Doctoral Dissertation

Understanding the role of cytoskeletal component, actin in prolonged moderate high temperature mediated stress response in *Arabidopsis thaliana*

Division: Bioresources Science

Specialty: Cellular Genomics

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2021. 12

Summary

With the upsurge of utilization of fossil fuel and emission of greenhouse gases after the beginning of industrialization, earth's average temperature has substantially increased resulting in global warming. This undeniable alarming phenomenon is primarily causing an increase in temperature and challenging the crop production world-wide. Recent discoveries show that high temperature stress physiology is a complex process that is regulated by multiple factors including phytohormones and cellular protein trafficking. Among the phytohormones, auxin has been shown to play an important role in regulating the high temperature stress response pathway. Another important regulatory factor is intracellular protein trafficking. In general, intracellular protein trafficking is a fundamental cellular process required for normal growth and development, which also influences various biotic and abiotic stresses, including high temperature stress response. Cellular auxin homeostasis is largely dependent on the intracellular trafficking of the auxin transport carriers required for their polar localization. Not surprisingly, the moderate high temperature- induced cellular auxin homeostasis was also found to be regulated by altered intracellular protein trafficking. The cellular protein trafficking activity largely relies on the cytoskeletal component, actin. Filamentous actin provides the track for vesicle movement and thereby facilitates the cellular trafficking. Obviously, any alteration in actin filaments impair vesicle movement and cause mistransport of various proteins. Actin is also interconnected with phytohormone auxin. Auxin transport inhibitors have obvious impact on actin organization. Similarly, actin polymerizing and depolymerizing drugs affect auxin distribution. Lack of certain actin isovariants shows altered auxin distribution. Taken together, these results raise a possibility that actin may function as an upstream regulator of the temperature response pathway. Nevertheless, the relation of actin cytoskeleton in high temperature mediated response has not

been studied yet. Therefore, the focuses of this research were to understand the thermomorphogenesis of root under prolonged moderate high temperature (Chapter 3), involvement of actin isovariants in this process in root (Chapter 4) in Arabidopsis.

To achieve the research goals, firstly, I focused on the underground part root as it is easily observable and experimentally exploitable. The root elongation behavior was observed for a prolonged period under moderate high temperature (29 °C for 3 days). In contrast to short-term treatment, under long term moderate high temperature root losses normal elongation pattern, which was found to be linked with perturbed auxin transport. Auxin influx transporters PIN1 and PIN2 abundances were reduced, resulting in hampered auxin homeostasis in the root. Long term moderate high temperature treatment also directly affects the actin dynamicity in terms of filament bundling, parallelness and orientation in the epidermal root cells. This finding suggests that prolonged moderate high temperature stress can modulate the actin dynamicity and abundance of auxin transporter proteins and thereby affects the root growth (Chapter 3).

Actin is a highly conserved protein ubiquitously present across the kingdom. In Arabidopsis, actin is largely divided in two subclasses, vegetative and reproductive classes. Since in this study, I primarily focused on vegetative growth, I examined the role of vegetative actin isovariants, ACT2, ACT7 and ACT8 in high temperature mediated root elongation and plant phenotype. ACT2 and ACT7 were found to be essential for normal root elongation under prolonged moderate high temperature stress. Lack of ACT2 and ACT7, influence auxin transport negatively by diminishing PIN1 and PIN2 abundance and drastically hamper auxin homeostasis. Actin quantification reveals that actin dynamicity is altered in *act2-1* and *act7-4* mutants under prolonged moderate high temperature stress. These results indicate that lack of ACT2 or ACT7 makes plant more sensitive towards prolonged moderate high temperature through auxin mediated pathway (Chapter 4).

In summary, this work identifies ACT2 and ACT7 as potential upstream regulators of moderate high temperature stress response pathway and can be exploited for developing plants tolerant to high temperature stress in future.

Chapter 1

General Introduction

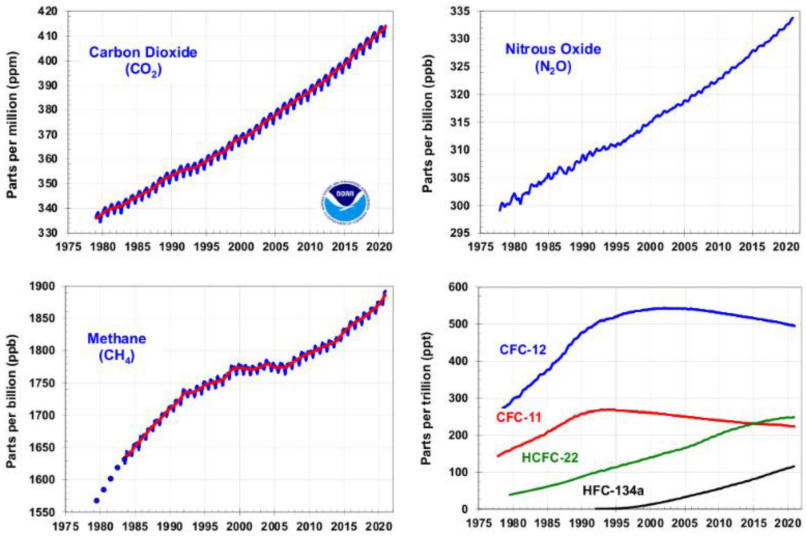
Heat stress affects crop production

Global warming is one of the burning questions since last century which is sharply going up along with greenhouse gases emission. From 1980 to 2020 concentration of (CO_2, CH_4, N_2O) is climbing every year without any decrease in air except CFC (Figure 1A). For example, the global average level of CO_2 in 2020 was 412.5 parts per million (ppm), a 2.6 ppm increase over 2019 (https://research.noaa.gov/article/ArtMID/587/ArticleID/2626/Warming-influence-of-greenhouse-gases-continues-to-rise-NOAA-finds). Global average temperature is also rising which is directly proportional to greenhouse emission from data shown in (Figure 1B). 2020 was the second-warmest year on record based on NOAA's temperature data. Averaged across land and ocean, the 2020 surface temperature was 0.98 °C warmer than the twentieth-century average of 13.9 °C and 1.19 °C warmer than the pre-industrial period (1880-1900) according to the latest data of National Centers for Environmental Information, USA (NOAA, https://earthobservatory.nasa.gov/world-of-change/global-temperatures).

Along with other abiotic stresses, high temperature stress is an inevitable issue caused by global warming. NOAA temperature statistics correlate with the agricultural economic data published by developed countries in the past years. Heat, drought, low temperature, and flooding caused damages with more than 1 billion USD between 1980 and 2012(Mittler, 2006; Suzuki et al., 2014). The combined effect of drought and heat stress is detrimental on agricultural production due to their antagonistic mechanism of response. Together they cause 200 billion USD production loss from 1980 to 2012 (Suzuki et al., 2014). High temperature alone caused annual production loss for

wheat, maize and barley which is together equal to 5 billion dollars since 1981 to 2002 (Lobell et al., 2011). From 1964 to 2007, 7.6% of cereal yield declined due to Extreme Weather Disasters (EWDs) for extreme heat (Lesk et al., 2016). Therefore, to reduce the drastic effect of high temperature stress on crop production, we need to study the cellular and molecular mechanisms of mild to extreme heat stress on plant growth and development from seed germination to adult stage as well as in reproductive stage. Better understanding of the molecular and cellular mechanisms will help to develop crops for future tolerant to heat stress and help in securing the food security.

A



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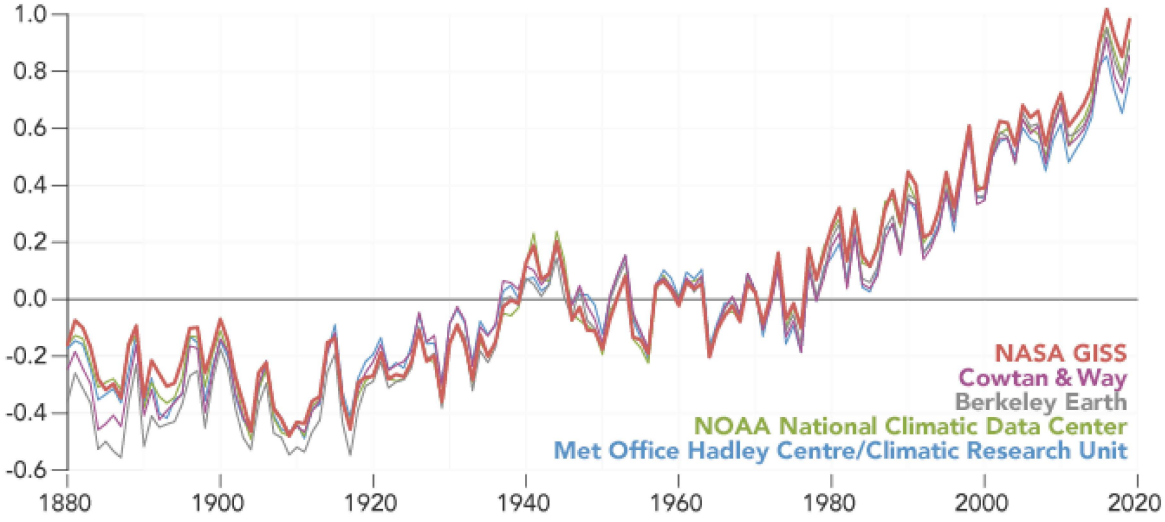

Figure 1. Global warming. A. Emission of greenhouse gases from 1980-2020. B. Temperature anomaly of earth's surface relative to 1951-1980 in °C according to five different reports. (Source: https://earthobservatory.nasa.gov/world-of-change/global-temperatures)

Effect of high temperature on plant growth and development

High temperature stress affects growth and development of plant during entire life cycle. Vegetative growth, reproductive growth, crop yield, crop quality, seed germination are the basic phases of plant which are influenced by high temperature stress ranges from 30-50 °C (Figure 2). An obvious effect of heat stress is water deficiency. Water content is reduced because of excessive transpiration under high temperature and plant biomass is diminished (Morales et al., 2003; Hasanuzzaman et al., 2013). The primary sites of injury due to heat stress are photosystem, membranes where ROS is produced. Excess ROS production generates oxidative stress which is one of the major ways of action of heat stress. High temperature stress (35-45 °C) can reduce drastically photosynthetic rate where photosystem is damaged by excessive production of electron and proton (Morales et al., 2003). Heat stress also alters the availability of large and small subunits of Rubisco and Rubisco Binding Proteins (Demirevska-Kepova et al., 2005). Lipid peroxidation in chloroplast and thylakoid membrane under high temperature can reduce chlorophyll pigments (Halliwell 1987). Consequently, sucrose content is reduced in plants (Sumesh et al., 2008). Extreme heat stress (>45 °C) can cause programmed cell death or gradual death of plants due to protein denaturation or oxidative stress (Rodríguez et al., 2005). High temperature also affects reproductive organs destructively, for example, flower and flower bud abortion, reduced pollen production and fruit dehiscence where anther dehiscence and pollen fertility are highly damaged (Dai Vu et al., 2019; Erwin and Warner, 2005). As a result, crop yield is reduced by high temperature. High temperature stress (>32 °C) during seed germination reduces germination rate (Lim et al., 2013; Toh et al., 2008), which can increase crop production cost. Elevated temperatures significantly decreased the total lipid and aluminum concentration by 7.1% and 15% in barley. The concentrations of total non-structural carbohydrates, starch, fructose and raffinose were

reduced in barley grown at high temperatures (Högy et al., 2013). The panicles or the whole plants under high temperature either at night or day reduces grain quality of rice (Krishnan et al., 2011). These findings suggest that heat stress can affect the plants growth and development at all developmental stages. Therefore, understanding plant stress physiology even at early stages is as important as reproductive stage.

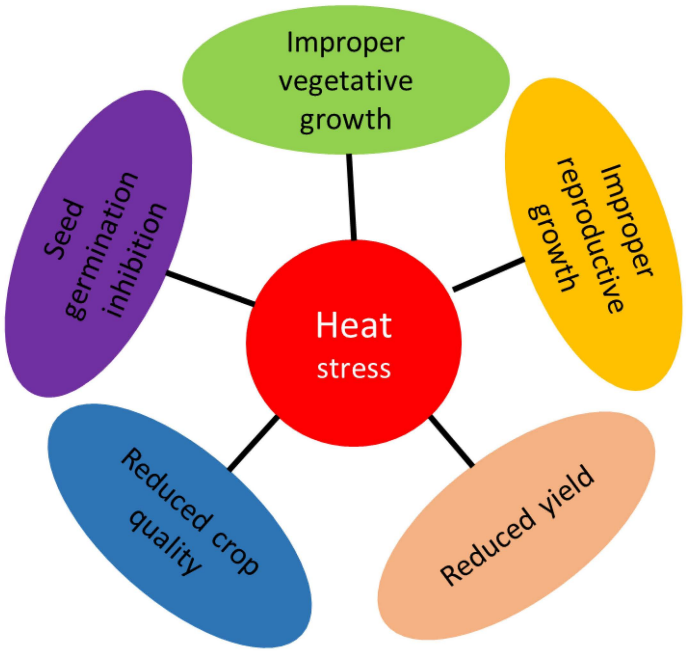

Figure 2. Effects of heat stress on plant's major physiological processes

Effect of Moderate high temperature on Arabidopsis growth

To study the effect of high temperature on plant root, I have chosen to use Arabidopsis as a model plant to work with. Arabidopsis is the first higher plant whose whole genome has been completely sequenced (Bevan and Walsh, 2005). Therefore, there are many accessible comprehensive databases for Arabidopsis for example TAIR (https://www.arabidopsis.org), AtDB (http://genome-www.stanford.edu/Arabidopsis), NCBI (https://www.ncbi.nlm.nih.gov). A large number of mutants are available which allows genetic and molecular analysis of cell biology, biochemistry and physiology. Most of the research studies in Arabidopsis correspond to other higher plants, which made Arabidopsis the standard reference plant for all areas of biology (Meinke et al., 1998). Optimum temperature for Arabidopsis natural growth is low temperature condition as it germinates in the late fall and early spring. Typically, Arabidopsis grows well either around 23 °C for both day and night, or at day temperature 22–23 °C and night temperature 16–18 °C (Dolzblasz and Dołzbłasz, 2018). Heat stress response of Arabidopsis has been studied in mainly three different set up such as suboptimal/moderate temperatures (around 30 °C), heat shock temperatures (37–45 °C) and acclimatized at 35–37 °C to treat around 45°C for acquired thermotolerance (Dolzblasz and Dołzbłasz, 2018). I have selected to study the effect of moderate high temperature stress on Arabidopsis as this condition is most likely to happen due to upcoming climate change. Under moderate high temperature stress, seedlings show some adaptive morphogenetic changes called thermomorphogenesis which includes hypocotyl elongation, leaf hyponasty, early flowering, rapid fruit setting, and root elongation in Arabidopsis (Li et al., 2018; Gray et al., 1998; Sun et al., 2012; Franklin et al., 2011; Park et al., 2019; Martins et al., 2017; Hanzawa et al., 2013; Dai Vu et al., 2019) (Figure 3). One of the highly studied factors for thermomorphogeneic growth of leaf, hypocotyl and petiole is *Phytochrome Interacting Factor 4*

(PIF4) which is upregulated by high temperature and interacts with many signaling pathways like red/far red, blue or UV mediated light signaling pathway (Dai Vu et al., 2019). Moderate high temperature also can inhibit seed germination where abscisic acid and gibberellic acid are involved (Toh et al., 2008). Increased *PIF1* by high temperature results in a secondary dormancy during seed germination (Martel et al., 2018). It is well known that moderate high temperature mediated responses of Arabidopsis regulated by plethora of hormone for example auxin, gibberellic acid, brassinosteroid, abscisic acid and their cross interactions (Dai Vu et al., 2019). Molecular mechanisms of high temperature-mediated plant growth and development are complex which include extensive cross talk with different pathways, such as hormonal regulation, light signaling and circadian pathways.

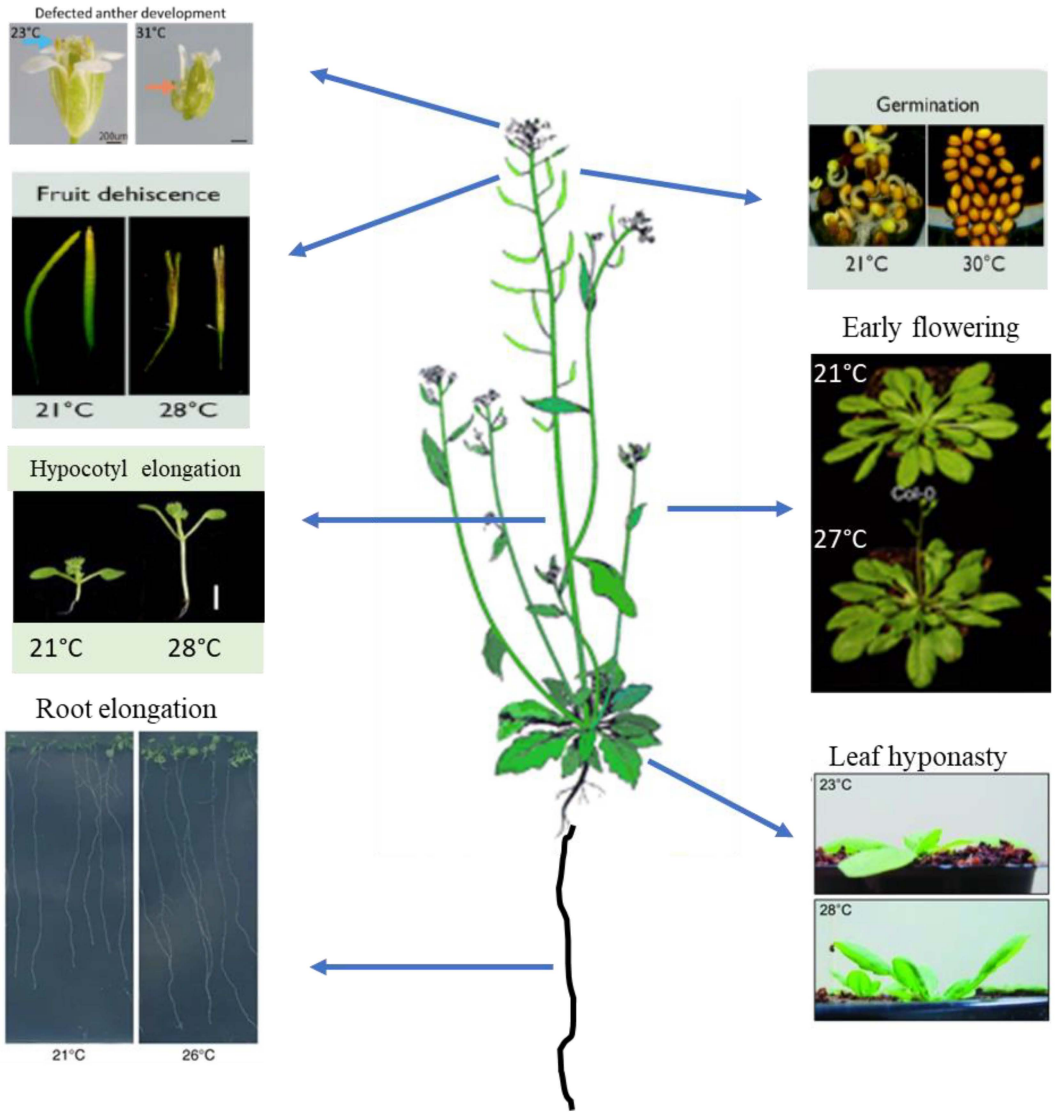

Figure 3. Effect of moderate high temperature on Arabidopsis.
(Sources: Sakata et al., 2010; Li et al., 2018; Koini et al., 2009; Martins et al., 2017; Hills et al., 2003; Balasubramanian et al., 2006; Park et al., 2019; Dai Vu et al., 2019)

Phytohormone auxin as major player under high temperature stress

In the hormonal crosstalk associated in moderate high temperature stress physiology, auxin plays an essential role in both reproductive and vegetative stages. High temperature influences reproductive organs destructively, for example, flower and flower bud abortion, reduced pollen production and fruit dehiscence (Dai Vu et al., 2019). High temperature stress (33 °C) for 24 hours suppresses auxin level in anther by reducing the expression of auxin biosynthetic genes *YUCCA2* and *YUCCA6* that results in inhibition of pollen production. This incident could be reversed by exogeneous application of auxin (Sakata et al., 2010). Exposure to the moderate high temperature stress at the seedling stage results in some adaptive thermomorphogenesis responses such as floral transition, leaf petiole and hypocotyl elongation, leaf hyponasty and root elongation (Dai Vu et al., 2019). High temperature stress-induced early flowering is linked to the upregulation of the flowering promoting gene *FLOWERING LOCUS T (FT)* regulated by *PIF4* (Kumar et al., 2012). Similarly, high temperature-induced increase in auxin biosynthesis has been attributed to the thermoresponsive transcription factor *PIF4* Under high temperature condition, PIF4 binds to the promoter region of auxin biosynthetic genes *YUC8, TAA1,* and *CYP79B2* and enhances their expression. The resulting alteration in the intracellular auxin level promotes the leaf and hypocotyl hyponasty (Sun et al., 2012; Franklin et al., 2011). *PIF4* promotes the expression of *PINOID* kinase which controls polar localization of PIN protein and inhibition of PIN mediated auxin redistribution can reduce thermomorphogenic petiole movement (Park et al., 2019). In contrast to hypocotyl, the effect of high temperature stress on root is not studied extensively. I mainly focused on the responses of root under moderate high temperature as root performs inevitable roles such as absorption of ions, water, anchoring in the soil etc. (Shekhar et al., 2019; Qiao and Libault, 2013). It has been shown that the short term (36 h) moderate high temperature stress stimulates

the root elongation (Hanzawa et al., 2013). Both the auxin transport and signaling have been implicated in the root elongation response under moderate high temperature. Auxin signaling mutant *tir1-1afb2-3* showed lack of the stimulatory root elongation under high temperature stress. HSP90 stabilizes auxin receptor and enhances auxin signaling TIR1 to increase root elongation and number of lateral roots under high temperature (Wang et al., 2016). Similarly, auxin transport mutants, *eir1-1* and *aux1-7* showed reduced root elongation response. Under moderate high temperature, the observed intracellular auxin homeostasis was shown to be linked to the *Sorting Nexin 1 (SNX1*) mediated recycling of PIN2 proteins from vacuole to plasma membrane (Hanzawa et al., 2013) However, root response for long term moderate high temperature stress remains elusive.

Protein trafficking plays essential role under various stresses including high temperature stress

Study of different components of endosomal trafficking pathways demonstrated that it plays vital roles in development as well as abiotic and biotic stress management in plants. Endosomal trafficking can be broadly divided into vesicle formation, movement and fusion. ARF-GEFs are essential for vesicle formation. Weak mutant of one ARF-GEF GNOM confers defective hydrotropism, temperature stress and fungal infection. GNOM-mediated vesicular trafficking plays an essential role in hydrotropism of seedling roots (Miyazawa et al., 2009). Recently it has been reported that over expression of GNOM ARF-GEF could make Arabidopsis resistant to cold stress (Ashraf and Rahman, 2019). GNOM-mediated trafficking is required for reorganization of PM material to the site of fungal attack for papilla formation which leads to penetration resistance against powdery mildew fungus (Nielsen et al., 2012). Soluble N-ethylmaleimide-sensitive factor

attachment protein receptors (SNAREs) are essential molecules in membrane trafficking. Three Q-SNAREs on a target membrane and one VAMP/R-SNARE on transport vesicles form a ternary SNARE complex for membrane fusion (Fasshauer et al. 1998). Plant syntaxin, SYP121 together with soluble N-ethylmaleimide-sensitive factor (NSF) adaptor protein (SNAP)33 and vesicle-associated membrane protein (VAMP)721/VAMP722 forms PM-localized ternary SNARE complexes which is required for penetration resistance of barley powdery mildew (Collins et al., 2003). Thus, it is clear that different components of endosomal trafficking are involved in pathways related to stress in plant.

Actin cytoskeleton controls auxin distribution by trafficking auxin transporters

Actin cytoskeletons is a component of endosomal trafficking. The trafficking of vesicles needs the filamentous actin as a track to be delivered to the sites of action. It is essential for various cellular, developmental and reproductive processes for example; establishing and maintaining cell shape and polarity, tip growth, cytoplasmic streaming and organelle movement (Kandasamy et al., 2009). Vacuolar trafficking of proteins from golgi complex and the relative vacuolar occupancy of the cell are also controlled by cytoskeleton actin in Arabidopsis (Kim et al., 2005; Scheuring et al., 2016). Auxin has been one of major player for thermomorphogenesis and high temperature influences PIN2 trafficking in Arabidopsis root (Hanzawa et al 2013). It has been demonstrated that recycling of PIN1 is actin dependent (Geldner et al., 2001). Vacuolar trafficking of PIN2 from plasma membrane under dark condition is also actin dependent (Rgen Kleine-Vehn et al., 2008). Actin dynamicity and polar auxin transport have been demonstrated to be interrelated as they have reciprocal effects on each other (Rahman et al., 2007). Actin depolymerizing drug latrunculin B causes intracellular accumulation of PIN2 and mislocalization of PIN1 and AUX1(Rahman et al., 2007; Kleine-Vehn et al., 2006). Auxin transport inhibitor, TIBA increases bundling of actin

filaments (Rahman et al., 2007) and inhibits plasma membrane localization of PIN2 by enhancing endocytosis (Zou et al., 2019). In contrast, NPA treatment abolishes polar localization of AUX1 and PIN1 as well as reduces actin dynamicity by stabilizing actin filaments (Kleine-Vehn et al., 2006; Zhu et al., 2016). These reports support the proposed model that the actin cytoskeleton is linked to correct transport and localization of auxin transporters and thereby controls auxin distribution (Figure 4) (Muday and DeLong, 2001), which makes actin cytoskeleton a target for high temperature mediated response of Arabidopsis root.

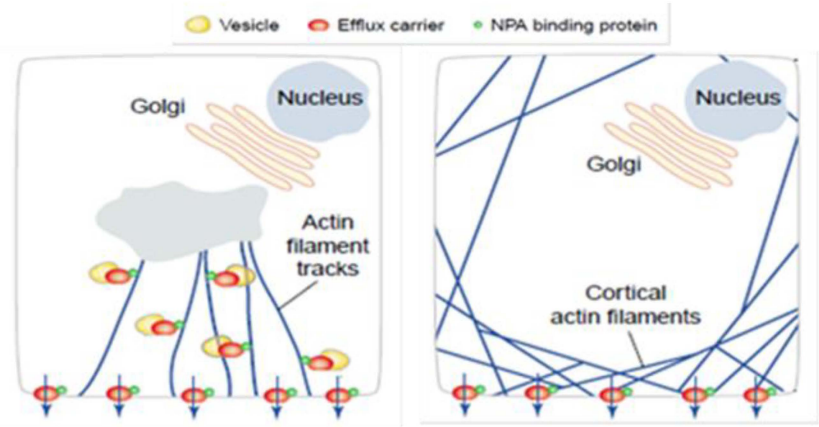

Figure 4. Model for the actin filaments on polar auxin transport. In cells that transport auxin, cortical actin filaments might serve to localize the auxin efflux carrier complex and/or actin filaments might act as tracks for vesicle delivery cycling to the plasma membrane. (Source; Muday and DeLong, 2001)

High temperature and actin cytoskeleton

Sensing changes in temperature in the cell is mainly observed by membrane fluidity, protein conformation, cytoskeleton rearrangement and metabolic reactions (Ruelland and Zachowski, 2010). High temperature treatment alters actin filaments in terms of isoform accumulation and organization in the subapex of heat-stressed pollen tubes (Parrotta et al., 2016). The actin cytoskeleton is a target of heat stress because heat shock depolymerized almost all filamentous actin independent of the cell type and developmental stage in the Arabidopsis root which indicates a higher sensitivity of actin towards heat stress (Müller et al., 2007). In tobacco BY-2 cultured cells, heat shock (50 °C for 5 min) induces depolymerization of actin filaments (Malerba et al., 2010). Heat shock (45 °C for 45 min) induced actin filament degradation leads to plant death (Fan et al., 2016). Therefore, actin cytoskeleton is considered as the important cellular components which shows early response to temperature change.

Even though actin cytoskeleton is altered by heat shock, the role of actin in root elongation under moderate high temperature in Arabidopsis is largely unknown. Arabidopsis actin gene family contains two ancient classes that diverged early in land plant evolution and have separated into vegetative and reproductive actin. Subsequent divergence produced five distinct subclasses of actin which shows distinct pattern of tissue specific expression (Mcdowell et al., 1996b; Kandasamy et al., 2009). There are eight actin isovariants in total including three vegetative and five reproduction actin isovariants (Figure 5). Three vegetative actin isovariants in Arabidopsis are ACT2, ACT7 and ACT8. ACT2 and ACT8 belong to subclass 1, and involve in root hair growth in Arabidopsis. ACT2 mutant exhibits wavy root phenotype but lack of neither ACT2 nor ACT8 affect the primary root growth. ACT7 is only member of subclass 2 which has been shown to be involved in root growth. ACT7 mutant displays delayed seed germination and dwarf phenotype at

seedling stage with twisted root (McDowell et al 1996a, Kandasamy et al 2001). Since different actin isovariants show specific function, it is also possible that some specific actin isovariant may be involved in high temperature mediated response of Arabidopsis root.

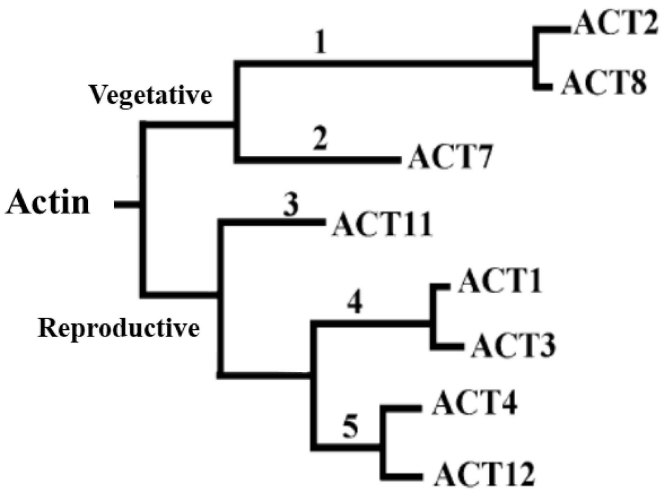

Figure 5. Arabidopsis actin family.

The actin tree of Arabidopsis consists of two major classes, vegetative and reproductive. Five subclasses (1 to 5) of protein isovariants were indicated. (Kandasamy et al., 2009)

Research objectives

In this thesis work, I focused on deciphering the role of protein trafficking component actin in root elongation under prolonged moderate high temperature and the objective is divided into two parts.

(1) Studying the effect of prolonged high temperature on root elongation and the involvement of auxin and protein trafficking component actin (Chapter 3).

(2) Finding the function of specific actin isovariants in root elongation under prolonged high temperature (Chapter 4).

Chapter 2

Materials and Methods

2.1. Plant Materials

All marker lines and mutant are in the Columbia background of *A. thaliana*. PIN2-GFP (Xu and Scheres, 2005) was provided by Ben Scheres (University of Utrecht, Utrecht, the Netherlands), PIN1-GFP was obtained from ABRC. AUX1-YFP was a gift from Malcolm Bennett (University of Nottingham, Nottingham, UK) (Swarup et al., 2001, 2004). Auxin marker lines IAA2-GUS was described earlier (Luschnig et al., 1998). For live cell imaging, the transgenic ABD2-GFP (Wang et al., 2004) was used. *act7-4* and *act8-2* were a gift from Rich Meagher (University of Georgia, Athens, GA, USA) and described earlier (Kandasamy et al., 2009; Swarup et al., 2001). PIN2-GFP, ABD2-GFP, AUX1-YFP, and IAA2-GUS maker lines in the mutant background were generated by crossing, and F3 homozygous lines were used for microscopy observations.

2.2. Growth Conditions

Surface-sterilized seeds were placed on modified Hoagland medium (Baskin and Wilson, 1997) containing 1% (w/v) sucrose and 1% (w/v) agar (Difco Bacto agar; BD Laboratories, Sparks, MD, USA) (http://www.bdbiosciences.com,). Two days after stratification at 4 °C in the dark, plates were transferred to a growth chamber (NK System; LH-70CCFL-CT, Tokyo, Japan) at 23 °C under continuous white light at an irradiance of 80–90 μmol m^{-2} s^{-1}. The seedlings were grown vertically for 5 days. For root growth assay, 5-day-old seedlings were transferred to new Hoagland plates and kept at 23 °C (NK System; LH-70CCFL-CT, Tokyo, Japan) and 29 °C growth chamber (NK System; LH-1-120.S, Tokyo, Japan) for 72-h treatment under continuous white light at an

irradiance of 80–90 μmol m^{-2} s^{-1}. Root growth elongation at different time point was carried out based on the procedure described earlier (Hanzawa et al.2013).

All phenotypic images were taken by Canon Power Shot A640 (Canon, Japan) without flash and using microfocus function.

2.3. Kinematic Analysis

The cell production assay was performed as described earlier (Rahman et al., 2007). Seedlings were grown vertically after stratification. Four-day-old seedlings were transferred to new medium. Cortical cell length was measured after 72 h. To ensure newly matured cells were scored, no cell was measured closer to the tip than the position where root hair length was roughly half maximal. The length of 10 mature cortical cells per root and eight roots used per treatment were measured. Cell production rate was calculated by taking the ratio of root elongation rate and average cell length for each genotype.

2.4. Chemicals

Cell clearing solution (Visikol) was purchased from Visikol Inc., Hampton, NJ, USA (https://visikol.com/, accessed on 30 May 2021). All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan.

2.5. GUS Staining

GUS staining was performed as described earlier (Okamoto et al., 2008). In brief, 5-day-old seedlings were transferred to a new agar plate and grown vertically at 23 and 29 °C under continuous white light. After 72 h, *IAA2-GUS*, *act2-1* IAA2-GUS, *act7-4* IAA2-GUS and *act8-2 IAA2-GUS* seedlings were transferred to GUS staining buffer (100 mM sodium phosphate, pH 7.0,

10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100) containing 1 mM X-gluc and incubated at 37 °C in the dark for 1 h. For cell clearing, Visikol was used as per manufacturer's instruction. The roots were imaged with a light microscope (Nikon Diaphot) equipped with a digital camera control unit [Digital Sight (DS-L2); Nikon, Tokyo, Japan (http://www.nikon.com/)].

2.6. Quantification of GUS Staining

For quantification of GUS staining in root, the region of interest (ROI) was drawn and integrated intensity was measured for each seedling at HSB stack (saturated level) using the ImageJ software (Béziat et al., 2017). The intensity/area, which is in arbitrary unit, was measured for columella cells and vascular tissue in meristematic region.

2.7. Live-Cell Imaging

To image GFP, the transferred seedlings were incubated at 23 and 29 °C under continuous light for 72 h. After mounting on a large cover glass, the roots were imaged using a Nikon laser-scanning microscope (Eclipse Ti equipped with Nikon C2 Si laser-scanning unit) with a 20X/40X water objective. Settings are as follows: HV 95, Laser 10, Pinhole 3.4 and Scan speed 0.06 for PIN1-GFP, HV 100, Lasser 7, Pinhole 2.3 and Scan speed 0.125 for PIN2-GFP, HV 100, Laser 10, Pinhole 2.3 and Scan speed 0.125 for AUX1-YFP, HV 100, Laser 10, Pinhole 2.3 and Scan speed 0.06 for ABD2-GFP. Fluorescence intensities were measured by drawing a ROI in the images (Approximately 250 μm from tip) for PIN1-GFP, PIN2-GFP and AUX1-YFP obtained from live-cell imaging using Image J software 1.8.0.

2.8. Actin Quantification

Actin quantification was performed using ImageJ software 1.8.0 as described earlier (Higaki et al., 2010). The confocal images were skeletonized and then actin features were measured as previously described (Higaki, 2017; Ueda et al., 2010).

2.9. Gene Expression Analysis

RNA was extracted from the root tissue of 5-day-old vertically grown Arabidopsis seedlings incubated at 23 °C and 29 °C for 72 h, using RNA Extraction Kit (QIAGEN, Hilden, Germany) with on-column DNA digestion to remove residual genomic DNA according to manufacturer's protocol. Extracted RNA was tested for quality and quantity. Each RNA concentration was normalized with RNase-free water. Then, 1 μg of RNA was used to make 20 μL cDNA using qPCR RT kit (TOYOBO CO., LTD). Semi-quantitative RT–PCR was performed using 1 μL of cDNA. PCR conditions for PIN1: 95 °C—5 min, 95 °C—30 s, 55 °C—30 s, 72 °C—30 s, 72 °C—10 min, 10 °C—hold, 30 cycles. PCR condition for PIN2: 95 °C—5 min, 95 °C—30 s, 59 °C—30 s, 72 °C—30 s, 72 °C—10 min, 10 °C—hold, 25 cycles.

EF1α was used as control. Data were obtained from three biological replicates.

Primer list used to perform PCR:

PRIMER	SEQUENCE
PIN1 F	ATCTTCACATGTTTGTGTGG
PIN1 R	TCGTCTTTGTTACCGAAACT
PIN2 F	GGAGACGGCTGGTTCAATTA
PIN2 R	ACCACCACGTGTAGCTTTCC
EF1α F	CTTGCTTTCACCCTTGGTGT
EF1α R	TCCCTCGAATCCAGAGATTG

2.10. Statistical Analysis

Results are expressed as the means ± SE from the appropriate number of experiments as described in the figure legends. Two-tailed Students *t*-test or Tukey–Kramer multiple comparison tests were used to analyze statistical significance using R (https://www.rproject.org/ 2021).

Chapter 3

Arabidopsis root growth under prolonged moderate high temperature

3.1. Introduction

Moderate high temperature stress for short duration affects plant shoot and root growth positively. Hypocotyl elongation, leaf hyponasty and early floral transition are the thermomorphogenic response of Arabidopsis shoot. In case of Arabidopsis root, moderate high temperature treatment for short duration (6-36 h) stimulates root elongation. Interestingly, for both root and shoot thermomorphogenic, auxin was found to be a key player. For root, auxin influx transporter AUX1 and efflux transporter PIN2 mediated shootward auxin transport was increased as well as *aux1-1* and *eir1-1* mutants showed reduced response towards high temperature in root (Hanzawa et al., 2013). Although the mechanisms of responses of seedlings germinated at high temperature and seedlings grown at optimal temperature and transferred to high temperature are likely to be different, it was shown that root length of the seedlings grown at 30 °C is half of root the length of those grown at 25 °C after 10 days with reduced root diameter, cell length and cell production rate (Yang et al., 2017). However, the effect of moderate high temperature for longer period on root elongation of seedlings which is germinated at control temperature remains elusive. Therefore, I examined the root growth pattern of Arabidopsis under prolonged moderate high temperature. Here, I show that root exhibits an unusual growth pattern under prolonged moderate high temperature which is linked to the disturbed auxin distribution and actin reorientation in Arabidopsis.

3.2. Results

3.2.1. Effect of prolonged moderate high temperature on Arabidopsis root growth

To check root behavior of Arabidopsis under prolonged moderate high temperature, I transferred 5-days old seedlings to 23 °C and 29 °C and analyzed root elongation for every 24 h for 3 days. At 23 °C, root elongation increases every day for 3 days whereas root elongation is increased for day 1 at 29 °C as reported earlier (Hanzawa et al., 2013). But root growth becomes slower and forms plateau for next 2 days (Figure 6). I have conducted the kinematic analysis to understand the reason for root elongation reduction. According to kinematic analysis (Table 1), cell elongation was decreased by 6.4%, while cell division was inhibited by 24.5% under prolonged high temperature treatment. Therefore, it can be concluded that prolonged moderate high temperature treatment affects more on cell division than cell elongation and thereby influences normal root elongation negatively.

3.2.2. Reduced abundance affects cellular auxin level

Auxin is one of the major players during thermoresponsive growth of plant hypocotyl and root (Hanzawa et al., 2013; Stavang et al., 2009). It has been shown that high temperature stimulates auxin level in the root using two different system DII-VENUS and DR5-GUS (Hanzawa et al., 2013). To understand the end-result of long-term high temperature treatment on the auxin distribution in the root, I used *IAA2-GUS* marker line, as it is a very sensitive auxin marker which is able to detect even a slight change in auxin response (Rusak et al., 2010; Luschnig et al., 1998). Comparing to 23 °C, *IAA2-GUS* expression at 29 °C is slightly increased in the vascular cylinder of the root which indicates more auxin response in this area (Figure 7A). On the other hand, *IAA2-GUS* expression at the root tip is decreased that means less auxin travels down from top to tip

under prolonged moderate high temperature (Figure 7B). This result suggests that auxin transport might be affected by prolonged moderate temperature stress.

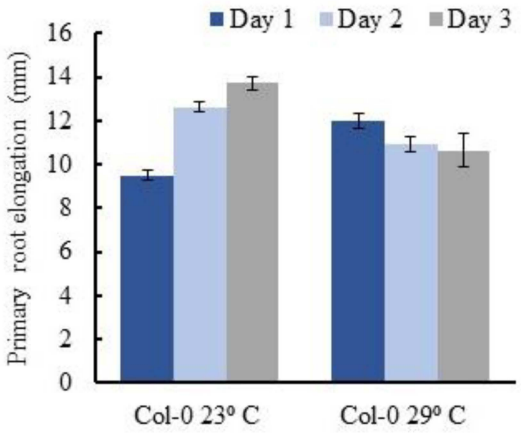

Figure 6. Effect of prolonged moderate high temperature on root elongation of Col-0.

Primary root elongation of Col-0 for 3 consecutive days at 23 °C and 29 °C. Five-day-old seedlings were transferred to new agar plates and subjected to high-temperature treatment (29 °C) and control temperature (23 °C) for 3 days under light condition. Vertical bars represent mean ± SE of the experimental means from at least three independent experiments.

Table 1. Effect of prolonged moderate high temperature stress on cell length and cell production rate in Col-0.

Genotype	Condition	Elongation rate (mm day^{-1})	Cell length (µm)	Cell length %	Cell production rate (cell day^{-1})	Cell production rate %
Col-0	23 °C	10.17 ± 0.09	193.54 ± 1.72	100	52.59 ± 0.5	100
Col-0	29 °C	7.19 ± 0.33	181.22 ± 1.67**	93.63	39.65 ± 1.48**	75.39

Four days old seedlings were exposed to the control (23 °C) and high temperature (29 °C) for 3 days and the measurements reflect the behavior over the third day of treatment. Data are means ±SE of three replicate experiments. Asterisks represent the statistical significance between control and high temperature treatment in Col-0.

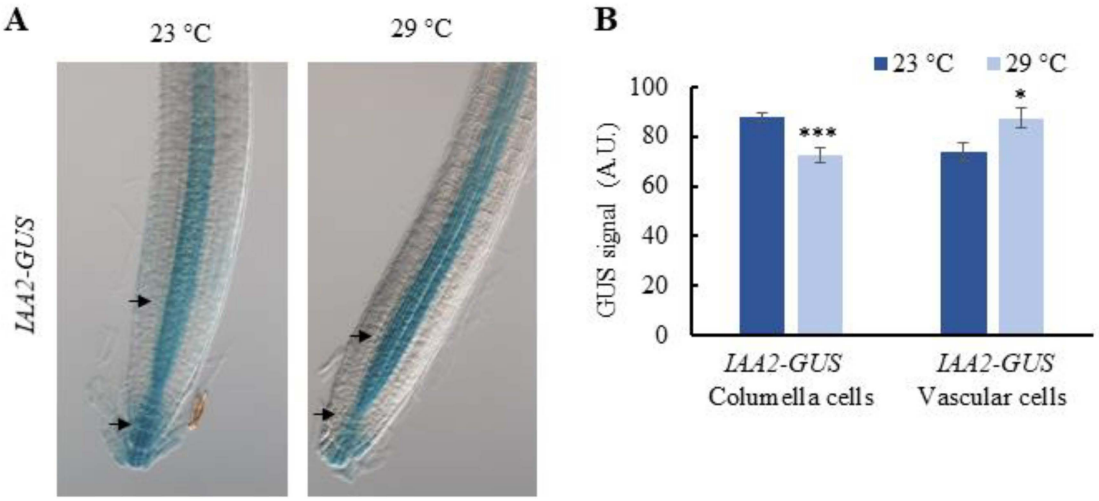

Figure 7. Prolonged moderate high temperature alters the auxin response in Col-0.

(A) *IAA2-GUS* at 23 °C and 29 °C.

Five-day-old *IAA2-GUS* seedlings were transferred to 23 °C and 29 °C for 72 h. Seedlings were stained in a buffer containing 1 mM X-gluc for 1 h at 37 °C and cleared for photography. These are representative images stained in at least three separate experiments. Bars = 100µm.

(B) Quantification of GUS signal in the root from (A).

Asterisks represent the statistical significance between the means for genotype specific treatment judged by the Student's t-test (*<0.05 and ***P<0.001). Vertical bars in the graph represent mean ± SE of the 15-20 seedlings from at least three independent experiments.

3.2.3. Effect of prolonged high temperature on auxin transporters

Since prolonged moderate high temperature affects the root elongation and also alters the cellular auxin homeostasis, I therefore examined whether high temperature affects the localization or the expression of auxin transporters PIN1 and PIN2 in Col-0 as they play major roles in cell-to-cell

efflux of auxin (Blilou et al., 2005). Both the PIN1 and PIN2 abundance, but not the localization was affected by prolonged high temperature treatment (Figures 8A and 9A). Quantitative data obtained from GFP fluorescence analysis show that PIN1 and PIN2 abundance is reduced by 53.5% and 35% respectively at 29 °C in wild-type comparing to 23 °C (Figure 8B and 9B). Expression of *PIN1* and *PIN2* at transcriptional level was checked in Col-0 under prolonged moderate high temperature where no significant difference was not found (Figure 8C and 9C).

I also checked the abundance of auxin influx transporter AUX1, which plays an important role for cellular uptake of auxin as well as participates in root development with PINs (Ugartechea-Chirino et al., 2010; Swarup et al., 2001, 2004). Under long-term high temperature treatment in Col-0, I did not find any alteration of AUX1-YFP abundance comparing to 23 °C (Figure 10). These results suggest that prolonged high temperature affects the cellular auxin efflux process without affecting influx.

3.2.4. Prolonged high temperature modulates actin dynamicity

It has been demonstrated that actin dynamicity controls PIN containing vesicle trafficking. Cytochalasin D, Lat B and TIBA induced filamentous actin degradation and bundling which inhibit removal of PIN1 from and its redistribution to the plasma membrane (Geldner et al., 2001). Actin filament bundling coupled with reduced PIN2 abundance on the plasma membrane was also observed under low phosphate condition (Pandya-Kumar et al., 2014; Kumar et al., 2015). Therefore, distribution and abundance of PIN is integrated with the changes in actin cytoskeleton. Nevertheless, prolonged moderate high temperature affects PIN1 and PIN2 abundance.

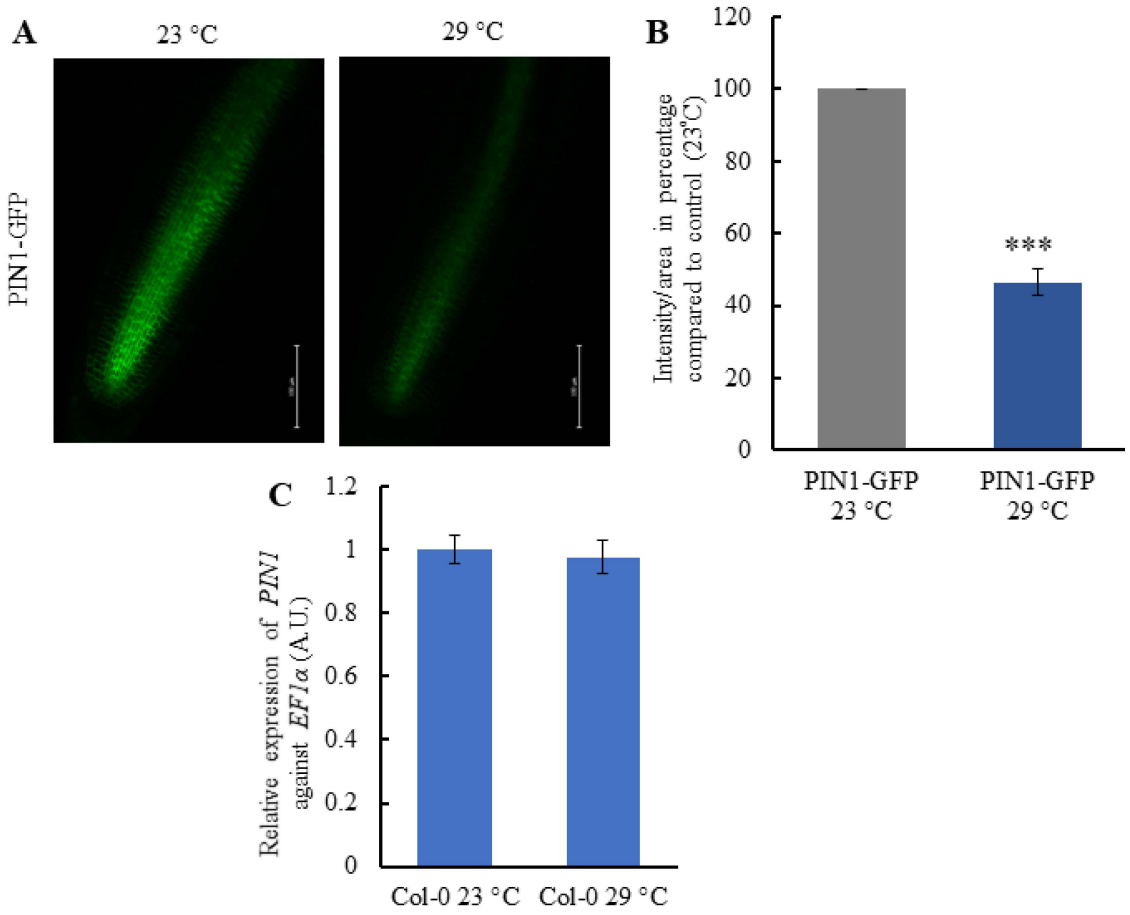

Figure 8. Prolonged moderate high temperature affects PIN1 abundance in roots of Col-0.

(A) PIN1-GFP at 23 °C and 29 °C. Bars = 100 µm.

(B) Quantification of GFP fluorescence intensity from (A). The images were captured using the same confocal setting. Vertical bars represent mean ± SE of the 15-20 seedlings from at least three independent experiments. (C) *PIN1* expression under prolonged high temperature in Col-0. Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before imaging or collecting sample for RNA extraction. Asterisks represent the statistical significance between the means of the percentage Col-0 and mutant judged by the Student's t-test (***P < 0.001).

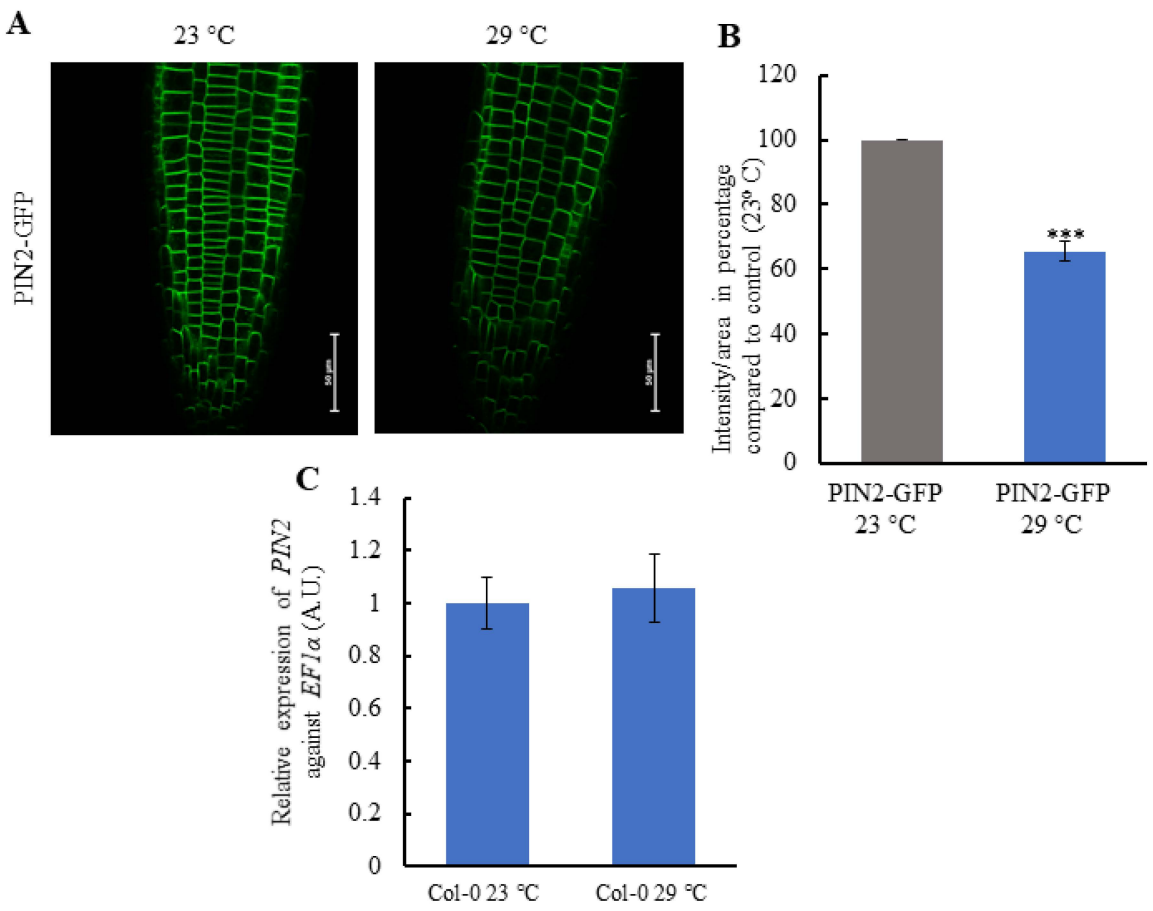

Figure 9. Prolonged moderate high temperature slightly reduces PIN2 abundance in Col-0 roots.

(A) Cellular imaging of PIN2-GFP at 23 °C and 29 °C, (Bar=50 µm).

(B) Quantification of GFP fluorescence intensity from (A). The images were captured using the same confocal setting. Asterisks represent the statistical significance judged by the Student's t-test (***P < 0.001). Vertical bars in the graph represent mean ± SE of the 15-20 seedlings from at least three independent experiments. (C) *PIN2* expression under prolonged high temperature in Col-0. Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before imaging or collecting sample for RNA extraction.

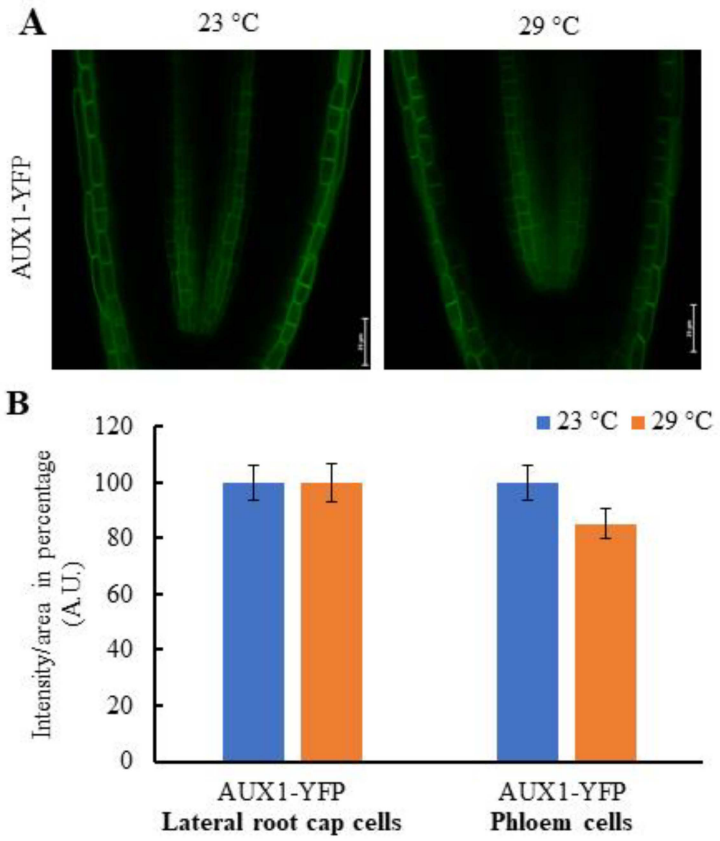

Figure 10. Prolonged moderate high temperature does not alter AUX1 abundance in Col-0.

Five-day-old light grown seedlings of AUX1-YFP were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before imaged with confocal microscopy using the same confocal settings. Images are representative of 15 to 20 seedlings obtained from three independent experiments.

Bars = 25 μm.

In addition, heat stress (37 °C for 24 h) reorganizes actin to transvers cables in hypocotyl and heat stress at 35 °C for 6 h induces thick bundles of actin filaments in root epidermal cells of Arabidopsis seedling (Fan et al., 2016; Müller et al., 2007). To find out the effect of prolonged high ambient temperature on actin dynamicity, I performed live cell imaging of ABD2-GFP marker line in Col-0 using confocal microscope (Wang et al., 2004). Typically, four different parameters are used for actin quantification, namely, 1) occupancy that represents filament density, 2) skewness that indicates filament bundling, 3) normAvgRad that represents parallelness of the filamentous actin and 4) $\Delta\theta$ that represents average angle against longitudinal axis (Higaki, 2017; Ueda et al., 2010). (Figure 11). Confocal microscopy shows visually bright of filamentous actin under prolonged moderate high temperature comparing to 23 °C (Figure 12A). Quantitative analysis of actin dynamicity reveals no significant difference in filament abundance (Figure 12B). Moreover, increased bundling, reduced filament parallelness and increased orientation in Col-0 were found significant under prolonged moderate high temperature using student's t-test (Figure 12C, 12D, 12E).

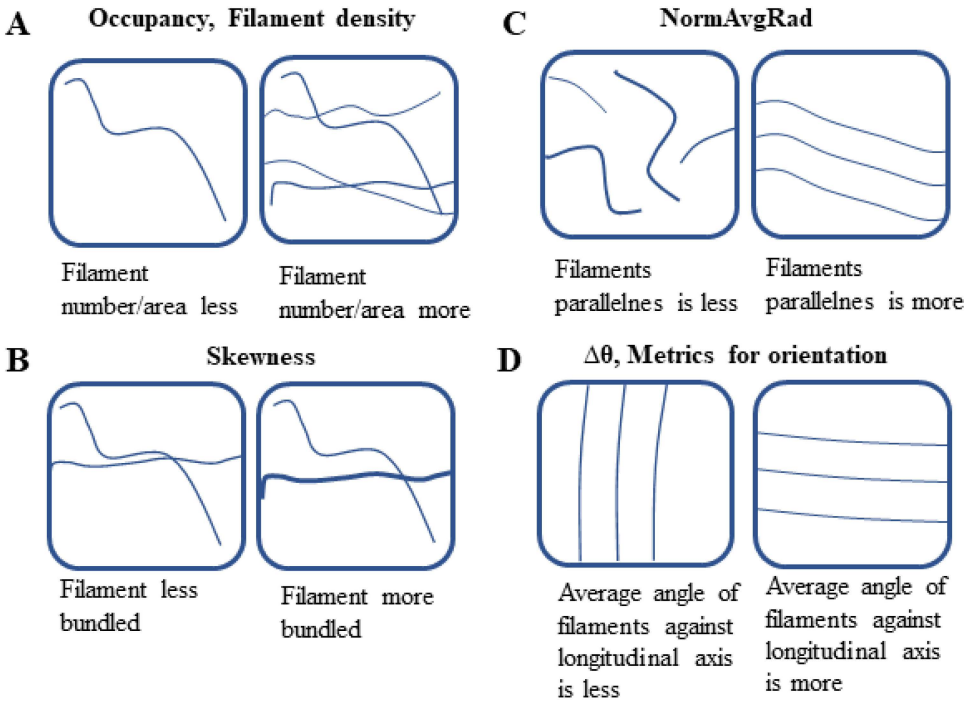

Figure 11. Four different parameters to measure the actin dynamicity. (A) Occupancy, filament density, number of filaments per area ($0 \leq$ occupancy ≤ 100), (B) Skewness, filament bundling ($-\infty <$ skewnesss $< \infty$), (C) NormAvgRad, filament parallelness ($0 \leq$ normAvgRad ≤ 1) and (D) $\Delta\theta$, filament orientation, average angle against the longitudinal axis ($0 \leq \Delta\theta \leq 90$).

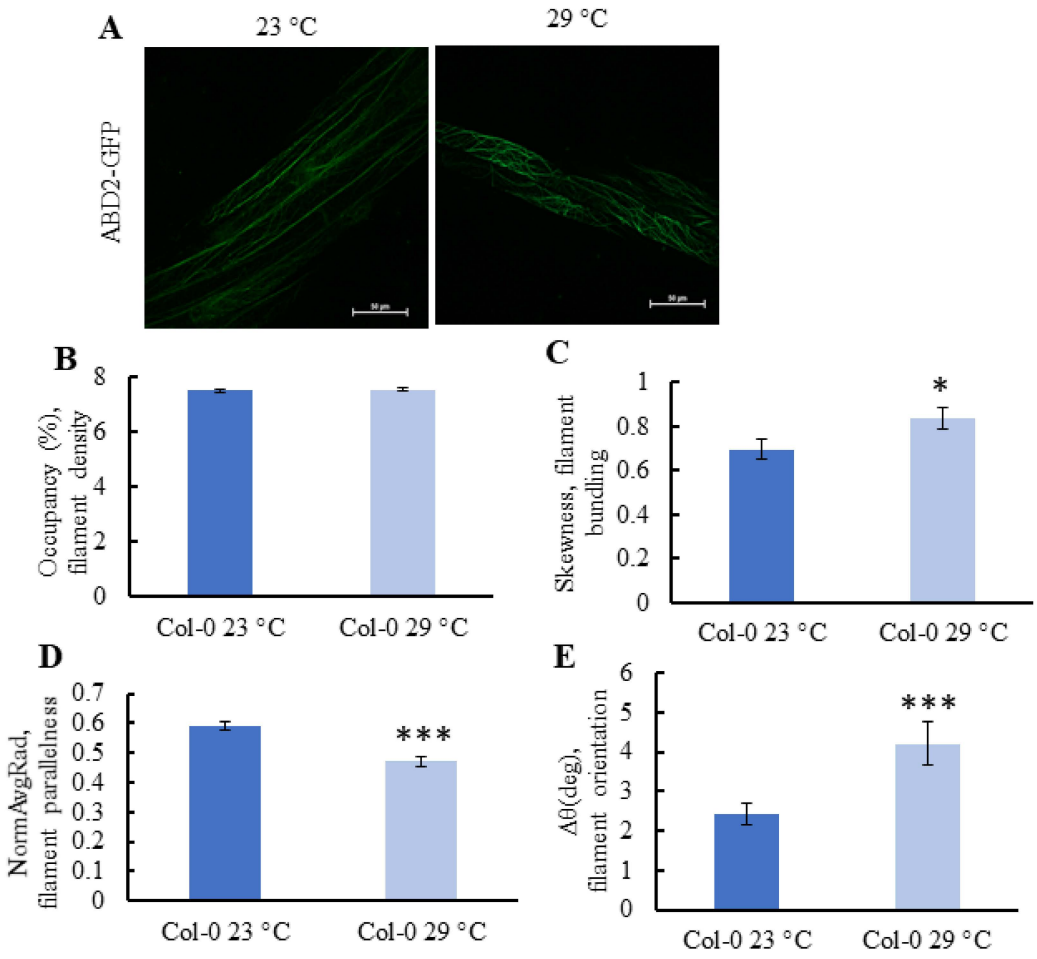

Figure 12. Effect of prolonged moderate high temperature stress on cellular actin organization in Col-0. (A) Confocal images of ABD2-GFP at 23 °C and 29 °C (Bars = 50 µm). Quantification of actin filaments (B-E). (B) Percent occupancy, (C) Skewness, (D) NormAvgRad and (E) Δθ in degree. Five-day-old seedlings were transferred to new agar plates and subjected to high-temperature (29 °C) and control temperature (23 °C) treatment for 3 days under light condition. Vertical bars represent mean ± SE of the experimental means from at least three independent experiments (n = 3 or more), where experimental means were obtained from 30–50 cells. Asterisks represent the statistical significance judged by the Student's t-test (* < 0.05, ***<0.001).

3.3. Discussion

Although increase in the root elongation was observed under short term effect of moderate high temperature, the prolonged effect of moderate high temperature on plant development remains obscure. In this chapter, I demonstrate that under prolonged high temperature, roots lose their ability to elongate, which is linked to the intracellular auxin homeostasis and actin dynamicity. Interestingly, phytohormone auxin was found to be the common target for short and long exposures to moderate high temperature. Short exposure at high temperature redirects the localization of auxin transporter PIN2 to plasma membrane in SNX1 dependent manner, that increases the shootward auxin transport and consequently resulting in root elongation (Hanzawa et al., 2013). On the other hand, long exposure to moderate high temperature inhibits the abundance of both PIN1 and PIN2 resulting in altered auxin distribution (Figure 9, Figure 8, Figure 7) and slows down the root elongation process (Figure 6). This concludes that moderately increased high temperature becomes a stress when the duration is longer, whereas it is a stimulatory factor for short duration. However, in both cases, phytohormone auxin is involved.

Opposite effects of moderate high temperature at initial and later stage were observed in Arabidopsis root. Moderate high temperature increases auxin level and auxin transport which facilitate root elongation (Hanzawa et al., 2013). On the other hand, depleted auxin level and abundance of PINs transporters were found under prolonged moderate high temperature, which slowed down root elongation in the Arabidopsis (Figure 9, Figure 8, Figure 6). In contrast to increased auxin level at the initial stage, there was no accumulation of auxin at root tip at the later stage (Hanzawa et al., 2013; Figure 7). The observed differences in auxin level during short and prolonged high temperature treatments could be due to increased auxin catabolism since this process is induced by auxin and various stresses. An auxin-inducible gene GRETCHEN HAGEN

3 (GH3) expression has been induced by drought, salinity, cold and heat which catalyzes amide conjugation of IAA (Ludwig-Müller et al. 2011, Park et al. 2007). Expression of GH3 or other auxin catabolic genes might be increased by the elevated auxin at initial stage which promotes the IAA catabolism resulting the reduction of free auxin level at the later stage. Additionally, at later stage, there might be changes in secondary messenger (Ca^{2+}, ROS)/metabolites (flavonoids) which can affect actin dynamicity and consequently abundance of PINs, although the effect of moderate high temperature at initial stage on actin dynamicity is currently unknown. These might be one of the survival strategies to deal with stress by modifying their morphology.

Despite PIN1 and PIN2 are homologous auxin efflux transport proteins, they have differential expression pattern as well as responsiveness to increased auxin levels (Vieten et al., 2005). Quantification of root images taken by confocal microscope shows prolonged high temperature reduces PIN1 abundance more than PIN2 in WT suggesting that PIN1 is more sensitive than PIN2 toward prolonged high temperature stress (Figure 8, Figure 9). Previous report showed that recycling of PIN2 from vacuole to plasma membrane is increased but PIN1 was not changed by high temperature for short exposure (Hanzawa et al., 2013). These data are also supported by other reports where differential response of PIN1 and PIN2 was shown during action of different stresses. Copper stress mediated auxin redistribution in Arabidopsis is regulated by PIN1 but not PIN2 or AUX1 (Yuan et al., 2013). Under low-B condition, accumulation of PIN1 is reduced without affecting PIN2 which causes accumulation of auxin in the stele of wild-type root meristems (Li et al., 2015). High levels of NO causes the disturbance of auxin transport and auxin response due to PIN1 reduction in the primary root, while PIN2 is not affected (Fernández-Marcos et al., 2011). These reports support the idea that PIN proteins can differ in the regulation of their proteasome-dependent turnover (Sieberer et al., 2000).

Additionally, filamentous actin organization has been altered in terms of skewness, parallelness and angle which could affect cell elongation and cell division under prolonged moderate high temperature. However, the mechanism is unclear. Cytoskeleton actin is also found to be a target of other biotic and abiotic stresses. During pattern-triggered immunity of Arabidopsis in response to pathogenic *Pseudomonas syringae pv.* tomato DC3000, an immediate increase of actin abundance was observed and latrunculin B- induced disruption of the host-cell actin filaments enhanced virulence (Henty-Ridilla et al., 2013). Infection with nematode *Meloidogyne incognita* up-regulates ADFs and filamentous actin turn-over in the giant feeding cells of Arabidopsis whereas knock down of ADF2 reduces filamentous actin turn-over and inhibits developing gall cells (Clément et al., 2009). Abiotic stresses, for instance, low and high salt stress (NaCl) promote actin filament assembly and disassembly after 18 h of treatment respectively in pavement cells in Arabidopsis (Wang et al., 2010). Under low phosphate condition, bundling of filamentous actin is increased comparing to high phosphate condition in Arabidopsis root (Kumar et al., 2015). Therefore, it is clear that prolonged high temperature stress affects actin assembly like other biotic and abiotic stresses. Furthermore, filamentous actin assembly regulating factors such as ADFs could be involved in this process.

Chapter 4

ACT2 and ACT7 isovariants are required to maintain root elongation under prolonged moderate high temperature

4.1. Introduction

Actin cytoskeleton is essential component of protein trafficking and maintains various cellular and physiological processes in all kind of organism (Kandasamy et al., 2009). It is also one of primary targets of various abiotic and biotic stresses including high temperature (Clément et al., 2009; Henty-Ridilla et al., 2013; Parrotta et al., 2016). Previous reports showed heat shock treatment degrades actin filaments (Müller et al., 2007; Malerba et al., 2010). In Chapter 3, I found that prolonged moderate high temperature hampers normal root elongation, reorganizes actin filaments and reduces abundance of PINs in Arabidopsis. Since filamentous actin can control trafficking of auxin transporter proteins, actin emerges as a clear focus to me. Therefore, I examined the role of different actin isovariants in root elongation in Arabidopsis under prolonged moderate high temperature.

There are two classes of actin isovariants in Arabidopsis; vegetative class and reproductive class. ACT2, ACT8 and ACT7, three vegetative actin isovariants, are grouped into two subclasses. Subclass 1, ACT2 and ACT8, involve in root hair growth in Arabidopsis. ACT2 mutant exhibits wavy root phenotype but lack of neither ACT2 nor ACT8 shows defect in root growth. Strikingly, ACT7, only member of subclass 2, implicates in root growth (Gilliland et al., 2003). ACT7 mutant displays delayed seed germination and dwarf phenotype at seedling stage with twisted root. Due to high expression level of ACT7 in rapidly growing vegetative organs and its promoter which contains many putative phytohormone responsive elements including auxin, environmental stimuli

can be reflected in ACT7 expression level (McDowell et al., 1996a; Kandasamy et al., 2009). Therefore, I examined the function of three vegetative actin isovariants, ACT2, ACT7 and ACT8 in root elongation under prolonged high temperature stress. In this study, using *act2-1, act7-4* and *act8-2* single mutants, I report that ACT7 and ACT2 isovariants are essential for root elongation, and are required to control actin dynamicity and the normal distribution of phytohormone auxin. Abundance of auxin transporter PIN1 and PIN2 were reduced in *act7-4* and *act2-1* mutant under prolonged moderate high temperature in Arabidopsis root.

4.2. Results

4.2.1. Screening of vegetative actin mutants

There are three vegetative actin isovariants ACT2, ACT7 and ACT8 in Arabidopsis which are expressed in the root tissues. Furthermore, lack of ACT2 and ACT7 exhibit alteration in actin filament in Arabidopsis root hair cells. Fragmented actin filaments and less actin filaments were observed in *act2-1* and *act7-4* (Kandasamy et al., 2009). Prolonged moderate high temperature stress alters actin filament organization (Chapter 3). Therefore, I tested the effect of prolonged moderate high temperature on root elongation of *act7-4, act2-1* and *act8-2*. Intriguingly, I observed that *act7-4* and *act2-1* are severely sensitive to high temperature treatment at second day and third day where *act8-2* mutant showed WT like response (Figure 13). To understand the reason behind the reduction of root elongation, I performed kinematic analysis for these two sensitive mutants under high temperature treatment. Consistent with their reduced root elongation, *act7-4* and *act2-1* showed drastic reduction in cell division by 67.21% and 77.68% and cell elongation by 44.6%

and 36.82% respectively (Table 2). Both in *act2-1* and *act7-4*, cell division is more affected than cell elongation like WT under prolonged moderate high temperature stress.

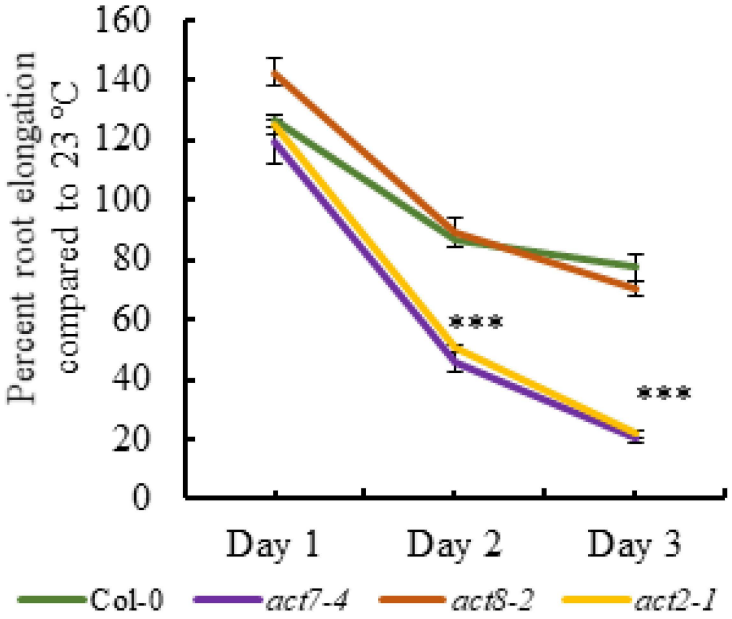

Figure 13. Effect of prolonged moderate high temperature on root elongation of Col-0, *act2-1*, *act7-4* and *act8-2*.

Percentage root elongation at 29 °C is calculated against of root elongation at 23 °C for each genotype. Percent root elongation of mutants were compared to that of Col-0 for statistically significance changes. Vertical bars represent mean ± SE of the experimental means from at least three independent experiments. Asterisks represent the statistical significance as judged by the Student's t-test (* $p < 0.05$; *** $p < 0.001$).

Table 2. Effect of prolonged moderate high temperature stress on cell length and cell production rate in Col-0, *act7-4* and *act2-1* mutant.

Genotype	Condition	Elongation rate (mm day^{-1})	Cell length (µm)	Cell length %	Cell production rate (cell day^{-1})	Cell production rate %
Col-0	23 °C	10.17 ± 0.09	193.54 ± 1.72	100	52.59 ± 0.5	100
Col-0	29 °C	7.19 ± 0.33	181.22 ± 1.67**	93.63	39.65 ± 1.48**	75.39
act7-4	23 °C	6.03 ± 0.15	149.49 ± 0.24	100	40.34 ± 1.07	100
act7-4	29 °C	1.09 ± 0.063	82.82 ± 3.26***	55.4	13.23 ± 0.66***	32.79
act2-1	23 °C	10.96±0.23	184.72± 1.44	100	58.93± 0.68	100
act2-1	29 °C	1.53±0.08	116.72± 0.66***	63.18	13.15± 0.80***	22.31

Four days old seedlings were exposed to the control (23 °C) and high temperature (29 °C) for 3 days and the measurements reflect the behavior over the third day of treatment. Data are means ± SE of three replicate experiments. Asterisks represent the statistical significance between control and high temperature treatment *in Col-0, act2-1* and *act7-4* are judged by the Student's t-test (**P < 0.01, ***P < 0.001)

4.2.2. Prolonged moderate high temperature affects cellular auxin level in *act2-1* and *act7-4*

Phytohormone auxin controls normal plant growth and development. Auxin also regulates biotic and abiotic stresses. It was previously shown that auxin is involved in high temperature mediated root elongation for short duration (Hanzawa et al., 2013). In prolonged moderate high temperature, auxin distribution was found to be perturbed in Arabidopsis root (Chapter 3). Hence, to check auxin level of theses mutants, I used the same auxin marker line *IAA2-GUS* which was crossed with these mutants. After prolonged high temperature treatment, *act7-4* and *act2-1* mutant but not *act8-2* showed altered GUS activity at the vascular cylinder and root tip compared to Col-0 (Figure 14). Quantitative data of GUS signal confirmed the highly significant alteration in *IAA2-GUS* signal in *act7-4* and *act2-1* under prolonged moderate high temperature. This result indicates ACT7 and ACT2 play important role in maintaining the proper auxin homeostasis under prolonged high temperature.

4.2.3. Effect of prolonged high temperature on auxin transporters in *act2-1* and *act7-4*

Auxin transport is affected by both high temperature and cold temperature (Hanzawa et al., 2013; Shibasaki et al., 2009). Cold temperature blocks PIN2 trafficking in Arabidopsis root cell (Shibasaki et al., 2009). High temperature stress for short duration increases auxin transport by enhancing retrieving more PIN2 from vacuole to plasma membrane where long duration moderate high temperature reduces PIN2 abundance in root (Hanzawa et al., 2013) (Chapter 3). I therefore examined the effect of prolonged high moderate temperature on PIN2 in the actin mutants. I checked the abundance of auxin transporter PIN2-GFP in *act7-4*, *act2-1* and *act8-2* mutants after long term high temperature treatment. According to quantitative data of the fluorescence intensity per area of PIN2-GFP, *act2-1* and *act7-4* mutants showed at least 75% reduction of PIN2-GFP

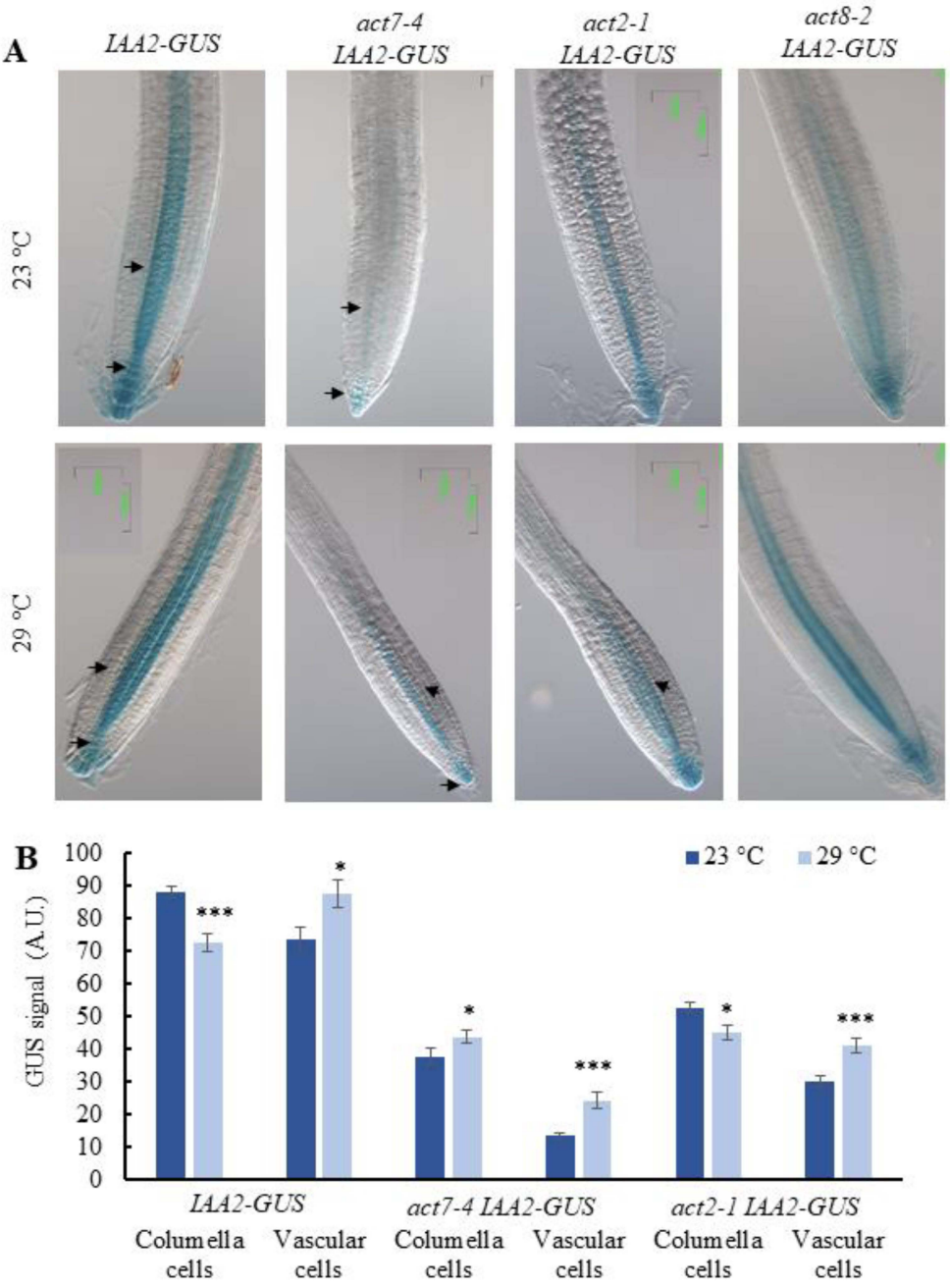

Figure 14. Prolonged moderate high temperature alters the auxin distribution in Col-0, *act7-4* and *act2-1* roots. (A) *IAA2-GUS*, *act7-4 IAA2-GUS* and *act2-1 IAA2-GUS* at 23 °C and 29 °C. Five-day-old *IAA2-GUS*, *act7-4 IAA2-GUS* and *act2-1 IAA2-GUS* seedlings were transferred to 23 °C and 29 °C for 72 h. Seedlings were stained in a buffer containing 1 mM X-gluc for 1 h at 37 °C and cleared for photography. These are representative images stained in at least three separate experiments. Bars = 100 µm. (B) Quantification of GUS signal in the root from (A). Asterisks represent the statistical significance between the means for genotype specific treatment judged by the Student's t-test (*<0.05 and ***P<0.001).

abundance which highly significant comparing to WT (Figure 15A, 15B). To understand whether this alteration in PIN2 abundance is the result of reduced transcription or translation, I check the *PIN2* expression level in *act7-4* and *act2-1* mutants under high temperature stress by semi-quantitative RT-PCR. This result shows no difference of *PIN2* expression in Col-0, *act7-4* and *act2-1*, which confirms that high temperature does not affect *PIN2* at transcriptional level (Figure 15C).

Nevertheless, absence of only PIN2 barely can inhibit root elongation. PIN2 and PIN1 together perform proper root elongation (Li et al., 2015). Therefore, to check the PIN1-GFP abundance in *act7-4* mutant, I have generated *act7-4* PIN1-GFP. Due to technical problem, I could not generate *act2-1* PIN1-GFP. PIN1-GFP abundance is reduced to 67% in *act7-4* which is significantly less than that of Col-0 (Figure 16A, 16B). To check whether *PIN1* expression is affected by high temperature in *act7-4* mutants, semi-quantitative RT-PCR was performed (Figure 16C). This result shows no difference of *PIN1* expression in Col-0 and *act7-4* which confirms that high temperature does not affect *PIN1* at transcriptional level in *act7-4* under high temperature stress.

High temperature mediated root elongation is reduced in *aux1-7* mutant (Hanzawa et al., 2013). Prolonged moderate high temperature does not affect auxin influx transporter AUX1 in WT (Chapter 3). To understand what happens in *act2-1* and *act7-4* mutants, next I checked the abundance of auxin influx transporter AUX1 under long-term high temperature treatment in the *act7-4* and *act2-1* mutants using AUX1-YFP marker. I did not find any alteration of AUX1-YFP abundance at 29 °C comparing to 23 °C (Figure 17A, 17B, 17C). These results suggest that prolonged high temperature mediated depletions of PIN1 and PIN2 at translational level depend on ACT2 and ACT7 actin isovariants, while AUX1 is unaffected.

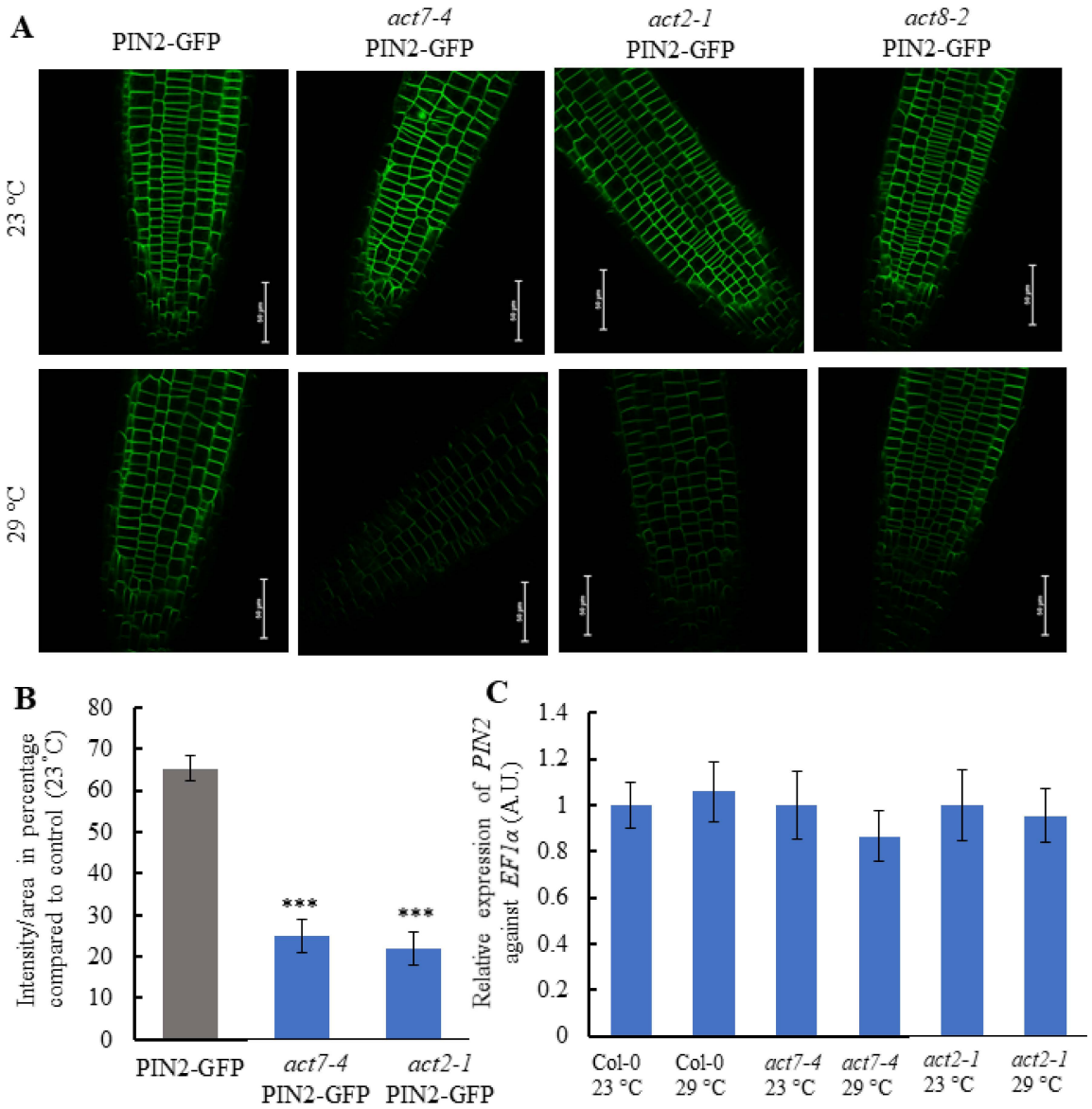

Figure 15. Prolonged moderate high temperature affects PIN2 abundance but not *PIN2* expression in roots of Col-0 and *act7-4* mutant. (A) Live cell images of PIN2-GFP, *act2-1* PIN2-GFP and *act7-4* PIN2-GFP at 23 °C and 29 °C. Bars = 50 µm. (B) Quantification of GFP fluorescence intensity from (A). The images were captured using the same confocal setting. Vertical bars represent mean ± SE of the 15-20 seedlings from at least three independent experiments. Asterisks represent the statistical significance between the means of the percentage Col-0 and mutant judged by the Student's t-test (*P < 0.05). (C) *PIN2* expression under prolonged high temperature in Col-0, *act7-4*, and *act2-1*. Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before imaging or collecting sample for RNA extraction.

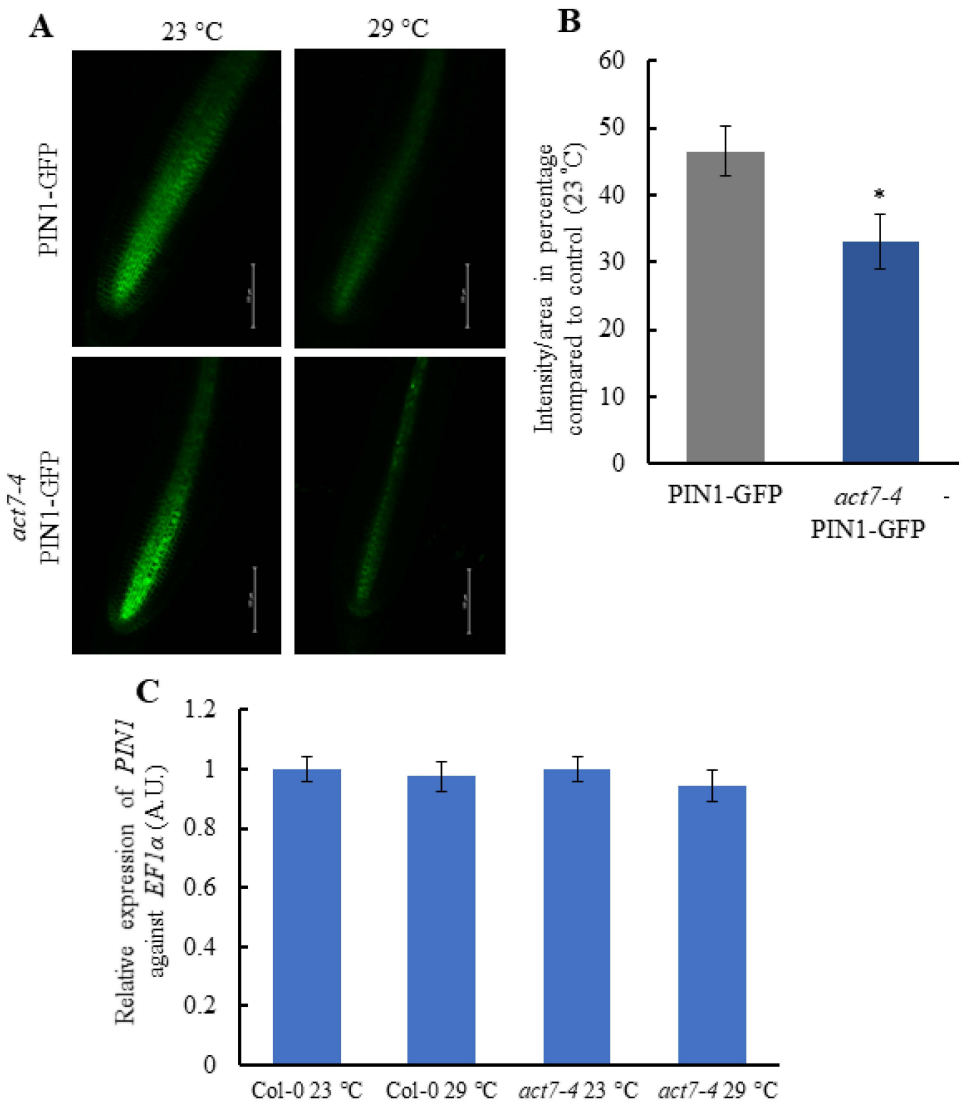

Figure 16. Prolonged moderate high temperature affects PIN1 abundance but not *PIN1* expression in roots of Col-0 and *act7-4* mutant. (A) Live cell images of PIN1-GFP and *act7-4* PIN1-GFP at 23 °C and 29 °C. Bars = 100 µm. (B) Quantification of GFP fluorescence intensity from (A). The images were captured using the same confocal setting. Vertical bars represent mean ± SE of the 15-20 seedlings from at least three independent experiments. Asterisks represent the statistical significance between the means of the percentage Col-0 and mutant judged by the Student's t-test (*P < 0.05). (C) *PIN1* expression under prolonged high temperature in Col-0 and *act7-4.* Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before imaging or collecting sample for RNA extraction.

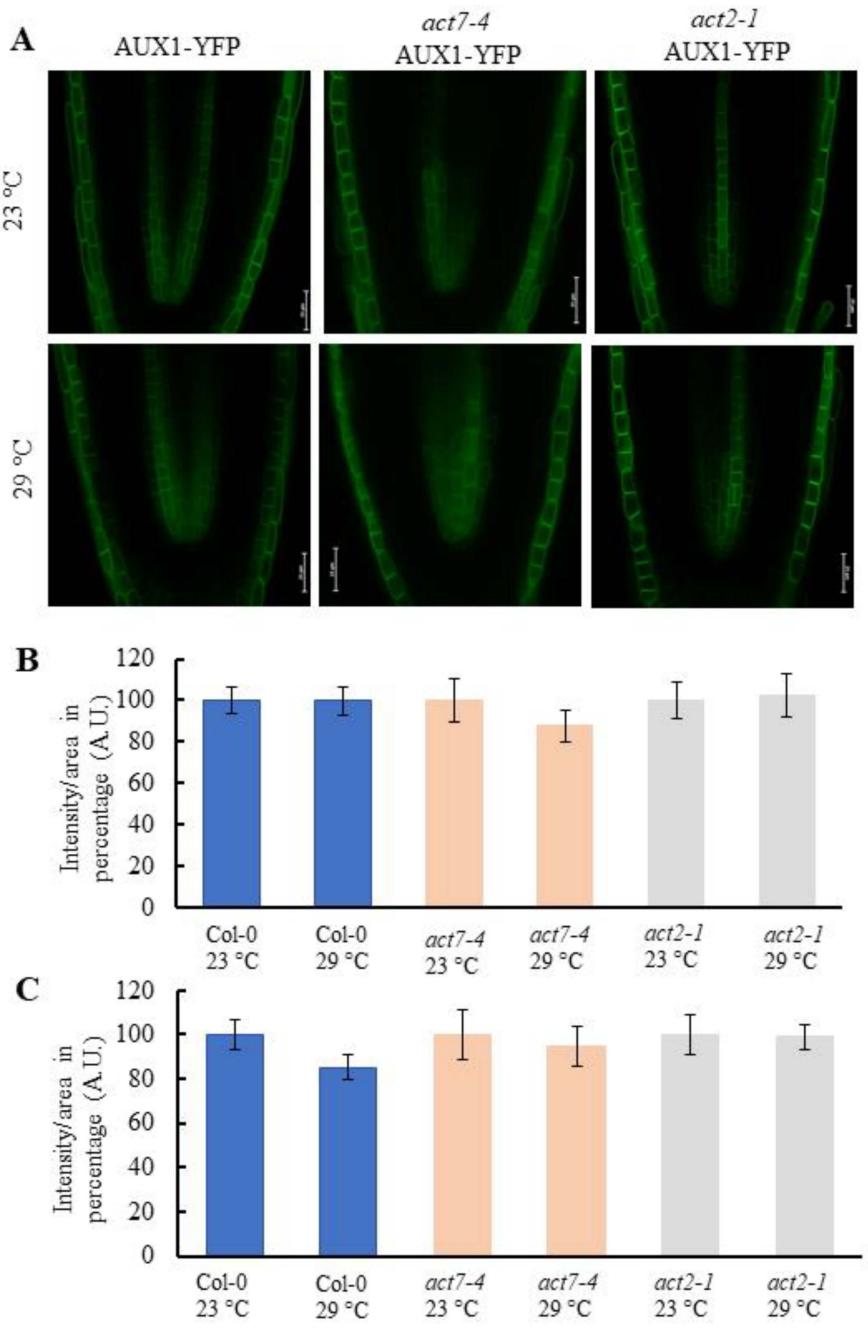

Figure 17. Prolonged moderate high temperature does not alter AUX1 abundance in Col-0, *act7-4* and *act2-1* mutant. (A) Live cell images of AUX1-YFP, *act2-1* AUX1-YFP and *act7-4* AUX1-YFP at 23 °C and 29 °C. Bars = 100 µm. (B) Quantification of YFP fluorescence intensity of lateral root cap cells from (A). (C) Quantification of YFP fluorescence intensity of phloem cells from (A). Vertical bars represent mean ± SE. Five-day-old light grown seedlings of AUX1-YFP, *act7-4* AUX1-YFP and *act2-1* AUX1-YFP were transferred to new agar plates and subjected to high-temperature treatment (29 °C) for 72 h before

imaged with confocal microscopy using the same confocal settings. Images are representative of 15 to 20 seedlings obtained from three independent experiments. Bars = 25 μm.

4.2.4. Prolonged high temperature modulates actin dynamicity in *act7-4* and *act2-1*

To check the actin dynamicity in *act7-4* and *act2-1* mutants, I crossed these with ABD2-GFP marker line. After confocal microscopy, quantification of picture was performed. Quantitative data revealed that occupancy is unchanged while skewness of actin filaments is significantly enhanced in *act7-4* and *act2-1* after prolonged high temperature stress (Figure 18A, 18B, 18C). In addition, alteration in actin filament parallelness and average angle were profound in *act7-4* comparing to WT. On the other hand, filament parallelness is slightly changed but the average angle remains unchanged like WT in *act2-1* mutant (Figure 18D, 18E). These data suggest that actin dynamicity is affected by prolonged high temperature particularly actin bundling when either ACT2 or ACT7 is missing in Arabidopsis root which perhaps influence the abundance of PIN1 and PIN2 causing altered auxin gradient.

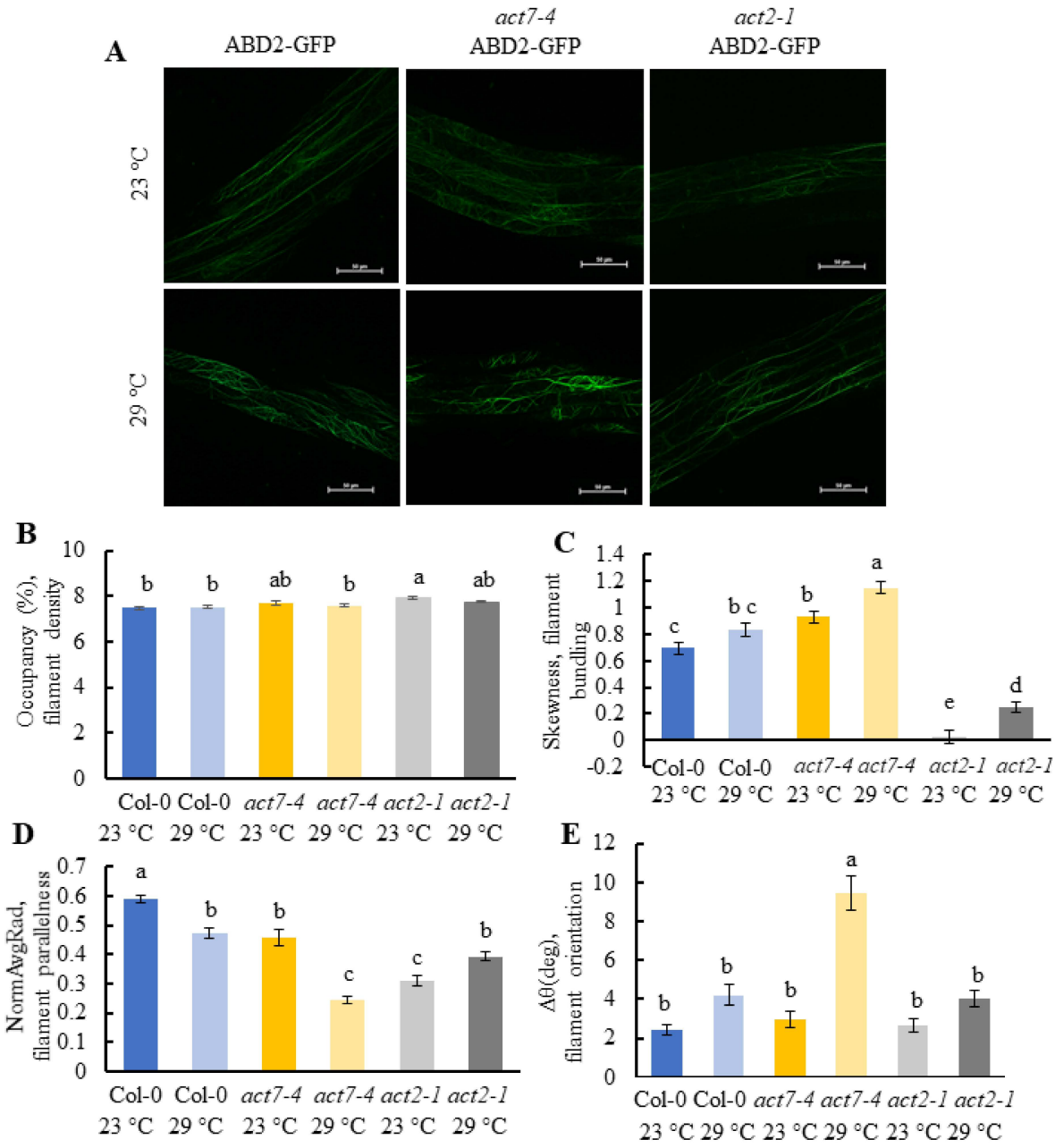

Figure 18. Effect of prolonged moderate high temperature stress on cellular actin organization in Col-0, *act7-4* and *act2-1*. (A) Live cell images of of ABD2-GFP at 23 °C and 29 °C (Bars = 50 µm). Quantification of actin filaments (B-E). (B) Percent occupancy, (C) Skewness, (D) NormAvgRad and (E) Δθ in degree. Five-day-old seedlings were transferred to new agar plates and subjected to high-temperature (29 °C) and control temperature (23 °C) treatment for 3 days under light condition. Vertical bars represent mean ± SE of the experimental means from at least three independent experiments (n = 3 or more), where experimental means were obtained from 30–50 cells. Comparisons between multiple groups were performed by analysis of

variance (ANOVA) followed by the Tukey–Kramer test. The same letter indicates no significant differences ($p < 0.05$).

4.3. Discussion

In this chapter, I examined root growth response of three vegetative actin mutants to identify whether the prolonged moderate high temperature response is actin isovariant specific. Here, I show that *act7-4* and *act2-1* exhibited no alteration in root elongation in case of initial time period of high temperature treatment, while prolonged treatment showed strong inhibition in root elongation comparing to WT (Figure 13). Therefore, it is clear that ACT7 and ACT2 both are required for root elongation under prolonged high temperature.

Analyses of different actin parameters further revealed that prolonged high temperature increases average angle as well as reduces parallelness of the actin filaments more in *act7-4* mutant while parallelness is decreased and average angle is unaltered *in act2-1* comparing to wild type (Figure 18D, 18E). This differential effect of prolonged moderate high temperature on parallelness and average angle of filamentous actin is probably due to their differential filament nature. Previous report showed that the actin filaments are thinner and longer in *act2-1* mutant whereas short, fragmented thick actin filaments were found in *act7-4* mutant which observed in my experiments under control condition (Kandasamy et al., 2009). Nevertheless, occupancy did not change in WT, *act7-4* and *act2-1* which is possibly due to reduced cell size under high temperature. Intriguingly, bundling of actin filaments under prolonged moderate high temperature both in *act7-4* and *act2-1* was found in this experiment (Figure 18C). This kind of impact of high temperature on filamentous actin has been reported earlier. Heat stress (35 °C 6 h) induces thick bundles of actin filaments in Arabidopsis root (Müller et al., 2007). An opposite effect was shown by heat shock on filamentous actin. Heat shock at 41 °C for 10 min depolymerizes the filamentous actin network in Arabidopsis

root (Müller et al., 2007). Heat shock (50 °C for 5min) induces depolymerization of actin microfilaments and changes in ER morphology accompanied by accumulation of the HSP70 binding protein (BiP) in BY-2 cultured cells (Malerba et al., 2010). Hence, it is clear that actin cytoskeleton is one of the core targets of heat stress where the nature of modification depends on the treatment intensity and duration. High temperature stress can modulate expression of different actin binding proteins such as ADFs, profilins and villins which could cause actin bundling especially in *act7-4* and *act2-1* mutant background since it was reported that high temperature increases expression of ADFs and profilins to bundle actin filaments in Arabidopsis hypocotyl (Fan et al., 2016). Biotic stress is also reported to induce the expression of ADFs to cause actin reorganization in Arabidopsis (Clément et al., 2009). Quantification of actin dynamicity revealed that the effect of prolonged high temperature on actin filaments in *act7-4* and *act2-1* is likely due to distinct biochemical properties of ACT7 and ACT2 which are essential to maintain actin dynamicity under high temperature stress (Zhu et al., 2016; Kijima et al., 2016)

The reduced abundance of PIN1 and PIN2 under prolonged moderate high temperature was also found to be linked with isovariant specific cellular actin organization (Figure 15, Figure 16). In agreement to these data, auxin inductive actin filament remodeling defective mutant *rmd-1*and *rmd-2* showed reduced plasma membrane localized PIN2 with increased internalization in rice (Li et al., 2014). Enhanced actin filament bundling results in reduced PIN2 abundance on the plasma membrane and vice versa depending on phosphate supply (Pandya-Kumar et al., 2014; Kumar et al., 2015). TIBA-induced polymerization of actin filaments also reduces plasma membrane localization and enhanced lytic vacuolar accumulation of PIN2 (Zou et al., 2019). Rho GTPase ROP2 and effector protein RIC4 induces fine filamentous actin and increase polar localization by inhibiting PIN1 endocytosis (Nagawa et al., 2012). In contrast, it has been shown that actin

filament stabilization through SPIKE1-ROP6-RIC1 inhibits PIN2 internalization in response to auxin (Lin et al., 2012). These results suggest that actin dynamicity governed by various regulators is influenced by environmental cues which can control PIN1 and PIN2 abundance. No alteration in *PIN1* and *PIN2* transcriptional expression under moderate high temperature confirms that prolonged high temperature either inhibits PINs at translational level or boosts ubiquitinated degradation or both. In *act7-4* root, intercellular accumulation of PIN1 and PIN2 was observed at 23 °C, which disappeared after long term high temperature treatment (Figure 16, Figure 15). Reduced plasma membrane localization of PIN1 and PIN2 without internal accumulation supports the idea that the reduction of the PIN abundance is possibly due to inhibition of protein synthesis. Post translational regulation of proteins by various stresses is not uncommon. For instance, prolonged high temperature has been reported to promote the ubiquitinated degradation of Brassinosteroid Receptor1 (BRI1) (Martins et al., 2017). On the other hand, PIN-LIKES6 is down regulated through post translational modification after long term high temperature treatment (Feraru et al., 2019). Cold stress down regulates SEC7 containing ARF-GEF GNOM at translational level in Arabidopsis (Ashraf and Rahman, 2019). Long-term (Cs^{+}) metal stress reduces expression of cesium uptake transporters ABCG37 and ABCG33 without affecting their transcription (Ashraf et al., 2021). E3 ubiquitin ligase IRT Degradation Factor 1 (IDF1) degrades ferrous Fe uptake transporter IRON-REGULATED TRANSPORTER1 (IRT1) depending on environmental condition to maintain Fe homeostasis (Shin et al., 2013). Although the detail mechanism is still unclear, these results suggest that translational regulation of proteins is an important regulatory mechanism for plant to combat various abiotic stresses.

Although all the actin isovariants have high homology, here I demonstrate that root thermomorphogenesis is linked to ACT7 and ACT2 but not ACT8. There is a subtle difference in

biochemical properties between ACT7 and ACT2/ACT8 whereas ACT2 and ACT8 have only single amino acid difference (Mcdowell et al., 1996b). Arabidopsis vegetative actin subclass II is in a sister group of the subclass I clade in the phylogenetic tree. Subclass I differs by 7% amino acid from subclass II, and these two classes of Arabidopsis actin are probably separated in their origin. ACT2/8 clade is closer to actin genes from maize and rice while ACT7 is more similar to the genes from pea, carrot, potato and pine, suggesting that they originated from different

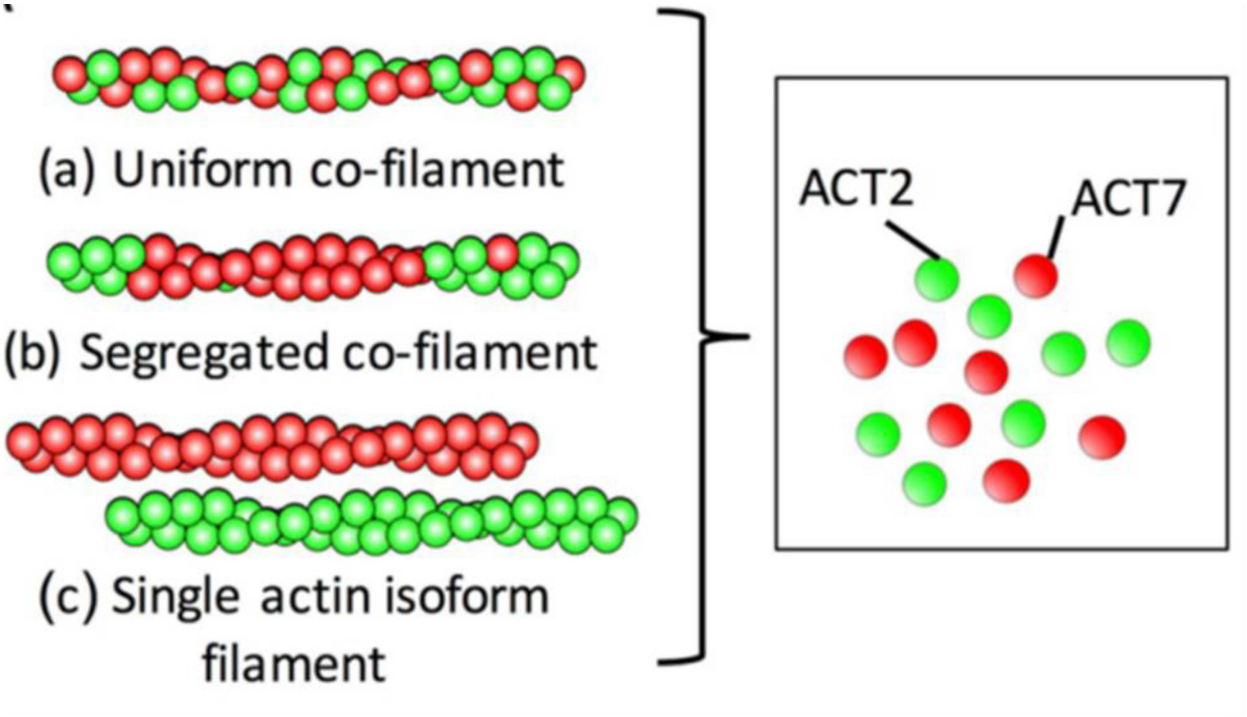

Figure 19. Possible polymerization patterns of ACT2 and ACT7 actin isoforms according to Kijima et al 2018.

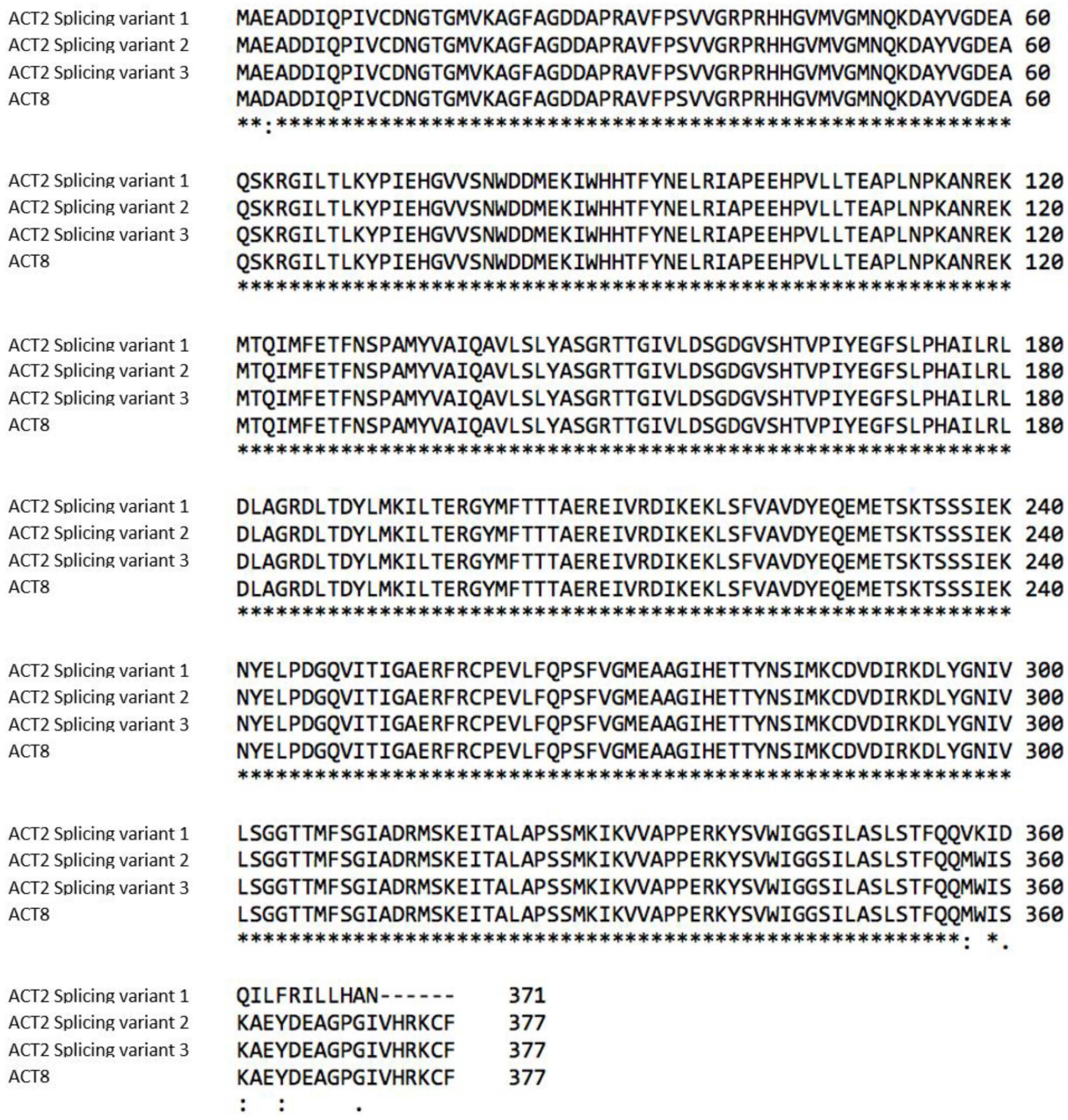

Figure 20. Comparison of amino acid sequences of ACT2 and ACT8.

Clustal Omega was used to compare the sequences of ACT2 and ACT8 proteins. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties - roughly equivalent to scoring > 0.5 in the Gonnet PAM 250 matrix. A . (period) indicates conservation between groups of weakly similar properties- roughly equivalent to scoring = < 0.5 and > 0 in the Gonnet PAM 250 matrix.

lineages (Mcdowell et al., 1996b). The differences between these two subclasses were also evident in their amino acid compositions. These subtle changes in amino acid compositions have the potential to alter actin-actin or actin-actin binding protein interactions (Mcdowell et al., 1996b). Differential biochemical properties of ACT2 and ACT7 had already been reported in terms of polymerization and phosphate release rates. Actin binding proteins, profilin 1 and profilin 2 inhibit the polymerization of ACT7 while the effect is minimal on ACT2 polymerization (Kijima et al., 2016). With these differences, ACT2 and ACT7 were found equally required for root elongation under prolonged moderate high temperature stress. Previously, they have been reported to response in the same way when treated with 2,4-D (Takahashi et al., 2017). However, the mechanism is not clear. It has been shown that ACT7 and ACT2 can generate single or mixed actin filaments (Kijima et al., 2018) (Figure 19). I hypothesize that mixed actin filaments generated with ACT2 and ACT7 is required to mitigate the changes generated by prolonged moderate high temperature.

On the other hand, ACT2 and ACT8, having very common characteristics, showed dissimilar responses under prolonged moderate high temperature which is supported by differential response of ACT2 and ACT8 under cold stress (poster presentation AAAS, 2021, https://aaas.confex.com/aaas/2021/meetingapp.cgi/Paper/28705). Highly homologues β and γ cytoplasmic actin isoforms showed differential function in mouse confirming that they defined by their nucleotide rather than their amino acid sequence (Vedula et al. 2017). There are three splicing variants of ACT2 and one for ACT8. ACT2 splicing variant 2 and 3 have completely same sequences of amino acid residues. Comparison among amino acid sequences of ACT2 and ACT8 with Clustal Omega showed ACT8 and ACT2 splicing variant 2 and 3 have only one amino acid difference (An et al., 1996) while the difference between ACT2 splicing variant 1 and ACT8 was more than one (Figure 20). Moreover, secondary structure prediction using CLC showed that one

beta strand at 55 amino acid position in ACT2 is missing in ACT8. Predicted ligand binding sites of ACT8 contains two Ca^{2+} binding sites which are not found in ACT2 while ACT2 has one extra ADP binding site than ACT8. These subtle differences may change the nature of ACT8 in terms of binding with actin binding proteins than ACT2. In addition, the promoter region of ACT2 and ACT8 differ largely in their binding elements (http://www.athamap.de/search_gene.php). Differential level of expression of ACT2 and ACT8 also could be the reason for their disparate response towards prolonged moderate high temperature. ACT2 has greater expression level than ACT8 in the Arabidopsis root (An et al., 1996). They have high variability in the introns (Mcdowell et al., 1996b) which also could regulate expression pattern and generating splicing variants as it is already reported that ACT2 promoter-GUS with its first intron showed 13-fold stronger expression in the shoot apical meristem region and root tips which was absent with only promoter (Jeong et al., 2009). It was also suggested that there is a translational mechanism which controls ACT2 and ACT8 expression in some tissues (An et al., 1996). All these lines of evidence suggest that ACT2 and ACT8 have some differences in regulation of expression and interactions with proteins/molecule, although they have high similarity in amino acid sequence, are responsible for their differential response.

In summary, these findings clearly identify that prolonged moderate high temperature response is actin isovariant specific. ACT2 and ACT7 are the upstream regulators of this pathway through which the cellular auxin homeostasis, required for proper growth is maintained. Manipulation of the actin isovariants may lead to develop high temperature resistant crops of future.

Chapter 5

General Discussion

High temperature stress is one of the most important abiotic stresses which affect plant growth, development and crop production. Eventually food crisis can occur due to the loss of yield due to this stress. To combat this global issue, we need to understand the response of plants under moderate high temperature stress. As plant root part absorb water and nutrients and soil temperature can affect root easily, I focused on the behavior of root under high temperature. In this thesis work, I have used *Arabidopsis thaliana* as a model system to understand the effect of prolonged moderate high temperature (Chapter 3) and decipher the cellular mechanism (Chapter 4).

This research shows that effects of prolonged moderate high temperature and short high temperature on root elongation are distinguishable. Previously, it was shown that moderate high temperature (36 h) accelerates root elongation which is linked to enhanced shootward auxin transport by efflux transporter PIN2 in Arabidopsis. Conversely, this initial root elongation does not continue under prolonged moderate high temperature (72 h). After first 24 h, root elongation becomes flat for next 48 h. Comparing to WT, root growth of *act2-1* and *act7-4* are more sensitive towards high temperature after day 1. Kinematic analysis revealed that cell division is the primary target by high temperature as cell division is more affected than cell elongation in WT. Short term and long term moderate high temperatures antagonistically influence cell division in WT. Cell division and cell elongation are drastically reduced in absence of ACT2 or ACT7 after prolonged moderate high temperature stress where cell division is more affected (Chapter 4). These findings indicate that cytoskeleton actin is profoundly associated in the root elongation under prolonged moderate high temperature stress in Arabidopsis.

Previously, actin cytoskeleton has been reported to reorganizes actin to transvers cables in hypocotyl and form thick bundles in root epidermal cells of Arabidopsis seedling under high temperature (Fan et al., 2016; Müller et al., 2007). This work shows that actin dynamicity is modified by prolonged moderate high temperature stress such as filament bundling, parallelness and orientation in the WT root (Chapter 3). Actin dynamicity are seriously refashioned in terms of bundling, parallelness and orientation in absence of ACT2/ACT7 comparing to WT (Chapter 4). Among these three parameters, bundling of actin filaments can be corelated with the reduced root elongation in two pathways together such as lack of actin regulation and through generating perturbed auxin homeostasis. A dynamic behavior of actin cytoskeleton has been found at different stages of cell division such as pre-prophase actin band, mitotic spindle actin filament cage and phragmoplast actin arrays in into suspension-cultured tobacco BY-2 cells (Yu et al 2006). Filamentous actin forms a structure called 'twin peaks' which guides cell plate formation at cytokinesis (Sano et al., 2005). YFP-mTn induced actin bundling inhibits cell division and IAA or NAA treatment can de-bundle actin filaments and initiate cell division in tobacco BY-2 cells (Maisch and Nick, 2007). Active actin dynamicity goes on during rapid cell elongation in epidermal and cortical cells in Arabidopsis (Takatsuka et al., 2018). Cell division and cell elongation both are regulated by actin cytoskeleton which is controlled by Cyclase-Associated Protein (CAP) (Barrero et al., 2002). These reports suggest that high temperature induced actin bundling might fail to have the dynamic behavior which is required for cell division and cell elongation where prolonged moderate high temperature mediated alteration in actin dynamicity influences cell division more than cell elongation (Table 1, Table 2). Another possibility is that bundled actin filaments reduces the abundance of PIN1 and PIN2 in WT, *act2-1* and *act7-4* where *act2-1* and *act7-4* are more affected rationally because actin filaments are more bundled compared

with WT. Consequently, auxin transport is blocked and altered auxin gradient has been found in WT, *act2-1* and *act7-4* which is the reason behind reduced root elongation. Polar auxin transport and auxin gradient are required for proper root elongation through maintaining cell division and cell elongation (Vieten et al., 2005; Overvoorde et al., 2010; Grieneisen et al., 2007).

Comparative analysis of actin dynamicity revealed that actin filaments in *act2-1* and *act7-4* are much more affected than WT by prolonged moderate high temperature but the mechanism is not clear (Chapter 4). Previous report showed that high temperature stress increases β CAP protein which stabilizes actin filaments by preventing addition or loss of new actin monomer and *atcp*β mutant shows increased survival rate after heat shock treatment (Wang et al., 2012). Other actin binding proteins, profilins and ADFs are increased in transcriptomes and control filamentous actin assembly under heat stress (Fan et al., 2016). In animal cell, it was found that small heat shock proteins can regulate filamentous actin (Mounier and Arrigo, 2002). These reports indicate that Actin Binding Protein (ABPs) or heat shock proteins might be involved in this process.

In summary, actin cytoskeleton plays vital role in the root elongation while ACT2 and ACT7 are essential to maintain optimal root growth under prolonged moderate high temperature stress.

This study provides primary idea about the actin mediated root growth of plant under prolonged moderate high temperature which could lead a fundamental, mechanistic exploration of how plants respond to moderate temperature for long duration. With this knowledge, crops would be tailored to faithfully match the projected climate change to combat future food crisis.

Acknowledgement

I would like to thank my major supervisor Dr. Abidur Rahman for his guidelines, critical discussion and review of the experimental data and manuscripts.

I am grateful to Dr. Matsuo Uemura of Iwate University and Dr. Wataru Mitsuhashi of Yamagata University for their support and suggestions throughout my research. I really appreciate Dr. Michiko Sasabe of Hirosaki University for her critical assessment of my thesis defense.

I am thankful to Japanese government to support my Ph.D. program through MEXT scholarship.

Finally, I would like to thank all the past and current members of Abidur Lab for their relentless support and discussion to improve my ideas about this project.

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