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Characterizing the role of β-amylase3 in cold stress response and recovery in Arabidopsis thaliana

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Characterizing the role of β-amylase3 in cold stress response and recovery in *Arabidopsis thaliana*

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Abstract

Starch is a polymer of glucose that is used as an energy store in plants. Mobilization of starch has implications in abiotic stress survival and recovery. While the importance of carbon and energy allocation in plant survival has been explored, the specific roles of starch degrading enzymes in plant responses to stress are still unclear. β-Amylase3, or BAM3, is the principle starch degrading enzyme at night and is transcriptionally upregulated in response to cold stress in the plant *Arabidopsis thaliana*. Using single and quadruple knockout mutant plants, I aimed to clarify the role of BAM3 in the response to cold stress over a 96-hour period. The difference between starting cold stress in the morning versus at night were compared to clarify any differences in BAM3 activity, reducing sugars content, or starch accumulation that could have implications in cold stress survival or recovery. Recovery from cold stress was also monitored to verify if BAM3 plays an essential role in recovery from cold stress as hypothesized. I found that BAM3 activity declined over 60% after 96 hours in cold stress regardless of when cold stress started, reducing sugars steadily increased, and starch degradation is halted within the first 24 hours of cold stress. Also, my data does not support that BAM3 plays an essential role in the recovery from cold stress. Activity does not change significantly in the first 24 hours of recovery and appears to decline by hour 48 of recovery from cold stress.

Introduction

Starch is a polymer of glucose that serves as the predominant energy reserve in plants. This reserve can be found in plant storage organs including roots, tubers, rhizomes, stems and seeds in which it serves as a long-term energy source for regrowth or seedling establishment (Santelia & Zeeman 2011). Starch is also temporarily stored in the chloroplasts of photosynthetic cells. This starch is called 'transitory' starch and is synthesized and degraded on a 24-hour cycle (Smith & Martin 1993). *Arabidopsis thaliana* leaves accumulate starch throughout the day and then degrade it throughout the night and by morning, the leaves are depleted of starch (Zeeman et al. 2007).

Storage starch and transitory starch granules are composed of the polymers amylose and amylopectin. Amylose is a straight-chain polymer of α-1,4-linked glucose, and amylopectin is a branched-chain polymer that has both α-1,4-linked glucose and α-1,6-linked branches (Pfister & Zeeman 2016). During the day in the presence of light, about half of the sugars produced by photosynthesis are used for biosynthesis and energy production (Smith & Stitt 2007). The remaining half is polymerized into starch granules (Zeeman et al. 2007). These granules are then broken down at night to supply carbon and energy to sustain plant function in the absence of light (Zeeman et al. 2010).

The biosynthesis and degradation of starch is under tight control as these metabolic pathways influence the growth, development, stress tolerance, and stress recovery of plants (Dong & Beckles 2019). Being sessile, plants are often exposed to a variety of abiotic stresses including heat stress, cold stress, drought, and high salinity. These stresses can signal for a change in metabolic pathways to remobilize starch and effectively respond to stress and buffer against the adverse effects of carbon depletion (Hare et al. 1998; Thomashow 1999; Wanner &

Junttila 1999; Krasavina et al. 2014). Sugars and other metabolites generated from starch degradation can support plant growth and function as osmoprotectants under stress (Krasensky & Jonak 2012; Dong & Beckles 2019). Transitory starch metabolism has major implications in plant fitness, and over the past years, regulation of starch metabolism in response to abiotic stress have been greatly elucidated but still require further investigation (Thalmann & Santelia 2017).

In Arabidopsis, the mobilization of starch involves the actions of multiple enzymes (Thalmann and Santalia 2017). First, outer glucose residues of a starch granule are reversibly phosphorylated by glucan water dikinases (GWD), phosphoglucan water dikinases (PWD), and starch excess 4 phophoglucan phosphatases (SEX4). These actions increase the hydration of the granule to grant access to principle starch degrading enzyme BAM3 as well as the glucan hydrolyzing enzyme isoamylase3 (ISA3) (Figure 1). ISA3 is a debranching enzyme that hydrolyzes the α-1,6 branches of starch into soluble malto-oligosaccharides that are then accessible to BAM3.

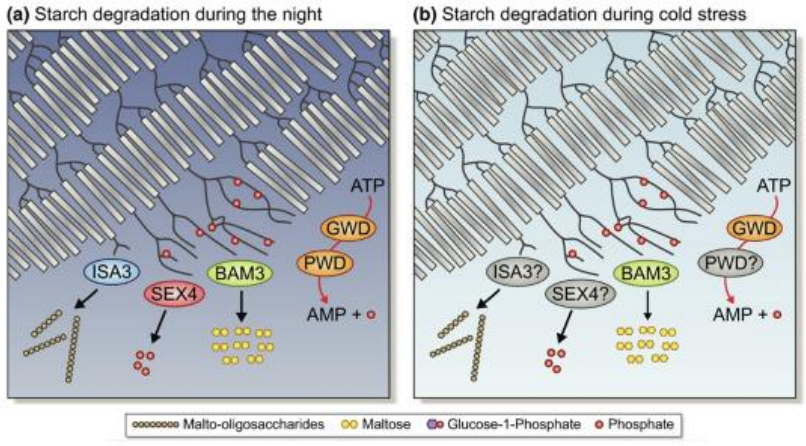

FIGURE 1. Pathways of starch degradation under different conditions. (a) During the night, GWD and PWD phosphorylate the starch granule surface, disrupting its semi-crystalline structure. Subsequently, BAM3 attacks the exposed ends of the glucan chains releasing maltose. As BAM cannot degrade past a phosphorylated glucose unit nor hydrolyze glucosidic bonds which are too close to a branch point, the activity of SEX4 and ISA3 are required to accomplish complete starch degradation. (b) GWD and BAM3 are also involved in starch degradation in response to cold stress, suggesting that a similar starch degradation pathway may operate under these conditions (Figure and legend taken directly from Thalmann & Santelia 2017).

BAM3 is the predominant nocturnal transitory starch degrading enzyme hydrolyzing glucan chains at α-1,4 linkages and releasing the disaccharide maltose (Zeeman et al. 2007). Maltose is then exported from plastids where it is further metabolized in the cytosol (Nittyla et al. 2004). Transitory starch metabolism is better understood on a diurnal cycle but is still not as well understood under abiotic stress conditions such as cold stress. BAM3 not only plays an integrative role in unstressed nocturnal starch metabolism but is implicated to play a large role in cold stress (Figure 1).

BAM3 is a member of a family of nine BAM proteins in Arabidopsis (Monroe & Storm 2018). BAMs are localized to three main cellular compartments. BAMs 1-4, as well as BAM6 and -9 are in plastids (Fulton et al. 2008; Lao et al. 1999; Zybailov et al. 2008; Stettler 2009). BAM5 is cystolic (Wang et al. 1995), and BAM7 and -8 are nuclear (Reinhold et al. 2011). The catalytic members of the β-amylase family that hydrolyze starch into maltose include BAM1 (Sparla 2006), BAM2 (Monroe et al. 2017), BAM3 (Lao et al. 1999), BAM5 (Monroe et al. 1990), and BAM6 (C. Torres & J. Monroe unpublished data). Of the remaining BAMs, BAM4 (Fulton et al. 2008), BAM7, -8 (Reinhold 2011), and BAM9 (Steidle 2010) are catalytically inactive. Of these inactive BAMs, BAM4 and -9 have unknown regulatory roles (Fulton et al. 2008; Li et al. 2009), but BAM7 and -8 are nuclear proteins that function as transcription factors (Reinhold et al. 2011).

BAM3 is the principle nocturnal starch degrading enzyme in Arabidopsis and is crucial for transitory starch mobilization on a diurnal cycle. It has also been thought to play a role in cold stress as it is transcriptionally upregulated in response to cold stress (Kreps et al. 2002; Kaplan & Guy 2004; Kaplan et al. 2006; Maruyama et al. 2009; Sicher 2011; Monroe et al. 2014). Elevated BAM3 mRNA and elevated levels of maltose are associated with the cold stress

response (Kaplan & Guy 2004; Storm et al. 2018; Monroe et al. 2014; Sicher 2011). From this work it was thought that the transcriptional upregulation of BAM3 would lead to increase amylase activity and increased starch degradation under cold stress conditions. However, cold stress was later found to cause BAM3 enzymatic activity to decrease despite transcriptional upregulation, and Arabidopsis plants were found to accumulate starch in extended cold stress conditions (Monroe et al. 2014; Storm et al. 2018). Despite the increase in mRNA, BAM3 activity did not increase with cold stress. This type of disparity has been observed with other proteins as a stress response strategy where mRNA transcript accumulates serving as a reserve pool of mRNA for rapid transcription after stress abates (Nakaminami et al. 2014). Decreased BAM3 activity suggested the influence of an additional regulator on BAM3 activity. Post translational modification has been explored as a potential explanation for these changes in activity levels as the BAM3 protein could be expressed and then deactivated by post translational modification (PTM) (Storm et al. 2018).

One such modification that could influence the BAM3 activity during cold stress is redox mediated cysteine modification. Nitric oxide signaling has been identified as a cold stress regulation mechanism in plants (Puyaubert & Baudouin 2014), and glutathionylation by GSNO, a nitric oxide signaling molecule, of BAM3 could help explain the disparity in transcription upregulation and enzymatic activity decrease (Storm et al. 2018). If BAM3 is translated and then deactivated by a PTM in response to cold stress, this deactivated BAM3 protein might serve as a reserve pool of amylase for cold stress recovery. This would account for the transcriptional upregulation observed as well as the functional role of the GSNO sensitivity of BAM3 (Monroe et al. 2014; Storm et al. 2018). However, there is currently no evidence that BAM3 is modified *in vivo*.

Studies using single gene knockout plants have been helpful in determining the function of some genes, but tissue extracts often contain multiple gene products that possess similar catalytic activity. In order to eliminate the masking influence of the other catalytic BAMs, the Monroe lab has constructed a quadruple knockout plant that lacks all of the catalytic BAMs except BAM3 (Monroe 2020). In addition to this quadruple mutant, the lab has also obtained a single mutant plant, *bam3,* lacking only BAM3 (Fulton et al. 2008). Using the BAM3 quadruple mutant plant (*BAM3Q*) and single mutant plant (*bam3*), I aimed to investigate the role of BAM3 in cold stress and cold stress recovery to provide a better picture of the cold stress response *in vivo*. I also aimed to clarify the differences in the cold stress response when cold stress was initiated in the morning, when leaves were devoid of starch, as compared to initiating the stress at the beginning of the night when leaves contained maximal starch, as this has not yet been explored. From this investigation, we hoped to gain a better understanding of the mechanisms by which plants regulate starch metabolism in response to cold stress and recovery from cold stress.

Methods

Plant material and growth conditions

I used three different genotypes of *Arabidopsis thaliana* ecotype Columbia-0 to conduct experiments. *BAM3Q* was generated by crossing homozygous single mutants (*bam1* (Salk_039895), *bam2* (Salk_086084), *bam5* (Salk_004259), and *bam6* (Salk_023637) (Monroe 2020). Seeds of *bam3* were a gift from David Seung and were previously described (Fulton et al. 2008). All plants were grown in 5-inch pots with 5 plants per pot a room at 23℃ under illumination of 130 µmol $m^{-2}s^{-1}$ on a 12-hour day/12-hour night light cycle. Plants under cold stress treatment were placed in a cold room at 4℃ under the same light conditions. The growth medium for the plants used to conduct the experiments to characterize cold stress started in the morning and cold stress started at night was ProMix BX (Premier Tech Horticulture) supplemented with macronutrients and micronutrients as described by LEHLE SEEDS. The growth medium used for the plants used to conduct the cold stress recovery experiments were grown in MiracleGro soil. All plants were collected for experiments between 6-8 weeks old after planting and stored at -80^{o}C until analysis.

Iodine starch staining of Leaves

Iodine stains starch black/blue, which is useful for monitoring starch accumulation in leaves. Leaf samples were boiled in 80% ethanol for 8-10 minutes until leaves were transparent and possessed no green color. The samples were then soaked for about 10 seconds in Lugol's iodine solution containing 5% elemental iodine and 10% potassium iodide dissolved in deionized water (Hostettler et al. 2011). Then, the leaves were rinsed with deionized water and placed on transparent sheets for photography. The dark regions on these samples indicate visually the levels of starch (Caspar et al. 1991).

Amylase Activity Assays

To quantify the enzymatic activity in leaves of the different plant genotypes (*BAM3Q*, *bam3*, and *WT*) leaf samples plants were ground in 3 volumes of extraction buffer (50 mM MOPS, pH 7.0, and 5 mM EDTA) with sand. After centrifugation, amylase activities were conducted in 0.5 mL containing 50 mM MES (pH 6.0) and 40 mg/mL soluble starch. Assays were conducted for 50 minutes in a 25°C water bath and were then stopped by immersion in a 100°C heat bath for 3 minutes. Reducing sugars were measured using the Somogyi–Nelson assay which is a colorimetric assay that stains reducing sugars in the products of the assays (Nelson 1944). Maltose was used as the standard. An unpaired two-tailed Student's t-test assuming equal variances was used to determine statistical differences in the amylase activity assays and reducing sugars assays.

Reducing Sugar Assays

Reducing sugars present in the extracts were quantified by pipetting 50 µL of each plant extract into 450µL of deionized water immersed in a boiling heat bath. These were then assayed using the Somogyi–Nelson assay described above (Nelson 1944).

Protein Concentration

Protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad) according to manufacturer's instructions with bovine serum albumin as the standard.

Results

BAM3 has been predicted to play an essential role in cold stress, but BAM3 activity under cold stress *in vivo* is not well characterized. I acquired single knockout Arabidopsis mutants lacking BAM3 as well as quadruple gene knockout plants that have only BAM3 and none of the other catalytic BAMs (Fulton et al. 2008, Monroe 2020). The amylase activity in these two mutants was compared to each other as well as to Wild Type Arabidopsis plants in their response to cold stress. To characterize and compare the cold stress response when started in the morning versus at night, three genotypes including *BAM3Q* (which excludes all catalytic BAMs except BAM3), *bam3* (which does not possess BAM3), and WT were used. Monitoring amylase activity and starch accumulation can reveal the presence of BAM activity *in vivo*. By measuring reducing sugars, increases in BAM activity or decreases in sugar utilization by the plant can be detected. The plants were grown at the same time and placed under 4^{o}C cold stress either at 9 AM or 9 PM. Under unstressed conditions, Arabidopsis leaves possess minimal and maximal levels of starch at 9 AM and 9 PM respectively. Also, under unstressed conditions, amylase activity has been shown to remain relatively constant throughout a diurnal cycle (Monroe 2020).

When 7-week-old plants were placed under cold stress in the morning, WT extracts exhibited a significant increase in amylase activity from 0 to 24 hours ($p=0.029$) and a significant decrease in activity from 24 to 48 hours after the start of cold stress ($p=0.037$) (Figure 2). Amylase activity rose from 0 to 96 hours in *bam3* extracts ($p=0.0036$), whereas *BAM3Q* extracts exhibited a steady decline in amylase activity from 0 to 96 hours ($p=0.0054$) (Figure 2).

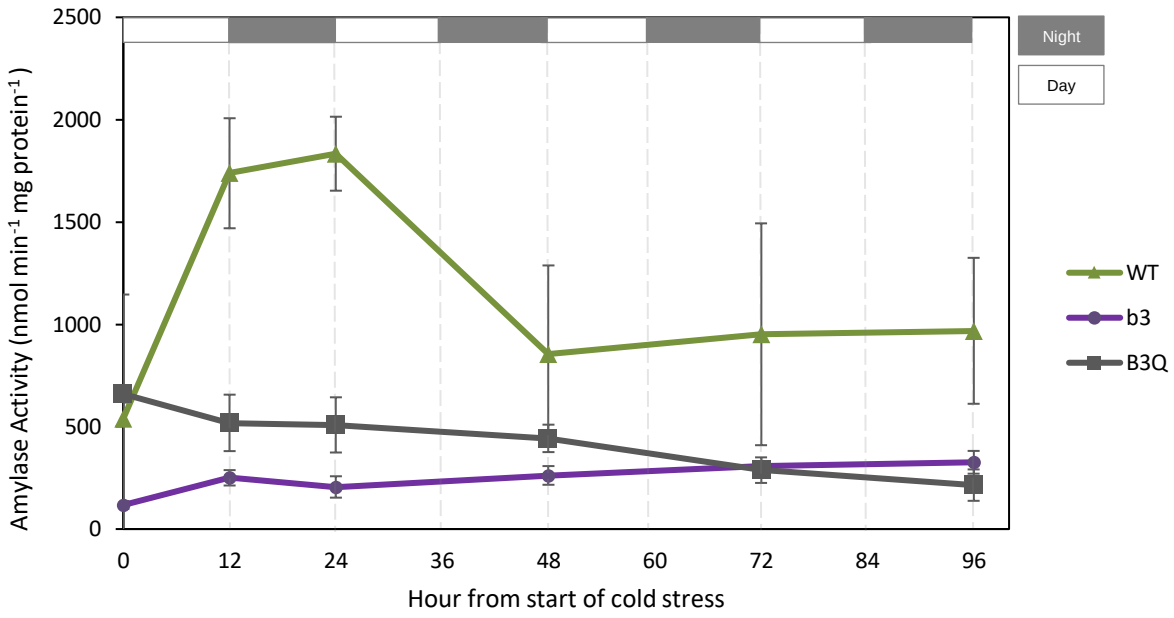

Figure 2. Amylase activity when cold stress was started in the morning. Amylase activity in crude extracts from leaves of WT (WT: green), *bam3* (b3: purple), and *BAM3Q* (B3Q: grey) plants were grown under a 12-hr day/12-hr night photoperiod and leaves were collected at 0, 12, 24, 48, 72, and 96 hours after start of cold stress, which was initiated in the morning. All extracts were assayed at 25°C in 50 mM MES buffer, pH6, with 40 mg/mL soluble starch. Values are means ± *SD* (n=3).

The reducing sugars in the WT extracts rose significantly across the 96-hour period from the start of cold stress (p=0.026) (Figure 3). Reducing sugars were all elevated and significantly higher than at hour zero of cold stress (from 0 to 12 hours, p=0.011, 0 to 24 p=0.042, 0 to 72= 0.0013, 0 to 96=0.026). Reducing sugars dropped significantly from 12 to 24 hours (p=0.032) and rose again by 72 hours (p=0.038). Reducing sugars in *bam3* rose from 0 to 12 hours of cold stress ($p=6.4x10^{-5}$) and continued to rise significantly until 72 hours in cold stress (p=0.0024). *BAM3Q* reducing sugars were all significantly higher than at hour zero (0 to 12 p=0.0061, 0 to 24 p=0.0084, 0 to 48 p=0.0036, 0 to 72 $p=3.1x10^{-5}$, 0 to 96 p=0.016). Reducing sugar levels dropped from 12 to 24 hours (p=0.045) and rose again from 24 to 48 hours (p=0.013) (Figure 3).

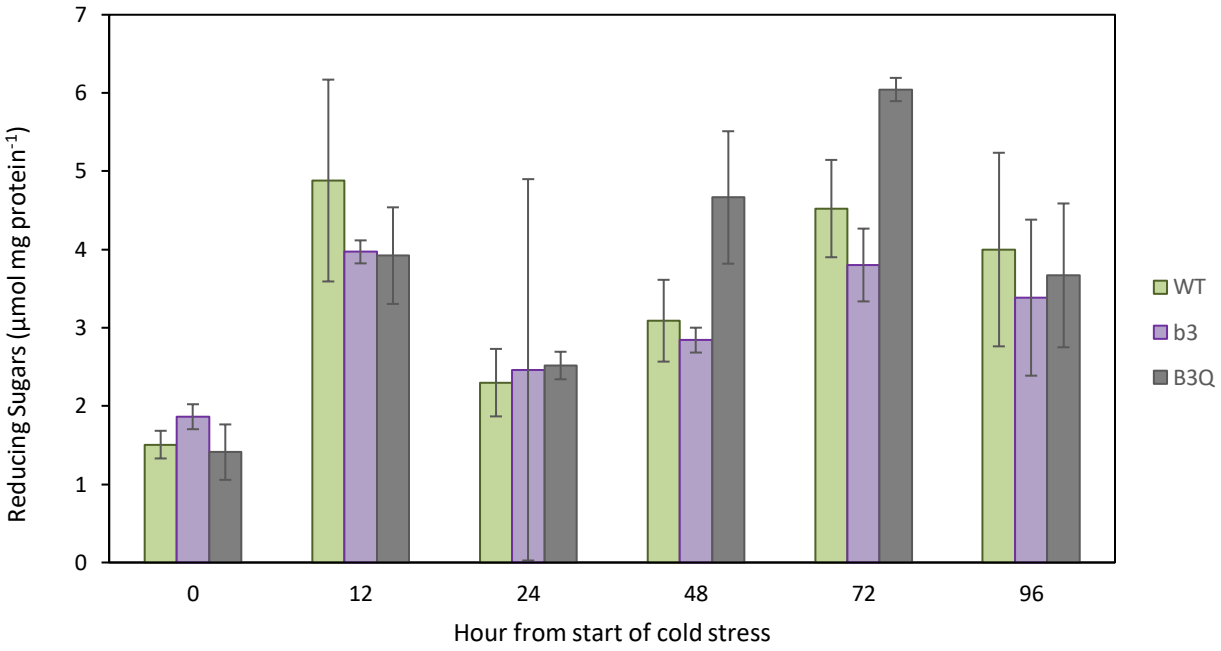

Figure 3. Reducing sugars when cold stress was started in the morning. Reducing sugars content in the same extracts used in Figure 2. Values are means ± *SD* (n=3).

In iodine starch stains, starch is dyed a dark blue/black color. Starch staining of WT plants show that WT plants with cold stress initiated in the morning started with no starch and accumulated some starch by 96 hours in cold stress (Figure 4). *bam3* plants exhibited a starch excess phenotype, and it did not change over the 96-hour period (Figure 4). *BAM3Q* plants did not contain visible starch when cold stress was started in the morning and may have accumulated some by 96 hours in cold stress (Figure 4).

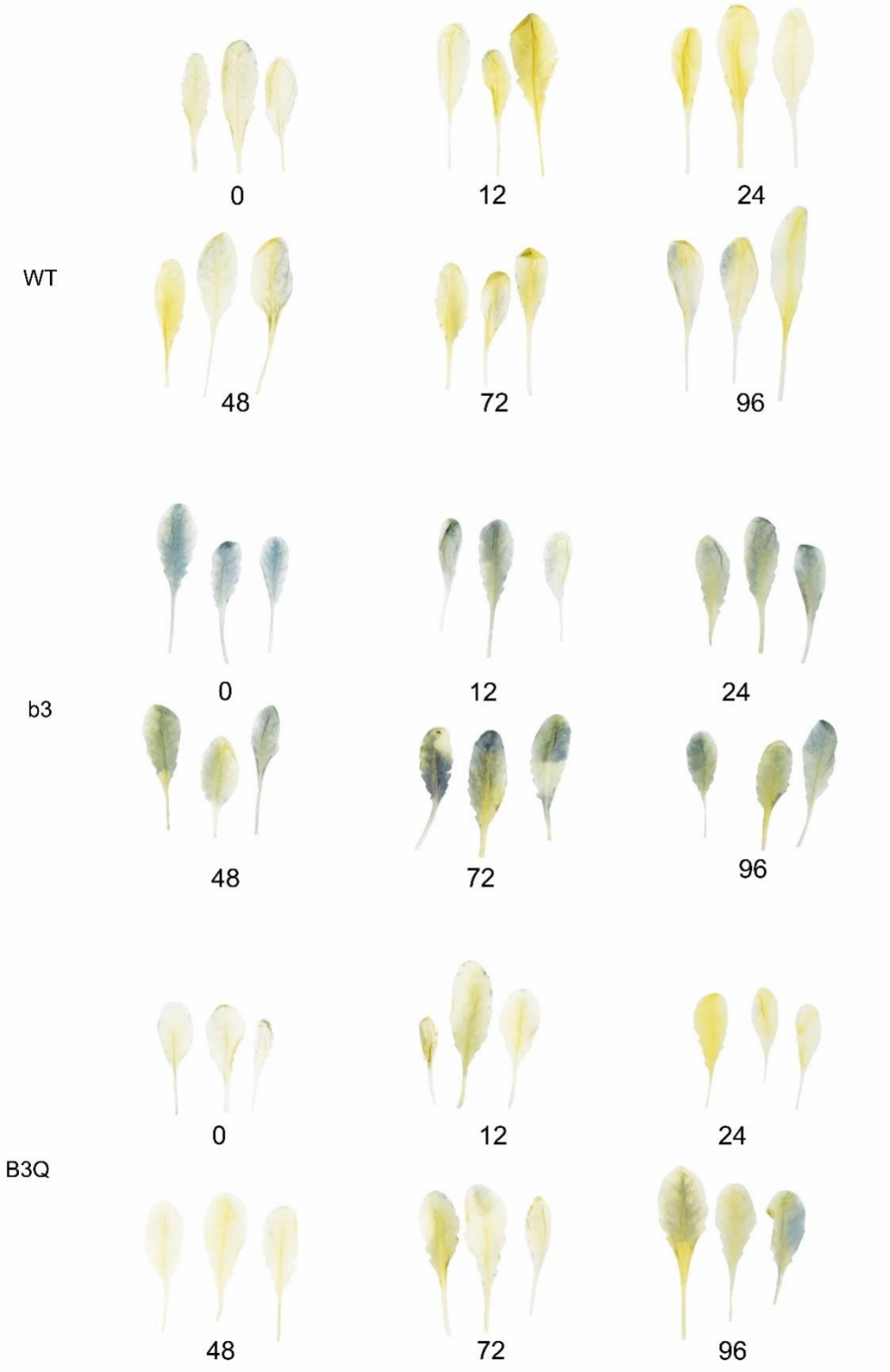

Figure 4. Starch accumulation when cold stress was started in the morning. Leaf starch accumulation, visualized using iodine stain, of WT, *bam3*, and *BAM3Q* plants at 0, 12, 24 48, 72, and 96 hours after cold stress started in the morning. Plants were grown under a 12/12 hr-day/night photoperiod, harvested at different hours after the start of cold stress.

WT plants that were placed into cold stress at the start of a night did not exhibit any significant change in amylase activity over the course of 96-hour cold stress (Figure 5). Amylase activity in *bam3* extracts decreased from 0 to 24 hours (p=0.037) and increased from 24 to 96 hours (p=0.00017). Amylase activity in *BAM3Q* extracts increased from 12 to 24 hours (p=0.020) and decreased 48 to 96 hours (p=0.00099) (Figure 5). Overall, *BAM3Q* extracts exhibited a decrease in amylase activity by 96 hours after cold stress compared to hour 0 (p=0.010).

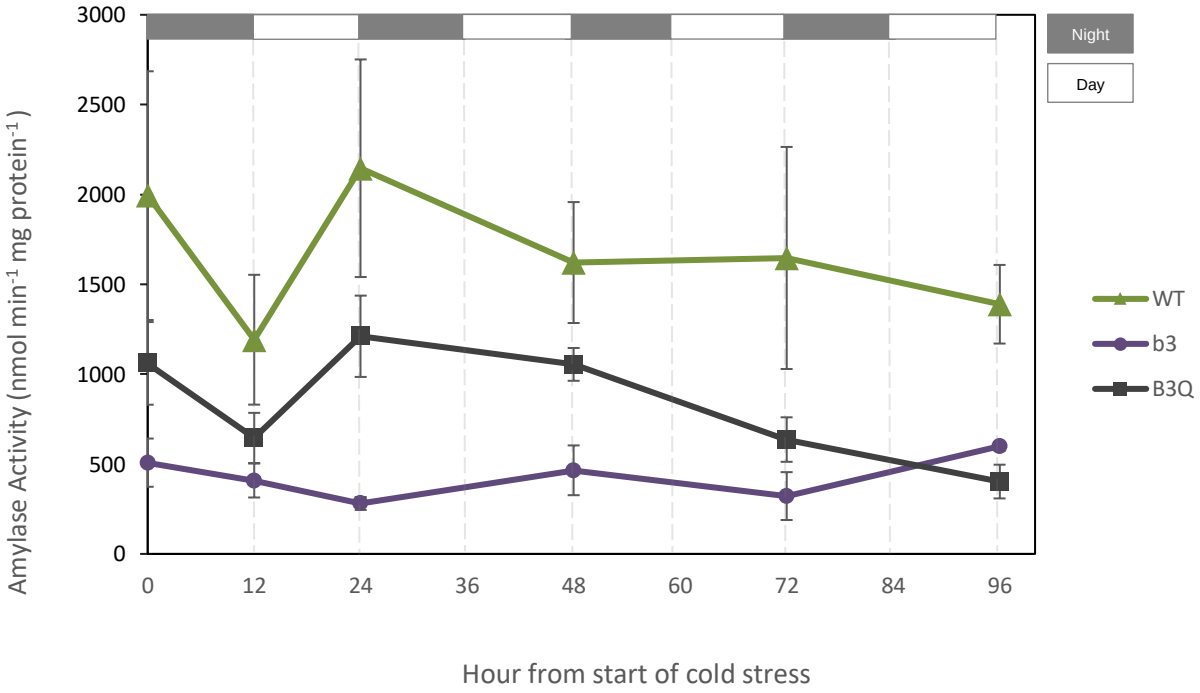

Figure 5. Amylase activity when cold stress was started at night. Amylase activity in crude extracts from leaves of WT (WT: green), *bam3* (b3: purple), and *BAM3Q* (B3Q: grey) plants grown under a 12-hr day/12-hr night photoperiod. Plants were collected at 0, 12, 24, 48, 72, and 96 hours after start of cold stress at night. All extracts were assayed at 25ºC in 50 mM MES buffer, pH6, with 40 mg/mL soluble starch. Values are means ± *SD* (n=3).

When cold stress was started at night, WT reducing sugars levels increased overall from 0 to 96 hours of cold stress (p=0.0086). Reducing sugars rose from 0-72 hours (p=0.0012) and then decreased slightly from 72 to 96 hours (p=0.034) (Figure 6). *bam3* reducing sugars

increased from 0 to 96 hours (p=0.0086). *BAM3Q* reducing sugars rose steadily throughout the 96 hours of cold stress. Reducing sugars were elevated in *BAM3Q* extracts at all time points compared to hour 0 (0 to 12 p=0.0047, 0 to 24 p=0.00081, 0 to 48 p=0.00089, 0 to 72 p=0.00014, and 0 to 96 p=0.00015) (Figure 6).

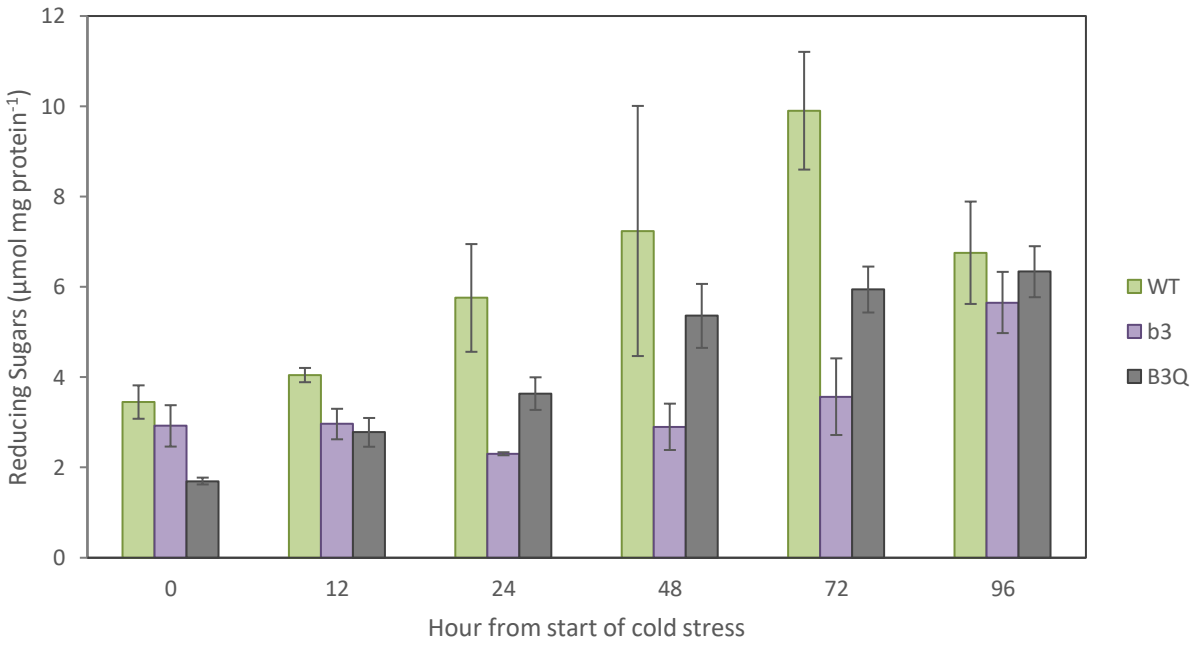

Figure 6. Reducing sugars when cold stress was started at night. Reducing sugars content in the same extracts used in Figure 5. Values are means ± *SD* (n=3).

The WT plants did not exhibit significant changes in starch accumulation in the 96-hour cold stress but started with some starch at hour 0 (Figure 7). *bam3* leaves exhibited a starch excess phenotype, but there was no noticeable change in starch accumulation from 0 to 96 hours (Figure 7). The *BAM3Q* plants did not exhibit significant changes in starch accumulation but started with some starch at hour 0 and appear to retain that starch throughout the cold stress (Figure 7).

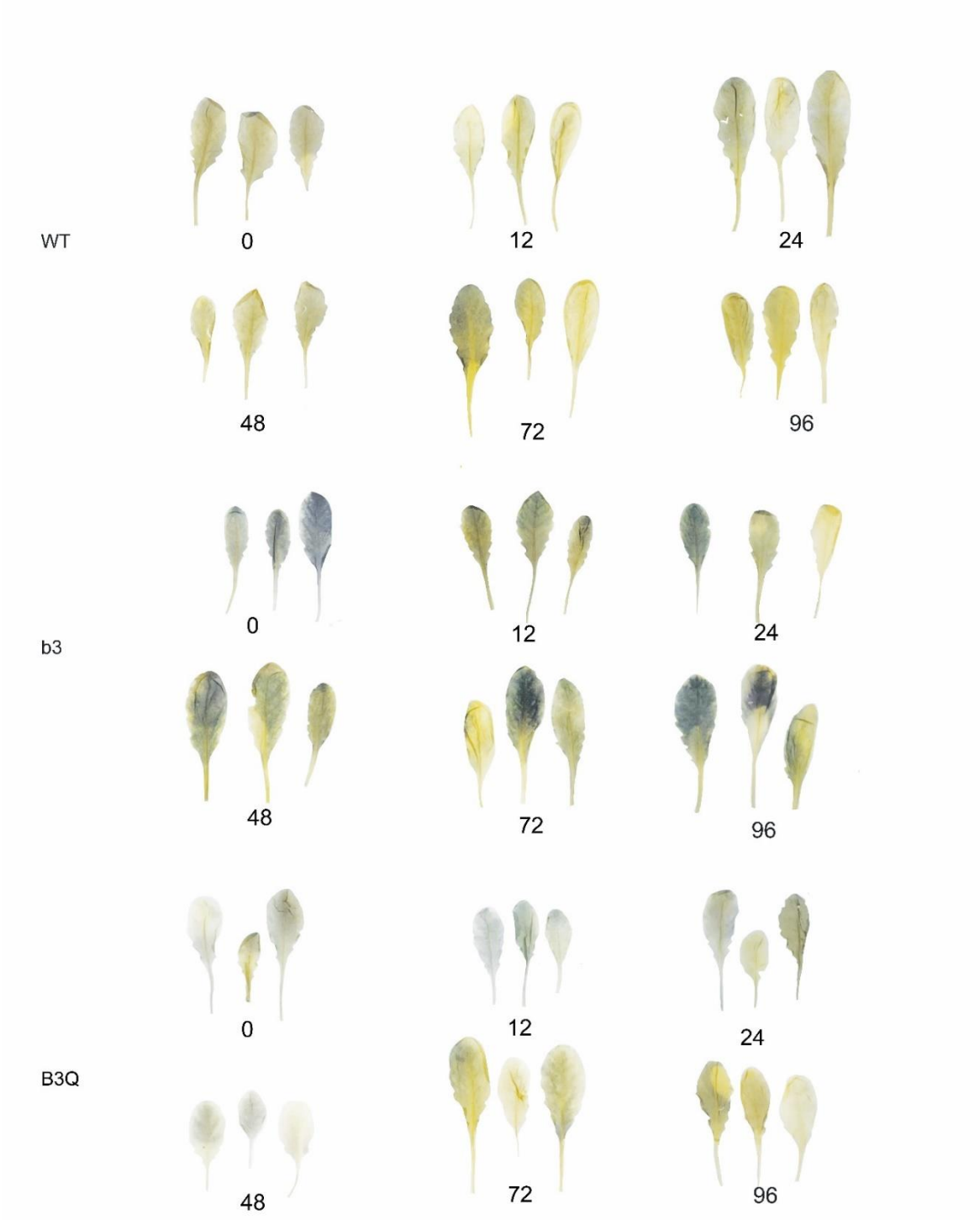

Figure 7. Starch accumulation when cold stress was started at night**.** Starch content, assayed by iodine staining, in WT, *bam3,* and *BAM3Q* plants at 0, 12, 24 48, 72, and 96 hours after cold stress started at night. Plants were grown under a 12/12 hr-day/night photoperiod, harvested at different hours after the start of cold stress.

To determine if BAM3 plays a role in recovery from cold stress, 7-week old WT, *bam3*, and *BAM3Q* plants were placed in cold stress for 96 hours. Amylase assays were conducted on leaves that were harvested after being taken out of cold stress in the first 48 hours of recovery. Amylase activity after cold stress abated appeared to decrease by 48 hours of recovery but did not change significantly over 48 (Figure 8).

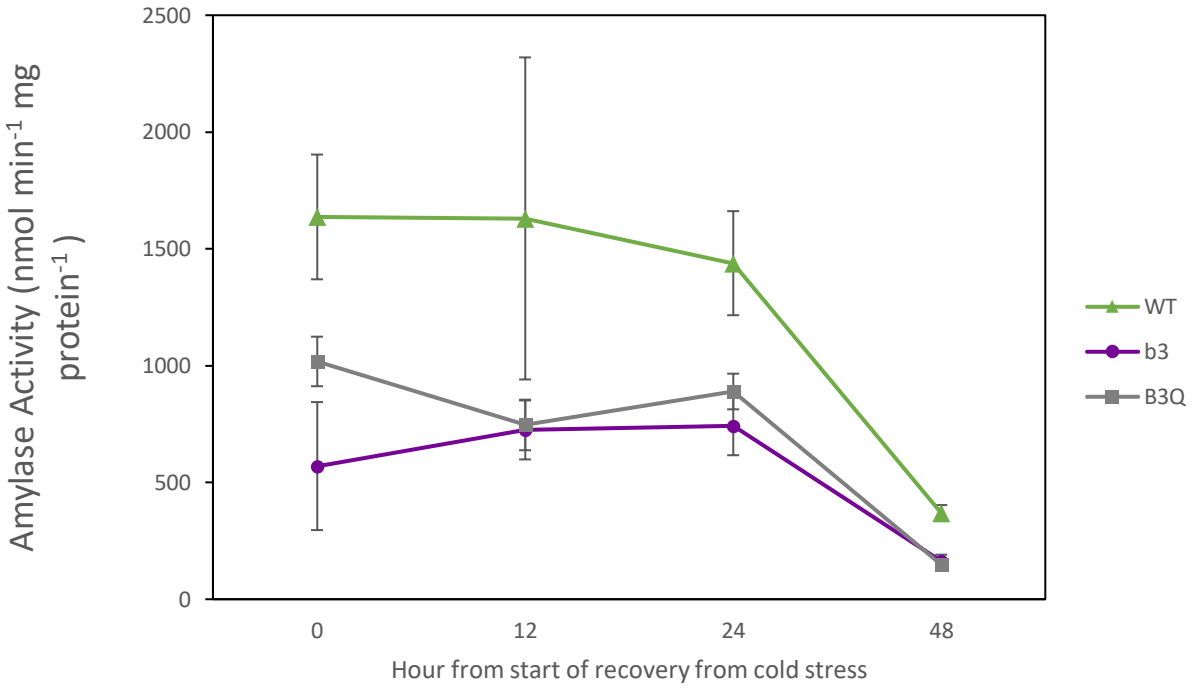

Figure 8. Amylase activity in recovery from cold stress. Amylase activity in crude extracts from leaves of WT (WT: green), *bam3* (b3: purple), and *BAM3Q* (B3Q: grey) plants grown under a 12-hr day/12-hr night photoperiod. Plants were collected at 0, 12, 24, and 48 hours after taken out of cold stress. All extracts were assayed at 25ºC in 50 mM MES buffer, pH6, with 40 mg/mL soluble starch. Values are means ± *SD* (n=3).

From the same extracts used in Figure 8, reducing sugars were measured in cold stress and recovery. In recovery from cold stress, reducing sugars decreased significantly by 48 hours in all genotypes (*WT* p=0.0029, *bam3* p=0.016, *BAM3Q* p=0.0012) (Figure 9).

Figure 9. Reducing sugars in recovery from cold stress. Reducing sugars content in the same extracts used in Figure 8. Values are means ± *SD* (n=3).

The same plants assayed in Figures 8 and 9 were also stained with iodine to estimate starch levels in recovery from cold stress. Plants were placed in cold stress at the end of the day when WT and *BAM3Q* contained a maximum level of starch (Fulton et al. 2008). At the start of cold stress, WT plants started with minimal starch but accumulated starch by 24 hours of cold stress (Figure 10). Plants were taken out of cold stress in the morning on the fourth day of cold stress. In recovery from cold stress, WT plants appeared to not break down starch in the first 12 hours of recovery but begin to break it down at night by 24 hours (Figure 10). *bam3* plants exhibited a starch excess phenotype throughout cold stress and recovery from cold stress

whereas *BAM3Q* plants started cold stress with starch but starch accumulation was variable and unchanging after 12 hours of cold stress as well as in recovery (Figure 10).

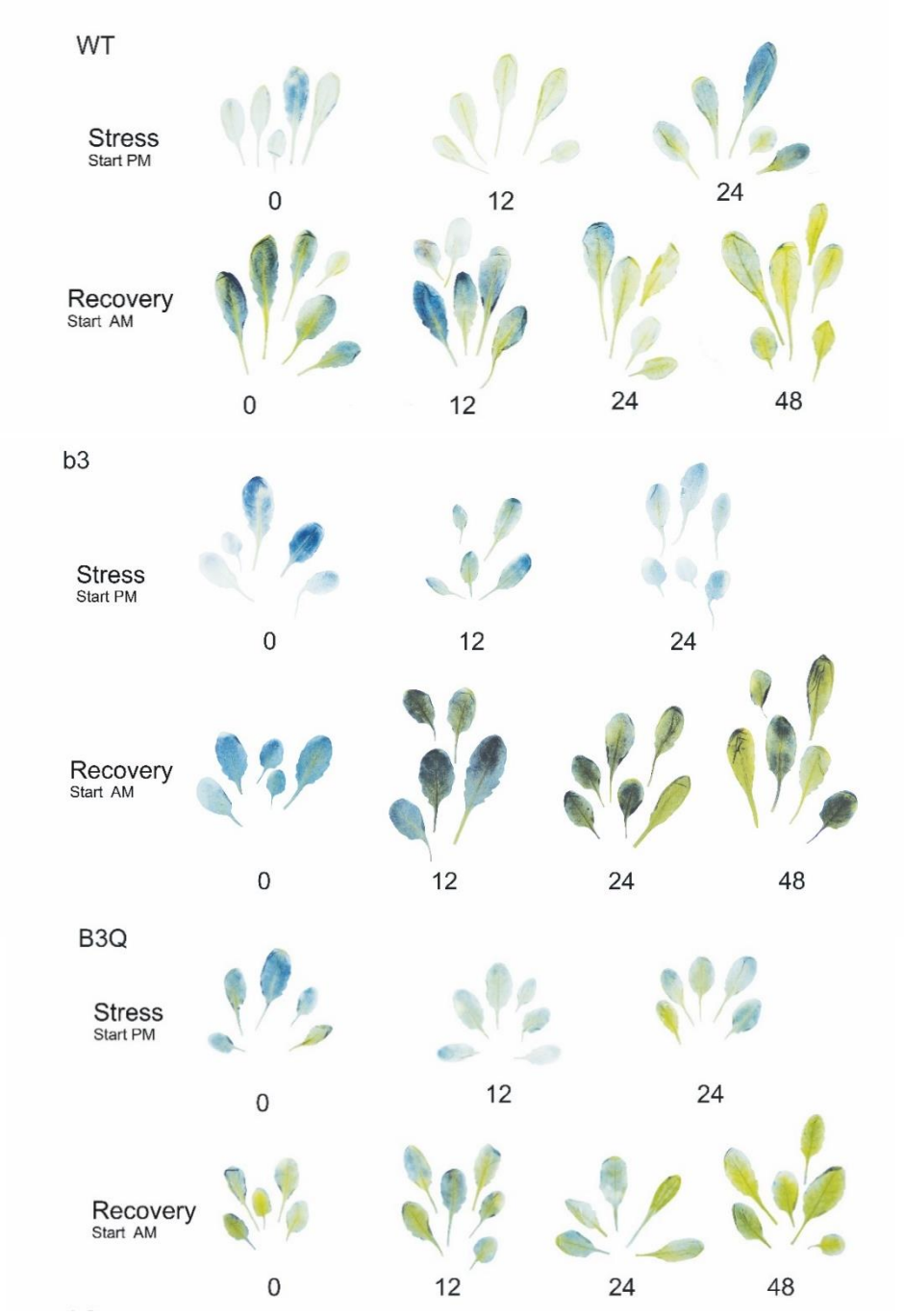

Figure 10. Starch accumulation in cold stress and recovery. Starch levels, assayed by iodine staining, in WT, *bam3*, and *BAM3Q* plants at 0, 12, and 24 hours into cold stress started at night and accumulation at hours 0, 12, 24, and 48 hours into cold stress recovery. Plants used in starch staining are from the same set of plants from Figures 7 and 8. Plants were grown under a 12/12 hr-day/night photoperiod, harvested at different hours after the start of cold stress.

Discussion

Transitory starch metabolism has major implications in plant fitness under normal and abiotic stress conditions. Over the past years, our understanding of the regulation of starch metabolism in response to abiotic stress, such as the response to cold stress, has increased, but still requires further investigation (Thalmann & Santelia 2017). Due to the increase in BAM3 mRNA in cold stress, BAM3 has been suggested to play a role in the cold stress response and possibly recovery in *Arabidopsis thaliana* (Kaplan & Guy 2004). Increased transcription of BAM3 suggested an increase in BAM3 activity to respond to cold stress. However, BAM3 activity was shown to decrease by 4 days in cold stress (Monroe et al. 2014). Also, the differences in cold stress started in the morning compared to at night have not been explored. To compare the difference in the cold stress response for plants depleted of starch, or containing maximum levels of starch, I monitored quadruple and single mutant BAM3 plants over 96 hours of cold stress started in the morning and started at night, respectively. I also monitored plants in recovery from 96 hours cold stress over a 48-hour period to verify BAM3's potential role in cold stress recovery.

I demonstrated that BAM3 activity decreased *in vivo* by 68% and by 62% in *BAM3Q* plants 96 hours into cold stress whether started in the morning or at night respectively (Figures 2 and 5). This indicates that the timing of the onset of cold stress relative to the starch content of leaves does not greatly impact the long-term outcome of BAM3 activity in cold stress. BAM3 activity did not appear to change *in vivo* significantly in the first 24 hours of cold stress in either test group (Figure 2 and 5). However, the significant decrease in BAM3 activity by 96 hours of cold stress is consistent with findings in current literature (Monroe et al. 2014; Monroe 2020). These data demonstrate that the decrease in BAM3 activity occurs gradually over a 96-hour

period of cold stress (Figures 2 and 5). Although both morning and night cold stress test groups experienced over 60% decreases in activity, overall activity levels were elevated in WT plants where cold stress was started at night (Figure 5). Elevated amylase activity could potentially impact recovery from cold stress. Assays would have to be conducted to compare the recovery of plants with cold stress started in the morning versus started at night to determine the impact of elevated amylase activity on recovery.

There was a distinct rise in reducing sugars in all genotypes in both test groups across the 96-hour period (Figures 3 and 6). The increase in reducing sugars cannot be attributed to an increase in amylase activity producing maltose as amylase activity increased in the *bam3* genotype, decreased in BAM3Q plants, and WT returned to baseline when cold stress was started in the morning and doesn't change when cold stress was started at night (Figures 2 and 5). The increase in reducing sugars is most likely due to a combination of the inhibition of growth and the associated use of reducing sugars, as well as sugars produced by the Calvin cycle. As found by Strand et al. in 1999, plants moved from room temperature of 23ºC to cold stress of ~5ºC exhibited an increase in Calvin Cycle enzymes including Rubisco, GADPH, and Aldolase. They also observed an increase in sugars such as sucrose, fructose, glucose, and free hexoses which could account for some of the increase in reducing sugars content observed in this data (Strand et al. 1999, Sicher 2011). My data are consistent with these findings as there was an increase of reducing sugars in both test groups by hour 96 of cold stress. This increase is most likely due to a combination of production from photosynthesis during the light periods and from the lack of use due to the slowing of growth. The increase in reducing sugars is most likely implemented as a cryoprotective measure (Krasensky & Jonak 2012; Dong & Beckles 2019).

Staining starch with iodine is not quantitative, but it can provide an indication of a relative starch levels in leaves and has been used to demonstrate the diurnal changes in transitory starch degradation (Fulton et al. 2008). Starch excess has been shown to occur in the absence of the BAM3 protein and can be indicated using iodine staining (Fulton et al. 2008; Monroe et al. 2014; Monroe 2020). Plants moved from room temperature to cold stress have also been shown to maintain unchanging starch levels indicating that starch mobilization as well as accumulation on a diurnal cycle is halted in at least the first 18 hours cold stress (Strand et al. 1999). This is consistent with my iodine stains as there is limited change in the starch accumulation until hour 96 of cold stress in both cold stress start times (Figures 4 and 7).

When cold stress was started at night, both WT and *BAM3Q* plants exhibited no change in starch. They also appear to potentially retain that starch whereas plants where cold stress was started in the morning did not accumulate any starch until hour 96 (Figures 4 and 7). The variation observed in the data, particularly in the starch stains (Figures 4 and 7), could be attributed to other stressors aside from cold stress. Additional starch stains comparing cold stress started in the morning versus at night should be conducted to investigate whether cold stress started at night impacts starch availability for the plant before 96 hours of cold stress. A difference in starch availability for plants experiencing cold stress initiated in the morning versus at night could have implications on the survivorship of plants long term.

BAM3 in cold stress recovery

The disparity between the increase in BAM3 mRNA and the decrease in activity was hypothesized by Storm et al. (2018) to be a cold stress response strategy in which mRNA transcript might accumulate, serving as a reserve pool of mRNA for rapid transcription after

stress abates. This has been observed in other plants (Nakaminami et al. 2014). BAM3 activity according to the *BAM3Q* extracts appeared to decrease by 48 hours of recovery but the change was not significant ($p=0.68$) (Figure 8). Iodine stains also did not indicate a significant increase in starch degradation during cold stress recovery. However, the stains also did not exhibit distinct starch accumulation at the end of cold stress which is not consistent with previous findings (Figure 10, Monroe et al. 2014; Storm et al. 2018). Reducing sugars in cold stress recovery decreased significantly by 48 hours of recovery indicating a mobilization of the free sugars (Figure 9). There was no significant increase in reducing sugars during the first 24 hours, which would be expected with an increase in BAM3 activity suggesting that BAM3 is not being rapidly translated for recovery. Maltose levels change diurnally with changes in BAM activity suggesting that a change in BAM3 activity would exhibit a subsequent change in maltose (Harmer et al. 2000; Smith et al. 2004). Monitoring maltose levels specifically instead of total reducing sugars would be useful in clarifying BAM3 activity specifically. The lack of change in BAM3 activity, lack of increase in reducing sugars, and lack of change in starch degradation in the first 24 hours of recovery indicates that a reserve pool of mRNA at the ready for transcription and subsequent starch mobilization is not likely. There is also no discernable change in starch mobilization which supports this, but starch stains should be repeated to confirm.

Future areas of investigation

These data provide a slightly better picture of the relationship between cold stress and BAM3 activity in the short term and in the long term, but they also raise more questions. I demonstrated that activity of BAM3 in *BAM3Q* plants in cold stress steadily decline over 96 hours of cold stress, reducing sugars increase steadily over 96 hours in cold stress, but starch

accumulation does not seem to coordinate to the changes in activity. Also, BAM3 does not appear to play the integral role in starch mobilization during recovery as previously hypothesized.

Whether cold stress is started at night or in morning, starch degradation on a diurnal cycle is immediately disrupted. My findings as well as those in Strand et al. (1999) support this claim, although starch stains should be repeated to confirm the halt in starch degradation by BAM3 in response to cold stress. Despite this halt of starch degradation in the first 24 hours, BAM3 activity was only shown to significantly decrease by 96 hours of cold stress which is not coordinated with the lack of starch degradation in the first 24 hours of cold stress (Figures 4 and 7; Strand et al. 1999). The rapid decline in starch degradation in the first 24 hours could be attributed to the rapid deactivation of BAM3 by GSNO, but this is not consistent with the lack of change in activity in the first 24 hours of cold stress observed (Figures 2 and 5). The change in starch degradation in the first 24 hours as well as the change in BAM3 activity only after 96 hours indicates that there are potentially two different influences on BAM3 activity.

First, as explored by our lab, BAM3 could be inhibited by a post translational modification in the first 24 hours of cold stress as an acute response to cold stress. Inhibition by GSNO could explain the immediate halt in starch degradation during cold stress to rapidly deactivate BAM3 in order to conserve starch. Starch levels have previously been shown to be constant under cold stress and are not degraded diurnally as in unstressed conditions (Stand et al. 1999). This is consistent with rapid deactivation of BAM3 at the start of cold stress. A future area of investigation could be to determine the presence of this PTM *in vivo*. DTT has been shown to reverse the effects of GSNO *in vitro* and could be a potential method for *in vivo* confirmation (Monroe et al. 2014). Another potential method could be to test different reducing

agents such as chloroplastic thioredoxins which have been shown to completely recover activity compared to DTT in other starch degrading enzymes such as SEX4 (Silver et al. 2012).

A second influence on BAM3 activity in cold stress could be the reduction in BAM3 protein over the 96-hour cold stress period. BAM3 has a short half-life of 0.43 days which, if BAM3 is not being transcribed, could contribute to the declining activity levels (Li et al. 2017). In order to determine if declining BAM3 levels influence long term cold stress, BAM3 levels specifically should be monitored over a 96-hour period.

Interestingly, BAM3 activity in *BAM3Q* plants has also been shown to remain constant over a diurnal cycle despite well characterized changes in starch levels on a diurnal cycle (Lu et al. 2005; Fulton et al. 2008; Monroe et al. 2014; Monroe 2020). BAM3 transcription is also regulated diurnally showing peaks at the transition from night to day or day to night (Bläsing et al. 2005; Smith et al. 2004) as well as being upregulated during cold stress (Kaplan et al. 2006; Kaplan and Guy 2004; Maruyama et al. 2009; Monroe et al. 2014; Kreps et al. 2002; Sicher 2011). Many signs point to an increase in BAM3 activity in the 12-hour period of nighttime and signs point to a decrease of activity during the first 12-hour period in cold stress. But, this is not what was observed in the assays from our quadruple mutant plant extracts. Mechanisms must be in place to prevent starch degradation during the day as well as during the first 12 hours of cold stress. These mechanisms are undetectable using the current assay and different methodologies or mechanisms need to be explored to determine true BAM3 activity.

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