Journal of Plant Development
Vol. **31**, Dec **2024**: 229-233
DOI: 10.47743/jpd.2024.31.1.956
Research Article

ISSN 2065-3158 print / e-ISSN 2066-9917
Available online: www.plant-journal.uaic.ro

ARABIDOPSIS ATMKK1 KNOCKOUT MUTANT FLOWERS UNDER SHORT DAY CONDITIONS

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Abstract: There are a variety of conditions that regulate flowering time in *Arabidopsis*, but there are no reported instances mitogen-activated protein kinase pathways playing a decisive role in flowering time. Our work has indicated that when long-day plant *Arabidopsis* mitogen-activated protein kinase kinase 1 (*AtMKK1*) was knocked out, *Arabidopsis* plants flowered under short day conditions. Possible mechanisms are discussed.

Keywords: flowering time, mitogen-activated protein kinase, photoperiod.

Introduction

Mitogen-activated protein kinase (MAPK) pathways represent a crucial regulatory mechanism in plant development [JAGODZIK & al. 2018; RODRIGUEZ & al. 2010]. Light, as a signal, is critical in plant growth and development and plants are acutely sensitive to seasonal, daily and moment-to-moment variations in solar radiation [MATSUBARA & al. 2014]. The light environment can convey information through variations in at least four dimensions, i.e. quality (the balance of photons of different wavelengths), quantity (energy flux), direction, and periodicity (relative length of day and night). However, with evidenced critical roles of both MAPK pathways and photoperiod in plant development, the involvement of MAPK pathways in photoperiod regulation is lacking. AtMKK1 (accession number AY050774; unique gene ID AT4G26070) is a stress response kinase that can activate the MAP kinases AtMPK3, AtMPK4 and AtMPK6 [RODRIGUEZ & al. 2010; MENG & ZHANG, 2013]. In our previous study, knockout of *AtMKK1* enhanced salt tolerance during both germination and adulthood and proteomic analysis indicated that the level of the α subunit of mitochrondrial H^+-ATPase, mitochrondial NADH dehydrogenase and mitochrondrial formate dehydrogenase was enhanced in *AtMKK1* knockout mutants upon high salinity stress [CONROY & al. 2013]. Here we report our observation of bolting in *Arabidopsis* AtMKK1 knockout line under a short day condition.

Material and methods

Selection of mutant plants and plant growth conditions were as described previously [CONROY & al. 2013]. Primers for SALK_027645 (*mkk1-2*) were selected from approximately 40 bp upstream of the insert, forward 5'-TATTTGGAGCTTGGGACTGG-3' and downstream of the insertion reverse 5'-GCGAGATGAAGGAGCAAAAC-3'. The third primer used to identify knockouts was the Signal LBA1 primer 5'-TGGTTCACGTAGTGGGCCATCG-3'. There were two rounds of PCR. One using the left and right primers to identify wild-type alleles

Received: 8 October 2024 / ***Revised***: 22 November 2024 / ***Accepted***: 26 November 2024

and the second using the left primer with the LBA1 primer to identify the T-DNA insertions. RT-PCR was carried out under the following conditions: 94 °C for 1 min; 1 min at 94 °C, 1 min at 61 °C, and 1 min at 72 °C for 25 cycles; and then 10 min at 72 °C.

For long day conditions the chamber was set to sixteen hours of light and eight hours of dark all at 22 ºC. For short day conditions, the lights were on for eight hours and off for sixteen hours again at a constant temperature of 22 ºC. Wild type and A31 knockout line seeds were all sterilized using 70% ethanol for two minutes followed by a solution of 30% bleach and 0.02% Triton X-100 for eight minutes. After surface sterilization was complete the seeds were rinsed eight to ten times with sterile water and the seeds were then stratified by placing the seeds on MS medium at 4 ºC for four days. They were then moved to growth chamber A (short day) and chamber B (long day). After ten days the plants were transferred from the plates into sterile soil and continued to grow in growth chambers.

Results and discussions

Arabidopsis development is photoperiod sensitive [GUO & al. 1998]. *Arabidopsis* as a long day plant followed normal growth patterns, bolting at 4-5 weeks and seeds were ready to be harvested by 8 weeks (Table 1). In contrast the short day plants followed normal short day growth and the wild type plants did not enter reproductive growth at any point. This can be contrasted to the appearance of the mutant plants. As can be seen in Figure 1C there was evidence that the plants had indeed entered reproductive growth. There was no significant difference in rosette leaf development (Table 1).

Table 1. Rosette leaf numbers (35-day-old) and percentage of plants that flowered (at 8 weeks). Data based on three repeats of experiments with standard deviation. LD: long-day; SD: short-day.

Plants	Rosette leaf number	Flowering plants (%)
Col-0 LD	13.84±0.26	100±0
A31 LD	13.36±0.47	100±0
Col-0 SD	13.45±0.83	0±0
A31 SD	13.62±1.06	41±2.16

The earlier flowering of AtMKK1 mutants under short days was unexpected. *AtMKK1* is commonly considered a defense responsive gene with few members of kinase cascades impacting upon the development of *Arabidopsis* [GAO & al. 2008; COLCOMBERT & HIRT, 2008]. This being said, there are still many roles and functions for MAPK pathway components that have yet to be identified. The impact of AtMKK1 on plant development has not been examined on any significant level, but the preliminary results showing the ability of *AtMKK1* mutant A31 to respond to changes in photoperiod indicated a significant phenotypic variation. More importantly, all of the bolted plants displayed fully formed siliques, flowers, as well as browning siliques ready for harvest.

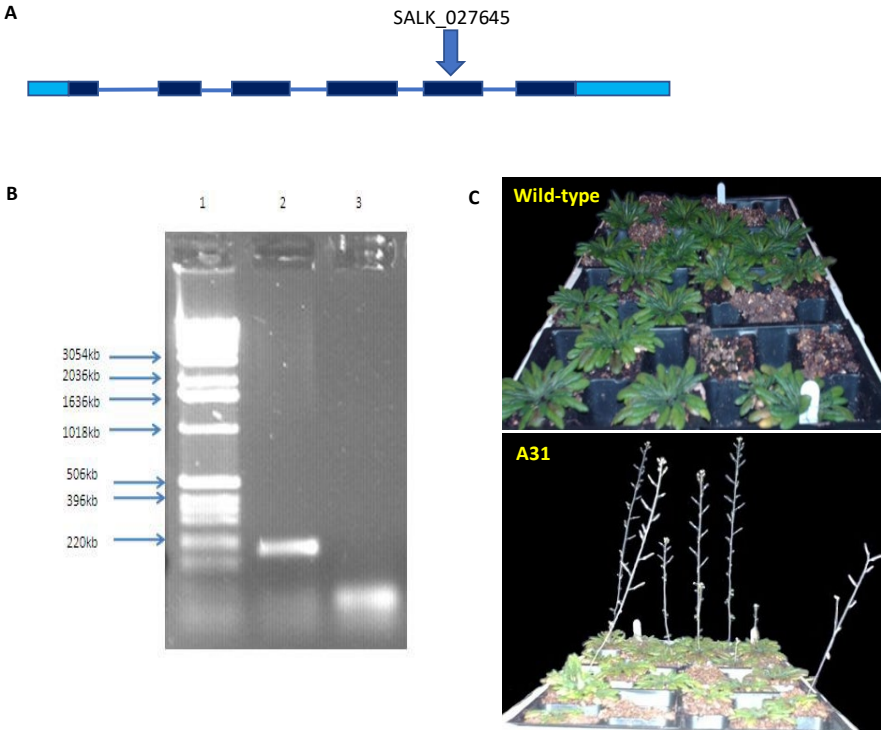

Figure 1. A. Insertion site of knockout mutant of *AtMKK1* (SALK_027645) is in the fifth intron. Line segments represent exons, dark boxes represent introns, and light boxes represent untranslated 5' or 3' flanking regions. **B.** Homozygous mutants for SALK_027645. Lane 1 shows the 1 kb ladder. Lane 2 shows the presence of the wild type AtMKK1 gene as seen in the band near the 220 kb marker. Lane 3 with no band shows that this sample contains the T-DNA insertion preventing PCR from running due to the size of the insert. PCR was performed three times to confirm the results. **C.** Wild type and knockout line A31 plants grown in 8 hours light and 16 hours of darkness after 8 weeks. This experiment was performed three times with similar results.

There are a variety of conditions that regulate flowering time in *Arabidopsis*, but there are no reported instances MAPKs, MAPKKs, or MAPKKKs playing a decisive role in flowering time. There are several environmental conditions, hormonal responses, and genetic variables that can lead to changes in the ability of *Arabidopsis* to thrive, or at least reproduce faster under short day conditions. These conditions are notable. Changes in light conditions, such as altering photoperiod or switching from short day to long day, can trigger bolting or planting in long day and then switching to short day can also affect bolting time [GUO & al. 1998; NAKATSUKA & al. 2009; SONG & al. 2013]. Changes to the intensity or wavelength of light can also cause *Arabidopsis* to bolt [CERDAN & CHORY, 2003]. Changes in the vernalization of *Arabidopsis*, through lowered temperatures during growth, or by increasing stratification time can also play a role in altering the ability of *Arabidopsis* to bolt [SONG & al. 2013]. Plants in this study, both wild type and *AtMKK1* mutants, were grown under the same temperature conditions and all seeds were stratified for the same amount of time and under the same conditions, 4 ºC in the dark. This rules out changes in the vernalization as the triggering factor in the early bolting of the *AtMKK1* mutants. Once the environmental effectors have been ruled out the only remaining

possibilities are that there needs to be a genetic or hormonal response in the *AtMKK1* mutant plants allowing the plants to break free from their short day induced vegetative state.

The primary hormones responsible for the initiation of bolting under short day conditions are gibberellins (GA) [WILSON & al. 1992; SONG & al. 2013]. There is little support for AtMKK1 interacting with GA. AtMKK1 is primarily linked with defensive hormones such as jasmonic acid or salicylic acid, as well as some recent links to ABA [Xing & al. 2008]. AtMKK1 appears to have no relationship with GA and in fact there are few if any MAPK cascade kinases that have been linked to GA [COLCOMBERT & HIRT, 2008]. The lack of characterization of MAPK cascades however does not rule out the possibility of AtMKK1 playing a role in a GA pathway directly or indirectly. AtMKK1 does play a role in regulating ABA sensitivity and *AtMKK1* mutants show reduced sensitivity to ABA [Xing & al. 2008]. There are ABA deficient and ABA insensitive mutants that exhibit early signs of bolting [BERNIER & al. 1993; BERNIER & PÉRILLEUX, 2005; DOMAGALSKA & al. 2010]. The ability of ABA deficient or insensitive mutants to bolt under short day conditions provides an interesting potential hypothesis to explain the effects of AtMKK1 upon early bolting. Due to the large numbers of short day AtMKK1 mutant plants that were capable of bolting, and due to the reported positive regulatory link to ABA in AtMKK1, the reduced sensitivity towards ABA could be the source of the early bolting.

Environmental factors, hormonal responses and genetic components all play a role in controlling the flowering time in *Arabidopsis*. There is a possibility that AtMKK1 does impact upon the expression level of photoperiod sensitive genes but there is little to no evidence to support that possibility at this time. A study with CRISPR gene editing of multiple members of MAPK cascade components in rice showed that loss-of-function mutations in *OsMPK1* and *OsMPK6* are unfavorable and an enrichment of inherited open reading frame-preserving mutations for *OsMPK1* and *OsMPK6* genes was found in T1 plants [MINKENBERG & al. 2017]. We could assume that a mutation of an essential MAPK pathway component may have a pleiotropic effect, which may include our observations in this study.

Acknowledgements

This work was supported by research grants to T.X. from Natural Sciences and Engineering Research Council of Canada and Bayer Crop Science Inc.

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How to cite this article:
CONROY C., NIZAM K. M. & XING T. 2022. *Arabidopsis* ATMKK1 knockout mutant flowers under short day conditions. *J. Plant Develop*. **31**: 229-233. https://doi.org/10.47743/jpd.2024.31.1.956