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ZUSAMMENFASSUNG

Struktur und Organisation des Genoms innerhalb des Zellkerns ist nicht zufällig und für den Programmablauf der Genexpression in der Zelle äußerst wichtig. Die Architektur des pflanzlichen Zellkerns ist variabel und kann sich aufgrund endogener Entwicklungssignale oder externer Stressfaktoren ändern. Die meisten Untersuchungen subnukleärer Struktur im Zusammenhang mit Genexpression wurden bisher mit fixiertem Material und isolierten Kernen gemacht. Solche Ansätze lieferten wertvolle Informationen über die Organisation des Chromatins, können aber die dynamischen Vorgänge in lebenden Zellen nicht berücksichtigen. Die Verfügbarkeit fluoreszenter Reporter und schneller Bilderfassungsmethoden in der Mikroskopie erlaubte es mir, Lebendbeobachtung an Zellkernen innerhalb des Wurzelgewebes von Arabidopsis Keimlingen durchzuführen und die Dynamik der Kernarchitektur nach Einwirkung interner und externer Trigger zu verfolgen. Mein ehrgeiziges Vorhaben, die Position spezifischer Genomabschnitte während der Aktivierung dort lokalisierter Gene in lebenden Zellen zu beobachten, wurde nicht erreicht, aber es führte zur Entwicklung eines Verfahrens, mit dem einzelne Zellkerne während der Differenzierung der Wurzelzelle unter dem Mikroskop verfolgt und die Umstrukturierung ihrer nukleären Architektur während der Differenzierung erfasst werden kann. Ich konnte zeigen, dass Unterschiede in der Identität der Epidermiszellen schon nahe an der Wurzelspitze in der Kernorganisation zum Ausdruck kommen, lange bevor sich die Zellanatomie weiter oben ändert. Als äußeren Faktor habe ich Hitzestress angewandt und gezeigt, dass die dadurch ausgelöste Dissoziation des Heterochromatins reversibel und mit Abrundung und Immobilisierung der Kerne verbunden ist. Mit Hilfe von Histonchaperon-Mutanten gelang es zu zeigen, dass die Aktivität von mehreren Histonchaperonkomplexen zum Erhalt der Kernstruktur und zur rechtzeitigen Reorganisation des Heterochromatins unter Hitzestressbedingungen erforderlich ist. Einige Langzeitfolgen des Hitzestresses auf die Kernarchitektur sind jedoch nicht auszuschließen. Das Erscheinungsbild der Wurzelzellkerne unter Hitzestress erinnert an das in Mutanten ohne einen funktionalen Komplex, welcher das Zytoskelett mit dem Inneren des Zellkerns verbindet. Das wirft interessante Fragen nach der Rolle dieses Komplexes für die Kontrolle der Zellkernarchitektur bei Hitze- und anderen Stressbedingungen.

SUMMARY

Structure and organization of the genome within the nucleus is non-random and of critical importance for the gene expression program of a cell. In plants, the nuclear architecture is plastic and can change in response to internal (development) or external (stress) cues. Most studies of subnuclear structures and its relation to gene expression so far were based on fixed material and extracted nuclei. These approaches have provided valuable information about chromatin organization but are unable to encompass the dynamics of nuclear organization in living cells. Taking advantage of recent progress in the availability of fluorescent reporter and fast acquisition microscopy, I applied live imaging of nuclei in cells within their native context in roots of *Arabidopsis* seedlings, to gain insight into the dynamics of nuclear architecture in response to internal and external cues. My ambitious attempt to achieve live imaging of specific single loci in response to changes in gene activation did not reach its goal, but it led to the development of a tracking tool allowing to follow individual nuclei during root cell differentiation under the microscope and to capture nuclear architecture remodeling during differentiation. I showed that differences in cell identity in the epidermis are reflected already close to the root tip by their nuclear organization, long before cell anatomy changes further up. As an external cue, I applied heat stress and could show that the resulting decompaction of heterochromatin is transient and accompanied with nuclear rounding and nuclear immobilization. Using histone chaperone mutants, I demonstrated that activity of several histone chaperone complexes is required to maintain nuclear integrity in response to heat stress and for timely restoration of heterochromatin after the stress. However, some long-term effect of the stress on nuclear architecture can occur. The phenotype of root cell nuclei in response to heat stress resembles that of mutants affected in a complex linking the cytoskeleton to the nuclear interior and open interesting question about the role of this complex and the control of nuclear architecture in response to heat and other stress conditions.

1 INTRODUCTION

1.1 MOLECULAR ORGANIZATION OF CHROMATIN

DNA contains all information required for the development of a cell or an organism. In eukaryotes, genomic DNA is enclosed in the nucleus. The nucleus, as the biggest organelle in a cell, was the first to be identified as a subcellular structure by A. Leeuwenhoek in 1682 or by F. Bauer in 1802 (Harris 1999). The term "nucleus" is documented since November 1st, 1831, and was coined by the botanist Robert Brown in a lecture to the Linnaean Society (Brown 1833). Nuclear DNA, if made linear, would be centimeters to meters long, but it is contained in a nucleus with a volume of a few hundred μm^3 . The DNA has thus to be compacted to fit in such a small volume. Such compaction is achieved through association of the DNA with proteins, mainly histones, into a complex called chromatin. The organization and distribution of chromatin within a nucleus as well as the shape of the nucleus will be referred as "nuclear architecture" further on.

The base unit of chromatin compaction is the nucleosome, consisting of ~ 147 base pairs (bp) of DNA wrapped around a histone octamer composed of two copies of each core histone, namely H2A, H2B, H3 and H4 (Luger et al. 1997). Its *in vivo* assembly occurs in two steps. First, an H3/H4 tetramer is loaded onto DNA (Smith and Stillman 1991), then the nucleosome is completed by two H2A/H2B subunits (Smith and Stillman 1991; Park et al. 2005). The extra-nucleosomal histone H1 then contributes to the stabilization of higher order organization of chromatin by binding neighboring nucleosomes and linker DNA (Carruthers et al. 1998; Syed et al. 2010). Overall, 70 to 90% of DNA is wrapped around nucleosomes (Kornberg 1974). Histones can be post-transcriptionally modified (reviewed in Pfluger and Wagner 2007; Berr et al. 2011), and histone variants can be exchanged for core histones in specific nucleosomes (reviewed in Jiang and Berger 2017). H2A, and H3 are the histone that comprise The most variants, and the best characterized ones, exist for histone H2A and H3 (reviewed in Jiang and Berger 2017), while no H4 variants have been identified so far, and only a few plant male germ line-specific H2B variants have been described to date (Yang et al. 2016).

Based on cytological observations by Emil Heitz in 1928 (Heitz 1928; Passarge 1979), chromatin was classified in two main types: euchromatin and heterochromatin. The first is lowly compacted and transcriptionally active, composed mainly of active genes and associated with active epigenetic marks such as histone acetylation and methylation of the lysine 4 on the tails of histone 3 (H3K4me). In contrast, heterochromatin is tightly compacted and was traditionally considered as transcriptionally inert (reviewed in Rosa and Shaw 2013; Pikaard and Mittelsten Scheid 2014; Allis and Jenuwein 2016). However, there is evidence that transcription is required for heterochromatin formation and maintenance (Volpe et al. 2002; Kato et al. 2005; reviewed in Castel and Martienssen 2013). Heterochromatin is subdivided into constitutive and facultative heterochromatin, depending on its maintenance through the cell cycle or upon differentiation (Brown 1966). Constitutive heterochromatin stays compacted throughout the cell cycle. It is formed by the gene-poor/repeat-rich regions of chromosomes and is enriched in repressive chromatin marks, such as heavy DNA methylation and di-methylation of the lysine 9 on the tails of histone 3 (H3K9me₂) (Fransz et al. 2002; Fransz et al. 2003). In *Arabidopsis*, it consists of pericentromeric repeats such as the 180 bp repeat, transposable elements, ribosomal DNA, and telomeres (Fransz et al. 2002). These sequences are compacted into discrete foci, organized around chromosome's centromeres and called chromocenters (CC), from which euchromatin loops emerge (Fransz et al. 2002). Eight to ten CCs can usually be observed in *Arabidopsis* interphase nuclei, appearing as bright spots when imaged with DNA dyes or chromatin reporters. Most CCs are localized at the nuclear periphery while telomeres and chromocenters containing the Nucleolar Organizer Regions (NORs) formed by the 45S rDNA loci are frequently associated with the nucleolus (Fransz et al. 2002; Fransz et al. 2006; Pontvianne et al. 2016). Facultative heterochromatin describes regions of chromatin that can be either open or conditionally silenced through heterochromatinization. It is associated with developmental regulation of gene expression in combination with specific chromatin marks, such as H3K27me₃ (reviewed in Schuettengruber et al. 2007; reviewed in Zheng and Chen 2011; Makarevitch et al. 2013).

The compaction of chromatin and tight association with nucleosomes restrain DNA accessibility to all processes that operate on it, including replication (reviewed in MacAlpine and Almouzni 2013), DNA repair (reviewed in Dona and Mittelsten Scheid 2015; Hauer and Gasser

2017), recombination (reviewed in Lieberman-Lazarovich and Levy 2011; Yamada and Ohta 2013) and transcription (reviewed in Li et al. 2007). Thus, the composition, positioning, assembly and disassembly of nucleosomes must be tightly controlled to express what should be expressed, to silence what should not be accessible, and to safeguard genome organization integrity. To this aim, specialized proteins called histone chaperones (Laskey et al. 1978) are in charge of many aspect of nucleosome dynamics, especially transport, storage, assembly and disassembly of nucleosomes. They are classed in H3-H4 and H2A-H2B chaperones depending on their preferential binding. I will focus here on the H3-H4 chaperones as H3-H4 histones are the first to be deposited onto chromatin (Smith and Stillman 1991), and mutations in genes coding for them lead to significant phenotypes.

In the cytoplasm, the Anti-Silencing Function 1 (ASF1) chaperone binds H3-H4 dimers (English et al. 2006; Natsume et al. 2007) and transfers them to the nucleus to either the Chromatin Assembly Factor 1 (CAF-1) (Smith and Stillman 1989) or to the Histone Regulator A (HIRA) complex (Tagami et al. 2004). Arabidopsis possesses two genes encoding for ASF1, *AtAsf1a* and *AtASF1b* (Ehsan et al. 2004). The Arabidopsis CAF-1 complex is composed of FASciata1 (FAS1), FASciata2 (FAS2), and Multicopy Suppressor of Ira1 (MSI1) (Kaya et al. 2001) and mediates DNA-synthesis-coupled deposition of the canonical H3.1, along with H4 (Smith and Stillman 1989; Tagami et al. 2004). The H3.3 variant is deposited in a replication-independent way by the HIRA complex composed in Arabidopsis of Histone Regulator A (HIRA), UBiNuclein 1/2 (UBN1/UBN2), and CALcineurin BINDing protein1 (CABIN1) (Nie et al. 2014; Duc et al. 2015).

Although most of H3-H4 histone chaperones mutants are viable in Arabidopsis, they show pleiotropic phenotypes, reflecting the wide implications in gene regulation through chromatin formation and dynamics. Indeed, simultaneous loss of function of the two Asf1 paralogues (Asf1a and b) leads to severe developmental defects, increased DNA damage sensitivity, and cell cycle mis-regulation (Zhu et al. 2011). *fas* mutants show impaired postembryonic development (Serrano-Cartagena et al. 1999; Exner et al. 2006; Schonrock et al. 2006), defects in maintenance of crucial genes for apical meristem organization, and reduced amounts of heterochromatin (Kaya et al. 2001; Schonrock et al. 2006). Due to an additional role of MSI1 in the Polycomb Repressive Complex 2, mutants in genes of this subunit are embryo-lethal (Kohler et al. 2003;

Guillon and Berger 2005). Hira mutants show mild developmental defects and mis-regulation of specific genes, reduced fertility, and impaired maintenance of transcriptional silencing associated with reduced nucleosome occupancy (Nie et al. 2014; Duc et al. 2015).

1.2 CHROMATIN ORGANIZATION WITHIN THE NUCLEUS

It has become clear in recent years that the structure and organization of the genome within the nucleus is non-random and of critical importance to determine the gene expression program of a cell (Cremer and Cremer 2001; Cavalli and Misteli 2013). In animals, the lamina, a filamentous meshwork formed by Lamins A, B and C at the nuclear periphery has wide implications on chromatin positioning, regulating gene expression and nuclear architecture (Aaronson and Blobel 1975; Gerace and Blobel 1980; reviewed in Dechat et al. 2010). Lamin-associated domains (LADs) are gene-poor, predominantly repressed and depleted of active epigenetic marks (Guelen et al. 2008). Artificial tethering of loci to the nuclear envelope showed that this relocation is sufficient to repress loci in somatic cells (Finlan et al. 2008; Reddy et al. 2008). During differentiation, the repertoire of expressed lamins is subject to changes, and many genes modify their association with the lamina in connection with changes in transcriptional activity (Peric-Hupkes et al. 2010; Solovei et al. 2013). Spectacularly, depletion of lamins A/C and lamin B receptors cause a drastic inversion of the nuclear architecture, causing peripheral heterochromatin to localize at the nuclear interior (Solovei et al. 2013). Moreover, the broad spectrum of diseases caused by mutations in lamin genes, called laminopathies (Nagano and Arahata 2000), underline the important role of chromatin positioning and tethering in gene expression and development. Lamin-independent positioning in connection with chromatin features has also been reported: in *C. elegans*, the CEC-4 protein tethers heterochromatin to the nuclear periphery through interaction with the H3K9me2 histone mark (Gonzalez-Sandoval et al. 2015). Its depletion releases the heterochromatin from the periphery without alleviating silencing, however, upon induction of muscle differentiation, mutant cells fail to fully commit to the induced fate (Gonzalez-Sandoval et al. 2015).

Several additional techniques shed new light on genome organization: chromatin conformation capture (3C) (Dekker et al. 2002) and its recent combination with massive parallel sequencing (Hi-C) (de Wit and de Laat 2012) revealed new features of 3D spatial interaction between multiple genomic loci. Particularly topologically associating domains (TADs), genomic regions preferentially interacting with each other rather than with external regions (Pombo and Dillon 2015), appeared as major structural features of most metazoan genomes (Dekker et al. 2013), and their disruption affect the expression of nearby genes, potentially leading to disease (reviewed in Lupianez et al. 2016). The compaction and position of chromatin have thus a dramatic effect on gene expression in metazoans.

Despite the progress in 3D organization analysis, we know too little whether transcription of individual genes has an effect on the position and/or chromatin modification of its genomic template, and about the sequence of events underlying gene activation. In mammalian embryonic stem cells, transcriptional activation is sufficient to induce gene repositioning toward the nuclear interior (Therizols et al. 2014). However, decompaction of chromatin by an acidic peptide is also sufficient to induce gene relocation to the interior without affecting transcription (Therizols et al. 2014). This suggests that in mammalian cells, chromatin remodeling would precede transcription and drive nuclear rearrangement, rather than transcription being the motor of reorganization in metazoan cell nuclei.

In *Arabidopsis* as a plant model, the situation is different, as no functional lamina homologues have been identified so far. Hi-C experiments showed that, unlike in mammals, TADs are not prominent in the small *Arabidopsis* genome (Feng et al. 2014; Wang et al. 2015a). Instead, the *Arabidopsis* genome is characterized by strong interaction between pericentromeric regions, long range interaction between heterochromatic foci (Feng et al. 2014; Grob et al. 2014), and chromatin loops around transcribed genes (Liu et al. 2016). At the single locus scale, alternative open and close chromatin states at the *GLABRA2* (*GL2*) transcription factor locus were identified corresponding to the transcribed or silent state of the locus, respectively. However, no change in its nuclear position was detected (Costa and Shaw 2006).

The most common and widespread techniques to study chromatin organization, namely Fluorescent *In Situ* Hybridization (FISH), Chromatin ImmunoPrecipitation (ChIP), and Chromatin

Conformation Capture and its derivative (3C, Hi-C) require fixation, the latter ones also a large pool of cells (Pinkel et al. 1986; Lysak et al. 2001; Dekker et al. 2002; Collas 2010; de Wit and de Laat 2012). They are consequently neither able to provide information on dynamic organization of chromatin in living cells nor on its cell-specific patterns (except for 3D FISH that conserves tissue information).

To overcome the need for fixation, approaches such as chromatin tagging or chromatin beacons use fluorescently labeled molecules to visualize the position of large (2–10 kb) transgenic insert within the nucleus in living organism (Kato and Lam 2003; Matzke et al. 2003; Matzke et al. 2005; Amini et al. 2011). Using such techniques, a genome-wide transposon tagging experiment revealed that the genomic position of a locus seems to have only a small impact on its expression (80% of insertion lines have less than 2 fold variation in expression), unless they are located close to known repressive regions like NORs and TEs (Rosin et al. 2008). Using FLC alleles tagged with the lacO repeat array, Rosa and colleagues (2013) provided the first evidence for a link between transcriptional activity with locus position in plants. Indeed, the clustering of FLC loci upon vernalization depended on its transcription (Rosa et al. 2013). Knowledge about dynamic locations of single loci and the relation with transcription in plants is scarce, and the example of the FLC locus remains, if not the only one, the best documented so far.

However, the lacO repeat arrays in the mentioned studies was 5-7 kb long, and the use of such large transgenic inserts has severe limitations. (I) The beacon array is randomly inserted because site-directed insertion is so far difficult to achieve in most plants. (II) The beacon insert can modify the surrounding chromatin environment, which can result in a modified 3D organization (Pecinka et al. 2005; Jovtchev et al. 2008; Del Prete et al. 2014). (III) Kilobase-long tandem repeats can induce repeat-induced transcriptional gene silencing of the array and/or adjacent genomic regions (Pecinka et al. 2005; Jovtchev et al. 2008; Del Prete et al. 2014).

To overcome the need for large transgenic repeats, visualization of endogenous non- or low repetitive regions with high resolution live imaging would be a major step forward. This option faces two problems: (I) specifically targeting the reporter protein to the locus of interest, and (II) concentrating enough fluorescent molecules to obtain a detectable signal over background.

The recent discovery of easily customizable DNA-binding protein such as Transcription Activator Like Effectors (TALE) proteins and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated protein 9 (CAS9) open new possibilities in genome editing but also in live imaging (reviewed in Mali et al. 2013; Moore et al. 2014; Adli 2018). By eliminating their original nuclease activity, reducing them to the DNA binding role, and fusing them with fluorescent proteins, TALE and CRISPR/CAS9 can be turned into easily customizable fluorescent reporters that can potentially target virtually any DNA sequence. Such strategy has been successfully used to image endogenous repetitive sequences in mammalian cells (Chen et al. 2013; Ma et al. 2013; Miyanari et al. 2013; Anton et al. 2014). *In vivo* imaging of repetitive regions has also been recently achieved using a nuclease-deactivated Cas9 (dCAS9) and transient transformation in plant cells (Dreissig et al. 2017). Imaging non-repetitive loci is more challenging because of the need to concentrate the signal at the locus and the high background caused by free-floating unbound fluorescently labeled proteins. Imaging of non-repetitive genomic loci was achieved in mammalian cells using the CRISPR/CAS9 strategy (**Chen et al. 2013; Anton et al. 2014**). The advantage of the CRISPR/CAS9 system, compared to TALE-based approaches, is that the binding target of CAS9 is defined by a small guide RNA (sgRNA) loaded into the protein, rather than by the protein itself (**reviewed in Bhaya et al. 2011**). This makes the design of the experiments much easier, brings down the costs for the components, and allowed non-repetitive locus imaging as the target loci could be covered with 26-36 sgRNA to enrich for the signal and detect it above the background signal (**Chen et al. 2013; Anton et al. 2014**).

At the time my PhD project started (2013), live imaging of defined genomic loci in plants was neither achieved with CAS9 nor with TALE strategy. My first goal was to use a live imaging approach to investigate the events underlying gene activation in plant cell nuclei, with the following key questions: Is it possible to achieve single locus imaging in living plant cells within their tissue context? How does it affect the expression of the locus? Does a locus change its position within the nucleus according to its activation status, as it is the case in mammals (**Therizols et al. 2014; Qin et al. 2017**)? Do all loci respond the same way, and what is the chain of events accompanying locus activation at the chromatin scale? Therefore, I started to develop

tools to achieve single locus imaging in stably transformed Arabidopsis plants, focusing on the CRISPR/CAS9 strategy because of its high versatility and easier customization.

1.3 LARGE-SCALE NUCLEAR ARCHITECTURE REARRANGEMENT DURING THE LIFE CYCLE OF PLANTS

Developmental transitions are crucial steps in a plant's life cycle, when transcriptional profiles are rearranged to cope with the new condition or requirement. Accompanying such transcriptome changes, nuclei can undergo mild to drastic rearrangement of their architecture. There are several examples: During female gamete formation, the megaspore mother cell, which will give rise to germinal cells, undergoes large chromatin decompaction with reduction in heterochromatin content, depletion of linker histones and changes in histone variants and modification chromatin landscapes (She et al. 2013). Upon seed maturation, nuclei in embryonic cotyledons undergo reduction of size and an increase in chromatin compaction, resulting in densely compacted chromocenters (van Zanten et al. 2011). In contrast, upon germination, nuclear size increases in a process depending on the nuclear matrix proteins CROWDED NUCLEI 1 and 2 (CRWN1/2), and heterochromatin get extensively dispersed (van Zanten et al. 2011). The formation of conspicuous chromocenters in cotyledons occurs in 4-5 days during the transition of the early seedling to a photosynthesis-competent state (Mathieu et al. 2003; Benoit et al. 2013). This process depends on light perception by the blue light receptors CRYPTOCHROM 1 and 2 as well as on the activity of CAF-1 (Bourbousse et al. 2015; Benoit et al. 2019). Reorganization of nuclear architecture can also be transient: nuclei in rosette leaves undergo a transient reduction of chromocenters and decompaction of chromatin in gene rich regions upon transition to flowering (Tessadori et al. 2007b).

Cellular differentiation can be considered as a developmental transition at the level of individual cells, as a newly generated cell will change its transcriptional signature upon acquisition of a defined identity (Birnbaum et al. 2003). The question thus rises whether differentiation would also be accompanied by similar changes in nuclear architecture and chromatin rearrangements as during developmental transitions.

Such correlations are documented for animal cells, where differentiation can involve drastic changes in chromatin compaction, dynamics of chromatin proteins and their subnuclear localization (Meshorer et al. 2006; Ueda et al. 2014; reviewed in Perino and Veenstra 2016). In mammalian cell nuclei, heterochromatin patterns have been suggested as a marker for differentiation states (Ueda et al. 2014). However, reports about nuclear architecture dynamics during differentiation in plants remain scarce. Cells in the Arabidopsis root present a variety of nuclear shapes that correlates with cell identity and differentiation, (Chytilova et al. 1999). Factors regulating nuclear morphology in plants started to attract attention only recently, and their link with cell identity remains elusive (Dittmer et al. 2007b; Walters et al. 2012; Sakamoto and Takagi 2013; Jevtic et al. 2014; Tatout et al. 2014). Positional information in the root epidermis, determining trichoblast (progenitors of root hair cells) and atrichoblast (cells remaining hairless) identity, was shown to induce alternative chromatin conformation at and correlating with the activity of the *GLABRA 2* locus, coding for a key determinant of cell identity (Costa and Shaw 2006). Histone H2B mobility and H3.1/H3.3 ratio had also been shown to be a marker of cell differentiation in the root. Indeed, a high H2B mobility and H3.1/H3.3 ratio is observed in undifferentiated, fast proliferating cells of the root apical meristem (Rosa et al. 2014; Otero et al. 2016). H3.1 is then evicted from nuclei of cells entering the elongation/differentiation zone of the root, and H2B mobility decreases as cells differentiate and reach a mature stage (Rosa et al. 2014; Otero et al. 2016). Additionally, dedifferentiation of mesophyll cells into protoplasts leads to chromocenter decondensation (Tessadori et al. 2007a). However, the dynamics of heterochromatin during cell differentiation remained elusive in plants. Therefore, I worked towards the development of tools and methods to achieve live imaging of nuclear architecture dynamic and subnuclear chromatin structures during differentiation in cells within their tissue context, using the transparent roots of young Arabidopsis seedlings expressing a fluorescently tagged Histone 2B (H2B-RFP) as a proxy to image chromatin.

1.4 CHANGES IN NUCLEAR ARCHITECTURE IN RESPONSE TO STRESSES

In addition to developmental transitions, response and adaptation to stresses also involve an elaborated transcriptional response, often concomitant with remodeling of the chromatin landscape (reviewed in Probst and Mittelsten Scheid 2015). Growth in low light lead to reversible decompaction of chromocenters in Arabidopsis (van Zanten et al. 2010), and growth under mild salinity in rice causes extensive decompaction of the 45S rDNA loci (Santos et al. 2011). Protoplast formation was shown to lead to chromocenter disruption. The reduction of heterochromatin occurred independent from changes in DNA methylation and H3K9me2 levels and included the 5S rDNA and 180 bp repeats, however, without reactivation of silent elements and only a partial decompaction of 45S rDNA loci (Tessadori et al. 2007a). Heat stress was also shown to modify nuclear organization. Indeed, in rice and/or rye, HS leads to decompaction of 45S and 5S rDNA loci (Santos et al. 2011; Tomas et al. 2013b). In Arabidopsis, prolonged heat stress (HS) was previously shown to lead to substantial but transient rearrangement and decompaction of heterochromatin (Pecinka et al. 2010). Study of selected targets showed that usually transcriptionally silent loci (180 bp centromeric repeats, 5S rDNA and certain transposable elements) became transiently activated upon HS, also here without changes in epigenetic modifications such as DNA methylation and repressive histone modifications. Instead, the extended heat stress induced a substantial but transient dissociation of nucleosomes from the DNA. Reloading of nucleosomes upon recovery after heat stress involves the activity of the H3-H4 chaperone CAF-1 (Pecinka et al. 2010). The heat-induced decompaction of chromocenters and release of transcriptional gene silencing (TGS) depends on HEAT-INTOLERANT 4 (HIT4) which re-localizes from chromocenters to the nucleolus upon heat stress (Wang et al. 2013b; Wang et al. 2015b).

The study by Pecinka and colleagues (2010) was conducted with fixed and extracted nuclei from whole seedlings and was limited to qualitative aspects of the nuclear response. Whether individual affected nuclei where indeed able to restore the previous chromatin configuration after the stress, or whether nuclei with regular features were exclusively originating from newly divided cells, remained an open question. To follow-up on the previous study and to better understand the dynamics of HS-induced chromocenter decompaction and the potential role of

histone chaperones in this response, I used live imaging of nuclei labeled with chromatin reporters, to study the quantitative and dynamic aspects of the nuclear response to HS in living nuclei within their native tissue context.

1.5 AIMS OF THE THESIS

Work described in this PhD thesis aimed at using live imaging as a tool to describe dynamic processes and to answer different biological questions. (1) I first attempted to achieve live imaging of single locus dynamics upon gene activation in stably transformed Arabidopsis plant. As an important prerequisite for this, I set out to (2) develop tools and methods to track individual nuclei in living cells during root growth for continuous microscopic image acquisition. I then (3) confirmed suitability of the system by documenting nuclear shape dynamics during a well-characterized differentiation process. Finally, (4) I applied this live imaging setup to study the dynamics of nuclear architecture and heterochromatin in response to heat stress *in vivo*.

2 RESULTS

2.1 TOWARDS LIVE IMAGING OF SPECIFIC SINGLE GENOMIC LOCI

The original plan for this PhD project was establishing a microscopy approach to investigate the events underlying gene activation in plant nuclei by imaging the position of defined single loci, aiming to answer several technical and biological questions: Is it possible to achieve single locus imaging in living plant cells within their tissue context and to track the locus over a long period of time, i.e. 10 h? If single locus positioning is possible, how does it correlate with the expression of the locus? Does a locus position change according to its activation status, as it is the case in mammals (Therizols et al. 2014; Qin et al. 2017)? Do all loci respond the same way to their activation? What is the chain of events accompanying locus activation at the chromatin scale? To answer these questions, I wanted to image the position of single locus within the nucleus of living plant organs, in order to visualize the sequence of events accompanying gene activation, with a focus on chromatin context rearrangement and dynamic movements upon changes of gene activity.

In order to correlate the transcriptional status of a gene with the gene's position in the nucleus, the plan was to combine a gene activation reporter with a gene position reporter. To find suitable gene activation reporters, I screened a GAL4-GFP enhancer trap collection (Haseloff 1999) for GFP patterns corresponding to gene activation in the root (Figure 1). Specifically, the GFP signal should be detected only in certain cells of the root, depending on developmental or tissue-specific cues. For the locus position reporter, I chose to use the CRISPR/Cas9 technology, engineering a nuclease-deactivated Cas9 (dCas9) fused with an array of 3 mCherry tags (3xmCherry), proteins fluorescing in the red spectrum and placed in an array to enrich for the signal. The binding of the dCas9-3xmCherry would be directed to the GAL4 locus by several gRNAs spanning this target, in order to enrich for the local mCherry signal. This design would allow to apply the method with the same gRNAs to the whole enhancer trap collection sharing the GAL4 sequence randomly inserted in different places of the genome in each line of the collection. It would also be feasible to apply the method to different organism where GAL4 enhancer trap collections are available, like in flies (Hayashi et al. 2002).

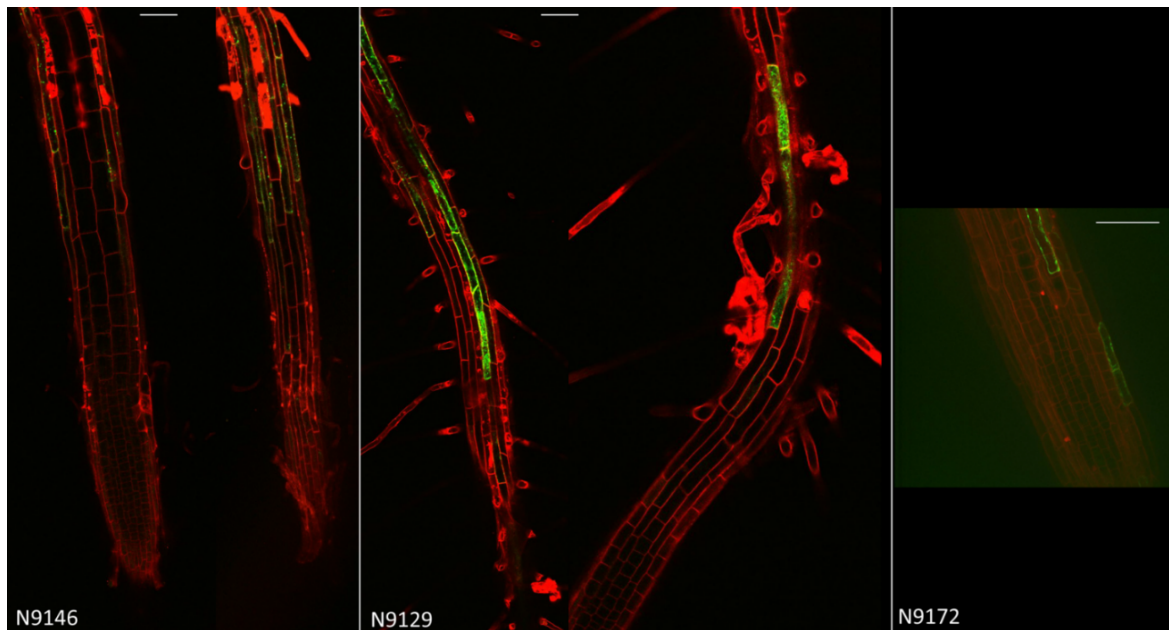


Figure 1 GFP reporting transcriptional activation in specific root cells of enhancer trap lines.

Representative pictures of some lines selected as suitable locus activation reporter. Propidium iodine staining is displayed in red, GFP signal in green, collection numbers in the left lower corner. Scale bar = 50 μ m.

Live imaging of a single, non-repetitive locus has been achieved in animal cells using different methods, from increasing the number of gRNA tiling along the target locus to increasing the number of fluorescent molecules bound to the Cas9 protein or the gRNA (Chen et al. 2013; Tanenbaum et al. 2014; Qin et al. 2017; Ye et al. 2017). In their study, Chen and colleagues determined the threshold for locus detection to the use of 26-36 sgRNAs tiling the locus, corresponding to the same number of green fluorescent proteins (EGFP) assembled on site. Considering the lower brightness and the concatenation of three mCherry proteins, this would correspond approximately to 18-25 dCas9-3xmCherry proteins, spanning the locus in my case. However, the authors noted that the required number of sgRNAs might be reduced by decreasing the background level of unbound dCas9-EGFP, which was quite high in their study. To follow up on this indication, I consulted Wolfgang Busch, and he suggested promoters based on data from Cartwright et al. (2009), to drive dCas9-3xmCherry expression based on their low constitutive expression in roots, in order to reduce potential background of unbound dCas9-3xmCherry (Table 1). The strong constitutive promoter UBQ10 was also included in the cloning scheme as well as an alcohol-inducible dCas9-3xmCherry construct. The plan was to first clone the promoters to

drive the 3xmCherry alone and, based on expression levels, to further select appropriate promoters. In the next step, the dCas9 coding sequence should be cloned between promoter and mCherry ORF in frame (Figure 2). To note: because the enhancer trap line collection is in the background of the C24 ecotype, all constructs were transformed into C24 as well as in Col-0

Pre-selected promoters	Short name
AT1G01790	101
AT1G02100	102
AT1G22910	122
AT1G28230	128
AT1G54460	154
AT1G67140	167
AT2G47850	247
AT3G08960	308
AT4G18600	418
AT5G57610	557
UBQ10 (AT4G05320)	U

Table 1 Table of the selected promoter to drive dCas9-3xmCherry

Selected promoter from the indicated genes and their short names used in construct labeling.

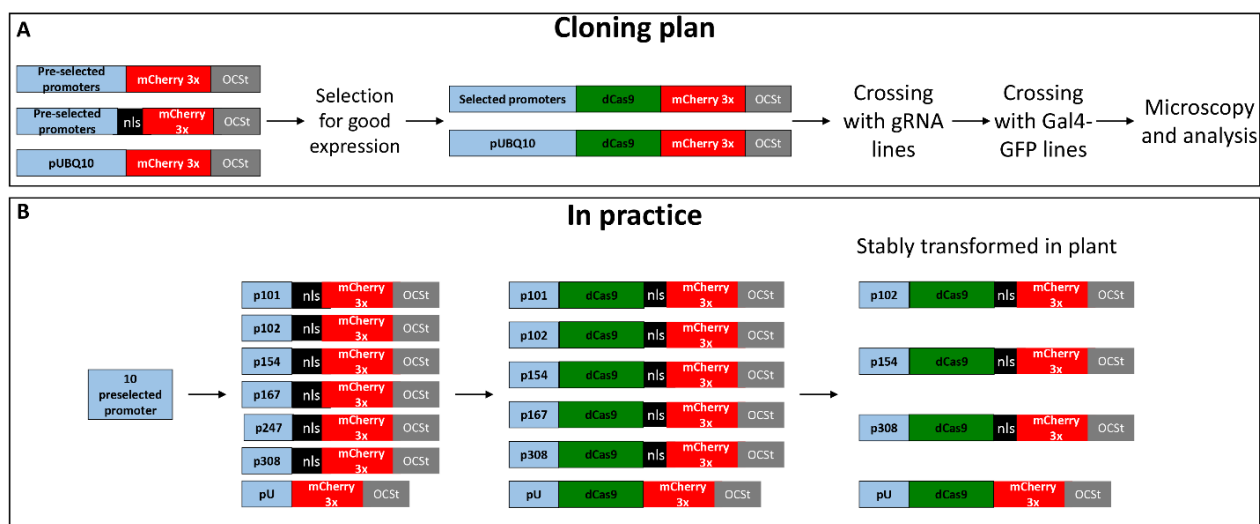


Figure 2 Scheme of the constructs designed for single locus live imaging.

(A) Constructs and transformations planned for the project. (B) Constructs generated during the project. Blue boxes represent promoters; green, black, and red boxes represent coding sequences (CDS), grey boxes represent the terminator.

Six out of ten pre-selected promoters were successfully cloned into the pGreen0229 vector, transferred to agrobacterium and transformed into plants. I managed to have T3 homozygous

from five of these promoters. Screening roots for mCherry signals in T2 lines was unsuccessful. I then decided to add a nuclear localization signal (NLS) to the mCherry construct to concentrate the signal in the nucleus. I repeated the screen with the NLS-containing constructs but could still not detect mCherry signals in roots. However, in tissues where the promoters were predicted to have higher expression like pollen grain or cauline leaves (according to the online eFP browser http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi), a nuclear mCherry signal could be detected (Figure 3). Fluorescent signals were also detected in lines with the UBQ10 promoter driving mCherry expression, but these lines were available only later in the project, due to problems in cloning and transformation (Figure 3). These data show that the constructs were functional but needed a certain expression threshold to result in fluorescence visible in the nucleus. Nevertheless, I proceeded by adding the dCas9 CDS to the constructs, based on the hypothesis that the reporter would still be expressed in the roots as “invisible background” but concentration of the dCas9-3xmCherry by gRNAs at the GAL4 site would allow detection. Although I did not finish generating plants with all constructs and all promoters, it became clear that no mCherry signal was detectable in any of these plants.

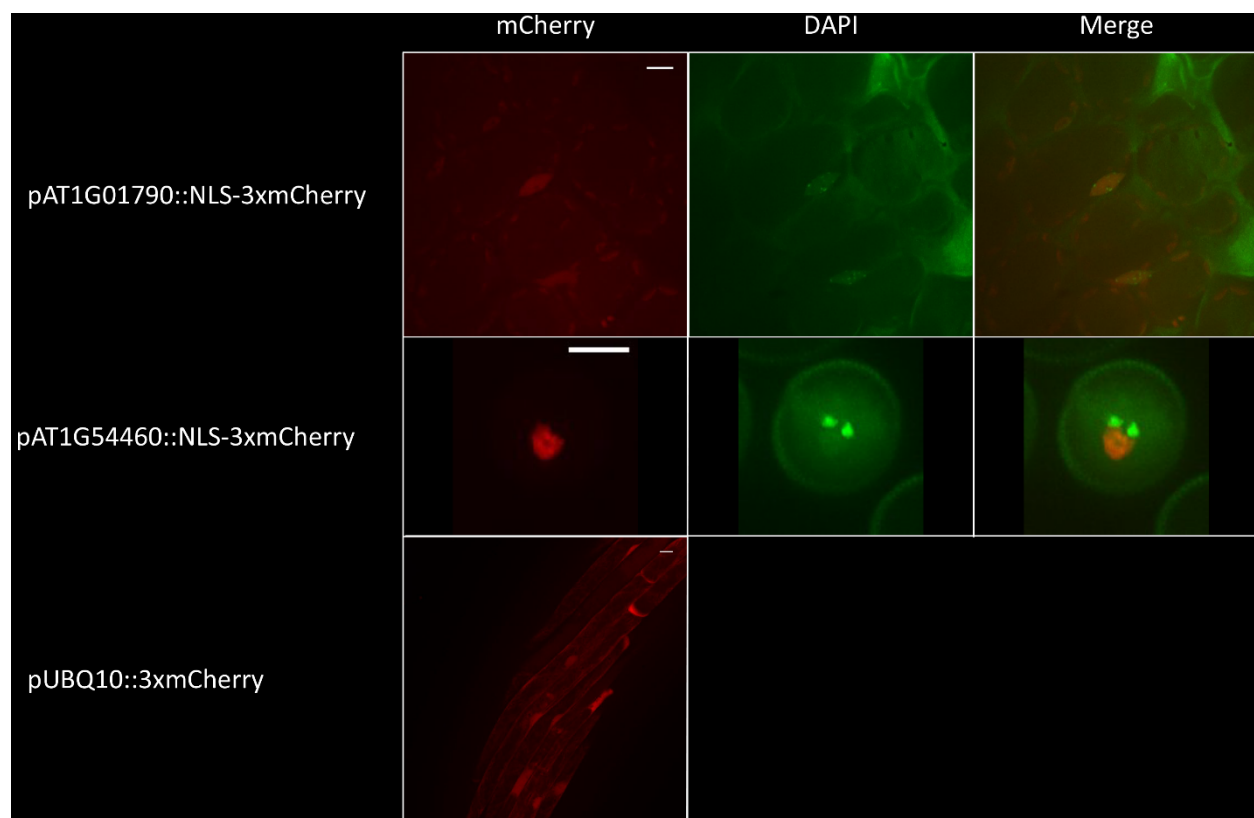


Figure 3 Detection of 3x-mCherry signal.

In living plants, mCherry fluorescence is detected in tissues with higher expression from the promoters. The name of the construct is indicated on the right side. pAT1G01790 is imaged in leaf, pAt1G54460 is imaged in pollen and pUBQ10 is imaged in root. Scale bars are 10 μ m.

Taking into account the low chance to observe a dCas9-3xmCherry signal and the time required to transform these lines with gRNA and introgress the resulting gRNA dCas9-3xmCherry lines into the selected Gal4-GFP reporters by crossing, it was decided in September 2016 to stop the attempt of stable expression and generate a proof of concept with transient expression. For this I used the dCas9-3xmCherry driven by the UBQ10 promoter to target telomers as endogenous repetitive sequences, in order to enrich for the signal. I could use gRNA sequences, kindly provided by Andreas Houben, that had been proven to work (Dreissig et al. 2017). Cloning the additional constructs was arranged to be done in the lab of Wolfgang Busch, but unfortunately, the person in charge unexpectedly left the institute for personal reasons and left neither material nor information. However, I could prove the functionality of the dCas9-3xmCherry construct for repetitive targets in collaboration with Idan Pereman from the lab of Avraham Levy at the Weizmann Institute. Idan used *Nicotiana benthamiana* plants transgenic for

LacOx256, a synthetic array consisting of 256 repeats of the short LacO sequence and co-infiltrated leaves with *Agrobacterium* strains containing a gRNA-LacO construct targeting the LacO array and my construct coding for dCas9-3xmCherry. He could observe 2-3 mCherry foci per nucleus if both constructs were co-infiltrated. In the absence of gRNA-LacO, Idan observed only a diffuse mCherry signal in the nuclei (Figure 4). This shows that my dCas9-3xmCherry construct was functional *in planta*.

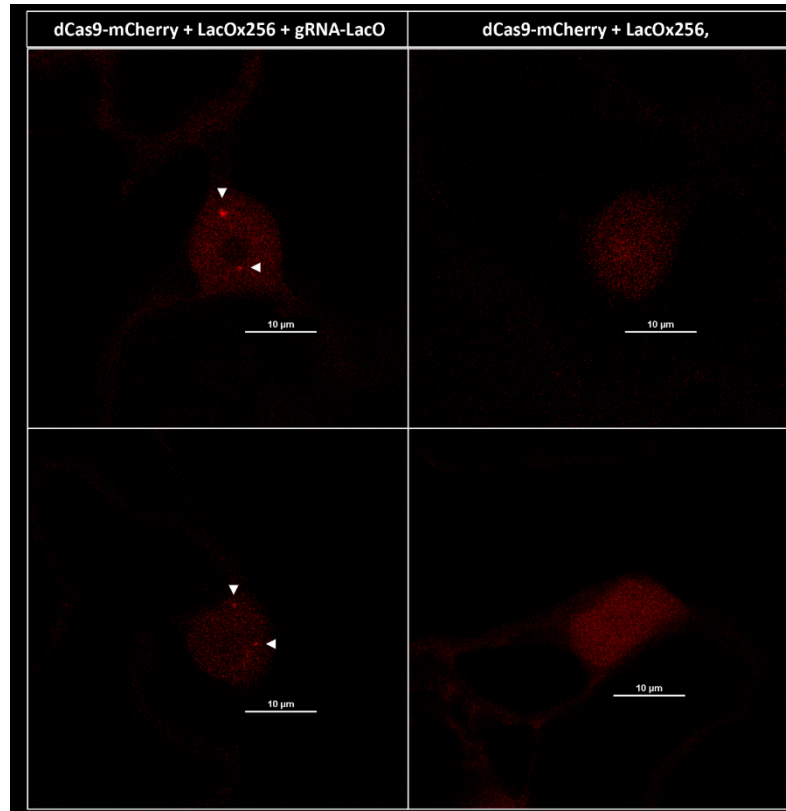


Figure 4 Detection of dCas9-3xmCherry foci in cell nuclei upon transient transformation. The plasmids used in the transient transformation by *Agrobacterium* infiltration of *Nicotiana benthamiana* leaves transgenic for lacO repeats are indicated at the top of the images. Images were taken by Idan Pereman (Weizmann Institute of Science).

However, the observed signal was much weaker than expected for expression by the AtUBQ10 constitutive promoter, even though this promoter is known to be relatively weak in *N. benthamiana* (Grefen et al. 2010). This low signal, despite the high enrichment at the 256 repeat array, in combination with the failed attempts to visualize dCas9-3xmCherry in transgenic plants *in vivo*, indicated limitations in the expression, possibly due to a non-optimal promoter-terminator combination or protein instability. Later, I managed to obtain one T2 line expressing pUBQ10::dCas9-3xmCherry where I could detect a weak nuclear mCherry signal (Figure 5). However, this line became available only in July 2017, when I had shifted to another project.

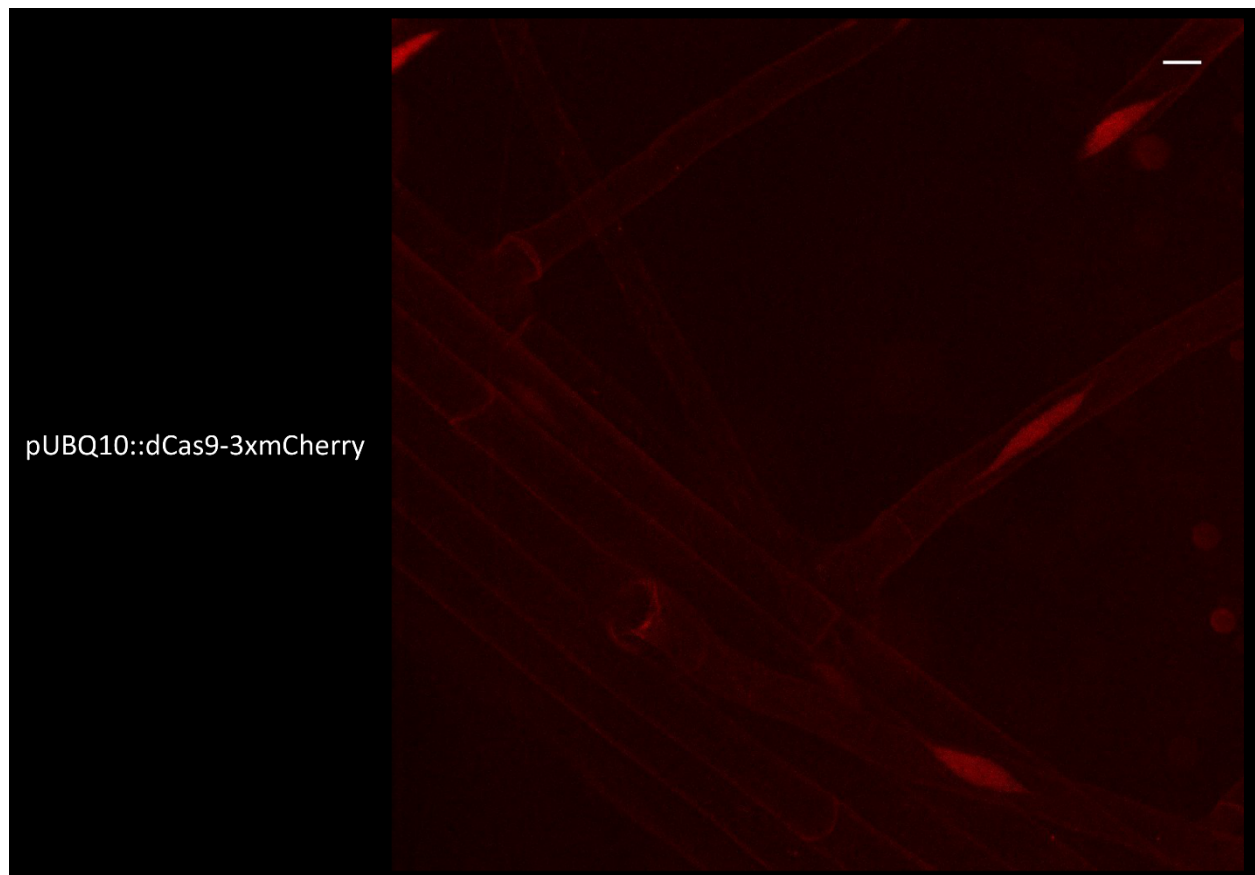


Figure 5 Expression of dCas9-3xmCherry in stably transformed plants.

The reporter protein is driven by the UBQ10 promoter and stains nuclei. Detection of the weak mCherry signal required strong illumination resulting in autofluorescence of the cell walls. Scale bar = 10 μ m.

2.2 ESTABLISHING LIVE IMAGING OF NUCLEAR ARCHITECTURE IN ROOT CELLS

Independent from the availability of reporter lines, achieving single locus imaging during gene activation requires to track one nucleus or a group of nuclei under the microscope over longer periods, with the potential to follow activation of the selected genes and to capture the potential changes in locus position and its chromatin architecture within the nucleus. The selected loci reported by the GFP enhancer trap lines express GFP depending on cell differentiation. Therefore, parallel to cloning the tools for single locus imaging described above, I worked on establishing a setup to achieve live imaging and tracking of root cell nuclear architecture during differentiation. For this I used chromatin reporters, kindly provided by the lab of Frederic Berger (GMI), consisting of histone genes fused with that for Red Fluorescent Protein (RFP) and controlled by the native promoters of the histone genes. Among the different reporters provided by the Berger lab, I chose histone 2 A10-RFP (H2A-RFP) because it gave the brightest signal in the root tip and stained the whole nucleus, except the nucleolus. I focused on nuclei in the root epidermis cells because they are most external and thus most accessible for microscopy. The epidermis has also the advantage to contain two distinct cell types next to each other in the same tissue, namely trichoblasts (root hair cells) and atrichoblasts (hair-less cells). This characteristic could be used to investigate the relationship between nuclear architecture and cell identity (see section 2.3).

Cell differentiation in the root is a constant process starting with the generation of new cells by the root apical meristem (RAM) located at the tip of the root. Newly generated cells progressively differentiate with growing relative distance to the root tip. Different developmental zones are defined by this distance and by characteristics of the cells. Briefly, in the RAM, specialized stem cells produce new cells which then undergo a series of cell divisions leading to the amplification of cell number (Scheres et al. 1994; van den Berg et al. 1998). Above this Division Zone (DZ), cells enter the so-called Transition Zone (TZ) where cell divisions become rare and cells slowly increase in size, while nuclei remain round and located in the middle of the cell. To the upper end of the TZ, nuclei undergo endoreduplication before the cells quickly elongate (Verbelen et al. 2006; Hayashi et al. 2013). This fast development in the Elongation Zone (EZ) is the primary factor of root growth, as cell size can quadruple in two hours, up to a 10-fold increase

in size over five hours. Only here starts also the bulging of root hairs from trichoblast epidermal cells. The nuclei are pushed to the side of the cells by expansion of the central vacuole and start elongating. At the upper end of the EZ, cell elongation slows down, and cells reach their final length. This zone is characterized by the full development of root hairs from trichoblast cells (Verbelen et al. 2006; Band et al. 2012; Hayashi et al. 2013).

I decided to start imaging at the upper end of the TZ to capture the most dramatic changes of nuclear architecture. The elongation/differentiation phase is known to last between 5 and 8 h (Band et al. 2012; Pavelescu et al. 2018), and I have set up the live imaging experiments to cover this period.

Establishing live nuclear tracking during Arabidopsis root cell differentiation

To image and track live cell nuclei over a long period of time, the microscopy system must fulfill the following requirements: 1) minimizing the amount of light, or photon dose, applied to the samples to reduce potential photodamage and bleaching of the chromatin reporter; and 2) a fast image acquisition that is able to record tissue context while achieving a 3D sub-nuclear resolution.

Three microscopy system could be considered: (1) widefield, (2) selective plane illumination (also called light sheet), and (3) spinning disc confocal microscopy (live imaging systems reviewed in Cole 2014). Widefield microscopy followed by deconvolution of acquired pictures would minimize the photon dose, but the thickness of the Arabidopsis root (100 μm diameter) would result in too much light outside the focus plane that could not be removed by deconvolution and would reduce spatial resolution (Swedlow and Platani 2002). The light sheet principle would be a superior microscopy system for long term imaging, however, preliminary experiments with an inhouse-built light sheet microscope revealed that the spatial resolution was not sufficient to resolve the chromocenters. Moreover, none of the current commercially available light sheet microscope systems, neither any super resolution light sheet systems (lattice light sheet) are suited for very long-term imaging of living plants. Therefore, I have chosen spinning disc confocal microscopy as a good compromise between fast image acquisition, reduced light exposure of the sample, and a good spatial resolution able to resolve subnuclear

structures. More specifically, I worked with an UltraView Vox Axio Observer Spinning Disc controlled by the Volocity® program.

Tracking individual nuclei during cell differentiation in the root required (1) that the root is able to grow in conditions suitable for microscopy; (2) that steps between consecutive timepoints are small enough to identify the same nuclei with certainty over time and in spite of root growth; (3) that the recording time is long enough to capture the transition between developmental zones.

For imaging living plants, I used imaging chambers, and I covered the root with a layer of solid medium with the same composition of nutrients as in the preculture of the seedlings. Care had to be taken not to let the roots dry out during the transfer, but my protocol ensured 100% root growth in the chambers (see Material and Methods).

Then, using H2A-RFP expressing lines, I started with time lapse imaging to have an overview of the root growth in this system. I imaged a region of interest (ROI) including the root tip and the free space where I predicted the root to grow. A ROI is composed of multiple field of views stitched together, resulting in a bigger picture while conserving the image resolution. An example of such time-lapse recording is given in Movie 1 and Figure 6. Movies from time-lapses produced in this thesis are available online, see Material and Methods. From these data, I determined the root growth rate for 3-5 d-old Arabidopsis seedlings growing under these conditions (Table 2) to be between 90 and 200 $\mu\text{m}/\text{h}$, with an average at 135 $\mu\text{m}/\text{h}$. In Movie 1, the red square represents the size of one field of view (FOV) with my selected settings, ca. 106 μm side length.

	Mean	Min	Max	S.D.
Growth rate $\mu\text{m}/\text{min}$	2.029	1.480	2.898	0.337
Growth rate $\mu\text{m}/\text{h}$	135.113	93.135	203.430	30.637
Growth rate $\mu\text{m}/10\text{min}$	22.519	15.523	33.905	5.106

Table 2 Measurement of root growth in live imaging condition.

Root growth speed measured from $n = 6$ roots, 4-12 measurement per root.

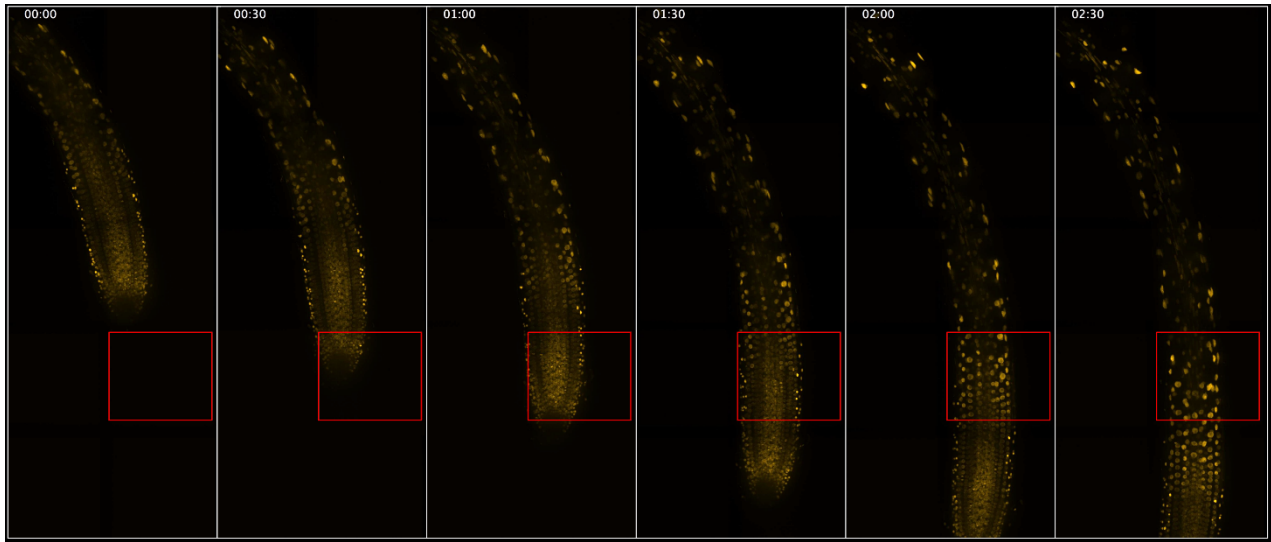


Figure 6 Root growth in live imaging condition.

Snapshot from Movie 1. The images are composed of multiple fields of view stitched together. The red rectangle represents one field of view. Time after the start of the experiment is indicated in h:min in the top left corner.

According to the average growth speed, cell nuclei located in or below the elongation zone will stay in the FOV for around 30-60 min. To keep track of an object, its position should shift as little as possible between two timepoints. Because of fast growth of the root and the goal of long-term imaging, I set the interval between successive imaging at 10 min. This left two options for long-term nuclear tracking: (1) imaging multiple FOV, stitching them together and designing a ROI including the root tip and enough space downwards to capture the differentiation process of newly generated cells upon root growth; (2) imaging one FOV and readjusting the microscope stage to maintain the cell nuclei of interest within the FOV.

Multiple FOV imaging presents a number of limitations. First, once the ROI is designed it is not possible to readjust the position of the sample if the root deviates from its predicted growth direction, not uncommon due to the horizontal position. It also increases the size of the final file (on average 8 h imaging with one FOV represents 1.5 Gb, multiplied with the number of necessary FOVs), which can cause issues with data storage and data handling (the Volocity software tends to crash when handling large files). Additionally, spinning disc images are not fully homogeneous in signal intensity. Intrinsically, the signal drops on the edges of the FOV. Multiple

FOV stitched together amplify this problem, as an object transiting through FOVs might vary in signal intensity, which will impair subsequent analysis.

I thus decided for single FOV imaging. This option allows the readjustment of the microscope stage so that the objects of interest are positioned in the middle of the image. It also reduces the probability of software crashes and allows monitoring and readjusting the sample position for potential z drift between acquisition timepoints, although this should be kept to a minimum as it increases the light exposure of the sample. However, single FOV also has limitations. It involves a frequent readjustment of the microscope stage to keep the object of interest in the center of the FOV and provides little information about the wider context of the image. An example of time-lapse without readjustment is presented in Figure 7. In this example, the nucleus of interest is almost out of sight after just 30 min of imaging. Additionally, nuclei on the edge of the FOV are less bright than those in a more central position.

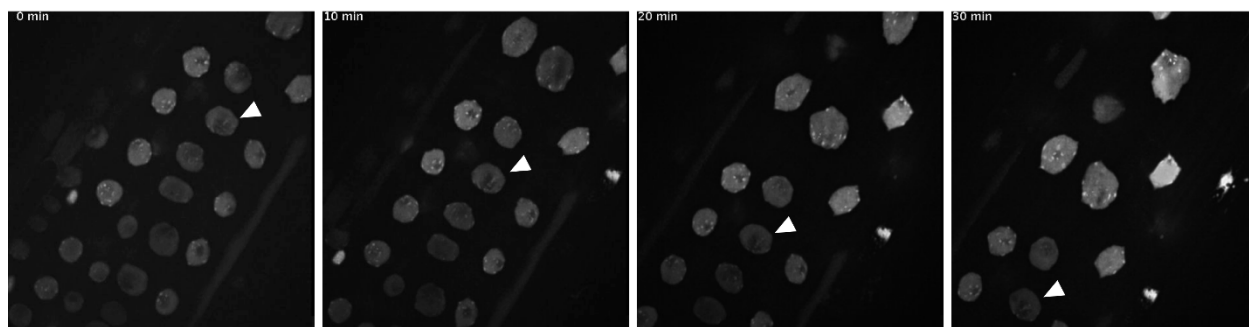


Figure 7 Objects of interest move out of the field of view quickly.

Snapshot of nuclei in a growing root without stage readjustment. The arrow indicates the same nucleus. Note that 30 min after the start of imaging the marked nucleus is almost out of the field of view and that nuclei close to the edge have lower intensity compared to the ones in the center.

In one experiment, I manually readjusted the position of the stage every 10-20 min for around 7 h. The results are shown in Figure 8 and Movie 2. This option is obviously not practicable for longer terms but the experiment highlighted factors to take into consideration. Indeed, I observed a shift in the z axis over time, partly due to the transition between the root tip and the elongation zone and partly due to the intrinsic root growth. This shift can lead to incomplete 3D information about nuclei of interest. I could also observe that after around 3 h of imaging, the growth direction changed and the root started to rotate around the axis, bringing the nuclei of

interest out of focus. Considering this, I increased the z interval of the 3D stacks and paid extra care to the mounting of the imaging chamber (see Material and Methods). Indeed, if the root is oriented as straight as possible in the chamber, chances that it starts turning are lowered.

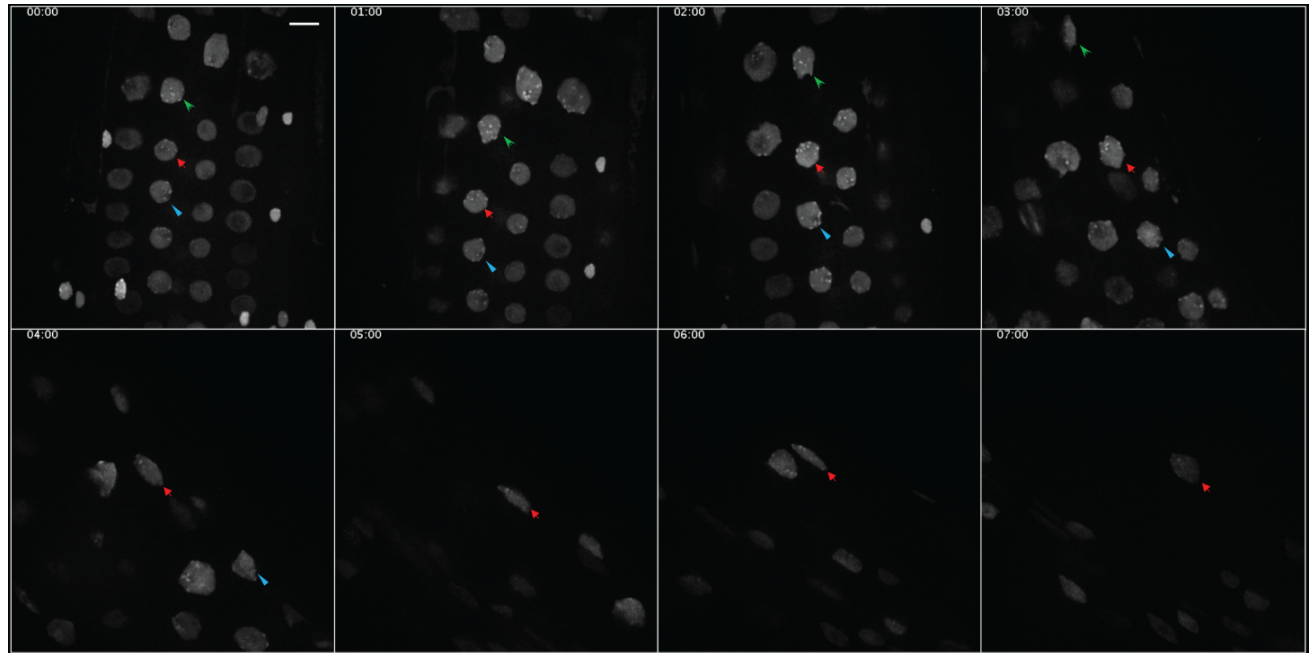


Figure 8 Manual tracking during root growth.

Snapshots from Movie 2 conducted on an H2A10-RFP expressing plant. Colored arrows indicate the same nuclei at the different timepoints. Time from the start of the experiment are indicated in h:min in the top left corner. Scale bar is 10 μ m.

Because manual tracking was not practicable on the long term, I tried to automatize the nuclear tracking. A tracking program called TARDIS (standing for Tracking Arabidopsis Root through Differentiation Stages) was created with the help of Christian Göschl in the lab of Wolfgang Busch, GMI. The program was imputing a manually calculated readjustment vector to the motorized stage controller to counteract root growth (see Material and Methods for details). Examples of such tracking experiments are shown in Figure 9 and Movie 3 and Movie 4. However, the initial readjustment vector needed to be recalculated over time, allowing only a semi-automated tracking for up to 7 h. The length of automatic imaging depended on multiple factors: "cooperation" of the root (straight growth and low z shift), stability of the software operation during the imaging, and the availability of the microscope. Because of the frequent need for

manual readjustment, it depended also on my availability and compatibility with working hours and lab routine. Therefore, I planned for a fully automated tracking system.

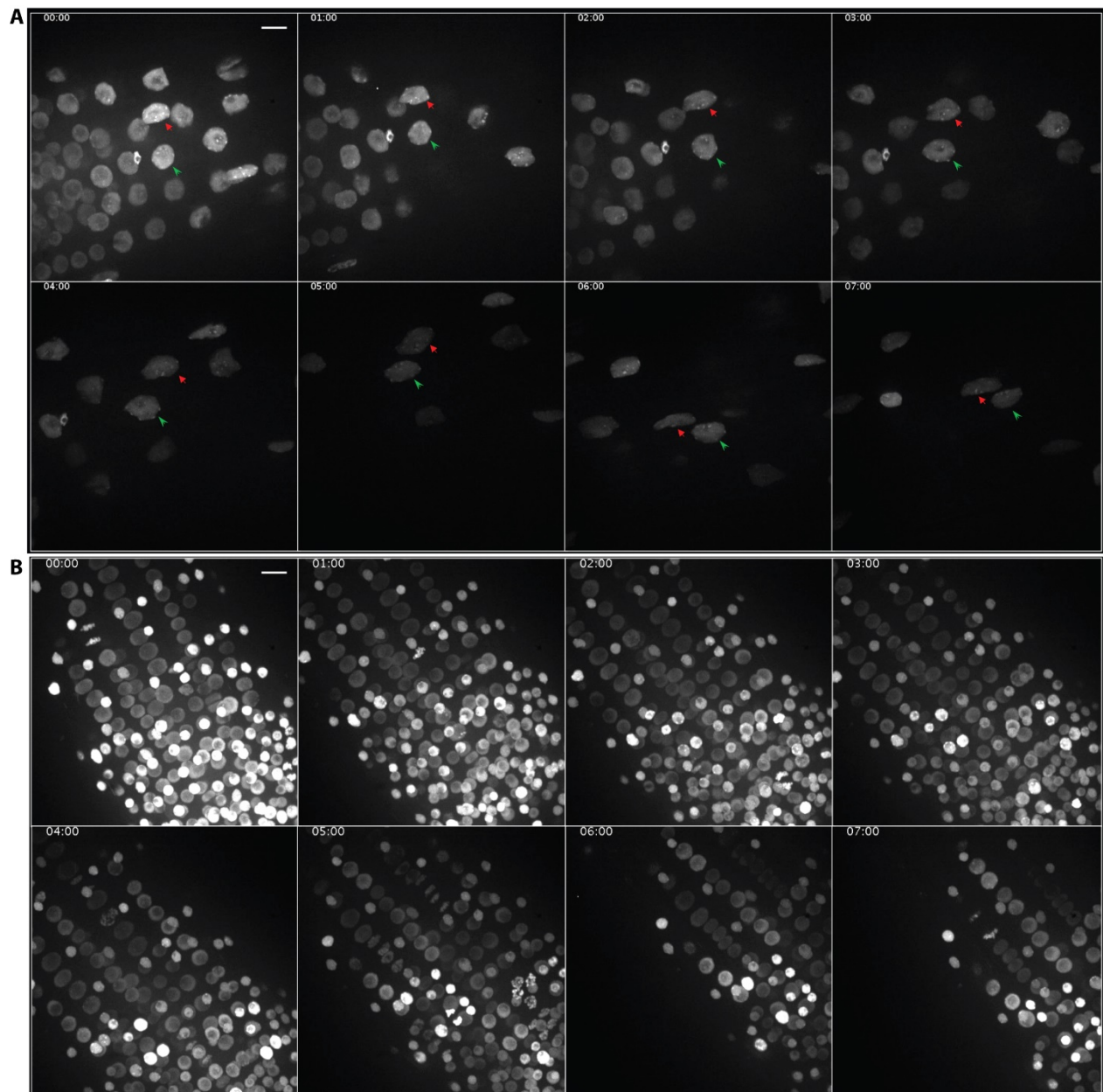


Figure 9 Semi-automated tracking during root growth.

Snapshots from Movie 4 (top) and Movie 3 (Bottom) conducted on H2A10-RFP-expressing plants. Colored arrows indicate the same nuclei at the different timepoints. Time from the start of the experiment is indicated in h:min in the top left corner. Scale bar is 10 μ m.

In collaboration with Miloš Šrámek (Scientific Visualization, GMI), we designed a second version of TARDIS (see Material and Method for details). Briefly, the program works by considering

each newly acquired timepoint and using the last two consecutive timepoints to predict the following. Examples of such tracking experiments are shown in Figure 10 and Movie 5, with another chromatin reporter consisting of a histone 2 B fused with RFP (H2B-RFP).

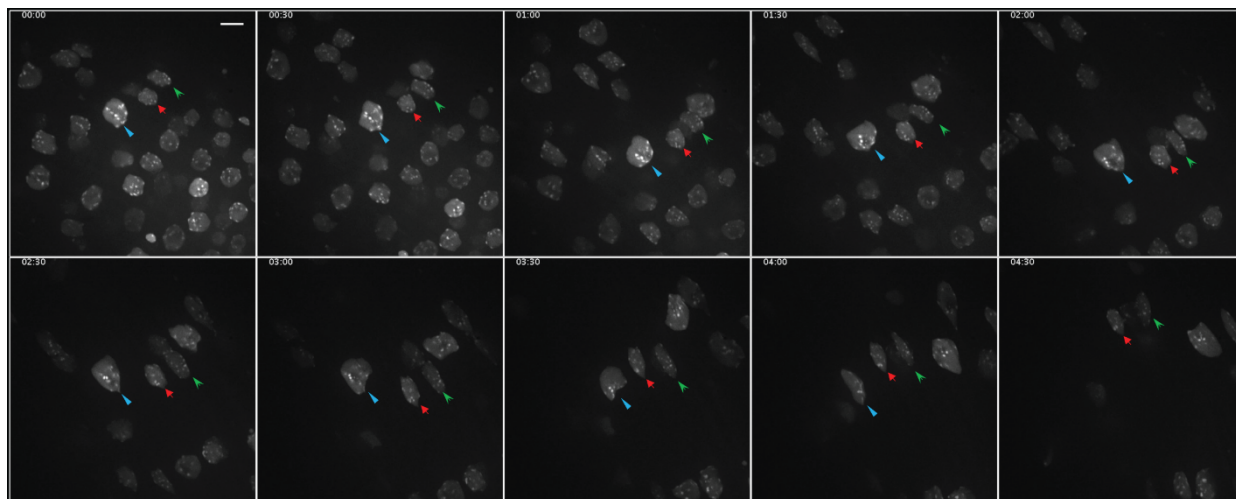


Figure 10 Automated tracking of RFP-stained nuclei during root growth.

Snapshots from Movie 5 recorded with H2B-RFP-expressing plants. The automated stage readjustment was achieved with the second version of the TARDIS software. Colored arrows indicate the same nuclei at different timepoints. Time from the start of the experiment is indicated in h:min in the top left corner. Scale bar is 10 μ m.

The second version of the TARDIS had the advantage to work fully automated, except for the initial prediction vector. It has the advantage to be completely independent of the imaging software and only requires access to recorded data and the motorized stage. It has nevertheless some limitations. The program needs to be adapted to the programming language or set of commands of the stage controller if different. Because the piezo controlling the z position of the stage cannot be remotely readjusted, shifts in the z axis for the nuclei of interest can move them out of sight. The shift of position between two consecutive timepoints must be small enough (around 20-50 μ m, the range shown in the tracking experiments in Figure 8-10 and associated movies) for the program to predict the next position. Currently, it is not possible to choose which element of the picture the program will pick to predict the next position. So far, the program seems to use the brightest object with the most distinguishable shape.

To sum up: The current microscopy setup, in combination with the use of an imaging chamber, allows tracking epidermal and cortex cell nuclei in a growing root in a fully automated

way over a period of 4-7 h with a 10 min interval between successive image acquisitions. With a magnification able to resolve subnuclear structures like compacted heterochromatin, it is possible to monitor the number of chromocenters over time and the relative heterochromatin fraction or volume (RHF or RHV). Examples are shown in Figure 11. The effective time of acquisition is usually about 4-5 h; later, the objects start to be significantly bleached or out of focus. However, this period is sufficient to capture the elongation of the nuclei and the rearrangement and movement of chromocenters within nuclei. To optimize tracking and acquisition, the use of seedlings 4-7 days after germination is recommended, as older plants will hardly fit into the chamber. Acquisition settings have to maximize contrast between objects of interest and the background while minimizing laser power used to excite the fluorescent proteins. An increase in exposure should be preferred over an increase in laser intensity. In the same way, single color imaging should be preferred as it reduces the amount of light exposure of the samples.

In the analysis of three neighboring epidermal nuclei shown in Figure 11, I noticed that one of the nuclei had a higher volume and lower RHV than the others. This difference between neighboring epidermal cell reminded me about the alternate pattern of trichoblast and atrichoblast cells in the differentiated root epidermis (Dolan et al. 1994; Berger et al. 1998). Based on this observation, I asked whether the identity of a cell could be deduced from nuclear features long before the cellular differentiation becomes apparent.

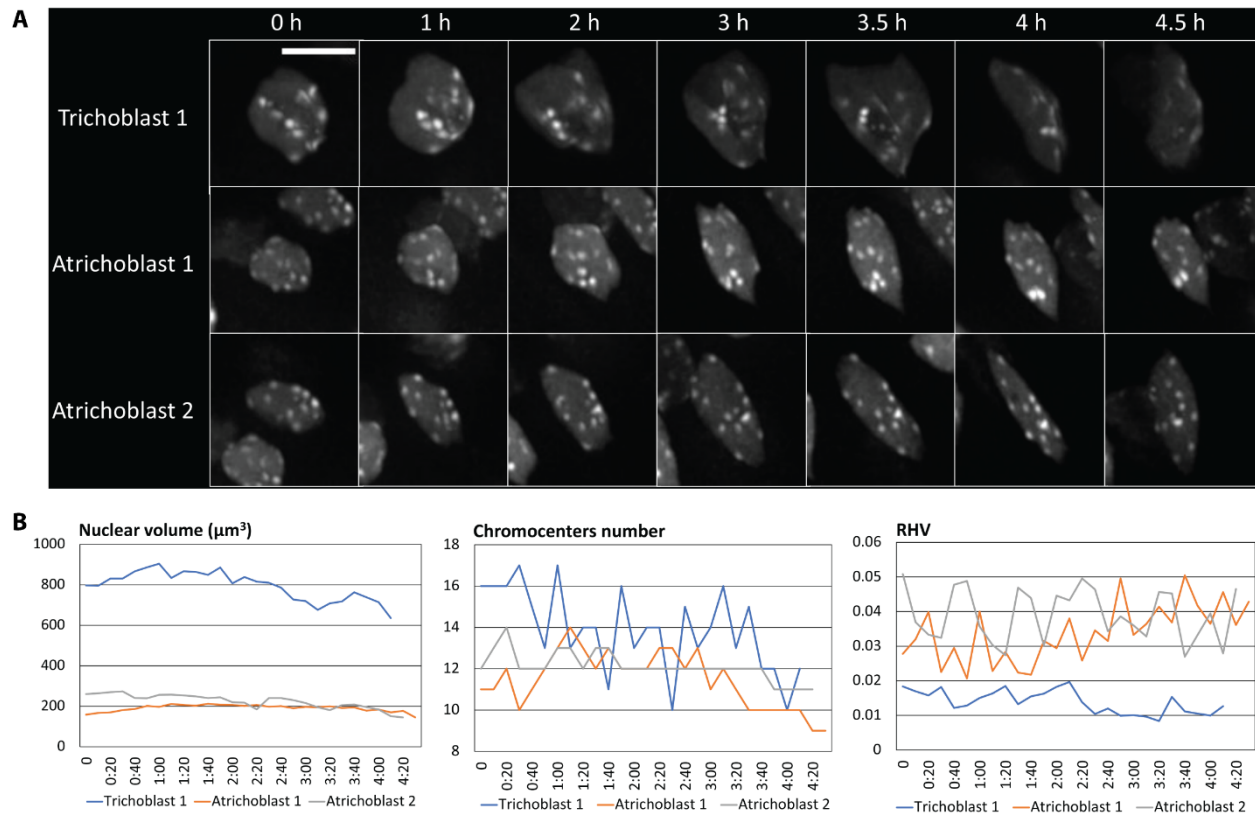


Figure 11 Tracking nuclear architecture dynamics during differentiation.

(A) Snapshots of nuclei from Movie 5 conducted on H2B-RFP expressing plants. Time from the start of the experiment indicated in h at the top. (B) Quantification of nuclear parameters over time: left, nuclear volume; middle, chromocenter number; right, relative heterochromatin volume. Scale bar is 10 μm .

2.3 APPLYING LIVE IMAGING TO CONNECT NUCLEAR ARCHITECTURE WITH CELL IDENTITY

Being able to identify the type of a cell by the features of its nucleus would be convenient, especially in high resolution single FOV imaging where tissue context is not visible. It would also support experiments based on extracted nuclei, as an alternative or addition to methods requiring specific labeling, like the INTACT method (Deal and Henikoff 2011).

Arabidopsis nuclei show a variety of shape, and different studies on living and fixed material already indicated a strong correlation between the type of cells, the shape of their nuclei, and changes during differentiation (Berger et al. 1998; Chytilova et al. 1999; Chytilova et al. 2000; Verbelen et al. 2006; Tamura and Hara-Nishimura 2011; Del Prete et al. 2014). I thus asked the question whether it is possible to predict the identity of a cell by the features of its nucleus,

focusing on the root epidermis and its two well-defined neighboring cell types, namely the hair and non-hair cells.

Relationship between nuclear architecture and cell identity, a hairy example:

Root hairs are protrusions of single cells that increase the cell surface for nutrient and water uptake by orders of magnitude (Grierson and Schiefelbein 2002). These root hairs are formed by trichoblasts that are arranged in vertical columns, or cell files, of the epidermis, and these columns alternate with columns of hairless cell, the so-called atrichoblasts. Tracking the columns vertically down from the zone where the hairs are already formed (or not), it can be predicted whether the progenitors close to the root tip are trichoblasts or atrichoblasts. Trichoblasts and atrichoblasts can also be determined according to their position over the underlying cortex cell layer (Dolan et al. 1994), but this is difficult to see when only nuclei are imaged.

With the help of Benjamin Kogelmann, a bachelor student supervised by me, we imaged roots with enough context information to identify the cell files and analyzed cellular and nuclear parameters in the different developmental zones. We showed that already in the meristematic zone, the architecture of the cell files could be used as identity reporter, as hair cell files contain more cells than non-hair files within a given interval (Dolan et al. 1994; Berger et al. 1998; Dolan and Costa 2001). This results in a phenotype where trichoblast nuclei appear piled up and are flattened along the transversal axis. This morphology, called here “stack phenotype”, can be reliably associated with the trichoblast cell identity and is not present in atrichoblast cell files (Figure 12).

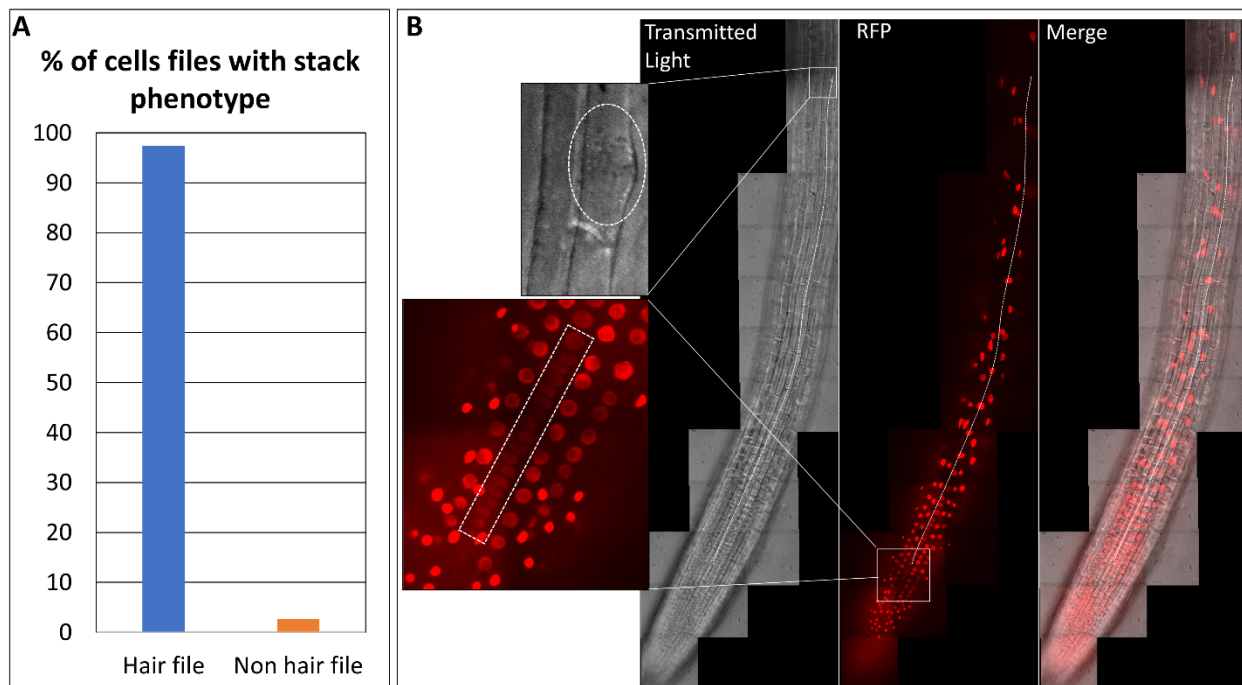


Figure 12 Association of cellular and nuclear features with cell identity.

Trichoblasts can be differentiated from atrichoblasts through the architecture of their cell file as early as in the root meristem. (A) Proportion of cell files presenting the “stack phenotype” according to their cell identity (n= 90 cell files from a total of 17 roots). (B) Schematic representation of the method to collect data in A. The stack phenotype is highlighted by the dashed box, the bulging root hair is highlighted by the dashed oval.

We measured different nuclear parameters and found that the nuclear volume allows distinguishing nuclei from future hair and non-hair cells already in the transition as well as in the elongation zone (Figure 13). Indeed, we found that trichoblast nuclei are on average 88% bigger than atrichoblast nuclei in the transition zone and 75% bigger in the elongation zone. This is in good agreement with the nuclear volumes measured from the tracked nuclei followed over time shown in Figure 11. It is also supported by the pattern in Movie 5 where one file of bigger nuclei is flanked by two files of smaller nuclei, matching the pattern of trichoblasts and atrichoblasts observed in root where one cell file of 2 or 3 is a trichoblast file (Dolan et al. 1994).

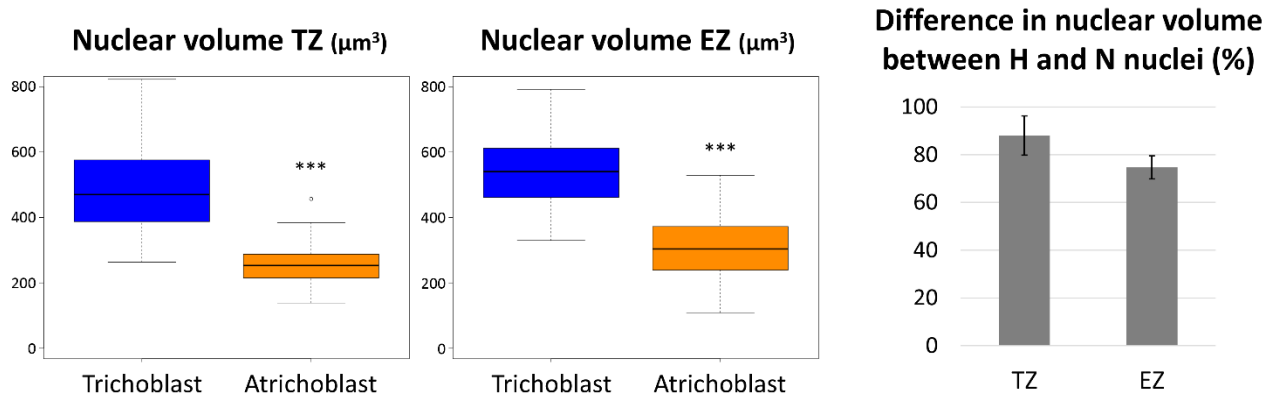


Figure 13 Association of nuclear volume with cell identity.

Trichoblasts have a larger nuclear volume than atrichoblasts. Box plots of nuclear volume of trichoblasts (H, blue) and atrichoblast (N, orange) in the transition zone (TZ) and elongation zone (EZ). Right panel: difference between trichoblast and atrichoblast nuclear volume in % in the indicated developmental zones. $n = 3$ roots, 135 nuclei for TZ, 111 nuclei for EZ. p values: $p < 0.01 = ***$, $p < 0.05 = **$ in a Welch two sample t-test.

However, in absolute values, the nuclear volume alone is not sufficient to distinguish the cell types as there is overlap between the two populations. The addition of other nuclear parameters could be useful to better characterize cell type-specific nuclei and allow better predictions. Based on the images from the tracked nuclei, RHV might be a useful parameter to take into account as it seems to be lowest in trichoblasts (Figure 11). The size of nucleoli might be another discriminating factor, between trichoblasts and atrichoblasts but also for the developmental zone where nucleoli almost disappear during the elongation/differentiation phase (data not shown). However, quantifying nucleoli size would require specific reporters that distinguish it from the chromatin reporter or DNA stain.

In summary, I could use the well-studied root development and particularly root hair differentiation (Dolan et al. 1994; Masucci et al. 1996; Wada et al. 1997; Berger et al. 1998; Dolan and Costa 2001; Otero et al. 2016) to confirm suitability of my live imaging system for acquisition of 3D images that capture nuclear and subnuclear features. I have then applied the setup to investigate the effect of prolonged heat stress on nuclear architecture.

2.4 USING LIVE IMAGING TO STUDY CHANGES OF NUCLEAR ARCHITECTURE IN RESPONSE TO HEAT STRESS

Nuclear architecture in response to heat stress in wild type *Arabidopsis* roots

It has been previously reported that in response to prolonged heat stress in *Arabidopsis thaliana*, several silent repetitive elements become transiently activated. This release of gene silencing is accompanied by a transient loss of nucleosomes at the affected loci and decondensation of heterochromatin (Pecinka et al. 2010). This chromocenter decompaction depends on HIT4 but its mechanism is unknown (Wang et al. 2013b; Wang et al. 2015b). Both studies used fixed and extracted nuclei for the cytological analysis and focused on the response directly after heat stress. However, the interpretation could be hampered by artefacts caused by the nuclei preparation, and it does not allow to follow the fate of individual nuclei after the stress period. Therefore, I used my live imaging approach to study the 3D architecture of living root cell nuclei within their tissue context at the single cell level during and after the stress treatment. In contrast to the previous publication (Pecinka et al. 2010), I compared all heat stress and recovery samples to their respective mock-treated samples, to take growth and developmental progression of the seedlings into account. I used the following timepoints: before heat stress (T0), after 30 h of heat stress or mock treatment (30 h HS/M), 2 days of recovery after heat stress or mock treatment (2 d R HS/M) and 7 days of recovery after heat stress or mock treatment (7 d R HS/M).

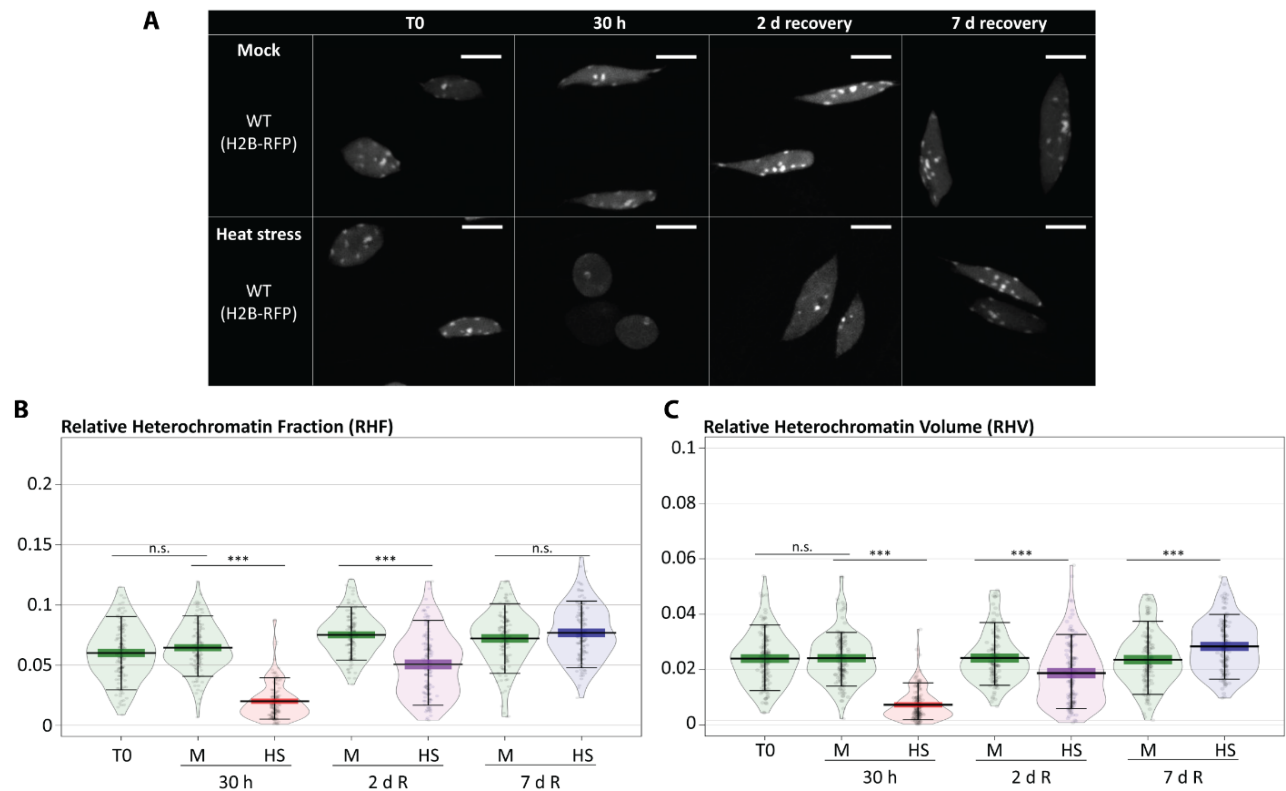
To monitor nuclear architecture and chromatin rearrangement in response to heat stress and upon recovery in living *Arabidopsis* seedlings, I used the setup described above with H2B-RFP as chromatin reporter. Histone 2B is incorporated in nucleosomes of all types in *Arabidopsis* root (Jiang and Berger 2017, Yang et al 2016) and can be used as a proxy to image chromatin. However, for experiments in which nuclear parameters were quantified, I always analyzed “fresh” samples, one from a series of parallel plant cultures kept under the respective conditions, to avoid photobleaching and additional stress effects. I quantified the changes in nuclear architecture upon heat stress and recovery by four main measurements, all on 3D-reconstructed nuclei. (1) The Relative Heterochromatin Fraction (RHF) is defined as the sum of fluorescence intensity of all H2B-RFP-stained chromocenters relative to the sum of fluorescence intensity of

the entire nucleus. It measures the proportion of heterochromatin in a nucleus. (2) The Relative Heterochromatin Volume (RHV) is defined as the total volume of H2B-RFP-stained chromocenters relative to the volume of the nucleus, measuring the space occupied by heterochromatin relative to the whole nucleus. (3) The Relative Heterochromatin Density (RHD) combines the two previous ratios by relating chromocenter density to nuclear density. Densities are defined as the sum of fluorescence signal intensity divided by the total volume of chromocenters or nucleus, respectively. (4) Nuclear sphericity is an index of ratio between longest and shortest diameters between >0 and 1, where 1 represents a perfect sphere. I considered nuclei as round if their sphericity was above 0.9, and as over-elongated if the ratio was below 0.7. The three abovementioned parameters (RHF, RHV and RHD) allow to monitor heterochromatin organization in response to heat stress under different perspectives and comparing nuclei of various shapes and sizes between timepoints and treatments. Measuring the sphericity captures changes in nuclear shape, a poorly investigated aspect of nuclear architecture in living and moving nuclei.

During the observed period of eight days, chromocenters in mock-treated plants were visible at all times and the RHF increased by $\approx 20\%$, while RHV remained stable (Figure 14, Table 3). The intensity of the fluorescence signal in chromocenters and in total nuclei increased during this time by 92% and 85%, respectively (Table 3). Maximum values were reached already at the time point corresponding to two days of recovery (2 d R) and remained the same till 7 d R, indicating that the amount of nucleosomes loaded into chromatin was not changing after ca. 8 days of stress-free growth after germination. The volume occupied by chromocenters remained stable after ca. 6 days of stress-free growth, while nuclear volume did not vary significantly. Taken together, I observed a progressive increase of total chromatin in chromocenters and nuclei during stress-free growth, while the relative volume of heterochromatin and the shape of the nuclei remained the same.

Directly after heat stress, however, I observed a substantial decondensation of chromocenters and a reduction in their numbers, reflected in a mean decrease of RHF and RHV by about 70% (Figure 14, Table 4). Return to regular growth conditions was followed by a progressive recondensation of heterochromatin and reformation of chromocenters, reaching an

intermediate RHF and RHV levels at 2 d R and RHF level of mock controls at 7 d R. RHV was $\approx 20\%$ higher than mock at this timepoint, indicating that the expansion of the chromocenters was more



pronounced than that of the nuclei and not fully back to unstressed levels (Figure 14, Table 4).

Figure 14 Heat stress lead to chromocenter decompaction in living nuclei.

(A) Representative nuclei at the given timepoint in mock and heat stress conditions. Scale bar = 10 μm . (B) Pirate plots of the relative heterochromatin fraction measured for root cell nuclei at indicated time points after heat stress or upon mock treatment in wild type plants. (C) Pirate plots of the relative heterochromatin volume in corresponding samples. $n = 122-141$, grey points represent raw data. Beans represent smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. Y-axis shows the RHF or RHV ratio. M = mock treatment, HS = heat stress treatment, R = recovery time point.

Variation Mock-Mock (in %)				
	Wt			
	30h/T0	2d/30h	7d/2d	7d/T0
RHF	7.33	16.52	-3.98	20.09
	2.74	2.49	2.64	3.30
	<i>0.08222</i>	<i>6.22E-06</i>	<i>0.2439</i>	<i>1.76E-05</i>
Chromocenters intensity	1.44	76.64	7.48	92.59
	5.05	8.89	6.57	11.77
	<i>0.8758</i>	<i>2.41E-12</i>	<i>0.369</i>	<i>3.54E-10</i>
Nuclear intensity	-0.75	70.95	12.15	85.70
	3.89	8.06	6.46	10.75
	<i>0.915</i>	<i>3.21E-13</i>	<i>0.2324</i>	<i>2.89E-11</i>
RHFV	0.62	0.30	-1.43	-1.70
	3.11	3.42	3.60	3.59
	<i>0.8926</i>	<i>0.948</i>	<i>0.6006</i>	<i>0.729</i>
Chromocenters volume	18.70	-6.48	-3.12	7.00
	4.72	2.79	4.27	4.72
	<i>0.01097</i>	<i>0.1851</i>	<i>0.4894</i>	<i>0.3386</i>
Nuclear volume	11.53	-2.09	-3.78	5.40
	3.74	3.21	3.89	4.26
	<i>0.1132</i>	<i>0.654</i>	<i>0.9857</i>	<i>0.6994</i>
RHD	5.51	20.84	-6.21	19.68
	0.01	0.02	0.02	0.02
	<i>0.01811</i>	<i>3.94E-14</i>	<i>0.01408</i>	<i>1.23E-11</i>
Chromocenters density	-32.17	100.32	16.51	54.19
	0.02	0.08	0.06	0.08
	<i>1.09E-07</i>	<i>< 2.2e-16</i>	<i>0.05188</i>	<i>9.93E-09</i>
Nuclear density	-30.84	67.02	23.66	39.02
	0.02	0.07	0.06	0.07
	<i>2.89E-07</i>	<i>7.49E-15</i>	<i>0.0049</i>	<i>7.13E-06</i>

Table 3 Variation of measured or calculated parameters between mock timepoints.

The mean variation is displayed in bold letters, the standard error of the mean (SEM) is displayed on grey background, and the p value is displayed in italics. Significant p values are displayed in green, non-significant p values are displayed in magenta.

The heat-induced decrease in RHF was indeed due to the decompaction of chromocenters, as the overall signal intensity in the nuclei remained the same as in their mock-treated

equivalents, while only chromocenter signal intensity changed dynamically (Figure 15, Table 4). Chromocenter volume followed the same dynamics as chromocenter intensity, except that they were higher than unstressed controls at 7 d R. I also observed a slight increase ($\approx 20\%$) in nuclear volume right after heat stress, likely linked with the decondensation of heterochromatin in the nucleus. With some fluctuations, heat stress nuclei had also higher volume than mock at 7 d R. (Figure 15, Table 4)

The difference of dynamics between intensities and volume upon heat stress is supported by the measure of chromocenter and nuclear densities and their ratio, the RHD. Relative to their respective mock control, chromocenters and total nuclei had 15-25% reduced density after heat stress, except 2 d R HS where both densities were not significantly different from their respective unstressed control (Figure 15 A, Table 4). Interestingly, once samples have experienced heat stress, their RHD seems to remain similar even upon recovery (Figure 15 C).

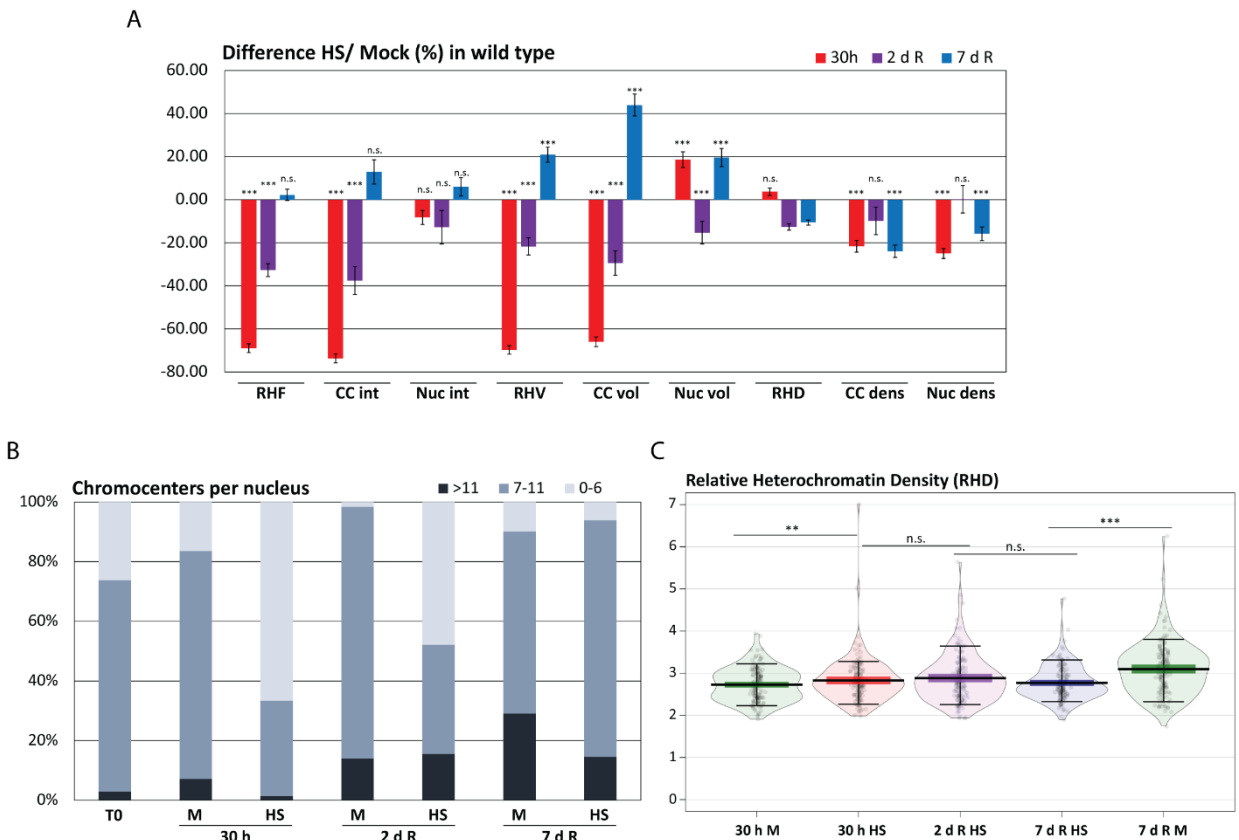


Figure 15 Nuclear architecture dynamics during HS.

(A) Bar plot of the enrichment or depletion of the indicated parameter in HS relative to mock condition in %. (B) Distribution of chromocenter number per nucleus at the different timepoints of HS and mock treatment. (C) Pirate plot of the Relative Heterochromatin Density (RHD) during heat stress and recovery. n = 122-141, grey points represent raw data. Beans represent a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: p<0.01 = ***, p<0.05 = **, n.s. = non-significant in a Welch two sample t-test. Int = intensity, vol = volume, M = mock treatment, HS = heat stress treatment, R = recovery time point.

Variation HS-Mock (in %)					
	Wt				
	30h	2 d R	7 d R	2d R HS/ 30HS	7 d R HS/ 2 d R HS
RHF	-68.97	-32.71	2.28	152.68	51.98
	2.03	2.98	2.62	11.11	3.77
	2.20E-16	2.20E-16	0.094	2.20E-16	2.20E-16
Chromocenters intensity	-73.70	-37.58	12.91	319.21	94.43
	2.07	6.50	5.64	43.34	9.42
	2.20E-16	8.23E-06	0.123	3.46E-11	2.11E-10
Nuclear intensity	-8.26	-12.77	6.00	59.32	34.33
	3.26	7.79	4.21	14.12	5.18
	0.108	0.111	0.489	8.41E-05	9.45E-04
RHFV	-69.74	-21.69	20.91	156.33	51.60
	1.98	3.98	3.51	12.95	4.41
	2.20E-16	2.19E-05	7.66E-05	2.20E-16	4.09E-13
Chromocenters volume	-65.96	-29.42	43.97	92.84	90.99
	2.23	5.68	5.10	15.40	6.56
	2.20E-16	6.49E-06	3.98E-10	1.39E-07	2.20E-16
Nuclear volume	18.58	-15.31	19.59	-29.86	31.62
	3.64	5.26	4.21	4.32	4.49
	2.23E-04	1.68E-02	8.09E-04	6.69E-08	7.02E-06
RHD	3.71	-12.62	-10.61	1.98	-4.13
	1.73	1.51	1.19	1.75	1.24
	0.083	3.10E-07	9.15E-07	0.421	0.070
Chromocenters density	-21.65	-9.87	-23.93	124.33	-1.56
	2.67	6.34	2.84	15.58	3.57
	6.17E-07	0.108	2.60E-05	2.81E-12	0.835
Nuclear density	-24.98	0.22	-15.83	116.90	3.92
	2.30	6.41	3.18	13.68	3.81
	2.01E-09	0.746	0.00624	1.43E-13	0.611

Table 4 Variation of measured or calculated parameters in heat stress (HS) samples relative to their respective mock treated samples.

The two last columns display the variation of measured or calculated parameters between indicated heat stressed samples. Negative values indicate that HS values are smaller than mock. The mean variation is

displayed in bold letters, the standard error of the mean (SEM) is displayed on grey background, and the p value is displayed in *italics*. Significant p values are displayed in green, non-significant p values are displayed in magenta.

Parallel to heterochromatin rearrangement in response to heat stress, I also observed a drastic change in nuclear shape. In mock-treated plants, most nuclei have a spindle-like shape throughout the observation period, but nearly 80% of nuclei were rounded at the end of the heat stress (Figure 16). As for heterochromatin organization, this change in nuclear shape is transient, as heat-stressed recovered nuclei have equivalent sphericity proportion as mock-treated controls after 7 days of recovery (Figure 16).

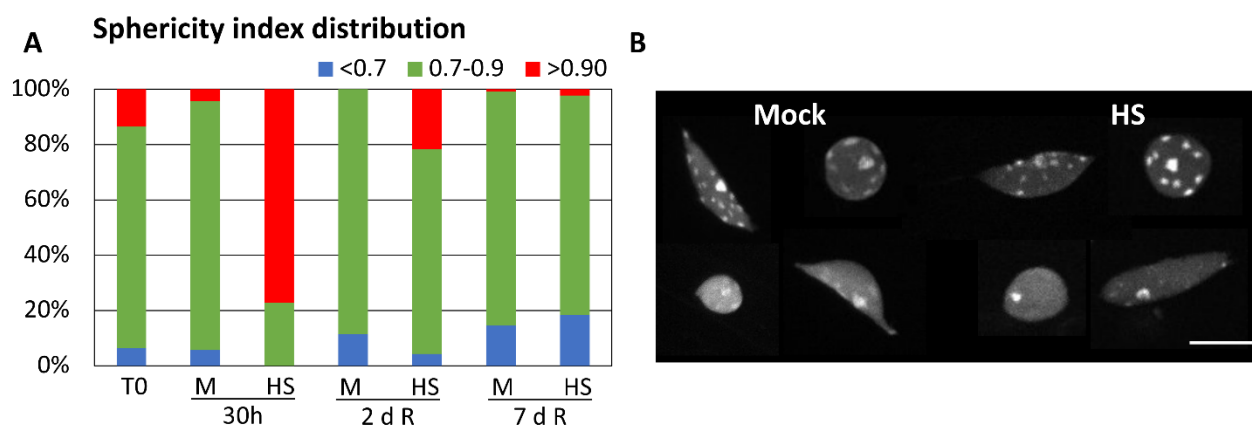


Figure 16 Nuclear rounding is independent from chromocenter decompaction.

(A) Distribution of nuclear sphericity classes during HS and Mock treatment. (B) Representative pictures of round and elongated nuclei presenting decompacted and conspicuous CC in heat stress and mock condition. Scale bar is 10 μ m.

While the analysis of high numbers of nuclei suggested simultaneous changes in nuclear shape and heterochromatin compaction, these parameters are not necessarily interdependent, as I observed round nuclei with conspicuous chromocenters as well as elongated nuclei with decompacted chromocenters (Figure 16). Therefore, to monitor the same individual nuclei during the heat stress period, I took advantage of a setup that allows continuous image acquisition with subnuclear resolution while applying heat stress directly at the microscope. I acquired 3D confocal sections of living *Arabidopsis* roots every 30 minutes during the 30 h of heat treatment. These image series revealed that rounding of nuclei starts after 4 h of heat exposure and is nearly complete after 10 h, followed by strong reduction of nuclear movement within the cells (Movie 6 and Movie 8). I also observed some nuclear deformation towards the end of the heat stress

(Movie 8 white squares). Nuclei under regular temperature remain elongated and exhibit high intercellular mobility throughout the observation period (Movie 7).

Measuring RHF dynamics for individual nuclei during long-term live imaging resulted in decreased values over time even in mock samples, likely due to loss of signal intensity by bleaching, but confirmed the much more pronounced loss of heterochromatin condensation during heat stress. It also revealed that, with or without heat stress, RHF fluctuates between timepoints (Figure 17), suggesting that chromatin organization in interphase nuclei is rather dynamic.

In summary, while tracking individual nuclei with long-term live imaging was limited by the unavoidable loss of signal strength, it provided complementary insight into dynamics and diversity of nuclear parameters and confirmed the significant changes of nuclear shape and heterochromatin condensation during extended periods of heat stress. Regarding nuclei deformation and impaired nuclei movement in HS condition, I cannot exclude with certainty that a cumulative effect between HS condition and repeated imaging could cause those phenotypes. However, no nuclear deformation was observed in mock experiments, and attempts to image the recovery phase indicated that nuclear deformation and static nuclei are as transient as the other HS-related phenotypes observed (Figure 18, Movie 9).

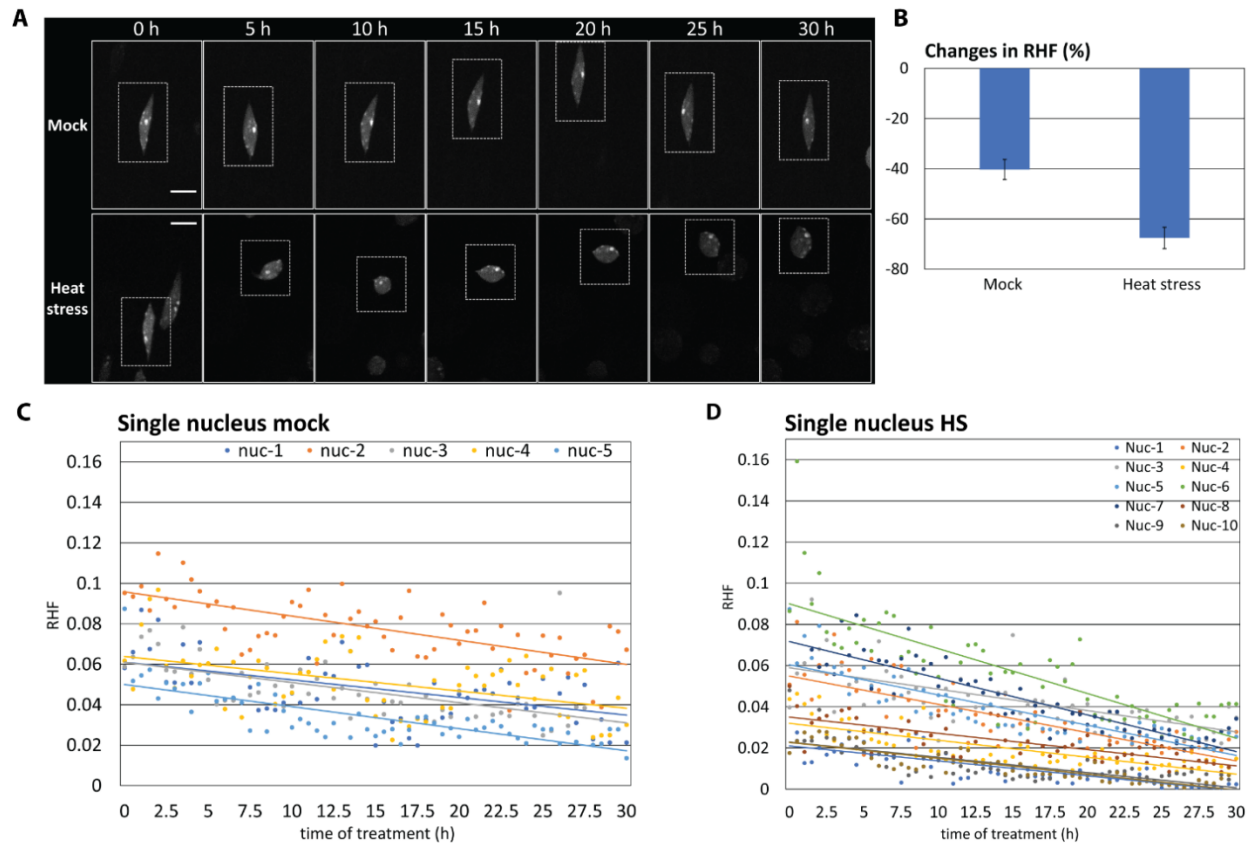


Figure 17 Live imaging of single nuclei during heat stress and mock treatment.

(A) Snapshot of Movie 6 and Movie 7 showing one nucleus during the 30 h of treatment. (B) Relative change in mean RHF between the start and the end of the indicated conditions. The five first timepoints were compared to the five last. (C-D) RHF dynamics during mock (C) and HS (D) experiments. Points of the same color represent individual RHF values for the same nucleus, the correspondingly colored line is the linear regression for these data. Scale bar is 10 μ m.

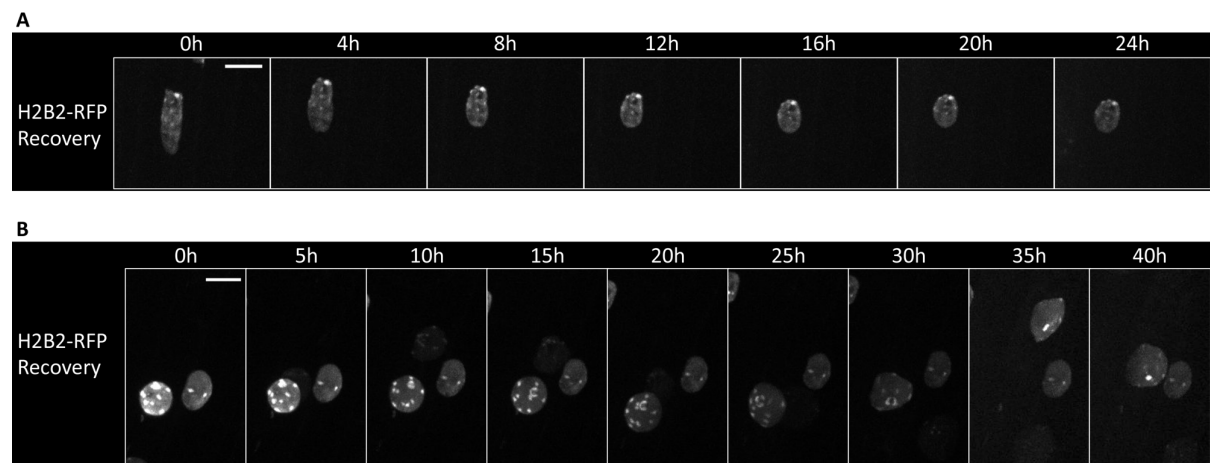


Figure 18 Plasticity of nuclei during the recovery period.

(A) Reformation of a deformed nuclei in 6-10h after heat stress. (B) Restoration of nuclear movement and elongation upon recovery. It is to note that chromocenter loss during recovery is likely an artifact of the imaging condition which is not found in control experiment in plates. Scale bar is 10 μ m.

To conclude, prolonged heat stress in *Arabidopsis* roots leads to transient decompaction of heterochromatin, rounding of nuclei, and impaired nuclear movement. While nuclear shape and signal intensity recovered to non-stress levels after 7 days of recovery from the stress, volumes were higher than in unstressed controls. This points towards a potential longer-term effect of heat stress leading to lower compaction of chromatin both in chromocenters and total nuclei even days after heat stress. Whether complete restoration occurs after longer recovery time or whether this modified architecture has a role in heat stress memory remains to be investigated.

Nuclear architecture in response to heat stress in histone chaperone mutants

Earlier work indicates that the restoration of histone occupancy upon recovery after heat stress depends on the histone chaperone complex CAF-1 (Pecinka et al. 2010). To further investigate the role of histone chaperones in heat stress response and recovery, I conducted the heat stress experiments also with lines affected in H3/H4 histone chaperone activity. As H3/H4 histone dimers are the first to associate with DNA to form nucleosomes (Smith and Stillman 1991), I hypothesized that investigating other mutants affected in this process might also have problems to restore chromatin after heat stress. I thus crossed several histone chaperone mutants to the line with the chromatin reporter (H2B-RFP) and selected lines homozygous for mutation and reporter in the segregating progeny. Unfortunately, the crosses involving CAF-1 mutants (*fas-1-4* and *fas2-5*) were made at a time when the focus was on another chromatin reporter (H2A-RFP) that later appeared to be poorly incorporated in mature root cell nuclei, compared to the H2B reporter (Figure 19). CAF-1 mutants are thus not included in the investigation here as their combinations with H2B-RFP could not be obtained later due to a lack of time. Investigations were thus conducted only with the *asf1ab* double mutant from the ASF1 complex, and the *hira* and *nuclein1* single mutants from the HIRA complex.

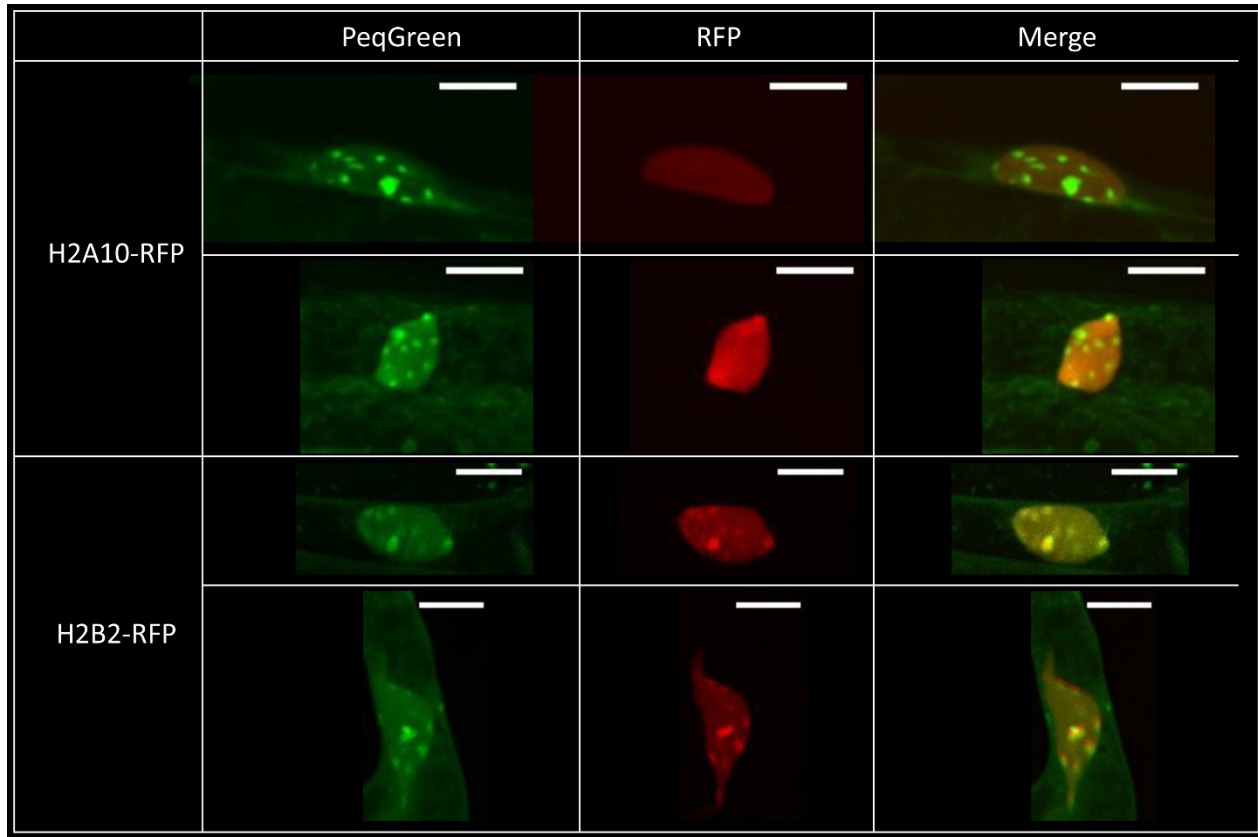


Figure 19 H2A10 is poorly incorporated into chromocenters of mature root cell nuclei compared to H2B. Left panel, DNA imaged in living root cell nuclei with PeqGreen. Middle panel, RFP signal from the indicated histone reporter. Right panel, merged images. Images are maximum intensity projections. Scale bar is 10 μ m.

Already under regular growth conditions, nuclei of *asf1ab* and *hira* mutants differed from those in wild type plants while *nuclein1* nuclei were more similar to those in wild type. Indeed, *asf1ab* and *hira* had an increased proportion of elongated nuclei (sphericity < 0.7) (Figure 20). The *hira* mutant also showed an increasing proportion of round nuclei, increasing with age. In both *asf1ab* and *hira*, the RHF and RHV was reduced by 10-40% compared to wt, depending on the timepoint, and more pronounced in older *hira* mutant plants (Figure 21, Table 5). More specifically, in *hira* mutants I observed a small but constant decompaction of heterochromatin during stress-free growth, indicating a possible instability of chromocenter organization in *hira* mutants (Table 5). The *nuclein1* mutant however did not differ from wt overall, except for nuclear volume which was slightly reduced compared to wt in all three mutants (Figure 21, Table 5).

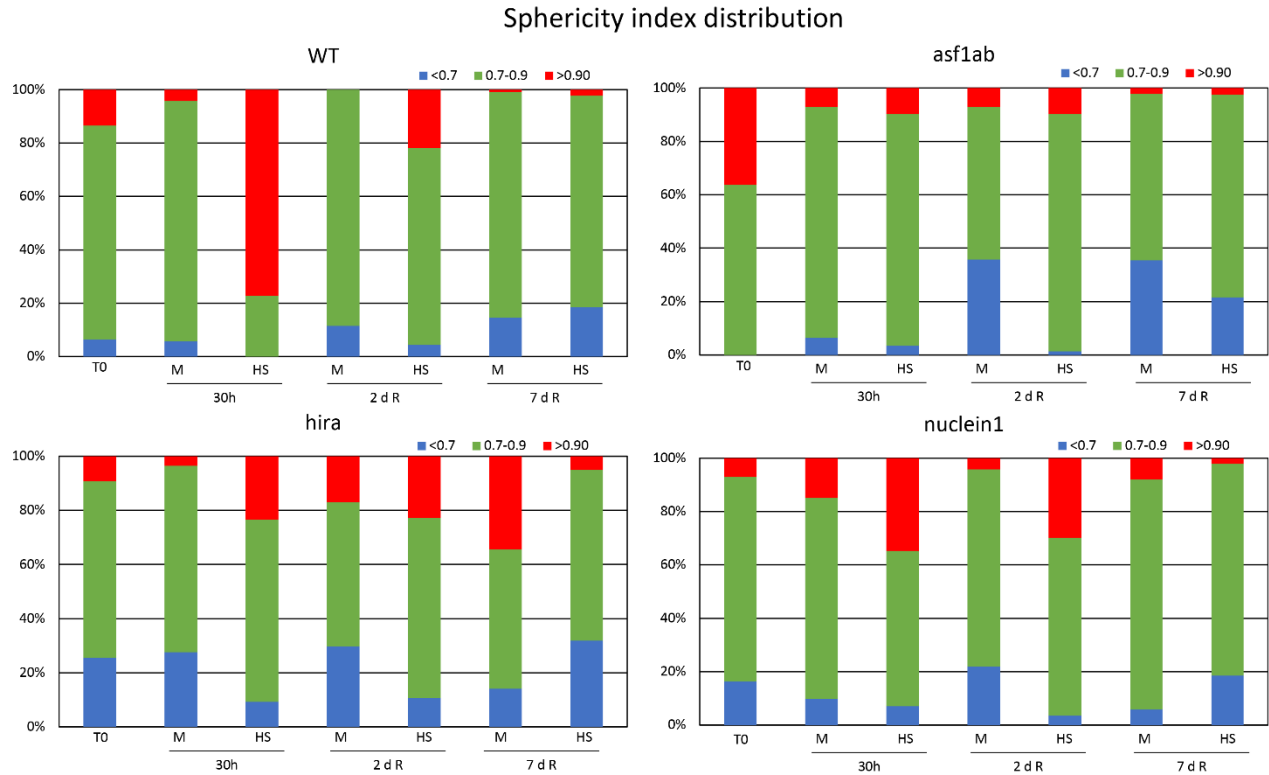


Figure 20 Histone chaperone mutants are impaired in nuclear rounding upon HS.

Ratio of nuclei in the different sphericity classes in wt or histone chaperone mutant background during heat stress (HS) or mock (M) treatment at different time points.

Upon heat stress, the differences to wt were even more eminent. No rounding of nuclei after the 30 HS period was observed in *asf1ab*, and only ca. 20% of nuclei showed this response in *hira* and *nuclein1* mutant (Figure 20). Also, the decrease in RHF and RHV observed in wt seemed to be suppressed (*asf1ab*) or reduced (*hira*, *nuclein1*) (Figure 22 A, Table 6). The images from mutant nuclei nevertheless indicated some decompaction of chromocenters (Figure 23). The discrepancy originated from a parallel loss of chromocenters and total nuclear fluorescence and volume, so that RHF and RHV remained unchanged (*asf1ab*) or alleviate its reduction (*hira*, *nuclein1*) with regards to wt (Figure 22 B, Table 6). Decreases in chromocenter intensity and volume were smaller than observed in wild type. However, histone chaperone mutants had already a reduce chromocenter intensity and volume compared to wt in mock condition (Table 5 A). I thus compared chromocenter intensity and volume after heat stress in mutants to wt. All mutants except *hira* showed significant differences to wild type directly after heat stress (Table

7). This showed that heterochromatin in *hira* mutants undergoes a decompaction in the same extent as in wild type but is accompanied by a diminution of nuclear intensity and volume that alleviates the variation in RHF and RHV. This suggest a role for *HIRA* in maintenance of nuclear organization with and without stress.

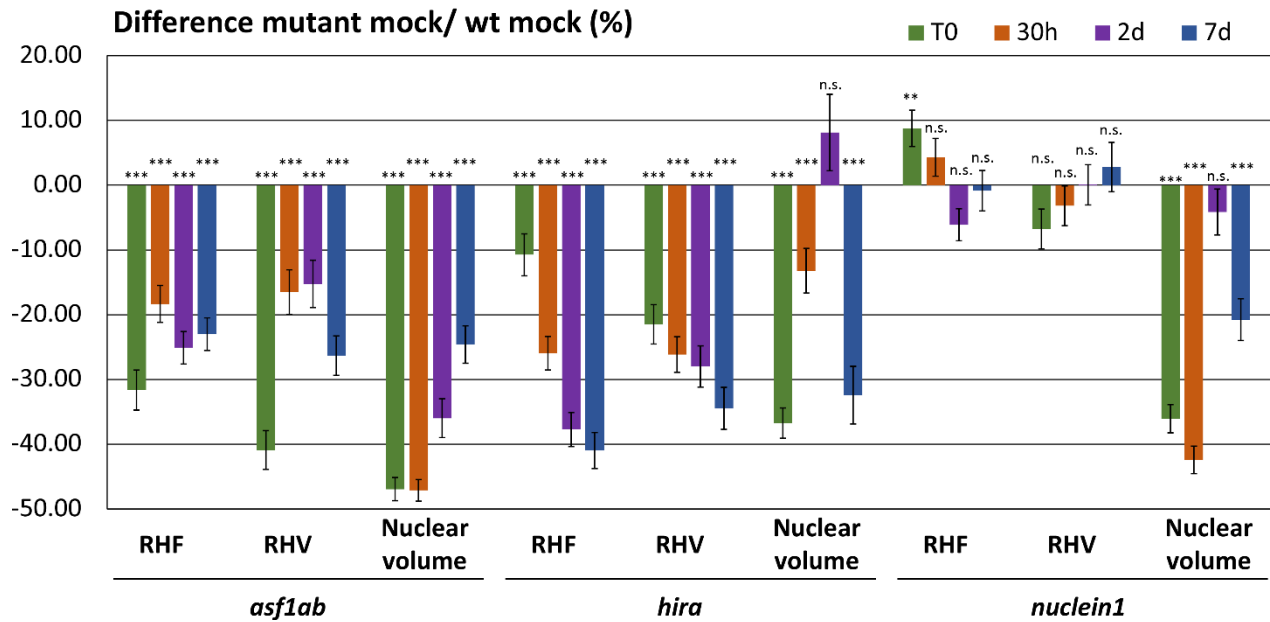


Figure 21 Histone chaperone mutants have altered nuclear architecture in stress-free conditions.

Bar plot of the difference (in %) of measured or calculated parameters between mock-treated wt and mutant samples. p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test.

A

Variation mutant Mock relative to wt Mock (in %)												
	<i>asf1ab</i>				<i>hira</i>				<i>nuclein1</i>			
	T0	30h	2d	7d	T0	30h	2d	7d	T0	30h	2d	7d
RHF	-31.64	-18.33	-25.11	-23.00	-10.75	-25.95	-37.73	-40.96	8.77	4.30	-6.11	-0.85
	3.09	2.85	2.50	2.52	3.23	2.60	2.63	2.78	2.80	2.92	2.48	3.12
Chomocenters Intensity	<i>7.87E-12</i>	<i>3.06E-06</i>	<i>6.10E-13</i>	<i>2.97E-09</i>	<i>0.01852</i>	<i>9.68E-12</i>	<i>< 2.2e-16</i>	<i>< 2.2e-16</i>	<i>0.03933</i>	<i>0.2698</i>	<i>0.1028</i>	<i>0.8381</i>
	-2.89	-43.83	-47.28	51.02	-27.00	-22.89	-54.28	-77.56	-4.72	17.37	29.51	15.36
Nuclear Intensity	11.32	4.06	3.06	13.28	5.33	5.33	3.35	2.13	5.72	7.45	6.39	5.77
	<i>0.8335</i>	<i>7.13E-11</i>	<i>1.00E-13</i>	<i>0.0006252</i>	<i>0.004308</i>	<i>0.001908</i>	<i>< 2.2e-16</i>	<i>< 2.2e-16</i>	<i>0.6234</i>	<i>0.05459</i>	<i>0.0003652</i>	<i>0.06978</i>
RHV	34.42	-30.70	-35.35	94.02	-14.03	7.44	-24.48	-62.41	-1.06	20.98	37.24	19.55
	11.81	3.78	3.08	14.99	4.41	5.70	4.26	2.29	4.55	5.65	5.98	4.60
Chomocenters volume	<i>4.80E-08</i>	<i>4.80E-08</i>	<i>5.78E-10</i>	<i>2.87E-08</i>	<i>0.05472</i>	<i>0.2834</i>	<i>5.56E-05</i>	<i>< 2.2e-16</i>	<i>0.2969</i>	<i>0.05208</i>	<i>0.0005578</i>	<i>0.1453</i>
	-40.89	-16.51	-15.24	-26.33	-21.49	-26.15	-28.00	-34.46	-6.77	-3.18	0.05	2.80
Nuclear volume	2.99	3.44	3.65	3.04	3.04	2.75	3.19	3.24	3.07	3.07	3.11	3.82
	<i>< 2.2e-16</i>	<i>0.000442</i>	<i>0.001289</i>	<i>5.81E-08</i>	<i>3.03E-06</i>	<i>1.10E-09</i>	<i>1.93E-09</i>	<i>1.27E-11</i>	<i>0.1362</i>	<i>0.4673</i>	<i>0.8066</i>	<i>0.656</i>
Chomocenters volume	-65.40	-53.05	-45.76	-43.77	-46.43	-34.19	-24.06	-57.21	-34.63	-42.55	1.99	-17.10
	2.10	2.66	3.32	2.89	2.69	3.24	4.18	2.93	3.19	2.82	4.25	3.91
Nuclear volume	<i>< 2.2e-16</i>	<i>< 2.2e-16</i>	<i>< 2.2e-16</i>	<i>1.34E-14</i>	<i>1.78E-12</i>	<i>1.64E-10</i>	<i>2.66E-06</i>	<i>< 2.2e-16</i>	<i>1.71E-07</i>	<i>4.41E-16</i>	<i>0.7761</i>	<i>0.004557</i>
	-46.94	-47.13	-35.98	-24.60	-36.74	-13.21	8.12	-32.42	-36.06	-42.41	-4.14	-20.76
Nuclear volume	1.79	1.67	3.00	2.89	2.34	3.44	5.90	4.45	2.18	2.11	3.54	3.22
	<i>2.42E-12</i>	<i>< 2.2e-16</i>	<i>4.50E-14</i>	<i>1.50E-06</i>	<i>6.64E-08</i>	<i>0.006433</i>	<i>0.2085</i>	<i>2.05E-07</i>	<i>9.13E-08</i>	<i>2.20E-16</i>	<i>0.4301</i>	<i>9.64E-05</i>

B

Variation Mock-Mock (in %)												
	Wt				<i>asf1ab</i>				<i>hira</i>			
	30h/T0	2d/30h	7d/2d	7d/T0	30h/T0	2d/30h	7d/2d	7d/T0	30h/T0	2d/30h	7d/2d	7d/T0
RHF	7.33	16.52	-3.98	20.09	28.20	6.86	-1.28	35.24	-10.94	-2.01	-8.97	-20.56
	2.74	2.49	2.64	3.30	4.48	3.59	3.23	4.42	3.11	4.08	4.28	3.74
Chromocenters intensity	<i>0.08222</i>	<i>6.22E-06</i>	<i>0.2439</i>	<i>1.76E-05</i>	<i>1.37E-05</i>	<i>0.1739</i>	<i>0.7849</i>	<i>5.84E-08</i>	<i>0.02325</i>	<i>0.7091</i>	<i>0.1356</i>	<i>0.0001055</i>
	1.44	76.64	7.48	92.59	-41.33	67.51	205.40	199.49	6.29	6.74	-47.15	-46.90
Nuclear intensity	5.05	8.89	6.57	11.77	4.25	9.75	26.86	26.34	7.32	7.70	5.02	5.02
	<i>0.8758</i>	<i>2.41E-12</i>	<i>0.369</i>	<i>3.54E-10</i>	<i>0.001117</i>	<i>5.28E-08</i>	<i>3.61E-12</i>	<i>4.50E-11</i>	<i>0.4929</i>	<i>0.6421</i>	<i>2.48E-07</i>	<i>1.52E-05</i>
RHV	-0.75	70.95	12.15	85.70	-48.08	55.47	234.18	170.79	24.08	17.88	-44.62	-19.15
	3.89	8.06	6.46	10.75	2.84	7.42	25.82	20.93	6.55	6.56	3.38	5.17
Chromocenters volume	<i>0.915</i>	<i>3.21E-13</i>	<i>0.2324</i>	<i>2.89E-11</i>	<i>4.22E-07</i>	<i>1.60E-09</i>	<i>< 2.2e-16</i>	<i>2.54E-13</i>	<i>0.004247</i>	<i>0.03652</i>	<i>9.48E-11</i>	<i>0.007788</i>
	0.62	0.30	-1.43	-1.70	41.40	0.31	-14.65	21.38	-5.36	-3.37	-10.43	-18.30
Nuclear volume	3.11	3.42	3.60	3.59	5.85	4.33	3.53	5.02	3.51	4.22	4.43	4.04
	<i>0.8926</i>	<i>0.948</i>	<i>0.6006</i>	<i>0.729</i>	<i>1.22E-07</i>	<i>0.914</i>	<i>0.009134</i>	<i>0.002273</i>	<i>0.3081</i>	<i>0.5452</i>	<i>0.07928</i>	<i>0.001272</i>
Chromocenters volume	18.70	-6.48	-3.12	7.00	59.50	8.68	1.62	74.36	45.81	7.37	-45.28	-14.39
	4.72	2.79	4.27	4.72	9.05	6.63	5.16	8.86	7.16	5.82	3.74	5.86
Nuclear volume	<i>0.01097</i>	<i>0.1851</i>	<i>0.4894</i>	<i>0.3386</i>	<i>7.72E-08</i>	<i>0.3759</i>	<i>0.947</i>	<i>6.06E-11</i>	<i>3.73E-07</i>	<i>0.319</i>	<i>1.66E-11</i>	<i>0.06435</i>
	11.53	-2.09	-3.78	5.40	10.83	18.62	13.21	49.23	53.00	22.35	-39.79	12.82
Nuclear volume	3.74	3.21	3.89	4.26	3.51	5.57	4.36	5.75	6.04	6.59	3.96	7.43
	<i>0.1132</i>	<i>0.654</i>	<i>0.9857</i>	<i>0.6994</i>	<i>0.02309</i>	<i>0.3759</i>	<i>0.947</i>	<i>2.38E-12</i>	<i>1.63E-12</i>	<i>0.002925</i>	<i>3.78E-09</i>	<i>0.1254</i>

Table 5 Histone chaperone mutants have altered nuclear architecture in mock conditions.

(A) Variation of indicated parameters in mock-treated indicated mutants relative to wt values for the same timepoint and treatment. (B) Variation of indicated parameters in mock-treated samples relative to previous mock timepoints in indicated mutants. The mean variation is displayed in bold text, the standard error of the mean (SEM) is displayed on grey background, and the p value is displayed in italics. Significant p values are displayed in green, non-significant p values are displayed in magenta.

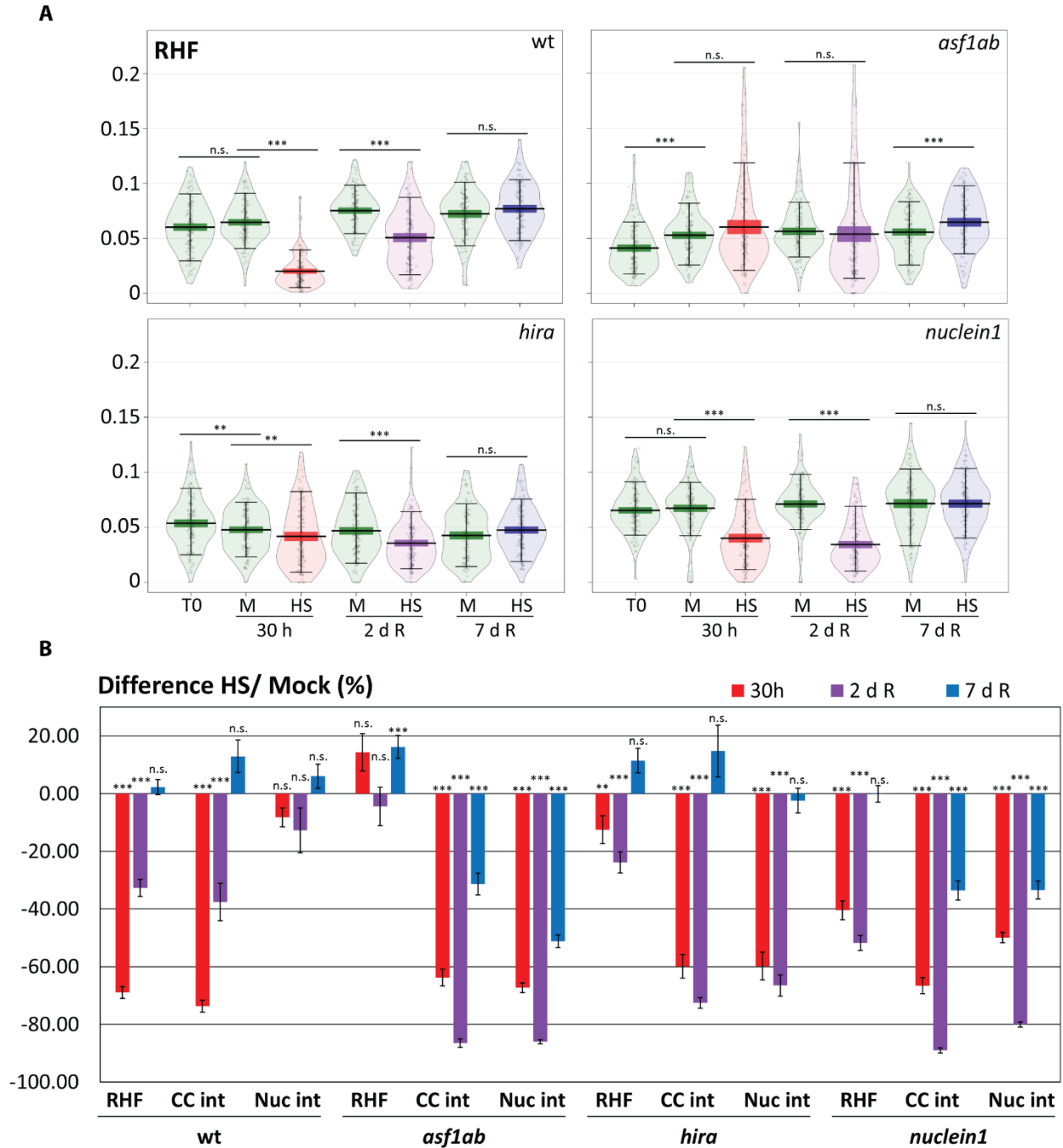


Figure 22 RHF dynamics in response to HS in histone chaperone mutants.

(A) Pirate plot of the relative heterochromatin fraction measured from root cell nuclei at indicated time points of heat stress or mock treatment in the indicated mutant or wild type background. $n = 122-141$, $118-144$, $129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1* respectively. Grey points represent raw data. Beans represent a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. Y-axis shows the RHF ratio. M = mock treatment, HS = heat stress treatment, R = recovery time point. (B) Bar plot of the enrichment or depletion in %, of RHF and its components between mock and heat stress time points, in the indicated wt or mutant background.

Variation HS-Mock (in %)										
	Wt					<i>asf1ab</i>				
	30h	2 d R	7 d R	2d R HS/ 30HS	7 d R HS/ 2 d R HS	30h	2 d R	7 d R	2d R HS/ 30HS	7 d R HS/ 2 d R HS
RHF	-68.97	-32.71	2.28	152.68	51.98	14.30	-4.49	16.19	-10.71	20.12
	2.03	2.98	2.62	11.11	3.77	6.47	6.65	3.94	6.24	3.69
	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>0.094</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>0.054</i>	<i>0.552</i>	<i>0.002</i>	<i>0.206</i>	<i>0.014</i>
Chromocenters intensity	-73.70	-37.58	12.91	319.21	94.43	-63.79	-86.54	-31.36	-38.04	1208.44
	2.07	6.50	5.64	43.34	9.42	2.94	1.50	3.75	6.91	64.73
	<i>2.20E-16</i>	<i>8.23E-06</i>	<i>0.123</i>	<i>3.46E-11</i>	<i>2.11E-10</i>	<i>4.38E-14</i>	<i>2.20E-16</i>	<i>3.02E-04</i>	<i>6.23E-04</i>	<i>2.20E-16</i>
Nuclear intensity	-8.26	-12.77	6.00	59.32	34.33	-67.32	-86.04	-51.21	-33.77	1068.09
	3.26	7.79	4.21	14.12	5.18	1.67	0.72	2.20	3.41	47.48
	<i>0.108</i>	<i>0.111</i>	<i>0.489</i>	<i>8.41E-05</i>	<i>9.45E-04</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>1.04E-10</i>	<i>9.26E-08</i>	<i>2.20E-16</i>
RHV	-69.74	-21.69	20.91	156.33	51.60	-5.78	-4.48	18.77	1.63	6.14
	1.98	3.98	3.51	12.95	4.41	5.90	6.67	4.84	7.12	3.92
	<i>2.20E-16</i>	<i>2.19E-05</i>	<i>7.66E-05</i>	<i>2.20E-16</i>	<i>4.09E-13</i>	<i>0.449</i>	<i>0.570</i>	<i>0.005</i>	<i>0.863</i>	<i>0.457</i>
Chromocenters volume	-65.96	-29.42	43.97	92.84	90.99	-59.94	-64.17	66.38	-4.45	367.36
	2.23	5.68	5.10	15.40	6.56	2.85	2.83	6.99	7.57	17.77
	<i>2.20E-16</i>	<i>6.49E-06</i>	<i>3.98E-10</i>	<i>1.39E-07</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>5.16E-13</i>	<i>0.669</i>	<i>2.20E-16</i>
Nuclear volume	18.58	-15.31	19.59	-29.86	31.62	-55.48	-63.65	42.85	-3.13	344.88
	3.64	5.26	4.21	4.32	4.49	1.26	1.19	4.75	3.19	13.40
	<i>2.23E-04</i>	<i>1.68E-02</i>	<i>8.09E-04</i>	<i>6.69E-08</i>	<i>7.02E-06</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>2.36E-11</i>	<i>0.463</i>	<i>2.20E-16</i>
	<i>hira</i>					nuclein 1				
	30h	2 d R	7 d R	2d R HS/ 30HS	7 d R HS/ 2 d R HS	30h	2 d R	7 d R	2d R HS/ 30HS	7 d R HS/ 2 d R HS
RHF	-12.53	-23.89	11.41	-14.73	33.24	-40.47	-51.79	-0.11	-14.34	108.33
	4.83	3.65	4.27	4.09	5.16	3.27	2.59	2.88	4.60	6.01
	<i>0.037</i>	<i>2.39E-05</i>	<i>0.075</i>	<i>0.034</i>	<i>3.59E-06</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>0.810</i>	<i>0.970</i>	<i>2.20E-16</i>
Chromocenters intensity	-59.91	-72.60	14.74	-27.53	120.04	-66.59	-89.09	-33.58	-36.35	482.87
	4.08	1.84	8.98	4.88	17.39	2.77	0.91	3.33	5.32	29.25
	<i>7.41E-13</i>	<i>2.20E-16</i>	<i>0.250</i>	<i>0.018</i>	<i>9.42E-10</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>6.91E-08</i>	<i>2.91E-04</i>	<i>2.20E-16</i>
Nuclear intensity	-59.81	-66.56	-2.45	-2.06	70.50	-49.92	-80.05	-33.44	-24.21	221.81
	4.83	3.65	4.27	3.43	11.84	1.81	0.91	3.12	3.44	15.11
	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>0.754</i>	<i>0.750</i>	<i>6.10E-08</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>9.41E-11</i>	<i>2.17E-06</i>	<i>2.20E-16</i>
RHV	-20.67	-20.72	12.05	-3.68	26.35	-46.67	-48.08	-1.17	-0.29	91.94
	4.39	3.69	4.58	4.48	5.22	2.85	2.97	3.21	5.70	6.23
	<i>3.97E-04</i>	<i>3.11E-04</i>	<i>0.081</i>	<i>0.607</i>	<i>1.92E-04</i>	<i>2.20E-16</i>	<i>2.20E-16</i>	<i>0.810</i>	<i>0.970</i>	<i>2.20E-16</i>
Chromocenters volume	-46.24	-26.18	38.85	47.33	7.25	-24.23	-59.04	13.90	-10.71	119.20
	4.00	4.18	8.18	8.35	6.39	4.91	3.44	5.01	7.51	9.65
	<i>3.73E-12</i>	<i>1.68E-04</i>	<i>6.11E-05</i>	<i>3.35E-05</i>	<i>0.395</i>	<i>5.84E-04</i>	<i>2.20E-16</i>	<i>0.046</i>	<i>0.283</i>	<i>2.20E-16</i>
Nuclear volume	-30.41	-16.01	16.80	47.80	-12.63	41.30	-25.95	16.36	-14.32	16.48
	3.90	3.13	6.31	5.50	4.77	5.48	3.24	5.17	3.75	5.17
	<i>1.05E-07</i>	<i>0.011</i>	<i>0.020</i>	<i>4.27E-09</i>	<i>0.037</i>	<i>1.91E-09</i>	<i>0.001</i>	<i>0.014</i>	<i>0.261</i>	<i>0.042</i>

Table 6 Variation of measured or calculated parameters in heat stress (HS) samples relative to their respective mock-treated samples in the indicated backgrounds.

The two last columns of each background display the variation of measured or calculated parameters between indicated heat stress timepoints. Negative values indicate that HS had smaller values than mock. The mean variation is displayed in bold text, the standard error of the mean (SEM) is displayed on grey background, and the p value is displayed in italics. Significant p values are displayed in green, non-significant p values are displayed in magenta.

The most prominent difference between wt and mutants was the delayed restoration of signal intensity in total nuclei and chromocenters in mutant nuclei during recovery after heat

stress (Figure 22, Table 6). Two days after the stress, both values were even lower than at the end of the heat exposure for the three mutants (Table 6). While *hira* plants reached mock levels after 7 d, *asf1ab* and *nuclein1* mutant nuclei regain signal strength at this time but values were still 30-50% below mock levels (Table 6, Figure 22, Annex 1, Annex 2). This indicates that reduced amounts of H3/H4 chaperones limit the speed of heterochromatin restoration during the observed period, with a stronger effect of the *asf1ab* and *nuclein1* mutant. However, I observed a different behavior regarding the volumes of chromocenters and total nuclei. While *hira* starts the recovery of its chromocenters 2 days after the stress, as in wt, *asf1ab* and *nuclein1* mutant showed a delay in chromocenter volume recovery similarly as observed for chromocenter intensity (Table 6, Annex 3, Annex 4). However, in contrast to chromocenter intensity, chromocenter volumes in the three mutants were higher than their respective mock volumes after 7 days of recovery (Table 6, Annex 3, Annex 4). Regarding nuclear volume, *nuclein1* nuclei showed an increase in volume directly after the stress as in wt, but of double amplitude (Table 6, Figure 24). Nuclei of *hira* mutant decreased in volume directly after the stress but started recovering 2 days after the stress, while *asf1ab* nuclei showed not any recovery in volume at this point. At 7 days of recovery after heat stress, nuclei of the three mutants had higher volume than their respective mock, similar to the observations in wt.

After 7 days of recovery after heat stress, both chromocenters and total nuclei intensities were equal (wt, *hira*) or below (*asf1ab*, *nuclein1*) mock level, while both volumes were higher than their respective mock levels, for both mutants and wild type, underlining a possible long term effect of heat stress.

p-values from Welch Two Sample t-test, mutant vs wt						
	chromocenters intensity			chromocenters volume		
	30h HS	2d HS	7d HS	30h HS	2d HS	7d HS
<i>asf1ab</i>	0.02	4.11E-14	6.91E-04	2.27E-08	6.49E-15	1.92E-13
<i>hira</i>	0.287	3.95E-12	< 2.2e-16	0.698	0.027	< 2.2e-16
<i>nuclein1</i>	0.001	1.87E-11	2.72E-07	0.009	2.68E-05	1.09E-12

Table 7 Chromocenter decompaction in *hira* mutant in response to HS is similar to wt.

p values of a Welch two sample t-test comparing values from indicated mutant background to wt. Significant *p* values are displayed in green, non-significant *p* values are displayed in magenta.

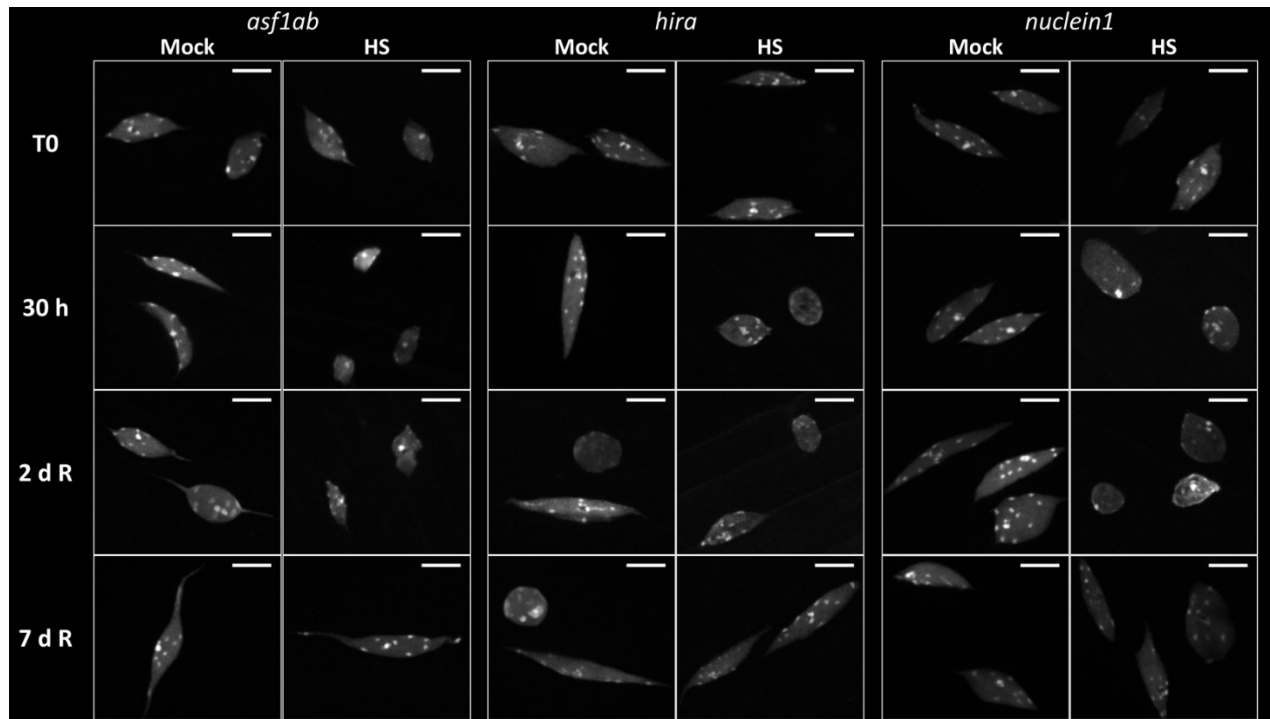


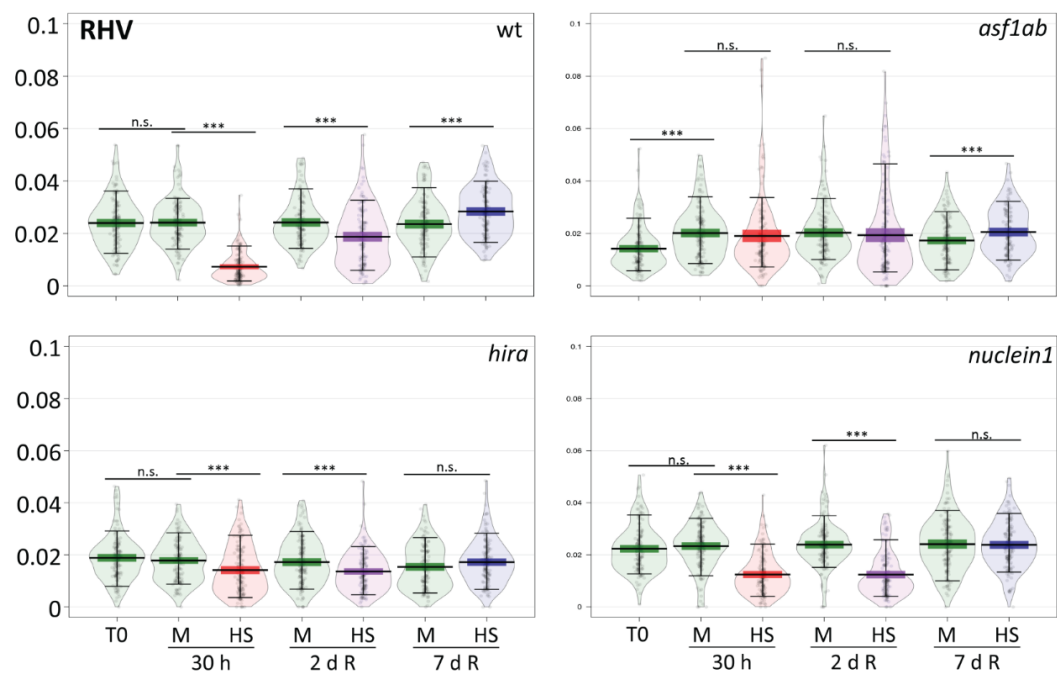
Figure 23 Histone chaperone mutants have altered nuclear architecture in HS condition.

Representative nuclei from the indicated histone chaperone mutants upon HS or mock treatment. Scale bar is 10 μ m.

In wt, heat stress leads to transient as well as long-term effects on nuclear architecture. Reduced histone chaperone activity affects nuclei already under regular conditions but mainly during the recovery period, likely contributing to long-term effects (Table 6, Figure 24). This indicates that, on the one hand, H3/H4 histone chaperons regulate nucleosome loading onto chromatin and their dynamics upon heat stress, on the other hand, chromatin compaction level through the control of chromocenter and nuclear volume. This might represent independent mechanisms. It is likely that there are more and redundant functions involved than could be addressed with the individual mutants used in this study.

In summary, the mutations in the chaperone genes affected some parameters of nuclear organization already under standard growth conditions, while they caused more pronounced differences under elevated temperatures and during recovery from these conditions. Therefore, both *HIRA* and *ASF1AB* complexes play a role in maintaining nuclear integrity, during regular growth, during and after heat stress periods.

A



B

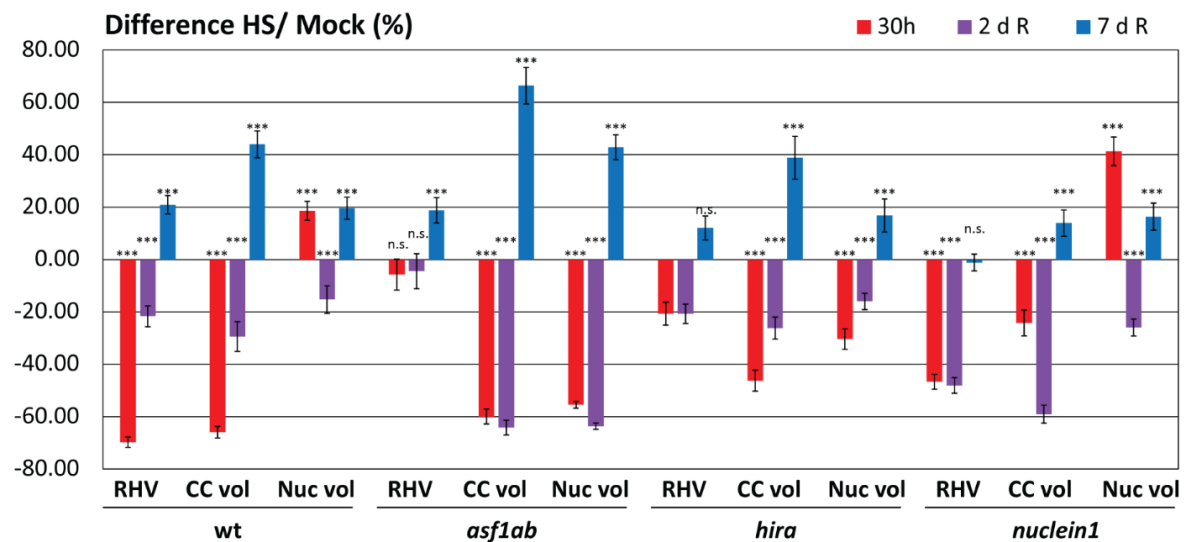


Figure 24 RVF dynamic in response to HS in histone chaperone mutant.

(A) Pirate plot of the relative heterochromatin volume measured from root cell nuclei at indicated time points of heat stress or mock treatment in the indicated mutants or wild type. $n = 122-141$, $118-144$, $129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1*, respectively. Grey points represent raw data. Beans represent a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean. (B) Bar plot of the enrichment or depletion in %, of RHV and its components between mock and heat stress time points, in the indicated wt or mutant background. p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. Vol = volume, M = mock treatment, HS = heat stress treatment, R = recovery time point.

3 DISCUSSION

3.1 LIMITATIONS IN LIVE IMAGING OF SPECIFIC GENOMIC LOCI

The experiments described in the corresponding part of the thesis were planned to address the question whether changes in gene activity were accompanied with chromatin rearrangement and/or locus movement, and what would have been the order of events. The molecular data generated in collaboration with Jasmin Bassler provided evidence that the restoration of transcription after perturbation occurs days before the restoration of nucleosome occupancy and chromatin organization (Bassler, Dumur et al., in preparation, Bassler PhD thesis, in preparation). Nevertheless, it remains interesting to visualize the process within living cells, and approaching single locus imaging was a timely and interesting idea but met several limitations and flaws in the project design that I will discuss in the following.

The use of the GAL4-GFP enhancer trap as activation reporter was a reasonable strategy. In case of successful imaging of its sequence, the approach could have been transferred to many other enhancer trap lines, in plant as well as in other organisms where such collections are available. This could have answered whether loci located at different places of the genome or in different chromatin context undergo the same sequence of events upon transcriptional activation. However, the strict connection between start of transcription and appearance of a GFP signal is to be taken with caution. On the one hand, GFP could diffuse to neighboring cells, causing false positive activation signals. To prevent this, a strict nuclear GFP version could have been used. On the other hand, the time needed for translation and accumulation of the GFP causes a delay between transcription of the locus and GFP detection. A method to measure this delay would be to use a line expressing non-diffusing GFP and run an RNA in situ hybridization time course, to

correlate the transcript appearance with GFP detection. Another solution would be to use the recently identified RNA targeting CRISPR/Cas13 system (Shmakov et al. 2015; Abudayyeh et al. 2016; Abudayyeh et al. 2017), using a nuclease-dead Cas13 fused with a fluorescent protein to image the messenger RNA and a dCas9-FP to image the locus. This would eliminate or at least reduce maturation biases.

Another, probably naïve and overambitious element was the attempt to go directly for stably transformed lines with lowly expressed promoters. It would have been advisable to establish the components of the system with tests in transient expression system, in *Nicotiana benthamiana* or *Arabidopsis* protoplasts, starting with Cas9 driven by strong promoters and readjusting expression levels afterwards. It would have further helped to include a chromatin reporter, which would have allowed to follow potential chromatin rearrangement upon gene activation.

During the time of my experiments, live imaging of genomic loci has been achieved in plants in a transient system (Dreissig et al. 2017). However, even here, only highly repetitive regions have been visualized so far. The problem of signal enrichment at the target locus over background of non-bound FP remains one of the main limiting factors for imaging single, non-repetitive, loci. The threshold for detection in mammalian cells was determined to co-localization of 26-36 EGFP molecules, depending on the background intensity (Chen et al. 2013). Methods and technologies around CRISPR/Cas9 have developed quickly in recent years and widened their use (Adli 2018). Combination of the recent advances like Cas9 fused with Sun-tag (Tanenbaum et al. 2014; Ye et al. 2017) and gRNA multiplexing (Ma et al. 2015; Xie et al. 2015) will likely increase the chance of achieving imaging of non-repetitive loci in plant cell nuclei in the future.

3.2 METHODOLOGICAL ASPECTS OF LIVE IMAGING IN ROOT CELL NUCLEI

One prerequisite for single locus imaging is the possibility to track nuclei of living cells over long-term periods with high spatial and temporal resolution. Tracking methods for root tip microscopy have been previously published (Campilho et al. 2006; Sena et al. 2011; von Wangenheim et al. 2017). They all are based on the same principle: comparing successive time

points to extrapolate root growth rates, predict the next position of the imaged region and readjust a motorized microscope stage in accordance. However, no tracking solution compatible with in-house spinning disc microscopes and allowing sub-nuclear 4D imaging existed when the project started in 2014. In collaboration with Miloš Šrámek, I worked to establish a tracking method, (i) fitting the constraints of the spinning disk acquisition software (VLOCITY) and hardware, (ii) considering the conditions at the Biooptics facility, and (iii) matching the necessary temporal and spatial scale for nuclei imaging. The setup allowed fast root tip growth (indicating physiologically adequate conditions) and relatively large distance between two timepoints, but together with the high magnification necessary to image nuclei, the third requirement was most challenging.

The TARDIS software allows on average 4-5 h of image recording that allows segmentation and analysis of nuclear and sub-nuclear features. Because the recording is not interrupted between time points, in contrast to the TipTracker developed by von Wangenheim and colleague (2017), the readjusted time-lapse is directly available at the end of the experiment and does not need the assembly step from single time points to 4D hyperstacks required with other tracking methods (von Wangenheim et al. 2017) or imaging software (VisiView). Such assembly can be demanding in time and computer resources and can be prone to mistakes (assembly in the wrong order or missing timepoints). Post processing is still possible with TARDIS-generated data and has been used for better representation of the time-lapse series in presentations but is not required. TARDIS has the advantage to be independent from the acquisition software and thus can be transferred to other platforms. It only requires real time access to recorded data (that can sometimes be tricky) and communication with a motorized microscope stage.

However, TARDIS still has limitations, as it has not been developed as a "plug and play" software. Thus, the transfer to another system requires the installation of a companion module. The script might also need adaptation to different microscope stages with a different programming language. The software requires also some anchor points in the images to register the drift between two timepoints. The main problem concerns potential drift along the z-axis, limiting the observation time for specific cells in some root zones. The drift is due to the horizontal position of the microscope and the resulting unnatural growth direction of the root. A

remotely controlled piezo for the z axis might be able to compensate the z drift. Alternatively, a vertically oriented microscope system, as used by von Wangenheim and colleagues (2017), could also reduce the z drift. However, the spinning disc principle is not compatible with a vertical position, as the necessary higher magnification objectives require liquid immersion medium between objective and sample. In a vertical position, the liquid immersion medium would drip down over time, not allowing long time-lapse imaging. The laser scanning microscope (LSM) used by von Wangenheim and colleagues (2017) allows imaging of vertically growing roots, but it remains to be investigated whether reaching an equivalent image resolution, with acquisition time fitting to root growth at this scale, will become possible.

3.3 LIVE IMAGING OF ROOT NUCLEI DURING DIFFERENTIATION

The goal of the tracking experiment was to capture dynamic remodeling of nuclear architecture. To verify the suitability of the tracking, I have chosen to apply it on the elongation zone of the root, where cell and nuclei start elongating and maturing (Verbelen et al. 2006) and where expression of fluorescent protein had earlier revealed correlations between nuclear morphology and cell type. For instance, cells of the root tip contain small spherical nuclei, while mature epidermal cell nuclei are fusiform (Chytilova et al. 1999; Chytilova et al. 2000).

I started nuclear tracking with a Histone 2 A-10 reporter because of its brightness. However, H2A-10 appeared later to be a poor reporter compared to H2B, particularly in mature nuclei (Figure 19). Thus, tracking experiments with H2A10 were not subjected to quantitative image analysis, however they indicated that H2A10 starts labeling chromocenters at the end of the transition zone and in the elongation zone but not in the RAM (Movie 2, Movie 3, Movie 4). However, chromocenters are mostly H2A-10-free in mature root nuclei (Figure 19). Using the H2B2 marker in addition (Movie 5), I could document the elongation of individual root epidermal cell nuclei with the tracking system, recapitulating the change in shape already documented with static observation within the expected time range of 5-8h (Chytilova et al. 1999; Chytilova et al. 2000; Band et al. 2012; Pavelescu et al. 2018).

Remodeling of the nuclear architecture was previously observed upon developmental stage transitions (Tessadori et al. 2007b; van Zanten et al. 2011; Bourbousse et al. 2015). Investigation of more subtle features linked to differentiation (Rosa et al. 2014; Otero et al. 2016) and cell identity (Poulet et al. 2017) only started recently. I could show that the differentiation within the root epidermis into cell files with trichoblasts and atrichoblasts is preceded by the acquisition of specific nuclear shape parameters already in the proliferation zone of the root meristem. It correlates well with changes of cell shape, as trichoblasts are more numerous and smaller than atrichoblasts in the RAM due to differential control of cell division (Berger et al. 1998). The flat shape of trichoblast nuclei is probably due to the smaller size of their cells and to their putative synchronous cell division, as suggested in Mov_TARDIS_1a. Thus, four cells must fit in the space previously occupied by two, and the new cells are wider than high, restricting also the space for the nuclei. Cell division in the RAM in trichoblast files seems to be more synchronous than in the other files. This had been suggested by observation of BrdU staining in neighboring epidermal cells (Hayashi et al. 2013; Kumpf et al. 2014), and Mov_TARDIS_1a supports this hypothesis. In the transition and elongation zone, nuclear volume can be used as one parameter clearly distinguishing the two epidermis cell types (Figure 13), although there is some overlap between values for hair and non-hair nuclear volume in the transition and elongation zone. However, the differences between nuclei populations are statistically significant, and additional parameters of chromatin organization, like RHF or RHV, are good candidates for further distinction (Figure 11). Therefore, the parameters could be used to identify and distinguish isolated nuclei, provided the differences are conserved after extraction. Whether the differences in chromatin conformation, gene expression and timing of entry into endoreduplication between trichoblast and atrichoblast nuclei contribute to or are a consequence of differences in nuclear volume (Costa and Shaw 2006; Wegel et al. 2009; Lan et al. 2013; Bhosale et al. 2018) remains to be investigated.

3.4 LIVE IMAGING OF ROOT NUCLEI IN RESPONSE TO HEAT STRESS

After setting up the conditions for long-term live imaging and validating the suitability of the system to detect biologically relevant parameters in nuclear architecture and chromatin

organization, I exploited the potential of a microscope equipped with an incubator for defined temperature to investigate the nuclear reorganization during heat stress in living cells.

Investigations of heat stress response in *Arabidopsis* are numerous and explore many different aspects (Bokszczanin et al. 2013; reviewed in Liu et al. 2015). One major limitation, however, of a general interpretation and discussion of HS experiments is the extraordinary multiplicity of heat regime, temperature, length of exposure and recovery time, applied by the different teams and uncovering different aspects of heat response (see Liu et al. 2015 table1 for an overview of the different kind of heat treatment used). Keeping conditions as controlled and reproducible was therefore of utmost importance.

This provided and using the chromatin reporter lines, I demonstrated the decompaction of chromocenters in living root nuclei upon heat stress, supporting previous publication on fixed nuclei (Pecinka et al. 2010; Wang et al. 2013b). I could also show that chromocenters were able to reform upon recovery, which contrasts with data in a previous publication where chromocenters were shown to remain decompacted 7 d after heat stress (Pecinka et al. 2010). However, these data were obtained with fixed nuclei from leaves, and the difference could indicate a tissue specificity of the heat stress response between roots and leaves. Another explanation could be the age of the plants, as my observations were made in roots of 7 day-old seedlings, while leaves of 21 day-old plants were used in the previous work. It is likely that at the 7 d recovery time point, when the plants are 28 days old, beginning senescence might have impaired the capacity to rebuild the chromocenters, explaining the difference between the two studies.

I measured volumes and intensity of the fluorescence-labeled chromatin from 3D living nuclei and calculated the ratio between total chromocenters and total nuclei in statistically significant numbers. Such quantification of parameters in 3D nuclei are rare in literature, and only one recent study used a semi-automatic segmentation approach to measure volumes of nuclei in fixed root hair cells (Poulet et al. 2017). My measurements of mock-treated nuclei gave about 4 or 3 times larger values for nuclear and chromocenter volumes, respectively, and a calculated RHV about 60% smaller, in comparison to the values measured by Poulet and colleagues. These discrepancies could be due to different factors. The fixation process could

shrink the nuclei, resulting in a lower volume. The pixel size used by Poulet et al. was about twice smaller in the xy axes and 2.5 smaller in the z axis, resulting in higher resolution. However, the thresholding has a significant impact on the final measurement (Del Prete et al. 2014), and thresholding methods were different between the two studies. As my goal was to compare nuclear architecture between two conditions, I optimized segmentation parameters to be comparable between experiments. This is supported by consistent mean values between all wt mock timepoints (Figure 25). Likely even more relevant for the differences, Poulet et al. used exclusively root hair nuclei, which are very compact, while I used mainly atrichoblast, and cortex nuclei for the heat stress experiments, trichoblast nuclei being frequently out of focus. Thus, my conclusions drawn from comparison between mock and HS treatment remain valid, although the measured absolute volumes are larger than reported elsewhere.

Nuclear volume	T0	30 h M	2 d R M	7 d R M
mean	487.02	553.14	534.07	532.95
SD	359.78	218.02	193.12	249.21

CC volume	T0	30 h M	2 d R M	7 d R M
mean	10.74	13.00	12.01	13.04
SD	7.18	6.04	3.96	7.11

RHV	T0	30 h M	2 d R M	7 d R M
mean	0.0238	0.0242	0.0242	0.0252
SD	0.0097	0.0090	0.0091	0.0104

Figure 25 Measurements are consistent between timepoints in mock-treated samples.

Mean and standard deviation of indicated parameters during mock treatment.

Heat stress causes a genome-wide release of TGS associated with chromocenter decompaction, largely independent from changes of known TGS characteristics like DNA methylation, RdDM pathway, histone marks, DDM1 or MOM1 (Pecinka et al. 2010; Tittel-Elmer et al. 2010). However, mutants defective in key players of the TGS pathway can have altered thermotolerance: *NRPD2* mutants, lacking a functional second largest subunit of Pol IV and V, or *HDA6* mutants, with impaired histone deacetylase activity, are hypersensitive to heat stress, as

(to a lesser extent) are *rdr2*, *dcl3*, and *ago4* mutants (Popova et al. 2013). This indicates that functional TGS components are required for thermotolerance, but that heat stress per se does not limit the activity of the trans-acting TGS factors, but interferes with TGS maintenance in a different (Tittel-Elmer et al. 2010).

The lacking impact of known TGS players on heat-induced TGS release could also be due to the possibility that the stress-reactivated elements are already transcribed to their maximum in the TGS mutants, so that transcription of these loci cannot be boosted any more by heat stress.

This let us consider cis-acting changes at sites of reduced TGS, and the transient nature of the heat stress-induced effects including nucleosome dissociation and involvement of H3/H4 histone chaperone complexes (Pecinka et al. 2010); Bassler, PhD thesis, in preparation) prompted to include the histone chaperone mutants into the live imaging experiments. CAF-1 mutants could not be considered for reasons described before, but I could show that mutants impaired in the other H3/H4 chaperone complexes, ASF1 or HIRA, had altered nuclear architecture already in stress-free conditions (Table 5). Upon HS, ASF1 and HIRA seem to be involved in the initial response (as the rounding of the nuclei was much less evident in the mutants) as well as in the restoration of chromocenters upon recovery, which was delayed in the mutants and reached or approached non-stress compaction levels only after longer periods. Interestingly, the heat-induced chromocenter decompaction was not substantially impaired in *hira* or *asf1ab* (Table 6, Figure 22, Figure 24). As there is likely some redundancy between the histone chaperones, the analysis of their role during heat stress and recovery needs extended analysis. So far, only the ASF1 complex was reported to have a role in basal and acquired thermotolerance (Weng et al. 2014). In yeast, Asf1 deletion leads to temperature sensitivity, and combined deletion of Asf1 and SNF2, a member of the ATP-dependent chromatin remodeling complex SWI/SNF, reduces the displacement of nucleosome during heat shock synergistically (Erkina and Erkinen 2015).

Similar to TGS factors, which are involved in thermotolerance but do not modify the extent of TGS alleviation, histone chaperones seem to be involved in the heterochromatin reconstitution during recovery but have little role in heat-induced chromocenter decompaction. However, they are necessary for correct gene regulation during heat stress response (Bassler, Dumur et al., in

preparation, Bassler, PhD thesis, in preparation). In brief, it seems that heat resistance and heat-induced TGS alleviation are controlled by two independent pathways.

More evidence for an influence of chromatin organization on heat stress resistance comes from a mutant with impaired HIT4 protein that is defective in basal heat tolerance and does neither decompact chromocenters nor release TGS in response to heat stress, but is involved in a new TGS pathway independent from MOM1 (Wang et al. 2013b; Wang et al. 2015b). This confirms that heat-induced chromocenter decompaction is a regulated process and suggests a link between chromocenter decompaction, TGS alleviation, and thermotolerance. The HIT4 protein relocates from CCs to the nucleolus upon HS, which is still possible for the protein in the point mutated *hit4-1* but without triggering CC decompaction or TGS release (Wang et al. 2015b). The most likely hypothesis to explain this phenotype is to postulate a partner protein for HIT4 for which the mutation in *hit4-1* impairs the interaction. Identifying HIT4 interactors could appear crucial to understand the function and mechanism underlying heat-induced CC decompaction and TGS release and its putative role in thermotolerance.

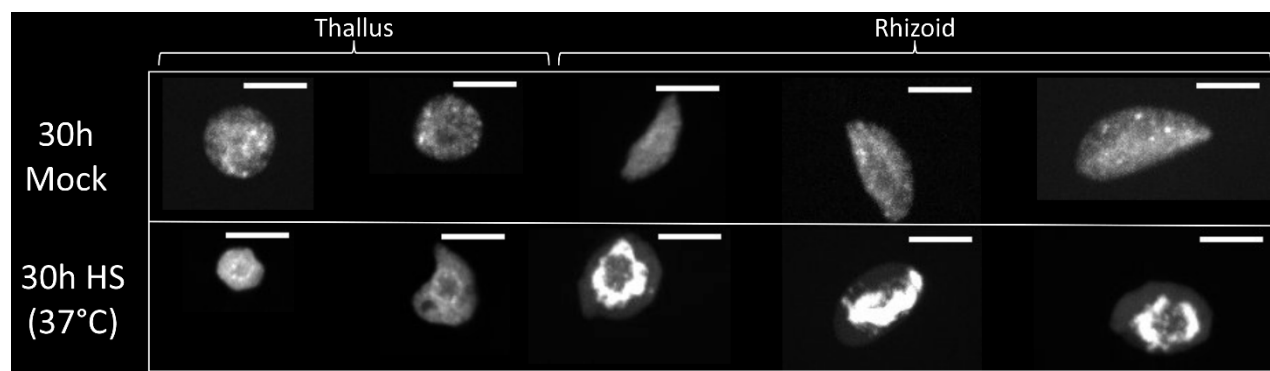


Figure 26 HS can induce striking heterochromatin formation in *Marchantia polymorpha*.

Maximum intensity projections of thallus (left) and rhizoid (right) nuclei expressing AtH2B-RFP after 30 h of mock (top) or HS (bottom) treatment. Scale bar = 10 μ m.

Why and how chromocenter decompaction and alleviation of TGS play a role in thermotolerance and whether they have an adaptive function remains elusive and is so far limited to speculation. It has been shown recently that the landing site of some TE families are biased towards H2AZ-containing sequences. *De novo* insertions of TEs are able to create new

epigenetically regulated alleles of neighboring genes (Quadrana et al. to be published <https://www.biorxiv.org/content/early/2018/10/21/447870>). H2AZ is enriched in genes responsive to different stimuli (Coleman-Derr and Zilberman 2012), and H2AZ-containing nucleosomes seems to have a role in heat response (Kumar and Wigge 2010). It might be possible that the transient heat-induced release of TGS increases the chance that TE insertions are guided by H2AZ to create new epigenetically regulated alleles with potential adaptive functions. Such nuclear response to heat stress might be the result of an evolution-driven adaptation specific to a given species: a pilot experiment in the liverwort *Marchantia polymorpha*, which does not have conspicuous chromocenters in its haploid vegetative phase, indicates that prolonged heat stress can cause striking heterochromatin formation (Figure 26).

My observations also revealed a lack of rounding upon HS in the investigated H3/H4 chaperone (Figure 20) and altered nuclear architecture in stress-free conditions most likely due to gene miss-regulation and impaired nucleosome deposition, supporting a role for these chaperones in maintenance and plasticity of nuclear architecture in stress and mock condition. The rounding of nuclei upon heat stress is visible in previous publication (Wang et al. 2013b; Wang et al. 2015b) however without further notice. The reason of nuclear rounding of root epidermal nuclei upon heat stress remain elusive. Shape is made mainly by nuclear envelope and forces applied to it, therefore comparison with nuclear envelope mutant was what made the most sense. More specifically the phenotype of round nuclei with impaired movements, what I observed in HS-time-lapse experiment, was observed in *kaku1* mutant lacking a plant specific myosin that interacts with outer-nuclear-membrane components of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex (Tamura et al. 2013). The LINC complex has been mostly characterized in mammalian cells. It is formed by Sad1/Unc84 (SUN) and Klarsicht/Anc1/Syne1 homology (KASH) proteins and forms the junction between cytoskeleton and the nucleus. SUN protein located in the inner nuclear membrane interact with Lamin A in the nucleoplasm and connect, in the perinuclear space with the KASH proteins located at the outer nuclear membrane, which interact with the cytoskeleton. SUN and KASH proteins have been recently characterized in plants, as well as CRWN proteins proposed to assume the Lamin function in plants (Crisp et al. 2006; reviewed in Tatout et al. 2014). Round shapes of nuclei is a common phenotype of mutant

affected in the LINC complex and associated proteins, some are affected in heterochromatin organization (Dittmer et al. 2007a; Zhou et al. 2012; Sakamoto and Takagi 2013; Wang et al. 2013a; Tatout et al. 2014). Data about LINC subunit regulation upon HS are contradictory. Indeed, RNA seq conducted on 21 d-old plants by Jasmin Bassler as part of her PhD project (Bassler, PhD thesis, in preparation) indicates that most of the subunits of the LINC complex are down-regulated upon HS, and few of them recover at 7 d R HS (Annex 5). In contrast, qPCR done on 7 d-old roots indicates that the subunits studied are not differently expressed between mock and HS-treated samples right after the stress or at 7 d R (Figure 27). The localization of Sun1 and 2, two component of the LINC complex at the inner membrane (Oda and Fukuda 2011; Graumann et al. 2014) was inspected, using GFP-tagged versions, and did not show differences between mock and HS conditions (Figure 28). The possibility of post-translational modifications is also to take into account as they could disrupt interaction between LINC subunits.

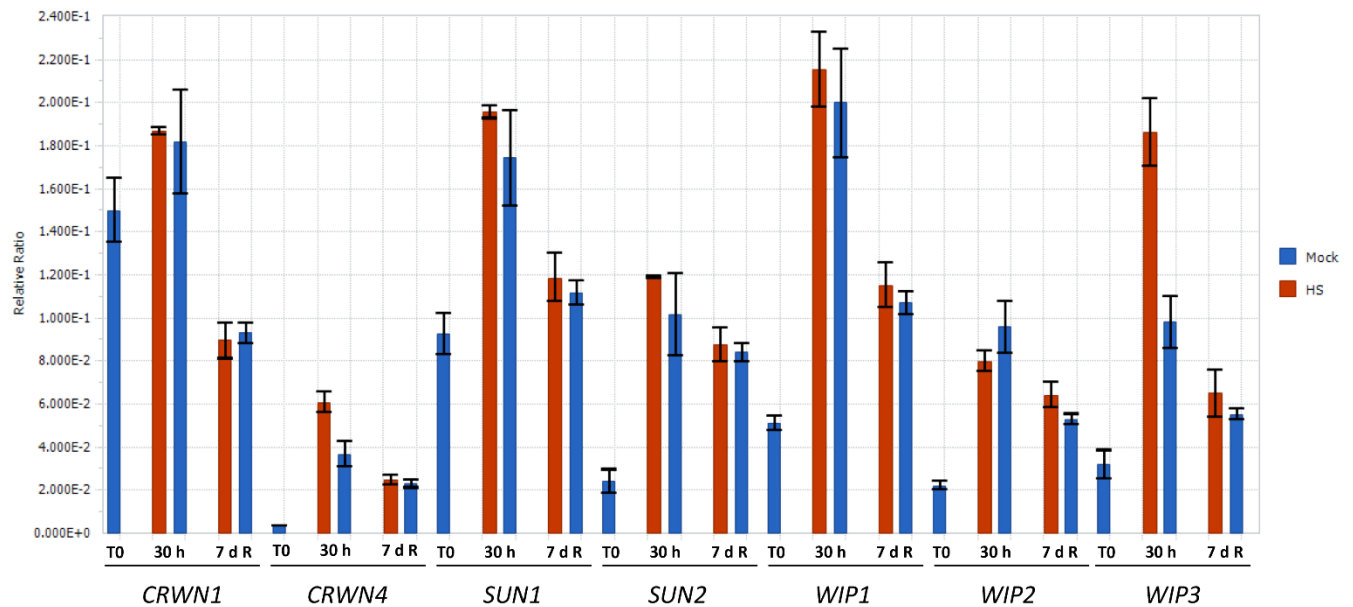


Figure 27 Selected members of the LINC complex are not differentially transcribed between HS and mock treatment.

Bar plot of relative expression of the indicated genes at T0 30 h and 7 d R of heat stress (red) and mock (blue) treatment.

CRWN1 encode for a nuclear protein thought to assume a lamina-like function in plant (Dittmer et al. 2007a; Ciska and Moreno Diaz de la Espina 2014). The *crwn1* mutant show round nuclei in stress-free condition (Dittmer et al. 2007a; Sakamoto and Takagi 2013). Its behavior in

response to heat stress was observed with a focus on nuclear sphericity. Around 30% of *crwn1* nuclei loss their round shape directly after HS and around 70% at 2 d R before restoring close to mock level at 7 d R (Figure 29). This support the idea that a the putative plant lamina, and LINC complex by extension, is involve in shape control in response to heat stress. In wild type some nuclear deformation is observed toward the end of the heat stress once nuclear rounding is complete (Movie 8). *crwn1* nuclei being already round it would be possible that a putative "deformation phase" comes earlier and cause the decrease in nuclear sphericity observed in *crwn1* nuclei. Taken together with the lack of nuclear rounding observed in the histone chaperone mutant, this would indicate that the conservation of nuclear integrity during heat induced deformation and its restriction to the stress period involve several factors including lamina-like protein CRWN1 and histone chaperone complex ASF1 and HIRA and other putative factors.

The cytoskeleton might be as well involve in the control of nuclear shape. Indeed actin-depolymerizing drugs is known to abolished nuclear movement in plants (Chytilova et al. 2000; Ketelaar et al. 2002) and to be involve in the shaping of the vegetative nucleus of germinating pollen grain (Heslop-Harrisson and Heslop-Harrisson 1989). Moreover *kaku1* encode for an actin motor Myosin XI-I responsible for shape and movement. However, pilot observation of Actin reporter in histone reporter line upon HS didn't show any obvious phenotype under HS (Figure 30). A study in BY-2 tobacco cells also indicated that both microtubules and actin filament are necessary for nuclear elongation (Yasuhara and Kitamoto 2014). Experiment with actin and microtubule inhibitor were conducted but the results were not conclusive and couldn't be repeated because of a lack of time.

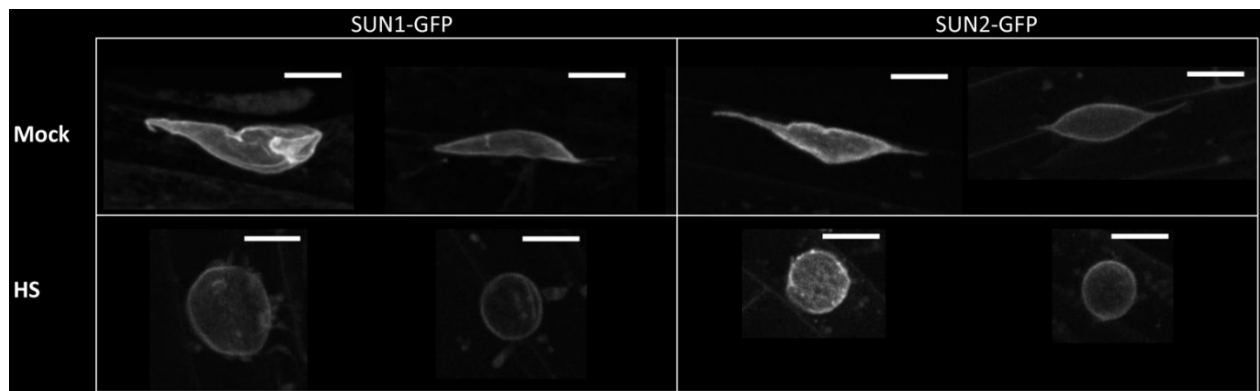


Figure 28 SUN proteins do not relocate upon HS treatment.

Representative images of nuclei expressing SUN1-GFP (left) or SUN2-GFP (right) after 30 h of mock (top) or HS treatment (bottom). Scale bar = 10 μ m

The different parts of this PhD project were focused on the use of live imaging to describe and bring element of answer to biological question. The more significant advance achieved is the development of software and setup to image the long-term process in a dynamic environment at the sub-nuclear scale. Different tracking solution have emerged in a relative short time (Campilho et al. 2006; Sena et al. 2011; von Wangenheim et al. 2017) illustrating the need of dynamic live imaging solution. I participated to the development of a solution adapted to in-house material allowing to describe and analyze chromatin dynamic during several hours to day long process. The major limitation in the current long-term imaging setup concerns the 30 h-long heat stress imaging. One problem is the unavoidable decrease of signal over time due to bleaching is still a thing but was nevertheless quite low for a confocal imaging of this length. The main limitation is that the heat stress imaging didn't manage to clearly capture the expected chromocenters decompaction. This is most likely due to the imaging conditions, where despite the LED panel installed the optimal growth conditions might not have been reach. The optimization of imaging condition, potentially with better illumination would be a solution. To conclude, this project illustrates the potential of live imaging to investigate dynamic and long-term processes with the often-underestimated advantage of fast and fixation-free image acquisition.

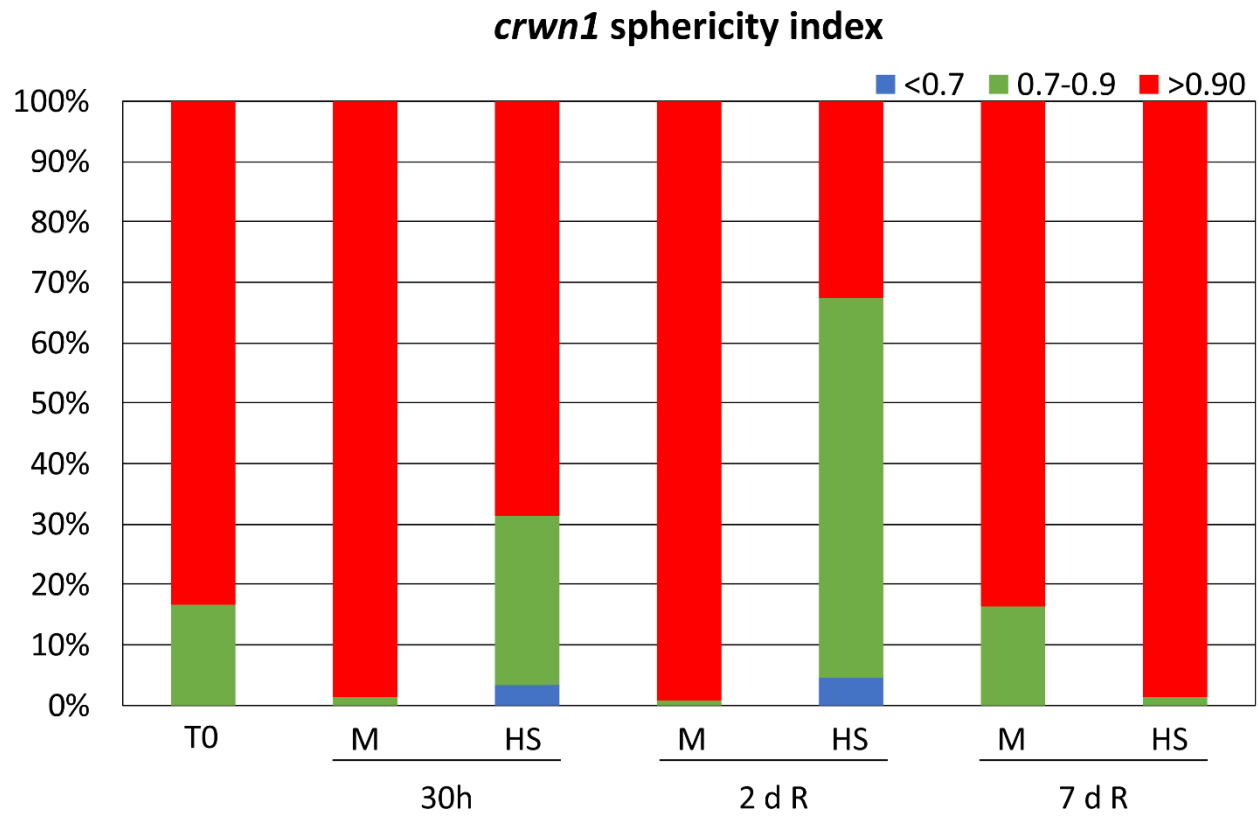


Figure 29 Heat stress alters nuclear sphericity in *crwn1*.
Distribution of sphericity classes in *crwn1* mutant.

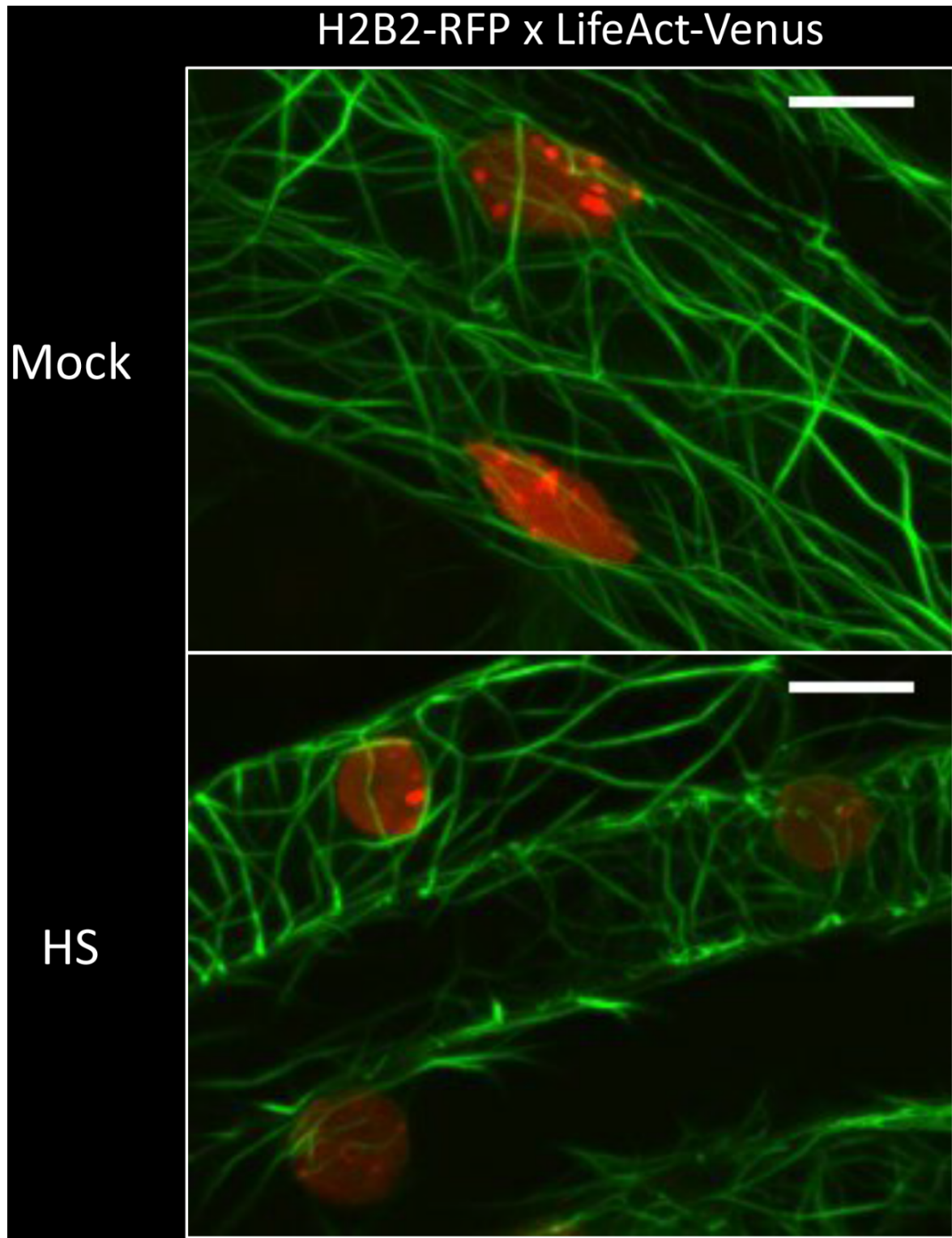


Figure 30 HS does not obviously perturb actin filament organization.

Maximum intensity projection of living root cells expressing a chromatin reporter (red) and an actin reporter (green) in mock (top) and HS (bottom) conditions. Scale bar is 10 μm .

4 MATERIAL AND METHODS

4.1 PLANT MATERIAL AND GROWTH CONDITIONS

The GAL4 enhancer trap collection (Haseloff 1999) was made in the C24 ecotype of *Arabidopsis thaliana*. All constructs created for this project were transformed into C24 and Col-0 ecotype. All other plant material is in *Arabidopsis thaliana* Col-0 background. Histone chaperone mutants in this study were *hira* (WiscDsLox362H05, Nie et al. 2014), *nuclein1* (insertion: GABI_018D02) and *asf1ab* (a double mutant combining *asf1a* (GABI_200G05) and *asf1b* (SALK_105822), (Zhu et al. 2011). The mutant for CRWN1 is *crwn1-2* (SALK_041774C) (Goto et al. 2014). Plant lines with SUN1-GFP and SUN2-GFP were graciously provided by the Fukuda lab (Oda and Fukuda 2011). For microscopy, I used lines expressing the chromatin reporter Histone 2B-2 or Histone 2A-10 tagged with Red Fluorescent Protein (H2B-RFP and HTA10-RP, respectively), cordially provided by the lab of Frederic Berger (Yelagandula and Berger, personal communication). H2B-RFP is referred to as wild type in the study of heat stress response. H2B-RFP was introgressed into *hira*, *nuclein1*, *asf1ab* and *crwn1* by crossing. F2 populations were screened for H2B-RFP expression with a stereomicroscope and by PCR for homozygosity of the insertion mutant alleles. Individual F2 lines homozygous for the mutations were screened for non-segregating H2B-RFP fluorescence in their F3 progeny, indicating homozygosity also at the H2B-RFP locus.

For imaging, seeds were surface-sterilized and sown on plates containing solidified Germination Medium (GM) (Masson and Paszkowski 1992 <https://www.oeaw.ac.at/gmi/research/research-groups/ortrun-mittelsten-scheid/resources/>). After 2 days of stratification at 4°C in the dark, plates were transferred to an *in vitro* growth chamber with constant 21°C and a 16 h light/8 h dark schedule and cultured in vertical position. For other purposes (seed amplification, genotyping or crossing), seeds were surface-sterilized, sown on soil (Einheitserde special profi substrat, Einheitserde GmbH) supplemented with ca. 10% perlite (premium perlite, Gramoflor GmbH & Co) and placed in a growth chamber (16 h light/8 h night at 21/16°C, respectively).

4.2 SEED SURFACE-STERILIZATION

Seeds were surface-sterilized in open Eppendorf tubes by a 10 min incubation in chlorine gas. Chlorine gas was generated by mixing 10% sodium hypochlorite with 1 molar hydrochloric acid in a beaker placed in a hermetic box within a fume hood.

4.3 PLASMID CONSTRUCTION AND CLONING

All constructs were assembled using the In-fusion strategy (Clontech), except stated otherwise.

A first plasmid was made containing the 3x-mCherry coding sequence in the pGreen0229 backbone (Hellens et al. 2000) opened with EcoRV using the In-fusion strategy (Takara Bio Inc). 3x-mCherry was taken from pGGC026 (Addgene plasmid # 48831, gift from Jan Lohmann. Then the octopine synthase terminator (OCSt) was cloned 3' of the 3x-mCherry CDS at the XbaI site. Originally, I aimed to clone all pre-selected promoters into the pGrMc3 vector (pGreen containing 3x-mCherry). When I found that a part of the 3x-mCherry was missing, I re-cloned the 3x-mCherry CDS and OCSt into pGreen0229 using circular polymerase extension cloning (CPEC, Quan and Tian 2009). The preselected promoters were then inserted in frame at the Bgl II site. I then I inserted an NLS (NLS a46, from Kosugi et al. 2009) in N-terminal position of the 3x-mCherry CDS in plasmids containing already the promoters. Cas9 gene mutations D10A/H840A to obtain a nuclease-inactive dCAS9 were generated with a site mutagenesis kit (Mattia Donà, personal communication) on a wt Cas9 template gene. Then the dCas9 without stop codon taken from pBSII dCas9-1 was inserted in frame in the 5' position of 3x-mCherry. Primer used in this study are listed in Annex1.

4.4 TRANSIENT TRANSFORMATION

Transient transformation was conducted by Idan Pereman (lab of Avraham Levy) at the Weizmann Institute of Science, Israel, according to standard protocols. Agrobacterium strains carrying plasmids containing pUBQ10::dCas9-3xmCherry, gRNA-LacO and the LacOx256 array were co-infiltrated into two leaves of three *Nicotiana benthamiana* plants. As a control, bacteria with a plasmid mix lacking the gRNA template were infiltrated in two other leaves of the same plants.

4.5 HEAT STRESS TREATMENT

After 7 days of vertical growth of seedlings on plates at regular temperature, heat stress at constant 37°C was applied in an incubator (model SE-41L from CLF PlantClimatics GmbH), under the same light regime as in the *in vitro* growth chambers. Mock-treated plates remained in the *in vitro* growth chambers. After 30 h of HS, plates for the analysis at recovery time points were transferred back to the regular growth conditions while plates corresponding the 30 h HS time point were imaged immediately. For time lapse imaging, HS was applied directly on a microscope equipped with thermoregulation, set at 37°C at least 2 h before imaging started. Mock-treated samples were imaged at thermostat-controlled room temperature (21-22°C).

4.6 SAMPLE PREPARATION AND MICROSCOPY:

Chamber mounting for time-lapse acquisition

For long term live imaging, three to seven day-old seedlings expressing fluorescent histone reporters (H2B-RFP or H2A10-RFP) previously grown on vertical agar plates were mounted into imaging chambers that have a glass coverslip at their bottom, can be autoclaved, and form a close environment suited to grow plants under axenic conditions. To prevent drying of roots and ensure root growth in the chamber, I used the following setup. I opened the plates with the seedlings under sterile conditions and cut a rectangle of agar slightly smaller than the size of the chamber's coverslip. Then I placed the seedling onto the rectangle with the root as straight as possible and the aerial part outside the block. Then I extracted the agar rectangle with the seedling from the plate with a spatula and gently flipped it into the chamber, with the roots facing to the coverslip and avoiding trapped air bubbles. The chambers were sealed using LEUCOPORE tape. The chambers were mounted preferentially one day or at minimum 2 hours before imaging. Between mounting and imaging, the chambers were placed in the *in vitro* culture chamber, in horizontal oblique position (ca. 45° angle), roots pointing downwards. This allows adaptation and to minimize z shift overtime when imaging, as the oblique position prevents the root from growing in spirals which would complicate the imaging.

Nuclear architecture during cell differentiation

For time lapse imaging in living plants I used MoBiTec 1CG imaging chambers (MoBiTec GmbH), see previous section for details. For short term imaging of each new sample, I mounted the seedling in a drop of tap water between slide and coverslip. Acquisition of RFP fluorescence was performed with an inverted spinning disk confocal microscope equipped with a PelkinElmer UltraView VoX spinning disc unit, installed in an incubator, and a 63× water immersion objective lens (plan apo, N.A = 1.4; Nikon) with settings for detection of RFP (excitation, 561 nm; emission filter mCherry 609/54). Fluorescence was acquired with an EMCCD camera (Hamamatsu 9100-3, 16 µm pixel, 512x512 pixels). Acquired Z stacks use 0.3 µm steps. Time-lapses were acquired with 10 min time points. Acquisition was monitored by Volocity (PelkinElmer).

Heat stress section

All experiments were performed on homozygous mutants, also homozygous for H2B-RFP or wild type seedlings homozygous for H2B-RFP. To compare fluorescence intensity in root nuclei, microscope settings were first established with the H2B-RFP wildtype line as a reference and kept identical to study fluorescence patterns in all mutant backgrounds. For time lapse acquisition, fluorescence was imaged 2 h to one day after mounting seedlings on Ibidi® µ-slide 2-well microincubation chambers (ibidi GmbH). Ibidi chambers were used because MoBiTec chambers were too small to fit into the VisiScope spinning disc. For series of images with always fresh samples, fluorescence was imaged immediately after mounting the living seedlings in a drop of tap water. Acquisition of H2B-RFP fluorescence was performed with an inverted VisiScope spinning disk confocal microscope (Visitron Systems GmbH, Puching, Germany) equipped with an incubator and a 60× water immersion objective lens (plan apo, N.A = 1.2; Nikon) with settings for detection of RFP (excitation, 561 nm; emission filter mCherry 609/54). Fluorescence was acquired with an EMMCCD camera (Andor Ixon Ultra 888, 13µm pixel, 1024x1024 pixels). Acquired Z stacks use 0.5 µm steps. Time-lapses were acquired with 30 min intervals for 61 timepoints. For comparison between DNA, H2A-RFP and H2B-RFP localization, DNA was stained using PeqGreen® (PeqLab) diluted 1:400 in tap water + 0.01% Triton X-100. Seedlings were dipped in PeqGreen® solution for 10 s and washed 3 times for 10 s in tap water and mounted on slides in tap water. PeqGreen® signal was collected using settings for detection of GFP (excitation, 488 nm; emission

filter GFP 500 LP). RFP signal was collected as stated previously.

4.7 IMAGE ANALYSIS AND PLOTS

Maximum intensity projection and time-lapse videos were made with Fiji (Schindelin et al. 2012; Schneider et al. 2012; Rueden et al. 2017). From images acquired with a spinning disk setup, 3D stacks around individual nuclei were defined with Fiji, in order to have a collection of 3D stacks each containing a single nucleus for analysis. Single nuclei 3D stacks were then converted to tiff format using a homemade Fiji macro before being imported into image analysis software. Greyscale images were analyzed with IMARIS software (<http://www.bitplane.com/imaris/imaris>). When relevant, nuclei and chromocenters were segmented using the surface function of IMARIS with background subtraction. For the heat stress investigation, a collection of 118-147 nuclei from at least 3 individual roots were analyzed for each timepoint and genotype. The result for each individual segmentation was manually saved and parameters of interest of the segmented objects (size, intensity sum, etc.) were automatically script-sorted into an Excel sheet for further quantitative analyses.

The relative heterochromatin fraction RHF, adapted from Soppe et al. (2002) and Tessadori et al. (2004) is calculated as the ratio between the sum of fluorescence intensity of total chromocenters and the sum of fluorescence intensity of the entire nucleus. The relative heterochromatin volume RHV is calculated as the ratio of the volume of total chromocenters to the volume of the entire nucleus. Densities of chromocenters or total nuclei were calculated as the ratio of the sum of intensities to the respective volume. The relative heterochromatin density RHD is calculated as the ratio of chromocenter density to total nuclear density.

All plots were created with Excel, with exception of the pirate plots. Pirate plots were made on RStudio using the yarr package (N.Phillips adapted from Kampstra 2008). On the plots, grey points represent raw data. Beams represent the smoothed density of the data. Rectangles represent the 95% mean frequentist confidence interval. Whiskers represent 90th and 10th quantiles. Black horizontal bars represent the mean.

4.8 NUCLEAR TRACKING

As Volocity, the microscope's proprietary control program, was not extensible, a homemade tracking system called TARDIS (standing for Tracking Arabidopsis Root through Differentiation Stage) had to be created. The first version of the tracking program was made in Java by Christian Göschl in the lab of Wolfgang Busch, GMI. This program consisted in a graphic interface which allowed to set a 2D readjustment vector and a timer that triggered the communication of the vector to the stage controller. The readjustment vector was calculated by recording the stage coordinates at t_1 , marking a specific nucleus on the monitor with tape, waiting to a defined later time point t_2 and then readjusting the sample position manually to bring the nucleus of interest to the marked same position as in t_1 . Stage coordinates at t_2 were then recorded and a readjustment vector was deduced, based on time difference and stage coordinate changes. The readjustment vector was fed to the TARDIS and a 10 min timer (corresponding to the chosen time interval between timepoints) was started around 30 seconds before image acquisition, to prevent stage movement during the acquisition.

The second version of the program was written in the Python language by Milos Sramek (Scientific Visualization, GMI) and worked by recording in a loop the directory where Volocity stored its files. In the loop, as soon as a new file with measured data was detected, it was loaded by TARDIS.py, analyzed, used to compute a new stage position and finally, the stage was adjusted accordingly. We adjusted the stage position after every second measurement: a pair of measurements t_{i-1} and t_i was used to predict the position of the stage in the next measurement t_{i+1} . Then the pair t_{i+1} and t_{i+2} was used, and so forth. The stage hardware enabled us to control the horizontal position (x, y) of the stage. In the analysis, we first obtained maximum intensity projection of the data in the z direction. The projection was subsequently smoothed by a recursive Gaussian filter with large σ (the value 23 pixels was used). Optimal stage displacement of measurement pairs was finally obtained by registration of the corresponding projections by means of an iterative steepest descent method, using normalized cross correlation metrics. The relative stage position of the previous pair was used as an initial estimate in the iterative procedure. For initialization of the first measurement pair, we used a manually estimated value of the growth speed vector, obtained by subtracting positions of a user-selected object in two

subsequent images. Specification of the initial growth speed vector enabled us to extend the time step between two measurements to desired values. A fully automatic optimization without initialization, owing to quasi-periodicity of the imaged structure, would be possible with shorter time point differences, i.e. 1 min, but too short time steps lead to strong bleaching. The program does not make any assumption about the shape or intensity of the tracked object and could be applied to various systems and samples.

For both versions of the tracking system, the programs were installed on a computer independent from the one managing the microscope (due to rules of the Biooptic facility managing the microscopy platform) and plugged in via USB to the automated stage controller (MS-2000-WK MULTI-AXIS STAGE CONTROLLER).

4.9 RNA EXTRACTION AND QPCR

Total RNA was extracted from ground roots of seedlings grown on vertical agar plates, 1 plate (c.a. 100 seedlings) per timepoint, using TRIzol RNA Isolation Reagents (Thermo Fisher Scientific). cDNA transcription was done with 2.5 µg RNA. Quantitative PCR assays were carried out using the “FastStart Essential DNA Green Master” kit from Roche. Reactions were performed using 1 ng (0.25 ng/µl) of cDNA in a final volume of 20 µl. Each sample was processed in triplicate and ran on the LightCycler 96 device (Roche). AtSAND gene was used as a reference gene and samples were normalized according to the comparative Ct method. Primers used for the qPCR are listed in Annex 6.

4.10 TIME-LAPSE MOVIES:

Time-lapses videos were acquired as mentioned in 4.6, they are available online:

Movie 1 Live imaging of root growth: <https://phaidra.univie.ac.at/o:925441>

Movie 2 Manual tracking of root growth: <https://phaidra.univie.ac.at/o:9254433>

Movie 3 Semi-automatic tracking in root tip : <https://phaidra.univie.ac.at/o:925445>

Movie 4 Semi-automatic tracking in elongation zone : <https://phaidra.univie.ac.at/o:925446>

Movie 5 Automatic tracking in elongation zone : <https://phaidra.univie.ac.at/o:925447>

Movie 6 Live imaging of heat stress treatment in root : <https://phaidra.univie.ac.at/o:925451>

Movie 7 Live imaging of mock treatment: <https://phaidra.univie.ac.at/o:925452>

Movie 8 Three assembled live imaging of heat stress treatment:

<https://phaidra.univie.ac.at/o:925453>

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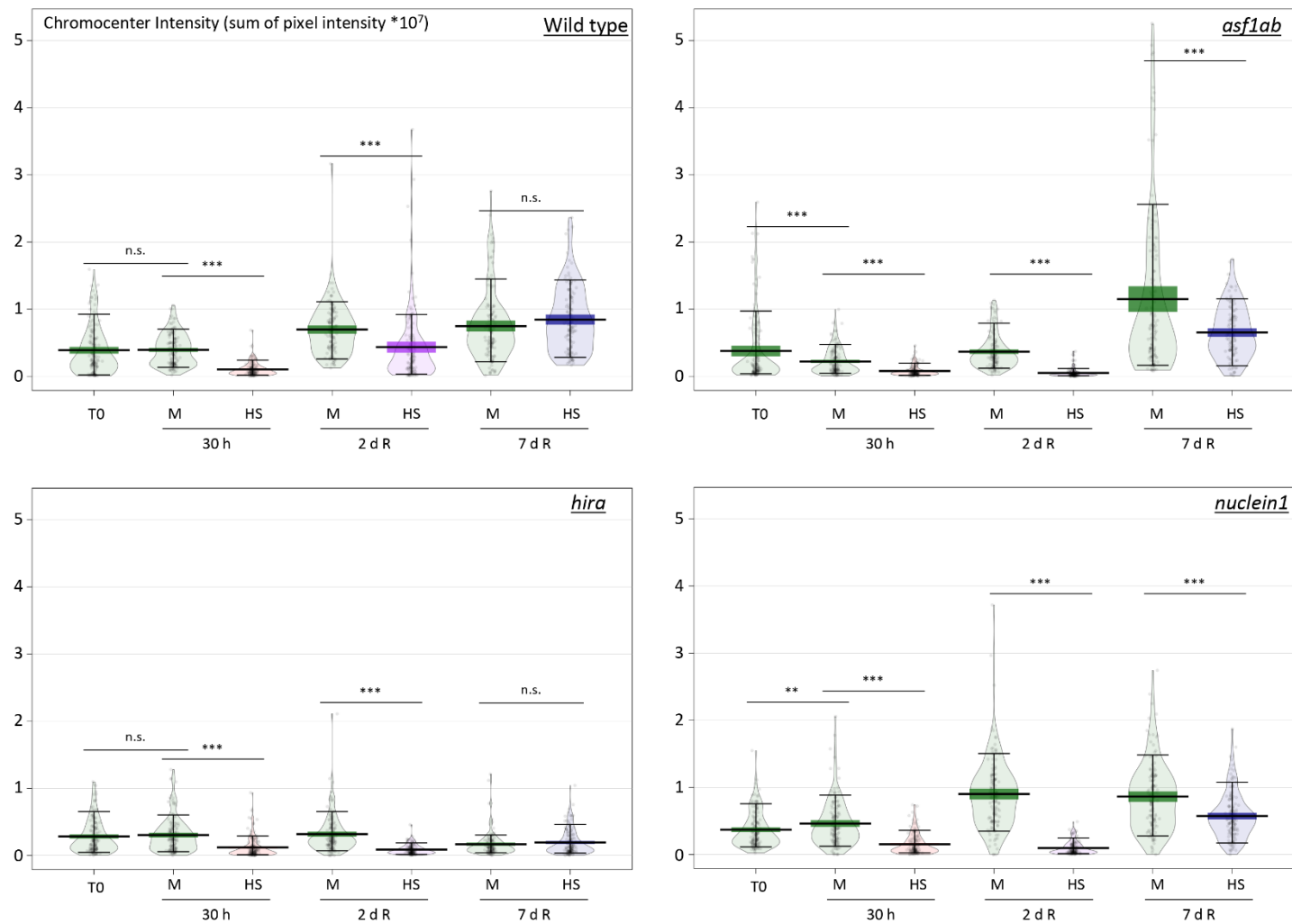
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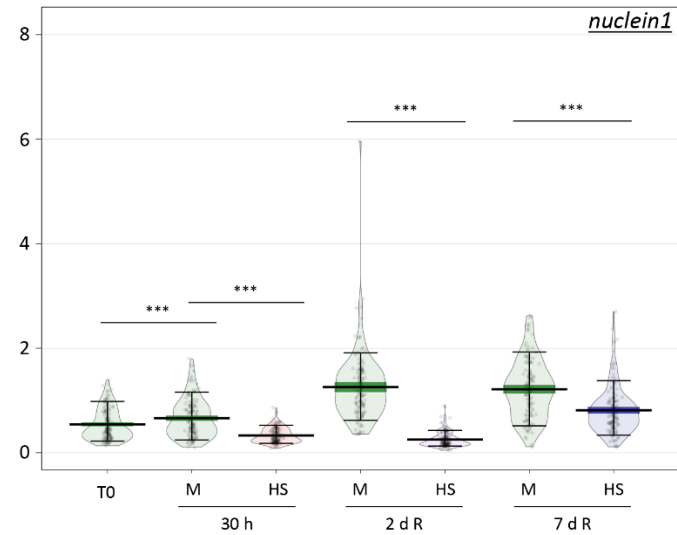
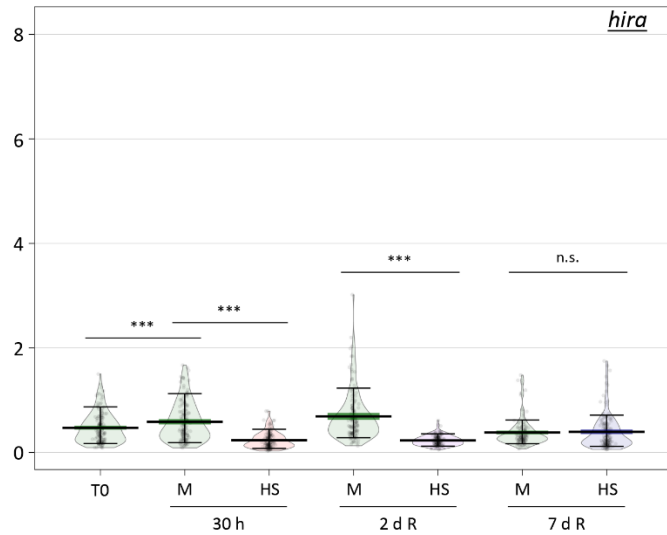
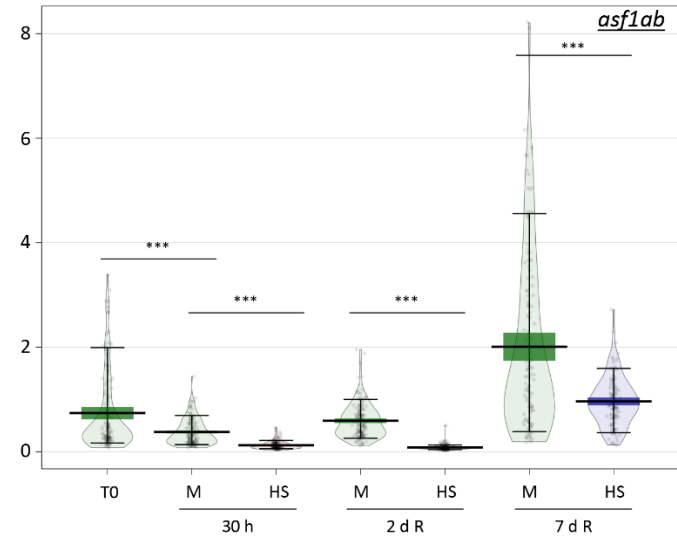
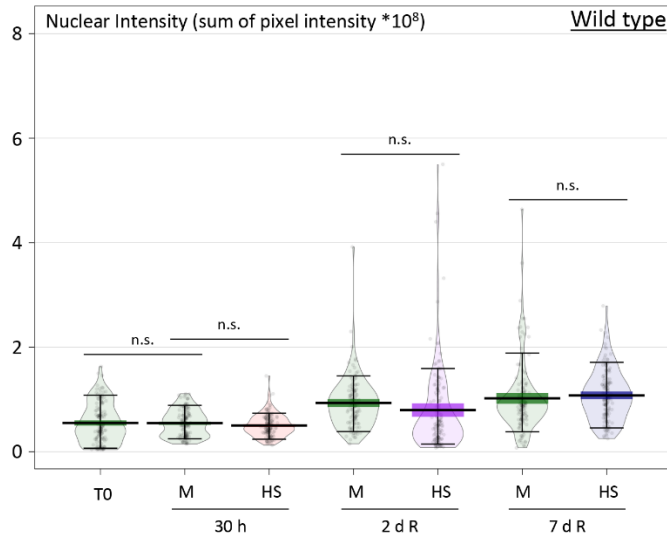
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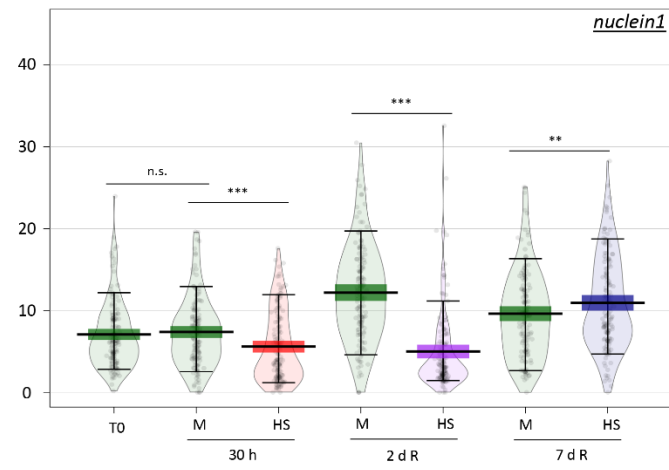
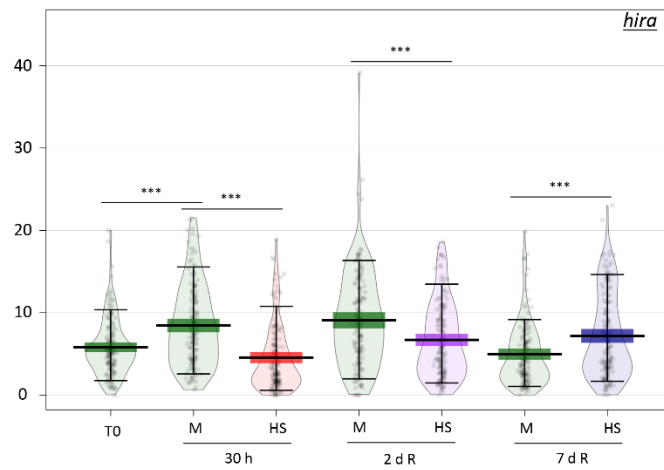
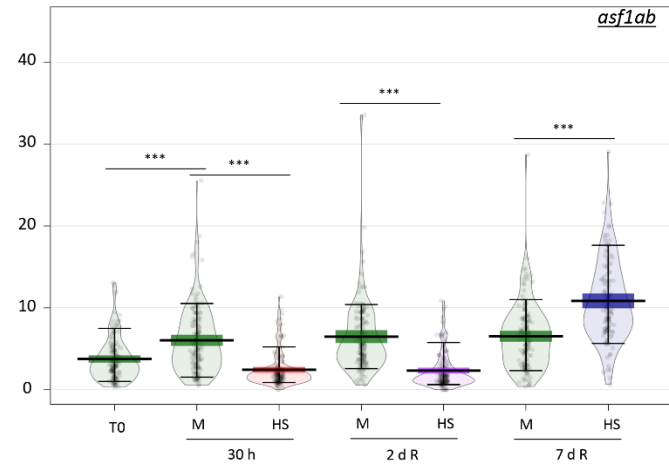
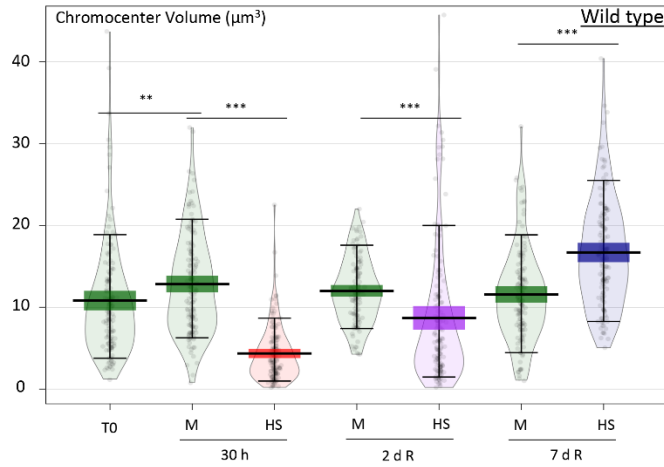
6 ANNEX



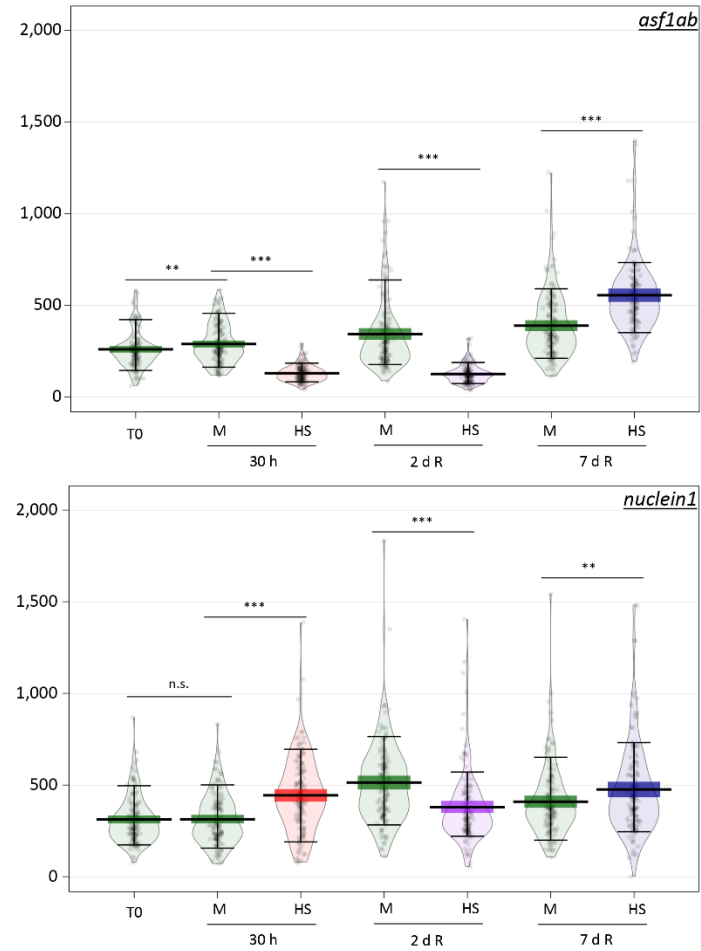
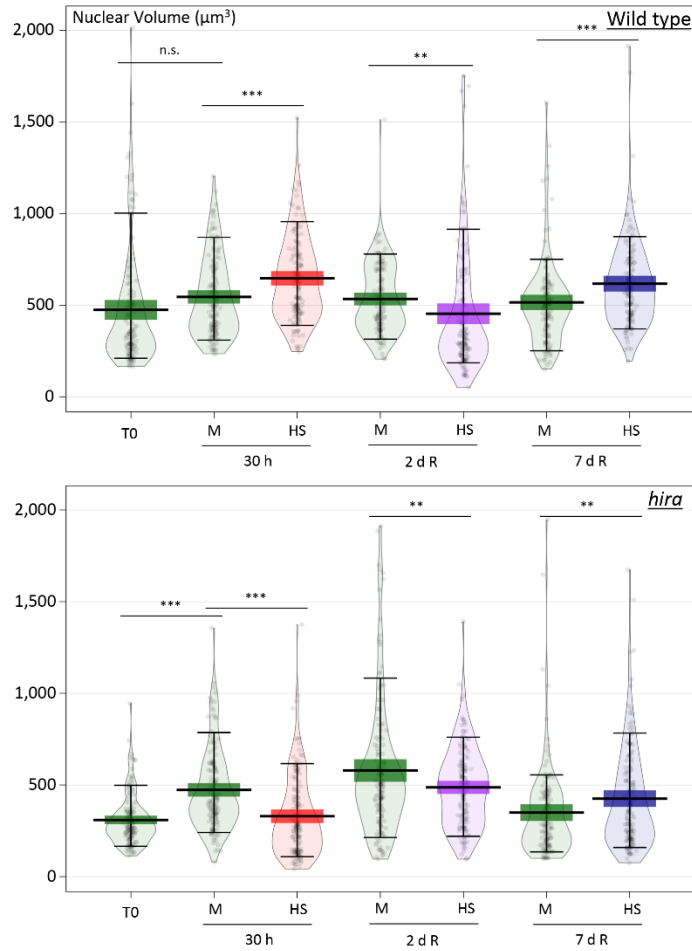
Annex 1 Pirate plot of total chromocenter intensity measured from root cell nuclei expressing H2B-RFP in wild type and histone chaperone mutants at indicated time points of heat stress or mock treatment. $n = 122-141, 118-144, 129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1*, respectively. Grey points represent raw data. Beans represent a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. M = mock treatment, HS = heat stress treatment, R = recovery time point.



Annex 2 Pirate plot of total nuclear intensity measured from root cell nuclei expressing H2B-RFP in wild type and histone chaperone mutants at indicated time points of heat stress or mock treatment. $n = 122-141, 118-144, 129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1*, respectively. Grey points represent raw data. Beans represents a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. M = mock treatment, HS = heat stress treatment, R = recovery time point.



Annex 3 Pirate plot of total chromocenter volume measured from root cell nuclei expressing H2B-RFP in wild type and histone chaperone mutants at indicated time points of heat stress or mock treatment. $n = 122-141, 118-144, 129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1*, respectively. Grey points represent raw data. Beans represents a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. M = mock treatment, HS = heat stress treatment, R = recovery time point.



Annex 4 Pirate plot of total nuclear volume measured from root cell nuclei expressing H2B-RFP in wild type and histone chaperone mutants at indicated time points of heat stress or mock treatment. $n = 122-141, 118-144, 129-147$, and $139-141$ nuclei per time point for wild type, *asf1ab*, *hira* and *nuclein1*, respectively. Grey points represent raw data. Beans represents a smoothed density of the data. Rectangles represent the 95% means frequentist confidence interval. Whiskers represent 90th and 10th quantiles. The black horizontal bars represent the mean, p values: $p < 0.01 = ***$, $p < 0.05 = **$, n.s. = non-significant in a Welch two sample t-test. M = mock treatment, HS = heat stress treatment, R = recovery time point.

RPKM values			T0	HS	HS + 2dR	HS + 7dR
Lamina-like structure						
AT1G67230	CRWN1		18.10692014	0.571227211	0.810541088	0.906132859
AT1G13220	CRWN2		13.48638503	1.83799194	0.652003248	0.36444887
AT1G68790	CRWN3		18.44333799	0.624226058	0.442871819	0.495102239
AT5G65770	CRWN4		7.779034908	0	0	0
AT4G31430	KAKU4		7.547742751	0.727814219	0.51636487	0.577262748
LINC complex and cytoskeleton						
AT5G04990	SUN1	<i>C-ter SUN</i>	23.86762894	6.223468878	4.415385977	0.987223567
AT3G10730	SUN2	<i>C-ter SUN</i>	17.23082812	1.295020232	0	1.027139782
AT1G22882	SUN3	<i>mid-SUN</i>	18.82009412	0	0.69632371	3.113780676
AT1G71360	SUN4	<i>mid-SUN</i>	20.39163184	0	2.790111606	1.559582755
AT4G23950	SUN5	<i>mid-SUN</i>	11.8131185	0.591893682	0	0
AT4G26455	WIP1	<i>KASH</i>	14.36092735	3.494962389	3.306110729	2.772014534
AT5G56210	WIP2	<i>KASH</i>	14.6543975	4.53903299	0	0
AT3G13360	WIP3	<i>KASH</i>	14.6543975	4.53903299	0	0
AT5G11390	WIT1		16.21767462	0	0.67590366	0.755616865
AT1G68910	WIT2		5.495986742	0.364782728	0.258803663	0.578651735
AT4G33200	Myosin XI-I		6.463017423	0.152918804	0.216983664	0.121286898
AT4G09550	GIP1		16.90851028	0	0	0
AT1G73790	GIP2		18.43032886	8.970633246	3.182213089	0
Nucleoporins						
AT3G10650	Nup136/Nup1		16.40128534	2.069675111	0.367094889	0.820777
AT1G33410	Nup160		11.69956293	1.205500174	0.855270375	0.191227466
AT5G05680	Nup88		16.65716845	5.900069939	2.391967437	1.337032955
AT1G54385	SINE1	<i>KASH</i>	9.715301239	0	0	0.437568524
AT3G03970	SINE2	<i>KASH</i>	8.517877128	1.004589395	0	0.7967858
AT3G06600	SINE3	<i>KASH</i>	15.16249111	0	0	0
AT4G24950	SINE4	<i>KASH</i>	27.22333988	0	0	0

Annex 5 RNA seq data of member of the LINC complex upon HS from Jasmin Bassler, PhD thesis, in preparation.

primer number (OMS database)	primer name	primer sequence (5'--> 3')	Purpose
670	p1.01-F	tcactctatgtgtgctgctta	Genotyping/sequencing for promotor cloning
671	p1.01-R	ttcctcgaaccttatataca	Genotyping/sequencing for promotor cloning
672	p1.02-F	gttttctttcttaccacact	Genotyping/sequencing for promotor cloning
673	p1.02-R	gtcgcctctctcagccaaaatg	Genotyping/sequencing for promotor cloning
674	p1.22-F	cacgaatttttgttggttat	Genotyping/sequencing for promotor cloning
675	p1.22-R	tttctctccgatctctttctct	Genotyping/sequencing for promotor cloning
676	p1.28-F	cgtacatgcggtatactgca	Genotyping/sequencing for promotor cloning
677	p1.28-R	tgtcctctgttttggccttt	Genotyping/sequencing for promotor cloning
678	p1.54-F	gatccttatgaaaagtctct	Genotyping/sequencing for promotor cloning
679	p1.54-R	taccactactacaacaacaac	Genotyping/sequencing for promotor cloning
680	p1.67-F	catctttcgactttctcagc	Genotyping/sequencing for promotor cloning
681	p1.67-R	agcaagcccagagttgaggag	Genotyping/sequencing for promotor cloning
682	p2.47-F	tgttggtttgtcaagtag	Genotyping/sequencing for promotor cloning
683	p2.47-R	aaatttagggatttgggtgac	Genotyping/sequencing for promotor cloning
684	p3.08-F	gtgtcttattcacgaaactca	Genotyping/sequencing for promotor cloning
685	p3.08-R	cagatccgacgatattgttg	Genotyping/sequencing for promotor cloning
686	p4.18-F	ttagttgatactttgtagttg	Genotyping/sequencing for promotor cloning
687	p4.18-R	tcccgagcagaaatgaaac	Genotyping/sequencing for promotor cloning
	Infus_pG_mCherry3_F	ATCGATAAGCTTGATATCATGGTGAGCAAGGGCGAGGAGGA	Infusion cloning of 3x-mCherry into pGreen0229
	Infus_pG_mCherry3_R	CTGCAGGAATTCGATATCCTTGACAACTCATCCATACCA	Infusion cloning of 3x-mCherry into pGreen0229
	pG0229_OCS_infus_F	ATCCACTAGTTCTAGACCTGCTTTAATGAGATATGCGA	Infusion cloning of OCS terminator into pGreen0229
	pG0229_OCS_Infus_R	TGGCGGCCGCTCTAGATCCTGCTGAGCCTCGACATGT	Infusion cloning of OCS terminator into pGreen0229
	At1g01790_infus1	CGGGCCCCCCTCGAGGGCAAGAATTGATGTTTTTTCAC	Infusion cloning of promoter into pGreen0229
	At1g01790_infus2	TACCGTCGACCTCGAGATCGGAAATCAAATAAAAAAAGTAGCC	Infusion cloning of promoter into pGreen0229
	At1g02100_infus1	CGGGCCCCCCTCGAGGTTTTCTTCTTACCACACT	Infusion cloning of promoter into pGreen0229
	At1g02100_infus2	TACCGTCGACCTCGAGCGTCGTCGCTCTCTCAGCCA	Infusion cloning of promoter into pGreen0229
	At1g54460_infus1	CGGGCCCCCCTCGAGGATCCTTATGAAAAGTCTCTGTTTGC	Infusion cloning of promoter into pGreen0229
	At1g54460_infus2	TACCGTCGACCTCGAGTACCACTACTACAACAACAAC	Infusion cloning of promoter into pGreen0229

	At1g67140_infus1	CGGGCCCCCCTCGAGCAATATTATACAAAGGGTACTTTGG	Infusion cloning of promoter into pGreen0229
	At1g67140_infus2	TACCGTCGACCTCGAGTAGCAGTGGAGAAACATAGAACGA	Infusion cloning of promoter into pGreen0229
	At2g47850_infus1	CGGGCCCCCCTCGAGTAAAAATTAGTTTATTTATTTTTTG	Infusion cloning of promoter into pGreen0229
	At2g47850_infus2	TACCGTCGACCTCGAGGTCCTTAAATTAGGGATTTGGTGT	Infusion cloning of promoter into pGreen0229
	At3g08960_infus1	CGGGCCCCCCTCGAGTCTTCTTCCTCTTCTAAGCGTGTC	Infusion cloning of promoter into pGreen0229
	At3g08960_infus2	TACCGTCGACCTCGAGCAGATCCGACGATATTGTTGACCT	Infusion cloning of promoter into pGreen0229
960	mCherF	gaattcctgcagcccATGGTGAGCAAGGGCGAG	Amplification of mCherry 3x for circular PCR (Cpec)
961	mCherR	cacctaggCTTGTAACAATCATCCATACAC	Amplification of mCherry 3x for circular PCR (Cpec)
962	OCStF	ttgtacaagCCTAGGTGAGTCTAGAGAGTTAATTAAG	Amplification of OCS for circular PCR (Cpec)
963	OCStR	actagtggatccccTCCTGCTGAGCCTCGACA	Amplification of OCS for circular PCR (Cpec)
1099	NLS-Nter-mCH-pF	CTCGAGGTCGACATGCC	Instering NLS in pGrPromOMc3KB, prom 1.01, 1.67, 2.47, 3.08
1100	NLS-Nter-mCH-R	GGGCTGCAGGAATTCAACCTTCTCTTCTTCTTAGGCATGTCGACCTCGAG	Instering NLS in pGrPromOMc3KB, prom 1.01, 1.67, 2.47, 3.08
1101	Infusion-pUBQ10-F	cgggccccctcgagCGACGAGTCAGTATAAACG	in fusion cloning of pUBQ10 in pGrOOMc3KB, XhoI site. To amplify from pGGN002
1102	Infusion-pUBQ10-R	taccgtcgacctcgagCTGAATACTGTTGTTAATCAGAAA	in fusion cloning of pUBQ10 in pGrOOMc3KB, XhoI site
964	McherSeqF1	tggacatcacctcccacaac	sequencing mCherry 3x
965	McherSeqR1	ttgatgttgacgtttaggc	sequencing mCherry 3x
966	McherSeqF2	AGATCAAGCAGAGACTTAAGCT	sequencing mCherry 3x
967	McherSeqR2	CCCTTGAGAGCACCATCTTCA	sequencing mCherry 3x
968	McherSeqR3	ACTTGAAGCCTCCCAACCCAT	sequencing mCherry 3x
969	McherSeqF3	ACCCAGGATAGTTCTCTCCAA	sequencing mCherry 3x
970	OCSseqR1	AATGGTGCCCGTAACTTTG	sequencing OCS
971	OCSseqF1	CCGGTCAAACCTAAAAGACT	sequencing OCS
1102	NLSgenot-F	ATGCCTAAGAAGAAGAGGAAG	genotyping NLS instertion
1157	TD_dCas9R1	TCTCATCCAAGTAGTGCT	For sequencing dCas9...
1158	TD_dCas9R2	CCGTTTGTCTCGATGAGT	For sequencing dCas9...
1159	TD_dCas9R3	CTCTGAGTGATGAGCTTA	For sequencing dCas9...
1160	TD_dCas9R4	TTAGCGAATCCATCAGAC	For sequencing dCas9...
1161	TD_dCas9R5	AAAGAGTGCTTAGGGAGC	For sequencing dCas9...
1162	TD_dCas9R6	CACTGAGGAGGATAGCAT	For sequencing dCas9...

1163	TD_dCas9R7	TTCTTCTGGTGTACCTTC	For sequencing dCas9...
1164	TD_pGrF1	CGGTATCGATAAGCTTGA	For sequencing dCas9...
1165	TD_NLS-F2	agagagatgattgttggG	Primer bindin NLSa46 for PCR amplification
1150	TD_NLS_NcoI_F	CCATGGTTCTTGGAAAGAGAAAGAG AGATGATTGTTGGGT	Cloning NLSa46 in NcoI site by restriction ligation
1151	TD_NLS_NcoI_R	CCATGACCCAACAATCATCTCTCTTTC TCTTTCCAAGAAC	Cloning NLSa46 in NcoI site by restriction ligation
1157	TD_dCas9R1	TCTCATCCAAGTAGTGCT	For sequencing dCas9...
1158	TD_dCas9R2	CCGTTTGTCTCGATGAGT	For sequencing dCas9...
1159	TD_dCas9R3	CTCTGAGTGATGAGCTTA	For sequencing dCas9...
1160	TD_dCas9R4	TTAGCGAATCCATCAGAC	For sequencing dCas9...
1161	TD_dCas9R5	AAAGAGTGCTTAGGGAGC	For sequencing dCas9...
1162	TD_dCas9R6	CACTGAGGAGGATAGCAT	For sequencing dCas9...
1163	TD_dCas9R7	TTCTTCTGGTGTACCTTC	For sequencing dCas9...
1164	TD_pGrF1	CGGTATCGATAAGCTTGA	For sequencing dCas9...
1166	TD_SUN1-RT-F	ATCAAGATTCGTTCCACAA	Primer couple spanning an intron for RT on Sun1: exp size:516pb
1167	TD_SUN1-RT-R	GTGATGGAGCATTGAGATCC	Primer couple spanning an intron for RT on Sun1: exp size:516pb
1168	TD_CRWN1-F	TCGTCAAGAAAGTCCAAAGC	Primer couple spanning an intron for RT on Crwn1: exp size:575pb
1169	TD_CRWN1-R	AAAGCAAGCACAGGTGAAAC	Primer couple spanning an intron for RT on Crwn1: exp size:575pb
2385	q-CRWN1-F	gacaaaagcgcgtgagcta	For q-RT-PCR on CRWN1 gene
2386	q-CRWN1-R	actagttgctgaactgccatctt	For q-RT-PCR on CRWN1 gene
2387	q-CRWN1-Fb	aagatcttgctttaagggaacaag	For q-RT-PCR on CRWN1 gene expected: 127nt
2388	q-CRWN1-Rb	actagttgctgaactgccatctt	For q-RT-PCR on CRWN1 gene expected: 127nt
2389	q-CRWN2-F	aaagcaatgtgtgcaagagc	For q-RT-PCR on CRWN2 gene expected: 109nt
2390	q-CRWN2-R	aaccagagcatttgcttcaac	For q-RT-PCR on CRWN2 gene expected: 109nt
2391	q-CRWN3-F	gagctccatgagttgaggag	For q-RT-PCR on CRWN3 gene expected :93nt
2392	q-CRWN3-R	tcttgaatctagaacagccttctg	For q-RT-PCR on CRWN3 gene expected :93nt
2393	q-CRWN4-F	ctggaggatgagaaggaacg	For q-RT-PCR on CRWN4 gene expected :68nt
2394	q-CRWN4-R	tctaatacgtcaaagactgctg	For q-RT-PCR on CRWN4 gene expected :68nt
2395	SUN1-F	tcaggctgagaggaccaatc	For q-RT-PCR on SUN1 gene expected :77nt
2396	SUN1-R	cgatctgtcgtaggctacactc	For q-RT-PCR on SUN1 gene expected :77nt
2397	SUN2-F	ggagacgggttcatagcaaa	For q-RT-PCR on SUN2 gene expected :93nt
2398	SUN2-R	catatccattgcttccttccaga	For q-RT-PCR on SUN2 gene expected :93nt
2399	SUN3-F	tctacgttagcgtacccaatcc	For q-RT-PCR on SUN3 gene expected :109nt
2400	SUN3-R	ccactaaacgattacacaggaca	For q-RT-PCR on SUN3 gene expected :109nt
2401	SUN4-F	gcaagtgttgaaagactgga	For q-RT-PCR on SUN4 gene expected :69nt
2402	SUN4-R	aacccgacacagattgtgaac	For q-RT-PCR on SUN4 gene expected :69nt
2403	SUN5-F	tcgttctcttactctggtgcttc	For q-RT-PCR on SUN5 gene expected :104nt

2404	SUN5-R	tccatcagtgtaattccaattc	For q-RT-PCR on SUN5 gene expected :104nt
2405	WIP1-F	cattaacagaggccatcaacg	For q-RT-PCR on WIP1 gene expected :73nt
2406	WIP1-R	tcctggaattgctgaacctc	For q-RT-PCR on WIP1 gene expected :73nt
2407	WIP2-F	caagaagccctccaaaaaga	For q-RT-PCR on WIP2 gene expected :108nt
2408	WIP2-R	tcctgcgtgtatgcaactg	For q-RT-PCR on WIP2 gene expected :108nt
2409	WIP3-F	acgcacttgacagattctatcag	For q-RT-PCR on WIP3 gene expected :76nt
2410	WIP3-R	tcctggaaactttgaacctctt	For q-RT-PCR on WIP3 gene expected :76nt

Annex 6 List of primer used in this study.

Numbers of primers (left column) correspond to the primer numbers in the lab primer database.