Journal of Hazardous Materials 432 (2022) 128704

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Research Paper

A long and stressful day: Photoperiod shapes aluminium tolerance in plants☆

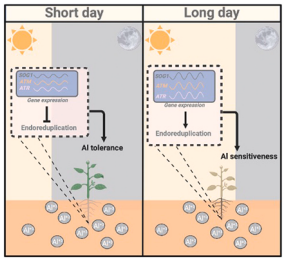
João Antonio Siqueira [a], Thiago Wakin [a], Willian Batista-Silva [a], José Cleydson F. Silva [b], Matheus H. Vicente [c], Jéssica C. Silva [d], Wellington R. Clarindo [d], Agustin Zsögön [a], Lazaro E. P. Peres [c], Lieven De Veylder [e,f], Alisdair R. Fernie [g], Adriano Nunes-Nesi [a], Wagner L. Araújo [a,*]

[a] Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil
[b] National Institute of Science and Technology in Plant-Pest Interactions, Bioagro, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil
[c] Laboratory of Hormonal Control of Plant Development. Departamento de Ciências Biológicas (LCB), Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, SP 13418-900, Brazil
[d] Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil
[e] Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent B-9052, Belgium
[f] VIB Center for Plant Systems Biology, Ghent B-9052, Belgium
[g] Max-Planck-Institute of Molecular Plant Physiology, Potsdam-Golm 14476, Germany

HIGHLIGHTS

- Aluminum (Al) toxicity is likely mitigated under short-day conditions.
- Our results reveal that photoperiod acts as a barrier for Al tolerance in plants.
- Both diel regulation and genetic diversity affect Al tolerance.
- Day-length orchestrates Al tolerance contributing to crop growth and productivity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Guillaume Echevarria

Keywords:
Cell-division
DNA-repair
Day-length
Genetic diversity
Photoperiodism

ABSTRACT

Aluminium (Al), a limiting factor for crop productivity in acidic soils (pH ≤ 5.5), imposes drastic constraints for food safety in developing countries. The major mechanisms that allow plants to cope with Al involve manipulations of organic acids metabolism and DNA-checkpoints. When assumed individually both approaches have been insufficient to overcome Al toxicity. On analysing the centre of origin of most cultivated plants, we hypothesised that day-length seems to be a pivotal agent modulating Al tolerance across distinct plant species. We observed that with increasing distance from the Equator, Al tolerance decreases, suggesting a relationship with the photoperiod. We verified that long-day (LD) species are generally more Al-sensitive than short-day (SD) species, whereas genetic conversion of tomato for SD growth habit boosts Al tolerance. Reduced Al tolerance correlates with DNA-checkpoint activation under LD. Furthermore, DNA-checkpoint-related genes are under

☆ Brief heading: Aluminium is a major constraint for crop yield worldwide. We reveal that photoperiod acts as a barrier for Al tolerance in plants. This could ultimately contribute to improving crop growth and productivity, particularly in many developing countries where the majority of acid soils reside and Al toxicity limits crop production.

* Corresponding author.
E-mail address: wlaraujo@ufv.br (W.L. Araújo).

https://doi.org/10.1016/j.jhazmat.2022.128704
Received 13 January 2022; Received in revised form 6 March 2022; Accepted 12 March 2022
Available online 15 March 2022

positive selection in Arabidopsis accessions from regions with shorter days, suggesting that photoperiod act as a selective barrier for Al tolerance. A diel regulation and genetic diversity affect Al tolerance, suggesting that day-length orchestrates Al tolerance. Altogether, photoperiodic control of Al tolerance might contribute to solving the historical obstacle that imposes barriers for developing countries to reach a sustainable agriculture.

1. Introduction

During the last decades, considerable research efforts have been placed to ensure global food safety and together achieve a sustainable agriculture, which are currently two of the major human necessities. These are central goals of the United Nations (UN) to be achieved over the next 15 years for extinguishing hunger worldwide (United Nations UN, 2015). Acidic soils (pH $\leq$ 5.5) promote the release of aluminum cations, imposing serious constraints on root development in farmland soils around the world. They not only impair nutrient and water uptake (Nunes-Nesi et al., 2014; Kochian et al., 2015) but also hinder food production which is required to meet the demands of the growing global population. Notably, soil acidity is a chronic problem in the temperate zones of eastern North America and throughout Europe (where acidic soils cover up to 80% of the total area). More than 50% of the potentially available arable land in the world is covered by acidic soils (Kochian et al., 2015), which results in significant losses in crop productivity.

Mechanisms that allow plants to cope with Al stress have previously been elucidated and include alterations in organic acid (OA) metabolism, cell wall modifications, vacuolar Al sequestration (de la Fuente-Martínez et al., 1997; Nunes-Nesi et al., 2014; Kochian et al., 2015), and modifications of DNA-repair checkpoints (Eekhout et al., 2017). Classically, the most accepted mechanism to increase Al tolerance describes metal neutralization with OA (e.g. citrate, malate and oxalate) which bind to Al that is neutralized (Nunes-Nesi et al., 2014; Kochian et al., 2015). Notwithstanding, higher levels of OA not always means enhanced Al tolerance, once Al sensitive genotypes produce and secrete large amounts of Al (Piñeros et al., 2005; Guimarães et al., 2014). Additionally, the most recent mechanism discussed to improve Al tolerance is based on genetic manipulations of DNA checkpoints, which enables cell cycle progression in presence of Al (Eekhout et al., 2017). Collectively, DNA checkpoint related proteins are responsible to monitor DNA integrity deciding whether maintain or arrest cell divisions during Al stress. Briefly, during chronic Al toxicity loss of quiescent-center (QC) identity occurs culminating in root death (Eekhout et al., 2017). In the Al-related DNA checkpoint pathway, the transcription factor Suppressor of Gamma Response (SOG1) is phosphorylated by the kinases Ataxia Telangiectasia Mutated and RAD3-related (ATR) and Ataxia Telangiectasia Mutated (ATM) (Eekhout et al., 2017). This phosphorylation enables the induction of DNA replication without the occurrence of mitosis, characterizing endoreduplication, a process that distinguishes Al sensitive from Al tolerant genotypes (Sjögren et al., 2015). Likewise, loss of function mutants for the genes *ATR* and *SOG1* are characterized by more cell divisions and root QC maintenance, which sustain root elongation under Al stress (Rounds and Larsen, 2008). Intriguingly, a multi-level response to Al-induced DNA damage was revealed, wherein loss of function mutants *atm and sog1* despite displaying a higher short-term Al tolerance also have a delay in root growth recovery following Al toxicity (Chen et al., 2019). Both mechanisms Al detoxification and DNA checkpoint are indispensable for plant growth under Al stressful conditions. However, when analysed individually, neither approach has been demonstrated to be sufficient to overcome Al toxicity. For example, Al-sensitive plants exudate large amounts of OA from root cells to the rhizosphere, whereas genetic manipulation of the DNA checkpoint machinery does not mediate plant survival in the presence of high Al concentrations (Piñeros et al., 2005; Chen et al., 2019; Siqueira et al., 2022). These findings reinforce the need to identify factors capable of regulating the overall plant response to Al.

Day-length is a remarkable factor modulating the growth and development of different organisms worldwide. For instance, water pH exhibits a diel fluctuation in Arctic summers with increases over day and declines during the night, and it has been shown that longer photoperiods sustain higher pH in the kelp forests supporting the expansion of Arctic marine vegetation in response to environmental variations (Krause-Jensen et al., 2016). Furthermore, in the soils of the African continent, a large pH (from alkaline to extremely acidic) range are usually found (Fig. S1), and photoperiod is a dominant factor that controls vegetation phenology and growing season (Adole et al., 2019). Indeed, in terms of root development, reduced cell death around root apical meristem (RAM) is observed in Arabidopsis seedlings growing under darkness, whereas shoot exposure to light imposes higher cell death levels in RAM cells (Raya-González et al., 2018). It seems reasonable to assume, therefore, that photoperiod is likely a candidate to modulate root development in responses to Al. Although different maps provide contrasting data for soil pH worldwide (Lin et al., 2012; Bian et al., 2013), by analysing them is consensus that most acidic soils occur around the Equator line. Despite certain exceptions, most Al tolerant species have their origin centre in regions with acidic soils. For instance, species such as rye (*Secale cereale*) from regions with alkaline soils, namely central Turkey and Syria, are characterized by higher natural Al tolerance than species from acidic soils (e.g. oat - *Avena sativa* from Northern Europe (for further details of origin centre see Khoury et al., 2016). Accordingly, an empirical correlation between the low-pH soils and mean annual day-length amplitude was noted, revealing that the centre of origin of cultivated plants exhibiting lower variations in day-length over year, generally summer days with 12 h or less, favour the natural Al tolerance across the plant kingdom (Fig. S1). Likewise, previous studies indicate that many Al-tolerant species are short-day (SD) plants (e.g., *Oryza sativa*, *Stylosanthes humilis*, and *Vigna unguiculata*), while long-day (LD) plants (e.g., *Hordeum vulgar*e, *Pisum sativum*, and *Lens culinaris*) are generally Al-sensitive (Table S1). On analysing the centre of origin of most cultivated plants, we hypothesized that day-length is a pivotal agent modulating Al tolerance across distinct plant species. Our findings revealed that diel regulation and genetic diversity affect Al tolerance, suggesting that day-length orchestrates Al tolerance.

2. Material and Methods

2.1. Plant material and growth conditions

Different plant species were used in this study. Briefly, we used: (i) Arabidopsis wild-type (WT) and mutant plants of DNA repair and cell cycle related genes, overall on the Columbia-0 ecotype (Col-0) background; (ii) tomato (*Solanum lycopersicum*) and loss of function mutants in the *SELF-PRUNING 5 G* (*SP5G*) and *SINGLE FLOWER TRUSS* (*SFT*) as well as near-isogenic lines harbouring the *S. pennellii* allele of *SP5G* ($SP5G^{pen}$), on cultivar Micro-Tom (MT); and (iii) different leguminous species, namely *Stylosanthes humilis*, *Vigna unguiculata*, *Lupinus albus*, *Crotalaria juncea*, *Pisum sativum,* and *Lens culinaris*. Previously to the realisation of our experiments we grew plants under the same conditions to obtain seeds with similar vigour and to avoid photoperiodic impacts in the progenies. Briefly, all species were grown in neutral day (12-h light/12-h dark) conditions, Arabidopsis wild-type and mutants grew at same conditions; tomato genotypes and leguminous species were grown in the same conditions (further details concerning genotypes are provided in Table S2). Briefly, seeds were surface sterilized by agitation in

30% (v/v) commercial bleach (2.7% [w/v] sodium hypochlorite) for 15 min, followed by three rinses with sterile distilled water and kept in darkness to synchronise germination for 4 days. Seedlings of all plant species, except *Arabidopsis thaliana*, were cultivated in hydroponics solution by using Hoagland medium (Hoagland and Arnon, 1950) at pH 4.0, with modifications. Plants were grown in a half-strength solution (pH 4.0) with 100 μM $AlCl_3$ (+Al) and 0 μM (-Al) for 5 days, the solution was exchanged five times and pH adjusted daily to 4.0. Plants were cultivated in a temperature-controlled chamber (20 ± 1 °C for Arabidopsis, and 25 ± 1 °C for other species) under 250 μmol photons m^{-2} s^{-1}, 60% relative humidity, and either under short-days (SD: 8-h light/16-h dark) or long-days (LD; 16-h light/8-h dark). Excluding *A. thaliana*, root systems were photographed and, although root growth was followed overall experimental periods, only the final elongation of the main tap root was measured using the ImageJ software (https://imagej.nih.gov/ij/). Thus, we obtained the ratio between SD and LD to assess differences associated with day-length allowing us to calculate the ratio between control and Al-treated plants. In addition, root systems were harvested 5 days after sowing (DAS) and further used for nutritional analyses.

For in vitro assays, seeds from *A. thaliana* were surface-sterilized and imbibed for 4 days at 4 °C in the dark on 0.8% (w/v) agar plates containing half-strength MS medium (Sigma-Aldrich; pH 4.0), with different $AlCl_3$ concentrations (0, 50, and 100 μM). Next, seedlings were cultivated for 10 days in a growth chamber (POL-EKO APARATURA® Climatic Chamber KK 1200) under SD and LD at 22 ± 1 °C, 60% relative humidity, and 150 μmol photons m^{-2} s^{-1}. In a second experiment, the steps described above were followed and in addition to the treatments mentioned, we used MS medium at pH 4.0 supplemented with zeocin (5 μM zeocin), methyl methanesulfonate (MMS) (50 ppm), or hydroxyurea (HU) (1 mM). The ratio of root elongation was determined in vitro in a similar manner to the hydroponics experiments. To investigate the long-term impacts of Al exposure, we transferred 10-day-old in vitro grown *A. thaliana* seedlings to pots containing washed sand. These plants were cultivated for more two weeks and received 7 mL of Murashige and Skoog (MS medium) (Murashige and Skoog, 1962) solution daily, with Al or +Al (50 μM $AlCl_3$) at pH 4.0.

2.2. Flow cytometry

Roots from *A. thaliana* seedlings were collected, and the root apical meristems (RAM) were excised. From ~30 RAM for each repetition, nuclei were isolated using grinding movements with a pestle in 0.2 mL OTTO-I lysis buffer (Otto, 1990) supplemented with 2.0 mM dithiothreitol (Sigma®), and then made up to 0.8 mL with the same buffer. The nuclei suspensions were then filtered through a 20 μm nylon mesh (Partec®) and centrifuged at 100 *g* × g for 5 min. The obtained pellet was incubated for 10 min in 0.1 mL of OTTO-I lysis buffer and stained with 0.5 mL of OTTO-I:OTTO-II (1:2) solution, supplemented with 75 μM propidium iodide and 2.0 mM dithiothreitol (Sigma®). The nuclei suspensions were incubated for 30 min in the dark at room temperature. Each suspension was analysed using a BD Accuri C6 flow cytometer (Accuri cytometers, Belgium) equipped with a 488 nm laser source. FL2 (585/640) and FL3 (670 LP) filters were used to detect propidium iodide fluorescence. The BD Csampler software (Accuri Cytometers, Belgium) was used for histogram analyses to determine the DNA ploidy level of each G_0/G_1 peak. For this, we considered only the histograms with G_0/G_1 peaks exhibiting a coefficient of variation below 5% for the G_0/G_1 peak and at least 5000 nuclei were counted for each nuclei suspension.

2.3. Nutrient analyses

To quantify the levels of Al, phosphorus, potassium, calcium, magnesium, the dry root tips (~0.5 cm) were subjected to nitric-perchloric digestion (65% and 70%) (Miyazama et al., 1999). The samples were analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 3000XL, Maryland, USA).

2.4. Expression analysis by qRT-PCR

Total RNA was extracted from root samples harvested (immediate snap-freezing in liquid nitrogen) at time points described in the specific figure captions. The RNA was isolated using the TRIzol reagent (Ambion, Life Technology), according to the manufacturer's recommendations. It was then treated with DNase I (RQ1 RNase free DNase I; Promega). RNA integrity was analysed on 1% (w/v) agarose gels by measuring the RNA concentration with a Nanodrop spectrophotometer. Real-time PCR was performed with cDNA using a sequence detection system (Applied Biosystems Applera) using the Power SYBR Green PCR Master Mix according to Piques et al. (2009). The calculations for transcript abundance were performed with standard curves of each selected gene and normalised using the constitutively expressed gene ACTIN (AT2G37620). Data analyses were performed as described previously (Caldana et al., 2007). The primer sequences used are shown in Table S3. Melting curves were checked for unspecific amplification and primer dimerization.

2.5. Genetic structure analysis

We selected 287 *A. thaliana* accessions from the 1001 Genomes Consortium database (https://1001genomes.org/) (Alonso-Blanco et al., 2016) based on variable day-length from 8:11–23:53 h of duration, calculated by http://www.solartopo.com/daylength.htm. Single Polymorphism Nucleotides (SNPs) were identified in genes related to cell cycle and DNA repair by using the POLYMORPH 1001 (https://tools.1001genomes.org/polymorph/). The genetic structure population was analysed using fast structure software (Raj et al., 2014). Unsupervised machine learning approaches were used to analyse the accession groups. The accession data were hierarchically clustered with the Orange Canvas software (http://orange.biolab.si/) using Pearson's correlation distance as the distance measure and the average linkage clustering option. The discriminant analysis of principal components (DAPC) was performed using the 'adegenet' package 1.4–1 (Jombart et al., 2010) in R studio V 2.3.2 (R Development Core Team, 2011) to confirm cluster numbers and to describe the global diversity for overlooking differences between groups.

2.6. Statistical analyses

All experiments were designed in a completely randomised distribution with a minimum of three biological replicates of each treatment. Additionally, the experiments were repeated at least three times (even in different growth facilities) with similar phenotypes observed each time. Data were statistically tested for normality and subsequently examined using ANOVA ($P < 0.05$). Differences in the means ($P < 0.05$) displayed in figures and tables were examined by Student's *t-test*. All statistical analyses were performed using R statistical software (www.r-project.org).

3. Results

3.1. SD favours Al tolerance regardless of flowering pathway

Since we observed that photoperiod may play a role in Al tolerance, it was hypothesized that an endogenous system also modulates Al-responses. Different species of leguminous plants, some that flower under SD conditions (*Stylosanthes humilis*, *Vigna unguiculata*, and *Lupinus albus*) and others that flower under LD (*Crotalaria juncea*, *Pisum sativum*, and *Lens culinaris*), were thus selected and cultivated in the presence of Al. In general, we observed that root elongation in SD species, which are generally Al-tolerant, was insensitive to photoperiod variations, showing similar root elongation rates under both SD and LD conditions,

regardless of the presence of Al. Meanwhile, LD plants, which are usually Al-sensitive, displayed higher root growth and Al sensitivity under LD than under SD conditions, revealing that these plants are indeed more sensitive to Al (Fig. 1A). It was further investigated whether fluctuations in day length altered the mineral-nutrient concentration. Our results revealed that photoperiod influenced Al uptake as revealed by higher Al levels in plants cultivated under SD conditions compared to those cultivated under LD (Figs. S2–3). Intriguingly, even though SD led to a higher Al-tolerance, higher levels of Al were observed in plants growing on SD than under LD, while the levels of other nutrients were less affected by day-length (Fig. S2).

Tomato (*Solanum lycopersicum*) is a day-neutral species, whereas its wild relative S. pennellii is an SD species (Soyk et al., 2017). The photoperiod-neutrality of *S. lycopersicum* is caused by the loss of function in *SELF-PRUNING 5G* (*SP5G*) (Fig. 1B), which is a flowering repressor that acts on the florigen paralog *SINGLE FLOWER TRUSS* (*SFT*) (Soyk et al., 2017). We analysed near-isogenic lines harbouring the *S. pennellii* allele of *SP5G* ($SP5G^{pen}$) or a loss-of-function mutation in *SFT* (*sft*) in tomato cv. 'Micro-Tom' (MT). Based on shoot apical meristem (SAM) analyses, we observed that $SP5G^{pen}$ modified *S. lycopersicum* into an SD plant, whereas sft modified it into a photoperiod-insensitive variety, which is not able to flower either under SD or LD conditions (Fig. S4). Additionally, $SP5G^{pen}$ plants exhibited a higher Al tolerance, as revealed by similar root growth under both SD and LD conditions and in the presence of Al, a response that was not observed in MT and *sft* plants (Fig. 1C, D, S5–11), distinguishing photoperiodic responses from the flowering pathway. Thus, Al tolerance in tomato seemed to depend on its ability of roots to respond to photoperiod, and not of flowering dependent on day-length.

3.2. Long-term Al tolerance occurs under SD

To further explore the molecular connections between photoperiodic response and Al toxicity, we cultivated Arabidopsis thaliana (L.) Heynh. ecotype Columbia 0 wild-type (WT) under SD (8 h light/16 h dark), neutral days (ND - 12 h light/12 h dark) and LD (16 h light/8 h dark), in the presence and absence of Al. Al-induced root growth inhibition was photoperiod-dependent, as neither SD nor ND reduced root growth in

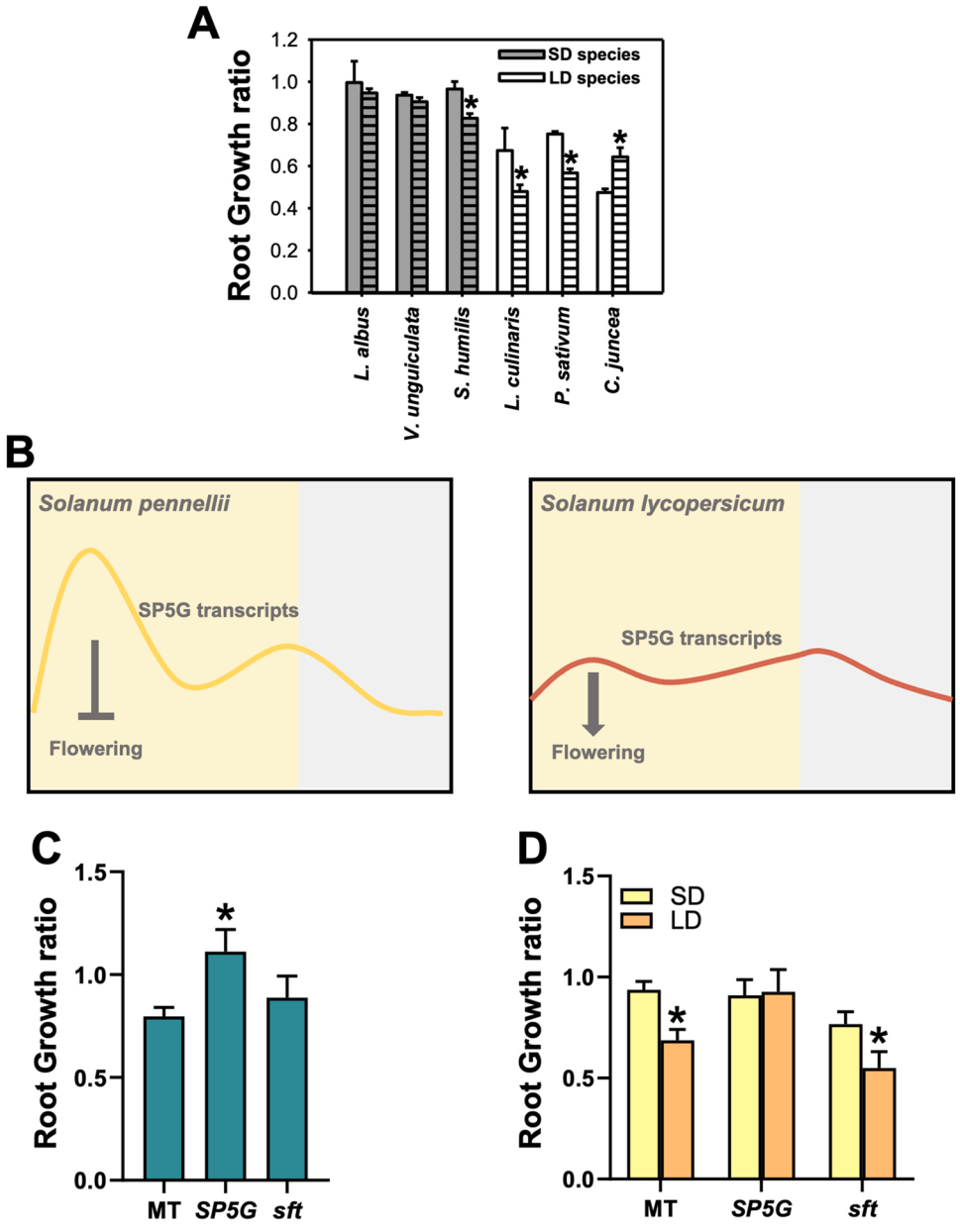


Fig. 1. Photoperiod responses modulate root elongation and aluminium (Al) tolerance. (A) Root growth ratio indicates the final root elongation measured in plants growing in the absence of Al^{3+} or the presence of 100 μM Al^{3+} under either SD (clear bar) or LD (hatches bar) conditions. Growth ratio was calculated as follow: SD (-Al) root elongation (cm) / SD (+Al) root elongation (cm) (clear bars); and LD (-Al) root elongation (cm) / LD (+Al) root elongation (cm) (hatches bar). An asterisk (*) indicates different values that were determined by the two-sided Student's *t*-test to be different ($P < 0.05$) between SD and LD. SD species: *Lupinus albus*, *Vigna unguiculata* and *Stylosanthes humilis*; LD species: *Lens culinaris*, *Pisum sativum* and *Crotalaria juncea*. (B) In the wild tomato *Solanum pennellii,* oscillations on the expression of *SELF-PRUNING 5 G* transcripts are observed arresting flowering under LD conditions, which does not occur in the domesticated tomato *Solanum lycopersicum*. (C) Root growth ratio was assessed by the final root elongation determined in plants growing at SD or LD under optimal control conditions. Root elongation was determined in 5-day-old seedlings cultivated on hydroponics culture (n = 12 plants for each condition), and the growth ratio was calculated as followed: SD root elongation (cm) / LD root elongation (cm). Asterisks (*) indicate different mean values that were determined by the two-sided Student's *t*-test to be different ($P < 0.05$) from the wild-type tomato Micro-Tom (MT). (D) Root growth ratio indicates the final root elongation measured in plants growing in the absence of Al^{3+} or the presence of 100 μM Al^{3+} under either SD or LD. This ratio was calculated as follow: SD (-Al) root elongation (cm) / SD (+Al) root elongation (cm) (yellow bars); and LD (-Al) root elongation (cm) / LD (+Al) root elongation (cm) (orange bars). An asterisk (*) indicate values that were determined by the two-sided Student's *t*-test to be different ($P< 0.05$) between growth conditions (SD and LD). Abbreviations: MT, *Solanum lycopersicum* cv. Micro-Tom; *SP5G*, *SELF-PRUNING 5 G* allele from *Solanum pennellii* introgressed into MT; *SFT*, introgression of a loss-of-function mutation on *SINGLE FLOWER TRUSS*.

the presence of Al. Whereas, 5 days after germination (DAG) plants growing under LD experienced the sensitivity to Al-toxicity, wherein root elongation began to be reduced (Fig. S12). It was further investigated whether day-length mitigated reduction in root elongation following either pH change or differential Al concentrations. SD did not ameliorate the reduction in root elongation evoked at lower pH (4.0) compared to the optimal pH (5.7) (Fig. 2A). SD-grown plants were also able to tolerate higher Al levels, showing lower reductions in root elongation than LD plants following increased Al levels (Fig. 2B). Consequently, a multi-level Al response was reported for *A. thaliana*. In addition, following long-term Al exposure, loss-of-function mutants for major DNA-checkpoint regulator genes were generally Al-tolerant, but showed a slower growth recovery after a short-term stress imposed by Al (Chen et al., 2019). Given this, we further investigated if SD would mitigate plant growth losses resulting from long-term Al exposure. No difference in rosette growth or root elongation was observed in plants growing under SD regardless of Al exposure (Fig. 2C), indicating a likely permanent Al tolerance under SD.

3.3. Photoperiod specifically mitigates Al toxicity

Cell cycle arrest and DNA damage resulting from Al toxicity are two major cellular alterations affecting plant growth. Al stress culminates in cell cycle blockage and ultimately alters root DNA endoploidy that induces differentiation of root apical meristematic (RAM) cells, a process known as meristem exhaustion (Nezames et al., 2012; Sjögren et al., 2015). Spatiotemporal control of DNA endoploidy was demonstrated across root tissues, indicating an elevated endoploidy in roots coping with low pH (4.6) (Bhosale et al., 2018). Thus, we isolated RAMs to assess the DNA ploidy levels in response to Al stress, and monitored cell divisions in the root meristems of young seedlings using flow cytometry. Our results revealed the maintenance of potential proliferative capacity (2–32 C cells) in roots exposed to Al-toxicity under SD, but not under LD conditions. It was also observed that SD suppressed the appearance of polyploid cells (4 C, 8 C, 16 C, and 32 C) on the RAM (Fig. 3A), indicating that Al tolerance under SD is likely associated with the down-regulation of endoreduplication. Endoreduplication promotes an increase in the DNA ploidy level in several cell types due to changes in cell cycle control. It only occurs in metabolically active and highly specialised cells and allows DNA replication in the absence of mitosis, which increases the levels of DNA endoploidy and regulates root-cell fates (De Veylder et al., 2011). Our results revealed that under SD, there was a strong reduction in the endoreduplication index in the presence of Al, but it was not so under LD (Fig. 3B). We next turned our attention to identify whether SD promoted reductions in endoreduplication, specifically triggered by Al toxicity, or whether it was a general mechanism of roots under genotoxic conditions. Previous studies have demonstrated that endoreduplication in quiescent-center (QC) cells of the RAM disrupt cell-cycle progression, which might be attributed to the effects of Al toxicity or drugs such as hydroxyurea (HU), methyl methanesulfonate (MMS), and zeocin, leading to a reduced root elongation phenotype (Sjögren et al., 2015; Adachi et al., 2011; Takahashi et al., 2019). Remarkably, the terminal impact of the aforementioned compounds is reducing the cell proliferation potential of roots. Thus, day-length did not mitigate root elongation limitations triggered by HU, MMS, or zeocin (Fig. 3 C), supporting our notion that photoperiod acted specifically in endoreduplication resulting from Al toxicity.

3.4. LD are required to arrest cell divisions under Al stress

Endoreduplication is an alternative pathway that avoids cell cycle arrest or cell death due to DNA damage (Endo et al., 2012). Thus, we investigated how SD promotes reductions in endoreduplication during Al toxicity. In response to Al, endoreduplication is mainly modulated by

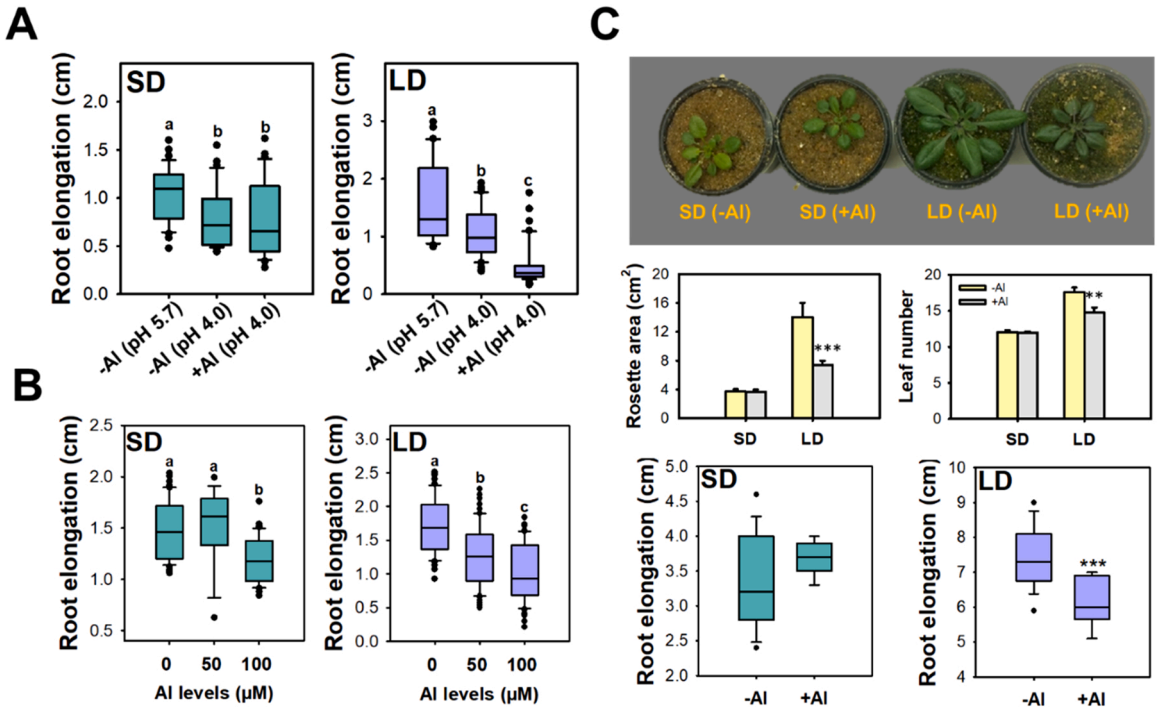


Fig. 2. Higher severity of aluminium (Al)-toxicity under long-days (LD) in *Arabidopsis thaliana*. (A) Root elongation was determined in seedlings growing at pH 5.7 (control) or 4.0 after 10 days, the seedlings were either cultivated with 0 (-Al) or 50 (+Al) μM $AlCl_3$ (n = 60 from three independent experiments). (B) Root elongation was determined in seedlings growing at differential levels of $AlCl_3$ for 10 days (n = 60 from three independent experiments). Statistical groups were determined using a Tukey honest significant difference (HSD) test ($P < 0.05$) and are indicated with letters. (C) Phenotype for Arabidopsis plants under Al treatment. Representative images of 3-week-old, short-day (SD) or long-day (LD) grown plants cultivated on the sand in the absence [–Al (0 μM)] or the presence of Al [+Al (50 μM)]. Measurements from rosette area (cm^2), leaf number and root elongation for *A. thaliana* plants cultivated on sand (n = 30). Asterisks indicate values that were determined by Student's *t*-test to be different at $P < 0.01$ (**) or $P < 0.001$ (***) between Al levels.

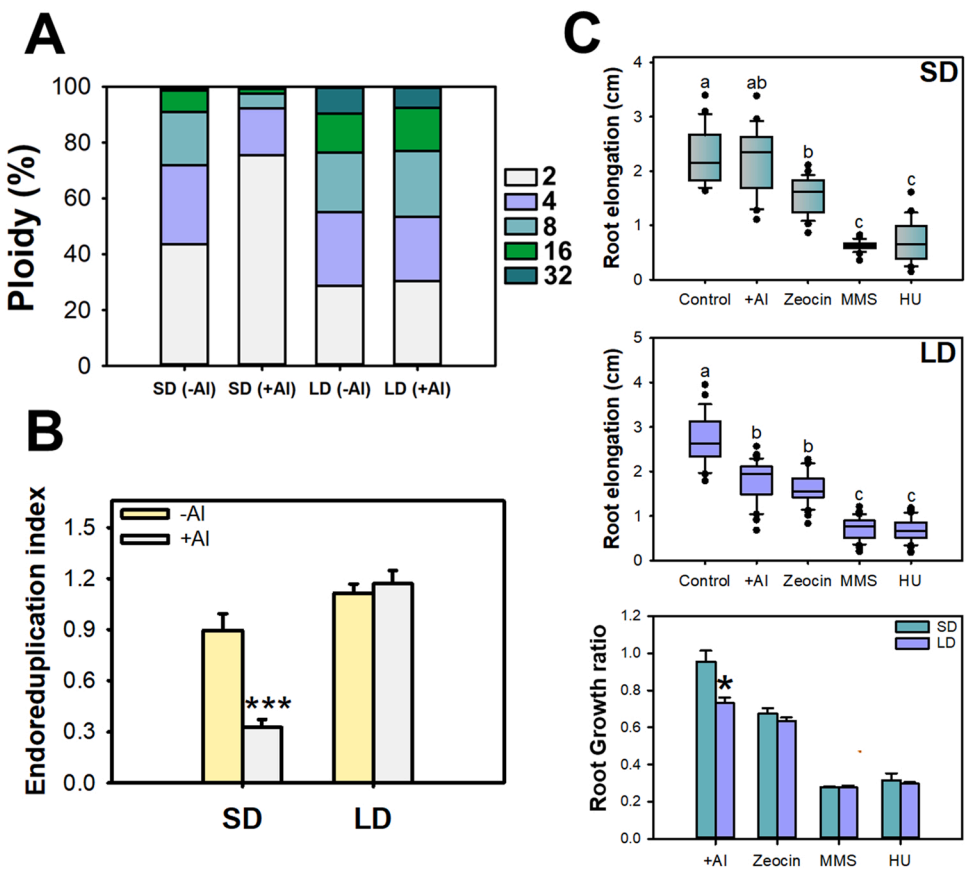


Fig. 3. Differential DNA ploidy level and cell cycle regulation modulated by day-length on *Arabidopsis thaliana* seedlings growing under aluminum (Al) stress. DNA ploidy level (%) (A) and endoreduplication index (B) from root apical meristem (RAM) cells of 10-day-old, short-day (SD)- or long-day (LD) grown seedlings cultivated on –Al (0 μM) or +Al (50 μM). Data represent measurements for five replicates obtained with cells of approximately 30 RAM. (C) Seedlings (10-day-old) were submitted to +Al (50 μM), zeocin (5 μM zeocin), methyl methanesulfonate (MMS) (50 ppm) or hydroxyurea (HU) (1 mM), and root elongation (cm) were determined (n = 60 from three independent experiments). Root growth ratio indicates root elongation in plants growing under cytotoxic and genotoxic treatments either under SD or LD. Root growth ratio was calculated as followed: SD (-Al) root elongation (cm) / SD (treatment) root elongation (cm) (green bars); and LD (-Al) root elongation (cm) / LD (treatment) root elongation (cm) (blue bars). Asterisks indicate values that were determined by Student's *t*-test to be different at $P < 0.05$ (*) or $P < 0.001$ (***) between Al levels. Statistical groups were determined using a Tukey honest significant difference (HSD) test (*P* value <0.05) and are indicated with different letters.

the transcription factor *SUPPRESSOR OF GAMMA RESPONSE1* (*SOG1*), which is activated by the kinases *ATAXIA TELANGIECTASIA MUTATED* (*ATM*) and *ATAXIA TELANGIECTASIA AND RAD3-RELATED* (*ATR*), enhancing the expression of genes associated with cell cycle stoppage and DNA repair (Sjögren et al., 2015). *A. thaliana* loss-of-function mutants for *SOG1* and *ATR* displayed higher Al tolerance due to the inhibition of early endoreduplication onset (Sjögren et al., 2015). In silico analyses further revealed that most of the genes involved in DNA repair and endoreduplication regulation exhibited a significant correlation with latitude/day-length in 32 *A. thaliana* accessions (Fig. S13). In

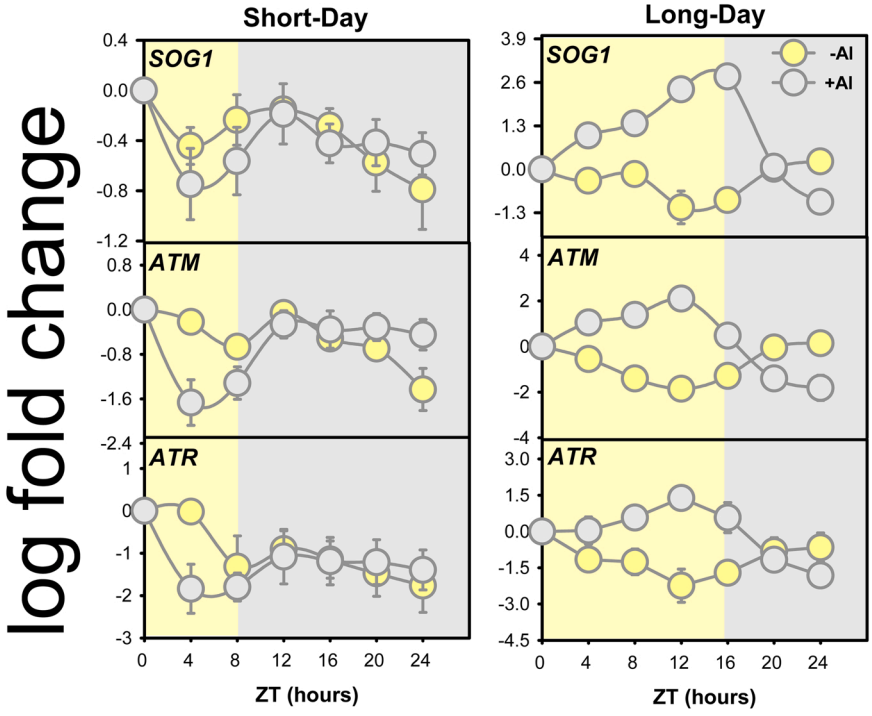


Fig. 4. Expression profile of regulators of DNA checkpoints is variable in response to photoperiod and Al. Diurnal oscillations of transcript levels from *SOG1* (*SUPPRESSOR OF GAMMA RESPONSE1*), *ATM* (*ATAXIA TELANGIECTASIA MUTATED)* and *ATR* (*ATAXIA TELANGIECTASIA AND RAD3 RELATED*) were determined in roots of 3-week-old plants of *Arabidopsis thaliana* ecotype Columbia (Col-0) cultivated at –Al (0 μM) or +Al (50 μM) under short-days (SD) and long-days (LD). Light and dark rectangles denote day and night periods in SD (8 h light/16 h dark) and LD (16 h light/8 h dark), respectively. Data represent the average expression of three biological replicates ± SE.

agreement with our in silico analyses (Fig. S14), qRT-PCR analysis of the major DNA-repair regulators (*SOG1*, *ATM*, and *ATR*) revealed a more coordinated gene expression under SD than under LD conditions (Fig. 4). Briefly, *SOG1*, *ATM*, and *ATM* expression increased in a light-dependent manner only during LD, reaching maximum expression peaks at points near dusk (Fig. 4). Based on the contribution of both *SOG1* and *ATR* checkpoint regulators on the Al-toxicity response, it was hypothesised that their diel regulation might have contributed to the observed photoperiodic Al-tolerance. We confirmed this by growing *A. thaliana* mutants defective in DNA checkpoint control, *sog1–1*, *atm-1*, and *atr-2*. As expected, *sog1–1*, *atm-1*, and *atr-2* showed an invariant Al tolerance following day-length variations, with similar root growth responses following SD and LD (Fig. S15). Collectively, SD seemed to support mitosis maintenance by repressing excessive DNA replication that would culminate in higher endoreduplication.

3.5. Circadian clock and genetic diversity impose constraints for Al-tolerance

Day-length is a more predictable, unperturbed, and non-oscillating factor than temperature, air humidity, and rainfall. Thus, A. thaliana accessions from high-latitude environments usually experience long photoperiods around the summer solstice, exhibiting longer circadian periods (Salmela and Weinig, 2019). Casein kinase 2 (CK2)

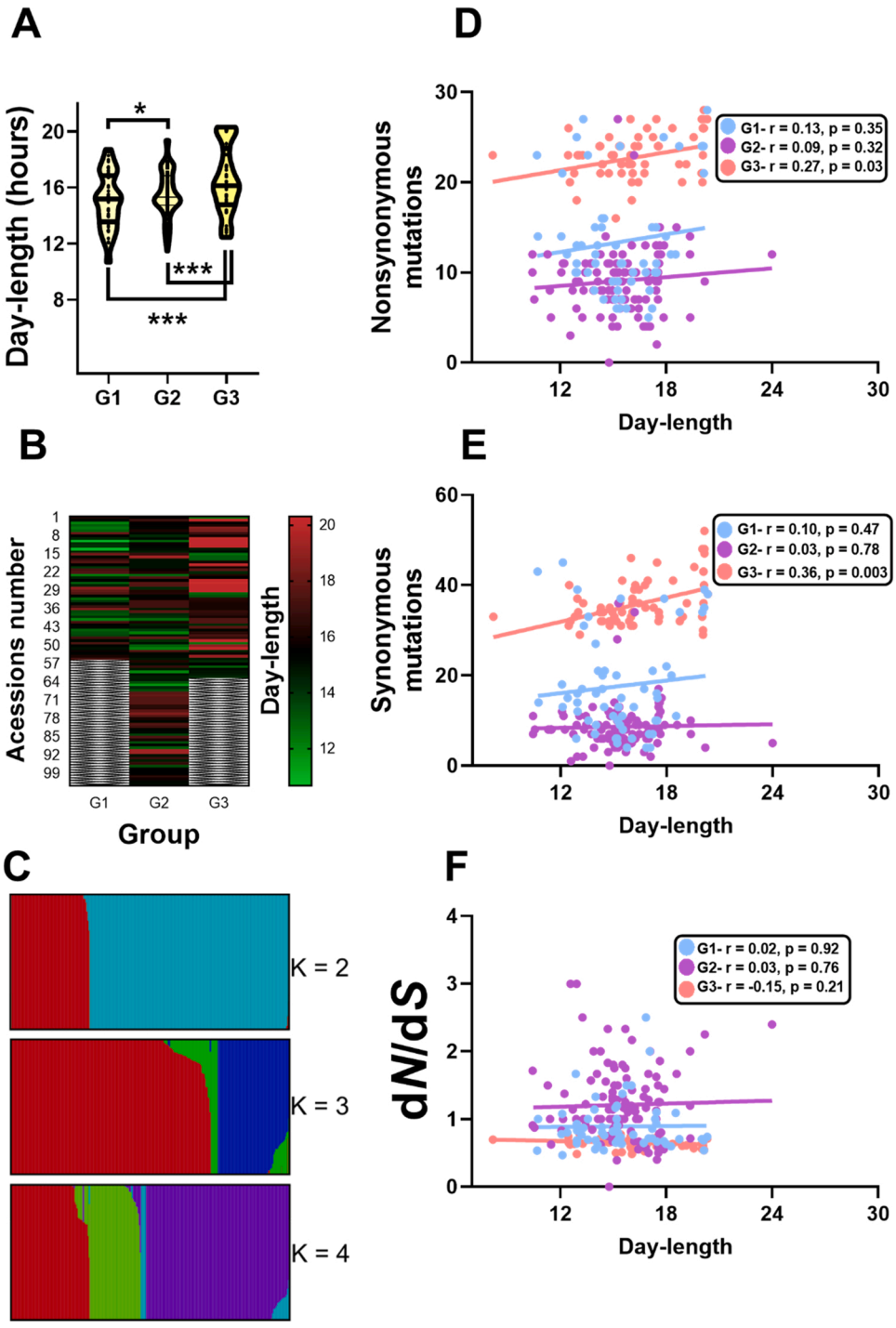


Fig. 5. Photoperiods discriminate *Arabidopsis thaliana* accessions according to genetic variants of genes related to cell cycle and DNA checkpoints. 287 *A. thaliana* accessions from the centre of origin varying in day-length from a duration of 8:11–23:53 were selected based on previous studies. (A) Violin-plots representing group analyses were clustered by the Orange Canvas software and discriminant analysis of principal components, in which three major accession groups G1 (n = 59), G2 (n = 112) and G3 (n = 63) based on distinct day duration were formed. Remarkably, some accessions were not grouped into these three groups. Asterisks indicate values that were determined by Student's *t*-test to be different at $P < 0.05$ (*) or $P < 0.001$ (****) between groups. (B) Heat-map highlighting the differential composition of day-length on groups. (C) Population structure of *A. thaliana* accessions in which each vertical bar represents an individual accession with single nucleotide polymorphism (SNP) on genes related to cell cycle and DNA checkpoints. K clusters indicate colours for fractional memberships. Further details concerning genes and accessions used in the analyses can be found in the supplementary material. (D-F) Accumulation of non-synonymous and synonymous mutations as well as the ratio of non-synonymous to synonymous fractions (*dN/dS*) and its correlations with day-length. By using the POLYMORPH 1001 (https://tools.1001genomes.org/polymorph/), polymorphism was detected on genes *ATAXIA TELANGIECTASIA MUTATED* (*ATM*), *ATAXIA TELANGIECTASIA AND RAD3 RELATED* (*ATR*), and *SUPPRESSOR OF GAMMA RESPONSE1* (*SOG1*) among groups G1, G2, and G3.

phosphorylates both circadian clock proteins related to the light cycle (Lu et al., 2011) and SOG1 protein on amino acid T423 (Wei et al., 2021). Correspondingly, the mutation of T423 into its phosphomutant A (T423A) mimics the Al-resistant phenotype of *A. thaliana sog1–101* mutant plants (Wei et al., 2021). Interestingly, *sog1–101* and *T423A-27* mutants displayed root elongation insensitive to day-length as well as higher Al-tolerance than WT plants, regardless of the photoperiod (Fig. S16). Therefore, we postulate that genetic diversity could occur in genes related to DNA and cell cycle in *A. thaliana*, which would be selected in response to day-length and light components of the circadian clock machinery. In keeping with this assumption, we analyzed two independent studies (Satbhai et al., 2017; Ristova et al., 2018) to verify whether latitudinal/day-length variation affected root elongation in A. thaliana accessions. Interestingly, we observed a positive and significant correlation between latitude/day-length and root length, revealing that accessions from higher latitudes displayed greater root length (Fig. S17). Regarding root development, only with light incidence on shoots, cell death was triggered around the RAM cells of *A. thaliana* seedlings (Raya-González et al., 2018). Furthermore, to identify the existence of genetic diversity for genes investigated here as members of the interface between Al-tolerance and day-length, we selected 287 *A. thaliana* accessions with the centre of origin regions widely varying in photoperiod. By approaches of grouping, we found three distinct groups (G1, G2, and G3) based on day-length in the region origin, in which G1 exhibited more accessions with photoperiod around 12–14 h while G3 had most accessions from regions with day-length of around 20 h (Fig. 5A-B). Assessing the relatedness of *A. thaliana* accessions using STRUCTURE, we were also able to discriminate individuals from three populations (Fig. 5 C), revealing an essential absence of a mixture of accessions due to photoperiod-barriers. Therefore, the higher fitness of *A. thaliana* accessions from low-latitudes under drought conditions (Exposito-Alonso et al., 2019) and more coordinated oscillatory behaviour in drosophilids (Bertolini et al., 2019) might be due to the lower photoperiod variability across the year. Likewise, the mapping of polymorphisms in genes involved in DNA repair, cell cycle, and endoreduplication revealed the existence of a characteristic genetic diversity for genes involved in Al tolerance in *A. thaliana*, which varied with day-length (Fig. S18). Furthermore, we found more non-synonymous and synonymous mutations in *ATM*, *ATR*, and *SOG1* genes in accessions derived from longer day regions (G3), the unique group in which these mutations were correlated with day-length (Fig. 5D-E and S19). In agreement with these findings, the global distribution of population genetic diversity across the animal and plant kingdoms reveal that only eudicots exhibit a significant correlation between population genetic diversity and latitude (De Kort et al., 2021). Notably, genetic diversity increases with distance to the equator within eudicots (De Kort et al., 2021). Strikingly, accessions from lower day regions (G1 and G2) were under positive selection ($dN/dS \geq 1$), meaning that the selective patterns are directly related to gene expression (Fig. 5F, S14, and S19). Therefore, photoperiod seems to have been imposing selection patterns in genes that are known to improve Al tolerance. Collectively, our results suggested that photoperiod is likely a factor that mediates plant Al tolerance.

4. Discussion

4.1. Day-length disrupts root growth enhancing Al sensitivity

Photoperiod has been extensively suggested as a pivotal factor modulating growth, development, and metabolic responses (Schaffer et al., 1998; Valverde et al., 2004; Lagercrantz, 2009; Gibon et al., 2009; Andrés and Coupland, 2012; Sulpice et al., 2014; Nunes-Nesi et al., 2016). This fact aside, the significance of photoperiod in overall stress responses has been suggested to be day-length dependent. In fact, the burst of oxidative stress responses in *A. thaliana* is dependent on the light period as evidenced by the higher efficiency to cope with reactive oxygen species (ROS) of plants growing under SD than LD (Queval et al., 2007, 2012; Abuelsoud et al., 2020). It is important to highlight that higher ROS production not necessarily will result in growth arrest since the ability of plants to cope with these molecules is highly variable across genotypes, and ROS scavenging mechanisms can clean the cells in specific regions mitigating the impact of these compounds. In addition, the significance of ROS during plant development has been demonstrated elsewhere (Yamamoto et al., 2003; Farnese et al., 2016). Notwithstanding, this knowledge is seemingly not well explored for abiotic stress responses since this apparent notion remained restricted for impacts of day-length on plant development. Here, by performing a multi-specie study, we explored the photoperiod relevance for Al tolerance. Our results suggested a crucial yet unreported significance of photoperiod and endogenous cues, which appear to play a critical role for photoperiod in shaping Al tolerance across the plant kingdom. Our data obtained mainly with seedlings that have only cotyledons, further indicated that SD higher Al-tolerance is mostly observed in SD-grown plants. In mature *A. thaliana* rosettes, net daily carbon gain is relatively similar for plants growing under 12 h and 18 h of photoperiod, whereas 4, 6, and 8 h of photoperiod reduce linearly carbon gain (Sulpice et al., 2014). This fact aside, cell proliferation depends on mature photosynthetic apparatus, which maximizes growth rates in plants with organs in late developmental stages (Sulpice et al., 2014; Siqueira et al., 2018). Notably, most of our assays were performed in plants harbouring only cotyledons wherein most of the carbon was provided almost exclusively from remobilisation of seed resources, reducing the potential impacts of differential carbon gain under SD and LD conditions. Intriguingly, plants growing under SD could uptake higher Al levels than under LD, but with less physiological impact. Higher Al content in the roots of plants growing under SD indicates an internal immobilisation of Al in the apoplast. Further work is clearly required to assess the significance of biochemical changes in photosynthesis and respiration, which are the major processes that produce and consume photoassimilates. Reprogramming of the mitochondrial OA metabolism may mediate Al tolerance in both microorganisms and plants (de la Fuente et al., 1997; Nunes-Nesi et al., 2014; Kochian et al., 2015). Although phosphorus (P) uptake was previously described to be dependent on red-light signalling on *A. thaliana* accessions (Sakuraba et al., 2018), here we have not found variations for P uptake between SD and LD conditions, which further suggest day-length as a specific factor modulating Al tolerance.

4.2. Long-term Al tolerance occurs under SD

Environmental stresses impact diverse aspects of plant development, arresting a wide range of traits associated with crop yield. Accordingly, Al toxicity have implications on metabolism and RAM meristem, as depicted by the Al sensitive genotypes that exhibit enhancement in responses classically described to improve Al tolerance, yet they are extremely sensitive to Al toxicity (Piñeros et al., 2005; Guimarães et al., 2014; Chen et al., 2019). Our results revealed that SD boosts root growth under Al stress, wherein this was not observed for variations in pH in absence of Al, once no growth mitigation was found comparing root elongation at low (4.0) and optimal pH (5.7). Increasing Al doses were less impacting for SD-grown *A. thaliana*, while these doses impacted more LD-grown plants during short- and long-term Al toxicity. Additionally, root growth is substantially elevated following day-length extension in *A. thaliana*, wherein at late night root elongation reach the maximum rates (Yazdanbakhsh et al., 2011). Root growth may be inhibited during light periods whereas at dark periods, growth may be recovered (Ruts et al., 2012). In consonance, Al toxicity is manifested differentially over time, since the same genotype may be tolerant to short-term Al exposure and sensitive to Al long-term exposure (Chen et al., 2019), which apparently occur due to the accumulation of light cycles that likely favour Al sensitiveness.

4.3. SD repress endoreduplication specifically triggered by Al toxicity

Cell divisions likely disarm replicative defences turning the genome more susceptible to replication errors and damages, a fact that is enhanced under Al toxicity that in turn activates DNA checkpoints to halt cell division progression (Hu et al., 2016). Usually, plant roots coping with Al toxicity experience the formation of a greater number of DNA polyploidy cells, reducing RAM cells competence for division (Nezames et al., 2012; Sjögren et al., 2015). Our monitoring of DNA ploidy at root meristems of *A. thaliana* seedlings revealed that SD boosts proliferative ability by arresting the emergence of polyploidy cells leading ultimately to higher Al tolerance. Natural populations of *Arabidopsis arenosa* are composed of diploids and tetraploids individuals widespread from Germany to Sweden (Monnahan et al., 2019), whereas the elevation on latitude/day-length seems to favour the natural occurrence of tetraploids. Accordingly, shifts in cell volume impact directly the final function of cells, in which RAM cells growing under LD conditions are characterized by reductions in proliferation rates triggered by low pH (4.0) (Pacifici et al., 2018). Although this pH range has a slight impact on RAM activity (Gujas et al., 2012), it generated a drastic rise in cell size at the meristematic zone indicating an altered cell differentiation programming (Pacifici et al., 2018). Notably, low pH induces a higher endoreduplication index on roots (Bhosale et al., 2018), and it seems to match with the higher cell size responsible for tailoring root developmental programs. Likewise, zeocin sensitivity directly implicates cell area enlargement with distancing from QC, which seems to induce endoreduplication further reducing root length (Adachi et al., 2011). Endoreduplication induced in response to genotoxic stress is assumed to be programmed (Adachi et al., 2011), wherein low pH seemingly disarm the natural programming. Thus, inducing QC cell proliferation the sensitiveness of QC cells to DNA damage is also elevated, resulting in impaired root growth after genotoxic stress (Cruz-Ramírez et al., 2013). We observed that plants with enhanced root elongation rates under LD are Al sensitive, indicating that root growth induction seems to turn those plants more sensitive to genotoxic events, emerging light relevance for stress responses modulation.

4.4. Diurnal gene expression and genetic diversity behind Al tolerance

The life of autotrophic organisms is most likely synchronized with light/dark cycles, supporting the notion of photoperiodism and development. A kingdom-wide evolutionary analysis of diurnal gene expression on photosynthetic organisms revealed that more than one-third of the genome is diurnally regulated (Ferrari et al., 2019). Despite the conserved diurnal regulation, precise gene expression peaks are likely able to orientate organism biology, and as such cell division and photoperiodism emerge as a divergent process across organisms (Ferrari et al., 2019). The momentary root growth rate displays a robust diel oscillation, wherein the minimum growth is reached at 8–10 h after dawn (Yazdanbakhsh et al., 2011). Interestingly, this is the exact period that we noted major peaks expression for genes *SOG1*, *ATM* and *ATR*. Thus, the extension of the light period subsequently to these 8–10 h upon dawn seems to be mandatory to arrest root elongation following endoreduplication induction under Al toxicity. Supporting this motion, it has been previously shown that SOG1 was not found among the targets of the kinases ATM and ATR (Roitinger et al., 2015). Accordingly, our results for the photoperiodic expression response of these genes may explain, at least partially, this previous controversial result. Mutations at *SOG1*, *ATM* and *ATR* culminate on an Al tolerance that is insensitive to day-length in Arabidopsis. Photoperiod is an extremely predictable factor and yet it is seemingly capable of modulating a differential Al tolerance across species. Taken together with our results it seems reasonable to indicate a potential role for photoperiodism in modulating genetic diversity on genes related to Al responses.

DNA checkpoint regulators were demonstrated to be under periodicity and oscillating widely across diverse animals affecting fatally DNA repair efficiency and cell divisions (Stewart-Ornstein and Lahav, 2017; Stewart-Ornstein et al., 2017). Plants are usually exposed to most distinct environmental conditions coping with shorter and longer days, wherein diurnal regulation of genetic features is rather contrasting on *A. thaliana* from low and high latitudes (Salmela and Weinig, 2019). Investigating 191 Swedish *A. thaliana* accessions it was revealed that the circadian period is dependent on both geographical localization and population sub-structure (Rees et al., 2021). Furthermore, a single non-synonymous polymorphism affects drastically the circadian period and flowering, turning both longer and generating selective pressure gradients across Sweden (Rees et al., 2021). Our genetic diversity mapping also suggested that (i) the lower competition among plant species at higher latitudes may enable the occurrence of species adapted to stressful conditions, and (ii) with increasing distance from the Equator, stressful environmental conditions may alter patterns of energy allocation from vegetative growth to reproduction, increasing the genetic diversity in populations from these regions (De Kort et al., 2021; LaManna et al., 2017). Day-length specifically influences cell cycle arrest and endoreduplication related to Al, modulating gene expression in a diel manner. Notwithstanding, it remains to be investigated whether specific soil types or day length would be the most relevant association governing Al tolerance evolution. Furthermore, since genetic variability within the sequence for these genes was altered according to photoperiod, it seems reasonable to question whether day-length regulates only Al responses, or it could also coordinate tolerance to other abiotic stresses. In addition, our results offer novel perspectives for understanding the underlying mechanisms of Al-tolerance in land plants. For example, under field conditions where plants are recurrently exposed to fluctuating conditions, Al-tolerance mechanisms involving different levels of complexity can play a pivotal role in helping plants to successfully cope with high levels of Al. Additional studies, which employ more sophisticated multi-disciplinary analyses, will likely be of fundamental importance in providing a holistic understanding of the underlying mechanisms that affect the evolution of Al-tolerance. Therefore, our findings suggest a wide yet unrecognised significance of day-length in orchestrating Al tolerance, paving the way to the development of the next generation of productive crops under higher Al conditions.

CRediT authorship contribution statement

J.A.S., and W.L.A. designed the research; J.A.S. performed most of the research with the support of T.W. and W.B-S.; J.C., and W.C. performed cytometry flow analyses; J.A.S., and J.C.F.S. realized bioinformatics analyses; W.B-S., J.C., W.C., L.D.V., A.R.F., and A.N-N. contributed new reagents/analytic tools; J.A.S., L.D.V., A.N-N., and W.L.A. analysed the data; and J.A.S., and W.L.A. wrote the article with input from all the others.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wagner Araujo reports financial support was provided by Serrapilheira Institute. Wagner Araujo reports a relationship with Serrapilheira Institute that includes: funding grants.

Acknowledgements

We thank Dimas Mendes Ribeiro and Cleberson Ribeiro for helpful comments and sound advice on an earlier draft of this manuscript. This work was made possible through financial support from the Serrapilheira Institute (grant Serra-1812-27067) (W.L.A.). We also thank the scholarships granted by the Coordination for the Improvement of Higher-Level Personnel (CAPES-Brazil) (W.B-S., and J.C.S.), the Foundation for Research Assistance of the Minas Gerais State (FAPEMIG-Brazil, Grant CRA-RED-00053-16) (A.N-N., and W.L.A.), and the

Foundation for Research Assistance of the São Paulo State (FAPESP-Brazil, Grant 2016/05566-0) (M.H.V., and LEPP). Research fellowships granted by National Council for Scientific and Technological Development (CNPq, Brazil) (A.Z., A.N-N., and W.L.A.) are also gratefully acknowledged.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128704.

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