salt stress conditions. The results indicate that DELLA-mediated growth reduction is beneficial and promotes survival.

The reduced growth response of the *della* quadruple mutant to stress conditions resembles the phenotype of *atchr12* or partially *atchr23* mutant (Mlynarova *et al.*, 2007, Chapter 2). In contrast, the *brm* mutant has a stronger reduction of growth upon stress similar to *AtCHR23* over-expression mutants. However, the root growth of *atchr12*, *atchr23* or *AtCHR12* over-expressing mutants was not affected by application of ABA, whereas the *della* quadruple mutant and *brm* mutant have altered ABA sensitivity. This suggests that AtCHR12- and probably also AtCHR23-mediated reduction of growth is ABA independent and differs from DELLA- or BRM-mediated growth responses.

AtCHR12 and AtCHR23 are involved in regulation of seed maturation genes

The response to environmental conditions is also important in developmental transitions during the plant life cycle. The first transition is germination, from dormant seed to growing seedling. In Chapter 3 of this thesis, the effect of *AtCHR12* and *AtCHR23* over-expression on this transition is studied. Over-expression of *AtCHR12* or *AtCHR23* reduced the frequency of seed germination compared to wild-type. The reduced germination of *AtCHR12* and *AtCHR23* over-expressing lines was associated with increased transcript levels of seed maturation genes during germination.

The maturation-related genes are highly expressed during late embryogenesis and relatively high levels of their transcripts are stored in dry seeds (Verdier *et al.*, 2008). In wild-type plants, the increase in expression of seed maturation genes during embryo maturation and the decrease of their mRNA levels during germination correlates with the expression pattern of *AtCHR12* and *AtCHR23*. This points towards a role of the two ATPases in the seed maturation program.

In dry seeds the transcript levels of seed maturation genes and *AtCHR12/AtCHR23* was similar in over-expressing mutants and wild-type. Upon germination the increasing transcript levels of *AtCHR12* and *AtCHR23* in the over-expressing mutants coincided with higher mRNA levels of seed maturation genes compared to wild-type. Thus it was suggested that over-expression of *AtCHR12* or *AtCHR23* interferes with degradation of mRNAs of seed maturation genes stored in dry seed during germination (Chapter 3).

During vegetative growth the seed maturation genes are thought to be repressed via chromatin modifications (van Zanten *et al.*, 2013). Increased expression levels of seed maturation genes were observed in roots of *pickle* (*pkl*) mutants (Rider *et al.*, 2004). PKL is a CHD3 chromatin remodeling ATPase, which represses the expression of the master regulators of embryonic identity *LEAFY COTYLEDON 1* (*LEC1*), *LEC2* and *FUSCA 3* (*FUS3*) (Dean Rider *et al.*, 2003). These were shown to repress seed maturation genes (Kroj *et al.*, 2003). This suggests that PKL affects the expression of seed maturation genes via *LEC1*, *LEC2* and *FUS3* repression. In contrast, BRM-containing chromatin remodeling complex was shown to be involved in repression of maturation-related genes during vegetative growth by targeting their promoters (Tang *et al.*, 2008). Compared to PKL, BRM represses the expression of maturation-related genes at a later developmental stage (Tang *et al.*, 2008).

The effect of *AtCHR12* and *AtCHR23* over-expression on the transcript levels of seed maturation genes is observed during early stages of germination. In addition, the expression of the master regulators of the embryonic identity was not affected in *AtCHR12* and *AtCHR23* over-expressing mutants. Furthermore, while BRM and PKL repress the expression of seed maturation genes, *AtCHR12* or *AtCHR23* over-expression interferes with the degradation of their transcripts.

This indicates that chromatin remodeling ATPases regulate the mRNA levels of seed maturation genes at different time points after germination and by different pathways. The data suggest, that during germination, the expression of *AtCHR12* and *AtCHR23* is reduced to allow degradation of mRNAs of seed maturation genes. Probably at a similar stage PKL represses the expression of *LEC1*, *LEC2* and *FUS3*, which further leads to repression of the expression of seed maturation genes. The repression of seed maturation genes seems to be further maintained during vegetative growth, among others, by BRM-containing chromatin remodeling complex.

Over-expression of *AtCHR12* affects flowering time via different mechanism than other Snf2-subfamily ATPases

AtCHR12 over-expression affected also the switch from vegetative growth to flowering (Chapter 4). Over-expression of *AtCHR12* altered the reprogramming of the main flowering repressor *FLC* gene during embryo development, leading to reduced *FLC* expression and early flowering. Also *AtCHR23* over-expression leads to early flowering due to reduced *FLC*

expression (own observations). However, due to time limitations the mechanism of this regulation was not studied in detail.

An effect on flowering time was also observed in tomato plants over-expressing a single tomato ortholog of *AtCHR12/AtCHR23*, *SlCHR1*. Surprisingly, the transgenic tomato plants were late flowering (Chapter 5). In transgenic Arabidopsis, reduced *FLC* expression causes early flowering. Although the *FLC* ortholog in tomato was identified (Jimenez-Gomez *et al.*, 2007), its function in flowering time control, if any, is not known. In Arabidopsis, *FLC* is the main flowering repressor, which is highly expressed to ensure that plant will not flower during winter (Kim *et al.*, 2009). After a prolonged period of cold (vernalization) the *FLC* locus is epigenetically silenced leading to reduced *FLC* expression that allows plant to flower. Since tomato originates from tropical/subtropical regions, the regulation of flowering time by FLC and vernalization is not expected (Samach *et al.*, 2007). This suggests that in tomato the over-expression of *SlCHR1* affects flowering time via a different pathway.

Also mutations in the other two Snf2-subfamily ATPase genes *BRM* and *SYD* affect flowering time control, as Arabidopsis *brm* and *syd* knockout mutants show early flowering (Farrona *et al.*, 2011, Wagner *et al.*, 2002). SYD affects flowering time via regulation of *FT* expression (Su *et al.*, 2006). It is not yet clear if it is a direct effect. *brm* mutants are early flowering at long-day (LD) conditions due to increased expression of photoperiod pathway gene CO, which activates *FT* and *SOC1* (Farrona *et al.*, 2011). In addition to the effect on the photoperiod pathway, BRM was recently shown to repress flowering by direct activation of the flowering repressor gene *SHORT VEGETATIVE PHASE* (*SVP*) (Li *et al.*, 2015). SVP forms a complex with FLC to directly repress *FT* expression. *brm* mutants have reduced *SVP* expression, therefore less SVP-FLC complex, leading to higher *FT* expression and early flowering. Interestingly, *brm* mutants displayed increased expression of *FLC* (Farrona *et al.*, 2011). The increased *FLC* expression does not delay flowering time at LD conditions due to increased expression of photoperiod pathway genes *CO*, *FT* and *SOC1*, or due to lower abundance of SVP-FLC complex (Farrona *et al.*, 2011, Li *et al.*, 2015).

The data discussed here suggest that the Snf2-subfamily ATPases, in addition to germination, are also involved in the induction of flowering. Similarly to germination, the Snf2-subfamily ATPases affect the transition into flowering by different mechanisms. Both BRM and SYD repress flowering, while *AtCHR12* over-expression accelerates the transition to flowering. BRM is involved in photoperiod pathway regulation (Farrona *et al.*, 2011) and it activates the flowering repressor SVP (Li *et al.*, 2015). SYD affects the FT expression, but further details are not known (Su *et al.*, 2006). In contrast, over-expression of *AtCHR12* interferes with *FLC* reprogramming during embryo development leading to reduced *FLC* expression (Chapter 4).

AtCHR12 over-expression may interfere with H3K27me3 removal

In Chapter 4 of this thesis it is shown that over-expression of *AtCHR12* is associated with reduced *FLC* expression, which correlates with increased H3K27me3 levels on the *FLC* locus. H3K27me3 is associated with gene repression and so far only Polycomb repressive complex 2 (PRC2), one of two classes of Polycomb group proteins (PcG), was identified in deposition of this mark (Xiao *et al.*, 2015). Proteins antagonistic to the PcG function are called Trithorax group (TrxG) proteins. TrxG proteins were identified in mutants that suppressed the polycomb mutant phenotype (Kingston *et al.*, 2014) and mutations in TrxG proteins lead to increased levels of H3K27me3 (Li *et al.*, 2015). TrxG proteins have been divided into three classes, SET domain-containing factors involved in histone methylation, ATP-dependent chromatin remodelers and factors binding specific DNA sequences (Schuettengruber *et al.*, 2011). Several proteins with TrxG function have been proposed in Arabidopsis, for example H3K27me3-specific histone demethylase RELATIVE OF EARLY FLOWERING 6 (REF6) or several ATP-dependent chromatin remodeling ATPases as BRM, SYD or PKL (Aichinger *et al.*, 2011, Li *et al.*, 2015, Lu *et al.*, 2011, Wu *et al.*, 2012).

The increased H3K27me3 levels in *AtCHR12* over-expressing lines suggest that *AtCHR12* over-expression could co-operate or promote the PRC2 function to increase the H3K27me3 levels. However, the increase of H3K27me3 levels on the *FLC* locus in *AtCHR12* over-expressing lines most probably happens during embryo development, which is largely independent of PRC2 action (Bouyer *et al.*, 2011, Xiao *et al.*, 2015). Therefore it is not very likely that *AtCHR12* over-expression is correlated with increased PRC2 function.

AtCHR12 over-expression could also interfere with the activity of proteins with TrxG function, like BRM or REF6. *brm* mutation is associated with increased H3K27me3 levels on several hundred genes and BRM was found to restrict the activity of the PRC2 complex during plant development (Li *et al.*, 2015). However, since BRM does not have H3K27me3-demethylase activity and PRC2 is not active during embryo maturation, it is not likely that *AtCHR12* over-expression interferes with BRM function. In contrast, REF6 is an H3K27me3-specific demethylase (Lu *et al.*, 2011), which is expressed during embryo development (Choi *et al.*, 2009). Although the role of REF6 in *FLC* reprogramming is not clear, it is possible that *AtCHR12* over-expression interferes with the function of REF6 during embryo maturation.

Another putative target that is affected by *AtCHR12* over-expression is EARLY FLOWERING 6 (ELF6), another H3K27me3-specific demethylase closely related to REF6 (Crevillen *et al.*, 2014). ELF6 is more likely to be affected by *AtCHR12* over-expression, because ELF6 is involved in *FLC* reprogramming during embryo development (Crevillen *et al.*, 2014). In addition, an *elf6* mutant is early flowering (Noh *et al.*, 2004) similarly as *AtCHR12* over-expressing lines. The similar phenotype of *elf6* and *AtCHR12* over-expressing mutant supports the hypothesis that *AtCHR12* over-expression interferes with ELF6 function.

Therefore I hypothesize that in contrast to BRM, which prevents the deposition of H3K27me3 by PRC2 complex (Li *et al.*, 2015), over-expression of *AtCHR12* disturbs the removal of H3K27me3 possibly by interfering with the function of H3K27me3-specific demethylase EFL6.

Potential applications of CHR12/CHR23 in crops

In this thesis it is shown that over-expression of *AtCHR12* or *AtCHR23* affects plant growth, especially in response to adverse environments (Chapter 2). Further, *AtCHR12* or *AtCHR23* over-expression influences two important developmental switches during the plant life cycle, germination and transition into flowering (Chapter 3, Chapter 4). These are all traits potentially interesting for agricultural and horticultural practices. However, in general the effects of over-expression of Snf2-subfamily ATPases are pleiotropic. To obtain more specific changes it will be important to know at what stage and in what cells the over-expression of a Snf2-subfamily ATPase causes the desired effect, and to use promoters that are active at such stage and cell type. For example to reduce growth of seedlings used as transplants, or to reduce growth during the vegetative stage to create more compact plants, *CHR12/CHR23* should be expressed specifically during seedling stage or during vegetative growth, respectively (Liu *et al.*, 2013).

It will also be important to select the right Snf2-subfamily ATPase. For example, over-expression of *AtCHR12* has no effect on growth under optimal growth conditions. However, it primes plants to respond faster and stronger to stress conditions (Mlynarova *et al.*, 2007). This response is a well-controlled physiological condition and plants are able to re-establish normal growth as soon as the stress is relieved. In contrast, over-expression of *AtCHR23* in Arabidopsis leads to increased variability of growth and expression levels of a subset of genes (Chapter 2). It is possible that variability in gene expression could cause sub-optimal physiological conditions in a plant. *AtCHR23* over-expressing lines respond to stress conditions with reduced growth. However it is possible that this is due to the reduced fitness (see 'Snf2-subfamily ATPases are involved in response to abiotic stress'). If this is indeed the case, it is of no use in agricultural applications.

To investigate if CHR12/CHR23 orthologs could be used to improve crop properties, the single tomato ortholog was over-expressed in tomato plants. In Chapter 5 of this thesis it is shown that over-expression of *SlCHR1* leads to reduced growth at all developmental stages with smaller seedlings and plants and compact reproductive structures. The transgenic tomato plants also manifested change in flowering time. However, compared to Arabidopsis, over-expression of *SlCHR1* led to delayed flowering. Surprisingly, the response of the transgenic tomato lines to abiotic stress was different compared to the response of transgenic Arabidopsis plants. The transgenic tomato lines showed less growth reduction than wild-type upon drought stress, while upon salt stress the transgenic tomato plants manifested similar reduction of growth as wild-type. These differences could be related to intrinsic differences

between Arabidopsis and tomato. However, also selection during tomato breeding, which has been mainly focused on production of tomato and not so much on response to stress, might have cause the loss of such traits. It may be possible that such traits remained in wild varieties, in which they could be identified and transferred into crops.

Growth responses can be modulated by changing the expression of genes that control the chromatin status of genes involved in growth and development. However, it would also be possible to change the expression of these target genes directly. The advantage of changing expression of chromatin remodeling genes is that a chromatin configuration might prime plants for growth arrest before the stress is perceived. Especially genes like *AtCHR12* are interesting for improvement of crops, since over-expression does not affect growth at normal conditions, however, leads to faster and stronger growth arrest upon stress (Mlynarova *et al.*, 2007). In contrast, modification of expression of transcription factors involved in stress response usually does affect growth at normal conditions (Seo *et al.*, 2012).

Concluding remarks

Chromatin remodeling complexes affect important aspects of both animal and plant life. The studies described in this thesis further extend the knowledge of functions of AtCHR12 and AtCHR23 ATPases during the plant life cycle. Over-expression of the two ATPases has impact on growth regulation, especially in response to abiotic stress, and affects the two most important developmental transitions in the plant life cycle, germination and flowering.

Although ATP-dependent chromatin remodeling has been studied for more than a decade, several questions still remain. One of these concerns the mechanisms by which they function, which is in most cases not clear. The results in this thesis suggest possible interplay between AtCHR12 and AtCHR23 with histone modification complexes. However, to understand the molecular mechanisms of these interactions further research will be required. For example targets of these ATP-dependent chromatin remodeling complexes have to be identified. Insight in regulatory mechanisms could also be interesting for agriculture and could be used in more precise future breeding programs.

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SUMMARY

Living organisms have to deal with changing environmental conditions during their whole life cycle. In contrast to animals, plants are sessile organisms. Therefore they have evolved multiple regulatory mechanisms that help them to cope with changing conditions. One of the first responses to stress conditions is reduction or arrest of growth. Therefore regulation of growth and development is essential to successfully complete their life cycle. To correctly time their development, plants need to integrate various environmental signals with intrinsic developmental programs. In this integration, regulation of gene expression plays a major role.

The genetic information of an organism is stored in DNA sequence. DNA forms a complex with histones and other proteins, which is called chromatin. Chromatin is a highly dynamic complex and modification of the chromatin structure makes DNA more or less accessible to the transcriptional machinery and other regulatory proteins. The modification of chromatin organization is called chromatin remodeling and it involves both covalent modifications of DNA and histone tails, and non-covalent modification of chromatin structure by ATP-dependent chromatin remodeling complexes. ATP-dependent chromatin remodeling complexes comprise multiple protein subunits with SNF2 ATPase as a catalytic subunit. Depending on the subunit composition, the complexes can perform different tasks at various places of chromatin, and can be active at different developmental stages (Chapter 1).

The SNF2 ATPases are conserved from yeast to plants. In Arabidopsis, 41 SNF2 ATPases have been identified. The focus of this thesis is on two of those ATPases – AtCHR12 and AtCHR23. It has been shown previously that AtCHR12 is involved in growth responses to environmental cues. We have extended these studies to its paralog AtCHR23 (Chapter 2). In contrast to over-expression of *AtCHR12*, which affects growth only during reproductive stage of development, over-expression of *AtCHR23* leads to smaller seedlings and reduced vegetative growth. Upon application of mild abiotic stress, the growth reduction is stronger than in wild-type plants. Moreover, the transgenic plants manifest increased variability of growth. The increased growth variability correlates with increased expression variability of genes associated with stress. The results indicate that accurate and controlled expression of *AtCHR23* is required for stability and robustness of growth, as well as gene expression.

Regulation of growth is important not only during vegetative or reproductive stage of development, but also during embryo development. The growth of the embryo is interrupted during the embryo maturation phase and it was suggested that AtCHR12 may be involved in this temporary growth arrest. Here we have shown that both *AtCHR12* and *AtCHR23* are expressed during embryo development, and that over-expression of *AtCHR12* or *AtCHR23* affects the embryo maturation phase with consequences on two important developmental transitions in plant life – germination and transition to flowering.

Over-expression of *AtCHR12* or *AtCHR23* leads to reduced seed germination, which is more pronounced under stress conditions (Chapter 3). The reduced germination of over-expressing lines is associated with increased transcript levels of seed maturation genes and reduced degradation of their mRNAs in germinating seeds. The results indicate that repression of *AtCHR12* and *AtCHR23* in germinating seeds is required for full germination.

The connection between embryo development and flowering time control was observed in transgenic lines over-expressing *AtCHR12* (Chapter 4). Over-expression of *AtCHR12* results in early flowering under both long- and short-day conditions. The early flowering phenotype correlates with reduced expression of the main flowering repressor, *FLC*. The reduced *FLC* expression correlates with increased levels of repressive histone mark H3K27me3 on the *FLC* locus. Additionally, *FLC* expression was affected already during *FLC* reprogramming, which takes place during embryo development. This leads to reduced *FLC* expression in mature embryos. The results show that *AtCHR12* over-expression affects flowering time by different mechanisms than other Snf2-subfamily ATPases. In contrast to AtCHR12, BRAHMA was shown to regulate flowering time via the photoperiod pathway, while SPLAYED affects flowering time by repressing *FT* expression.

We have observed that over-expression of *AtCHR12* or *AtCHR23* affects plant growth in response to stress, and play a role in germination and transition to flowering. These traits are also important for agriculture, and such genes are potentially interesting targets for breeding programs. To test, if such genes have a similar role in crops, we have studied the effect of the tomato ortholog of AtCHR12 and AtCHR23 on tomato growth.

Tomato (*Solanum lycopersicum*), as well as other crops, have only one ortholog of AtCHR12 and AtCHR23, which was suggested to possess a role of both ATPases. We have successfully cloned the tomato ortholog and over-expressed it in tomato plants (Chapter 5). The transgenic tomato plants have reduced vegetative growth and compacted reproductive structures, resembling the phenotype of *AtCHR23* and *AtCHR12* over-expression, respectively. However, in contrast to Arabidopsis, the tomato plants responded to abiotic stress similarly as wild-type, and they flowered later than wild-type plants. The results indicate that modification of expression of AtCHR12 and AtCHR23 orthologs could be used to develop novel methods to control plant growth.

Taken together, the research described in this thesis identifies AtCHR12 and AtCHR23 as regulators of plant growth, especially in response to environment, as well as of the seed maturation program with clear effects on seed germination and flowering time, and we show that such genes can be potentially interesting for agriculture and horticulture practice.

SAMENVATTING

Levende organismen moeten omgaan met veranderende milieu omstandigheden tijdens hun hele levenscyclus. In tegenstelling tot dieren zijn planten sessiele organismen. Daarom hebben zij meerdere regulerende mechanismen ontwikkeld die hen helpen om te gaan met veranderende omstandigheden. Een van de eerste reacties op stresscondities is de vermindering of stop van groei. Daarom is regulatie van groei en ontwikkeling essentieel om hun levenscyclus met succes te voltooien. Om hun ontwikkeling te timen, integreren planten verschillende milieu-signalen met intrinsieke ontwikkelingsprogramma's. In deze integratie speelt regulatie van genexpressie een grote rol.

De genetische informatie van een organisme is opgeslagen in de DNA-sequentie. DNA vormt een complex met histonen en andere eiwitten dat chromatine wordt genoemd. Chromatine is een zeer dynamisch complex en wijziging van de chromatinestructuur maakt DNA meer of minder toegankelijk voor het transcriptieapparaat en andere regulerende eiwitten. De modificatie van chromatine organisatie heet chromatine remodeling en omvat covalente modificaties van DNA en histonen, en non-covalente modificatie van de chromatinestructuur door ATP-afhankelijke chromatine remodeling complexen. ATP-afhankelijke chromatine remodeling complexen bestaan uit meerdere subunits met SNF2 ATPase als katalytische subunit. Afhankelijk van de subunit samenstelling, kunnen de complexen verschillende taken op verschillende plaatsen van chromatine uitvoeren, en kunnen de complexen actief zijn in verschillende ontwikkelingsstadia (Hoofdstuk 1).

De SNF2 ATPasen zijn geconserveerd van gist tot planten. In Arabidopsis zijn 41 SNF2 ATPasen geïdentificeerd. De focus van deze thesis ligt op twee van deze ATPasen - AtCHR12 en AtCHR23. Eerder is aangetoond dat AtCHR12 betrokken is bij groeirespons op omgevingsfactoren. We hebben deze studies uitgebreid tot zijn paraloog AtCHR23 (Hoofdstuk 2). In tegenstelling tot overexpressie van *AtCHR12* die groei alleen tijdens voortplanting ontwikkelingsstadium beïnvloedt, leidt overexpressie van *AtCHR23* tot kleinere zaailingen en verminderde vegetatieve groei. Bij toepassing van milde abiotische stress, is de vermindering van de groei sterker dan in wild-type planten. Bovendien laten de transgene planten een verhoogde variabiliteit van de groei zien. De toegenomen variabiliteit van groei correleert met een verhoogde variabiliteit in expressie van genen geassocieerd met stress. De resultaten geven aan dat nauwkeurige en gecontroleerde expressie van *AtCHR23* vereist is voor stabiliteit en robuustheid van groei en genexpressie.

Regulatie van de groei is niet alleen belangrijk tijdens de vegetatieve of reproductieve ontwikkelingsstadia, maar ook tijdens de embryonale ontwikkeling. De groei van het embryo wordt onderbroken tijdens de embryo rijpingsfase en het werd voorgesteld dat AtCHR12 betrokken zou kunnen zijn bij deze tijdelijke groeistop. Hier hebben we aangetoond dat *AtCHR12* en *AtCHR23* worden gemaakt tijdens de embryonale ontwikkeling en dat

overexpressie van *AtCHR12* of *AtCHR23* de embryo rijpingsfase beïnvloedt met gevolgen voor twee belangrijke overgangen in ontwikkeling - kieming en de overgang naar de bloei.

Overexpressie van *AtCHR12* of *AtCHR23* resulteert in verminderde zaadkieming, die meer uitgesproken is onder omstandigheden van stress (Hoofdstuk 3). De verminderde kieming van overexpressie lijnen is geassocieerd met een verhoogd aantal transcripten van zaadrijpingsgenen en een verminderde afbraak van hun mRNAs in kiemende zaden. De resultaten geven aan dat repressie van *AtCHR12* en *AtCHR23* in kiemende zaden is vereist voor volledige kieming.

Het verband tussen embryonale ontwikkeling en bloeitijd regulatie werd waargenomen bij transgene *AtCHR12* overexpressie lijnen (Hoofdstuk 4). Overexpressie van *AtCHR12* resulteert in een vroege bloei onder lange- en korte dag omstandigheden. Het vroege bloei fenotype correleert met een verminderde expressie van de hoofd bloei-repressor, *FLC*. De verminderde *FLC* expressie correleert met verhoogde niveaus van repressieve histon markering H3K27me3 op het *FLC* locus. Daarnaast werd *FLC* expressie reeds beïnvloed tijdens de *FLC* herprogrammering die plaatsvindt tijdens de embryonale ontwikkeling. Dit leidt tot verminderde *FLC* expressie in rijpe embryo's. De resultaten tonen aan dat *AtCHR12* overexpressie bloeitijd beïnvloedt via andere mechanismen dan andere Snf2-onderfamilie ATPases. In tegenstelling tot AtCHR12 werd aangetoond dat BRAHMA bloeitijd regelt via de fotoperiode signaaltransductieketen, terwijl SPLAYED bloeitijd beïnvloedt door FT expressie te onderdrukken.

We hebben waargenomen dat overexpressie van *AtCHR12* of *AtCHR23* plantengroei beïnvloedt in reactie op stress, en een rol speelt bij kieming en de overgang naar bloei. Deze eigenschappen zijn ook belangrijk voor de landbouw, en dergelijke genen zijn potentieel interessante targets voor veredelingsprogramma's. Om te testen of dergelijke genen een vergelijkbare rol in gewassen hebben, hebben we het effect van de tomaten ortholoog van AtCHR12 en AtCHR23 in op groei onderzocht.

Tomaat (*Solanum lycopersicum*), en andere gewassen, hebben slechts één ortholoog van AtCHR12 en AtCHR23, die werd voorgesteld om een rol van beide ATPases bezitten. We hebben de tomaten ortholoog met succes gekloond en tot over-expressie gebracht in tomatenplanten (Hoofdstuk 5). De transgene tomatenplanten hebben een verminderde vegetatieve groei en verdichtte reproductieve structuren, wat lijkt op het fenotype van over-expressie van respectievelijk *AtCHR23* en *AtCHR12*. In tegenstelling tot Arabidopsis reageerden de tomatenplanten op gelijke manier op abiotische stress als wildtype, en bloeiden zij later dan wildtype planten. De resultaten geven aan dat modificatie van expressie van AtCHR12 en AtCHR23 orthologen gebruikt kan worden om nieuwe methoden te ontwikkelen om plantengroei te regelen.

Samengevat, het onderzoek beschreven in deze thesis identificeert AtCHR12 en AtCHR23 als regulatoren van plantengroei, vooral in reactie op omgevingsfactoren, alsmede het zaadrijpingsprogramma met duidelijke effecten op zaadkieming en bloeitijd, en we tonen

aan dat dergelijke genen potentieel interessant kunnen zijn voor de land- en tuinbouw praktijk.

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Thank you all!

Adam Folta

23 September 2015, Ede

PUBLICATIONS

Folta, A., Bargsten, J.W., Bisseling, T., Nap, J.P. and Mlynarova, L. (2015) Compact tomato seedlings and plants upon overexpression of a tomato chromatin remodelling ATPase gene. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12400

Leeggangers, H.A., Folta, A., Muras, A., Nap, J.P. and Mlynarova, L. (2015) Reduced seed germination in Arabidopsis over-expressing SWI/SNF2 ATPase genes. *Physiol. Plant.*, **153**, 318-326.

Folta, A., Severing, E.I., Krauskopf, J., van de Geest, H., Verver, J.*, et al.* (2014) Over-expression of Arabidopsis AtCHR23 chromatin remodeling ATPase results in increased variability of growth and gene expression. *BMC Plant Biol.*, **14**, 76.

Bargsten, J.W., Folta, A., Mlynarova, L. and Nap, J.P. (2013) Snf2 family gene distribution in higher plant genomes reveals DRD1 expansion and diversification in the tomato genome. *PLoS ONE*, **8**, e81147.

Rogowski, K.J., Folta, A., Bargsten, J.W., Nap, J.P. and Mlynarova, L. (2013) Unexpectedly rapid IS1 transposition into an Arabidopsis chromatin remodeling gene. *Transgenic Res.*, **22**, 869-871.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Adam Folta
Date: 19 November 2015
Group: Laboratory of Molecular Biology
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Chromatin remodeling and growth: The role of SNF2-like ATPases in stress responses	May 27, 2011
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Bioinformation Technology (SSB-20306)	Nov 30, 2011
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Plasticity', Wageningen University	Dec 10, 2010
EPS theme 4 symposium 'Genome Biology', Wageningen University	Dec 09, 2011
EPS theme 4 symposium 'Genome Biology', Radboud University Nijmegen	Dec 07, 2012
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Jan 24, 2014
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 02-03, 2012
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 22-23, 2013
ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 14-15, 2014
► **Seminars (series), workshops and symposia**	
Seminar: Sir David Baulcombe: 'Mobile RNA silencing in plants'	Sep 27, 2010
Seminar: Jan Willem Borst: 'In vivo imaging and protein behavior' (CBSG Clustermeeting Arabidopsis & Brassica)	Sep 28, 2010
Seminar: KEYS 2010	Oct 20, 2010
Seminar: Prof. Peter Cook: 'Transcription factories as organizers of the genome: the role of fixed polymerases'	Oct 27, 2010
Seminar: Javier Palatnik: 'Biogenesis and function of plant microRNAs'	Aug 28, 2011
Seminar: Prof. Hong Ma: 'Molecular genetic, transcriptomic and genomic characterization of meiotic recombination in Arabidopsis'	May 29, 2012
Seminar: Sir David Baulcombe: 'Plant versus virus: Defence, counter defence and counter counter defence'	Oct 10, 2012
Seminar: KEYS 2012	Oct 24, 2012
Seminar: Andrew Sugden: 'Writing for high impact journal'	Feb 08, 2013
Seminar: Eric Kramer: 'Auxin and plasmodesmata: Measuring fluxes in the root'	Jul 02, 2013
Seminar: KEYS 2013	Sep 19, 2013
Seminar: LinkedIn for beginners	Mar 25, 2014
Seminar: Life Technologies	Jun 25, 2014
EPS Flying Seminar: Steven Penfield: 'Parenting in plants: maternal control of seed dormancy'	Jun 12, 2012
EPS Flying Seminar: Prof. Ruth Finkelstein: 'ABA signaling networks in Arabidopsis'	Nov 14, 2012
WEES seminar: William Ratcliff: 'Experimental Evolution of Multicellularity'	Aug 29, 2011
WEES seminar: Marc van Roosmalen: 'The Brazilian Amazon: Hotspot of Biodiversity'	Dec 07, 2011
Webinar: Detecting Native Protein Interaction and Phosphorylation: New Approaches and Novel Technologies	Nov 09, 2011
ExPectationS Career Day	Nov 19, 2010
ExPectationS Career Day	Nov 18, 2011
ExPectationS Career Day 'Creativity and inspiration in science'	Feb 01, 2013
Symposium 'Advances in Life Science Technology	Nov 25, 2010
CBSG Meeting Proteomics Hotel Projects	Dec 13, 2010
CBSG Summit 2011	Jan 31-Feb 01, 2011
CBSG Summit 2012	Feb 29-Mar 01, 2012
CBSG Clustermeeting Arabidopsis & Brassica	Nov 06, 2011
ServiceXS Symposium	Dec 08, 2011
Start symposium Plant Developmental Biology	Oct 14, 2013
Minisymposium: 'How to write a world class paper'	Oct 17, 2013
9th Dutch Chromatin Meeting, Groningen, NL	Nov 10-11, 2011
Joint Dutch Chromatin Meeting and NVBMB Fall meeting, Rotterdam, NL	Oct 29, 2013
iPlant Tools and Services Workshop, Cold Spring Harbor Laboratory, NY, USA	Dec 04, 2013

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►	**Seminar plus**	
►	**International symposia and congresses**	
	4th European Plant Science Retreat 2012, Norwich, UK	Aug 14-17, 2012
	Plant Genomes & Biotechnology: From Genes to Networks, Cold Spring Harbor Laboratory, NY, USA	Dec 04-07, 2013
	6th European Plant Science Retreat 2014, Amsterdam, NL	Jul 01-04, 2014
	11th EMBL Conference: Transcription and Chromatin, EMBL Heidelberg, Germany	Aug 23-26, 2014
►	**Presentations**	
	Tomato chromatin remodeling ATPases as breeding target for environmental stress tolerance' at CBSG Summit 2011, Wageningen, NL (Poster)	Jan 31-Feb 01, 2011
	ATP-dependent chromatin remodeling in flowering time control' at CBSG Summit 2012, Wageningen, NL, and 4th European Plant Science Retreat 2012, Norwich, UK (Poster)	Feb 29-Mar 01, 2012
	Over-expression of Arabidopsis AtCHR23 chromatin remodeling ATPase leads to increased growth variability' at Plant Genomes & Biotechnology: From Genes to Networks, Cold Spring Harbor Laboratory, NY, USA (Poster)	Dec 04, 2013
	Over-expression of Arabidopsis AtCHR23 ATPase increases growth variability' at ALW Meeting Experimental Plant Sciences, Lunteren, NL (Talk)	Apr 22-23, 2013
	Over-expression of Arabidopsis AtCHR23 chromatin remodeling ATPase leads to increased growth variability' at Joint Dutch Chromatin Meeting and NVBMB Fall Meeting, Rotterdam, NL (Talk)	Oct 29, 2013
	Over-expression of chromatin remodeling ATPase AtCHR12 results in early flowering due to decreased FLC expression' at 6th European Plant Science Retreat 2014, Amsterdam, NL (Talk)	Jul 01-04, 2014
	Over-expression of chromatin remodeling ATPase AtCHR12 results in early flowering due to decreased FLC expression' at 11th EMBL Conference: Transcription and Chromatin, EMBL Heidelberg, Germany (Poster)	Aug 23-26, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Nov 14, 2012
►	**Excursions**	
	CBSG Matchmaking Event Young Plant Scientists	Nov 18, 2012
	Rijk Zwaan Excursion	Sep 27, 2013
	Subtotal Scientific Exposure	*23.5 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Wellcome Trust Advanced Course: Protein Interactions and Networks, Hinxton, Cambridge, UK	Dec 11-17, 2011
	The power of RNA-seq, Wageningen, NL	Jun 05-07, 2013
	PhD course: Transcription Factors and Transcriptional Regulation, Wageningen, NL	Dec 17-19, 2013
►	**Journal club**	
	Member of a literature discussions group	2010 - 2014
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.6 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD Competence Assessment	Aug 30, 2011
	Improve your writing	Nov 01-Dec 06, 2012
	Scientific writing	Nov 22, 2012-Jan 24, 2013
	Voice Matters: Voice and Presentation Skills Training	Feb 12 & Feb 26, 2013
	Workshop Presentation skills	Mar 12 & 26, Apr 03, 2012
	TOEFL IBT Test	Feb 03, 2012
►	**Organisation of PhD students day, course or conference**	
	Organisation of ExPectationS career day	2011
►	**Membership of Board, Committee or PhD council**	
	Board member of EPS PhD Council	2012 - 2013
	Subtotal Personal Development	*6.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**44.4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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