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Research Article

Thermopriming-Induced Autophagy in Shoot Apical Meristem of Arabidopsis

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Background: Since embryogenesis, plants deal with environmental changes, which might affect their growth and development. Plant autophagy has been shown to function in various stress responses, immunity, development, and senescence. Acquired thermotolerance or thermopriming is enhanced resistance to the elevated temperature following heat stress.

Objectives: Potential contribution of autophagy mechanism after thermopriming was investigated in shoot apical meristem (SAM) of *Arabidopsis thaliana*.

Materials and Methods: Transcriptic expression of Autophagy related Genes (*ATG*s) were analyzed by qRT-PCR data in 5-day old *Arabidopsis thaliana* (Col0) seedlings at 4 h and 24 h after thermopriming. Autophagy induction was confirmed by confocal microscopy.

Results: Expression patterns of 39 ATGs and ATG-receptors were described and relevant thermopriming induced autophagy genes were identified according to their highest expression fold changes during the time after treatment. Significantly, *ATG8A*, *ATG8B*, *ATG8G*, *ATG8H*, *ATI1*, *ATI2*, *NBR1*, and *TSPO* genes were identified as the most relevant thermopriming-associated autophagy genes especially in SAM of young seedlings. This mainly implies the role of ATG8 core proteins and their receptor interactors in the regulation of autophagy in form of selective or non-selective during environmental stresses.

Conclusions: Autophagy, a conserved mechanism for cell survival in plants will be activated in response to the thermopriming which is a promoted acquired resistance stimulus. Determined key genes and components of autophagy associated with thermal priming signaling pathway could be noteworthily employed to study transcriptional regulation of autophagy and integrated defense system against environmental stresses for the improvement of plant thermal tolerance and resistance to the pathogens.

Keywords: Autophagy, Gene Expression, Meristem, Priming, Stress

1. Background

Plants have developed different mechanisms to resist pathogens and environmental stresses. Autophagy is a survival mechanism that protects cells against undesirable environmental conditions such as microbial pathogen infections, nutrient starvation, salt and drought stresses, oxidative stress, aggregated damaged proteins, etc. Autophagy proceeds the degrading of invading agents like bacteria and viruses and old and damaged organelles and therefore leads to reduce cell consumption and uses their raw to produce new components and material (1-5).

Studies regarding the role of autophagy in plant pathology are mainly limited to the function of some genes such as *ATG2*, *ATG5*, *ATG6*, *ATG7*, *ATG8*, *ATG9*, *ATG10*, *ATG18a* against model pathogens and abiotic stresses (6-12); However, some pathogens have obtained the ability to use their own or host autophagy mechanism to overcome the autophagic host defense as their pathogenicity factor (13, 14). Hence, it is important to study autophagy in immunity and defense responses upon plant stresses.

High temperature as an important climate change factor retards plant growth and notably reduces crop yields

(15). Therefore, uncovering the molecular basis of plant responses and tolerance to heat stress will help genetic breeders to maximize crop yields under adverse environmental conditions. One possible strategy for improving the plant's ability to withstand heat stress is to stimulate the plant by moderate heat stress treatment (heat priming). This helps the plant physiologically to cope with subsequent exposure to normally lethal levels of heat stress. In another word, it establishes a stress memory and plant acquire thermotolerance (16). While without priming, plants will meet dead or very weak states after a severe stress (17-19). Priming enhances multiple defence responses and primed plants display longer-lasting activation or attenuated repression of defence upon challenge than unprimed plants (20).

Autophagy is efficiently related to priming. Research has shown that autophagy mediates the specific degradation of heat shock proteins (HSPs) at later stages of the thermorecovery phase, leading to accumulation of protein aggregates after the second heat shock and a compromised heat tolerance. Also, autophagy mutants retain HSPs longer than wild type and concomitantly display improved thermomemory (21).

Plant response to stresses is somehow complicated and including transcriptomic changes which lead to physiological differences. Abiotic stresses may affect plant susceptibility to pests and pathogens. Activation of multidirectional defence signals would provide the beneficial effect of integrated disease-pest management. It is controlled by a wide range of molecular mechanisms working together in a complex regulating network. Forty ATG and receptor ATG (rATG) are recognized in Arabidopsis incorporating in autophagy mechanism, but their function, as well as transcription regulators, are mostly unknown. The first step to study the regulating of autophagy transcriptome is to find expression patterns of autophagy-related genes in response to a stimulus (thermopriming) which is preferably able to exhibit a balance of immunity and growth threshold. Transcriptomic changes versus an autophagy-inducing treatment start normally in few minutes to hours. Therefore, identifying the key genes responding in this system is aimed in this study.

Meristem is including a pool of pluripotent stem cells able to maintain themselves and produce cells needed for organ development. Hence, cell division, stem cell maintenance, and their integration into organ meristems are the basis of plant development after embryogenesis. Thereby, plant development under stress is dependent on the meristem (22, 23). In these conditions, the shoot apical meristem (SAM) of plant seedling is dominant to the other tissues and has a pivotal role in defence signalling and memorizing to survive the plant. SAM ultrastructural changes have been observed due to heat and oxidative stress in Arabidopsis (24) and salinity stress and autophagy activity in Canola (25), but the molecular integration of stress and autophagy in SAM has not been studied so far.

2. Objectives

In the context of plant-stress molecular interaction, investigation of autophagy and priming that has several aspects including temperature, growth and development, reproductivity, defence signalling, and also finding a balance between them is of great importance. Hence, this study aimed to investigate the autophagy genes expression level in given time-points after thermoprimig phases, to determine the effectiveness of autophagy on thermopriming induced defence mechanism in SAM of young Arabidopsis.

3. Materials and Methods

3.1. Plant Source and Seed

Shoot apices including SAM tissue of 5-day old Arabidopsis were aimed to study. For this, *Arabidopsis thaliana* Columbia 0 ecotype (Col0) seeds were disinfected for 10 min with sterilization suspension containing 5 mL ethanol 70% and two drops of Triton X-100. Seeds were stored for 2 days in the darkroom at 4 °C for stratification. Then, 60 seeds were sown in square plates (12×12 cm) containing 70 mL Murashige & Skoog (MS) culture media (pH~5.7) plus 1% sucrose. Seedlings were grown continuously for 5 days in long days conditions (16-h light/8-h dark cycle, start at 9 am, light intensity 160 μmoL m^{-2} s^{-1}), at 22 °C with 60% relative humidity.

3.2. Thermopriming

Each biological replicate was including 4 plates, of which for control (without priming) and others for priming treatment according to the protocol published in (18). Briefly, thermopriming treatment was consisting of 90 min at 37 °C, followed by 90 min at 22 °C, 45 min

at 44 °C. Waterproof Leukopor tape was removed and plates returned to phytotron 22 °C until sample harvest time.

3.3. Plant Tissue Harvest

Shoot apices of 5-day-old Col-0 seedlings (5 days after sowing) were harvested under the microscope at 4 h and 24 h after subjecting to the thermopriming treatment. For each time point, a primed (P) and unprimed (C) plate was harvested to their corresponding sample microtubes. Three biological replicates were collected for each sample and there contained approximately fifty SAM tissues. Finally, 4 samples were prepared for each biological replicate: 4 h control (4C), 4 h primed (4P), 24 h control (24C), and 24 h primed (24P).

3.4. RNA Isolation-cDNA Synthesis

RNA isolation of the three biological replicates was performed using Qiagen RNasy mini kit (Qiagen, Hilden, Germany). RNA quantity (ηg. μL^{-1}) and quality ($A_{260/280}$ ratio ~ 2.0) for each sample were calculated by NanoDrop system ND-1000 UV–Vis spectrophotometer (Nano-Drop Technologies, Böblingen, Germany). Genomic DNA digestion was performed via TURBO DNA- free™ kit (Ambion/ Applied Biosystems™, Lithuania, Vilnius), and cDNA synthesis was carried out by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific™/ Invitrogen, Darmstadt, Germany). All of these were performed according to the manufacturer's instructions.

3.5. Quantitative Real-Time /Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Real-time PCR detection system CFX Connect (Bio-Rad Laboratories, California, USA) was used to carry out qRT-PCR for cDNA qualification and gene expression measurements. The reactions were performed in triplicates with SYBR® Green-PCR Master Mix (Applied Biosystems/ThermoFisher Scientific, Massachusetts, Vereinigten Staaten, USA) and specific qRT-PCR primers. Name and accession number of 40 Gene of interest (GOI) *ATG* and *ATG*-receptors and 4 Reference Gene (Ref) in Arabidopsis thaliana was obtained from *The Arabidopsis Information Resource* (TAIR) database. Then, published resources were used for qRT-PCR primer sequences for GOI (21, 26, 27) and Ref. (28-30) and ordered to Eurofins genomics company (Berlin, Germany). Each measurement was performed on 10 µL volume sample solution including 5 µL 2× buffer, 4 µL mix forward and reverse primer (0.5 µM) and 1 µL cDNA in sterile 96-well qRT-PCR plates. Reactions were performed in a program including 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 10 sec followed by 60 °C for 45 sec. and 1 cycle of 95 °C for 10 sec. In order to qualification and measurement analysis, raw data were exported by CFX Manager Software version 3.0 according to cycle threshold (Ct) calculated in Excel 2010.

3.6. Data Analysis

Raw data were qualified and analyzed by Eq. 1 and 2 (30) and Eq. 3 (31). *ACTIN2* (AT3G18780) and *UBQ10* (AT4G05320) were used as marker genes for cDNA quantification (20< Ct <18) and *GAPDH* (AT1G13440) for qualification (Eq. 1). Data were normalized to the housekeeping (reference) gene *SAND* (AT2G28390) as the internal control. Gene expression values were calculated at 40-ΔCt, $2^{-\Delta\Delta Ct}$ and log2FCh using the comparative Ct method for approximately 40 *ATG* genes, relative to their biological control (31).

$2^{\Delta Ct}<3$ Eq. 1

$$\longrightarrow \quad 2^{-[mean(Ct_{GAPDH5'})-mean(Ct_{GAPDH3'})]} < 3$$

40-ΔCt Eq. 2

$$\longrightarrow \quad 40 - \left[mean(Ct_{GOI}) - mean\left(Ct_{Ref}\right)\right]$$

$2^{-\Delta\Delta Ct}$ Eq. 3

$$\longrightarrow \quad 2^{-\left\{\left[mean\left(Ct_{GOI}^{treatment}\right)-mean\left(Ct_{Ref}^{treatment}\right)\right]-\left[mean\left(Ct_{GOI}^{control}\right)-mean\left(Ct_{Ref}^{control}\right)\right]\right\}}$$

For each biological replicate, an average of three or two converge technical replicates were calculated for all of GOI and Ref. then, the relative expression of each gene was determined with an average of normalized Ct data. Statistical analysis was performed by a two-tailed Student's *t*-test. Data presented as the mean ± standard error (SE) and $p<0.05$ considered as significant differences between experimental and control treatments.

3.7. In Vivo Observation of Autophagy Induction

Seeds of *Arabidopsis thaliana* (Col-0) transgenic by *PromUBQ10:GFP-ATG8a* construct (21) (carrying coding region of the reporter gene of green fluorescent protein (GFP) fused to *ATG8A* as a molecular marker of autophagy) was sown and grown as described above for non-transgenic seeds. Plates containing 7-day old seedlings were subjected to thermopriming and then to the growth chamber for about 48 hours. Seedlings were treated in an MS culture medium containing 1 µM conconamycin A to raise the pH in vacuolar lumens to inhibit vacuolar hydrolases. This resulted in the accumulation of autophagic bodies in the vacuoles during imaging. Pictures were taken 48 h after thermopriming by Leica DM6000B/SP5 confocal laser scanning microscope (CLSM, Leica Microsystems, Wetzlar, Germany). SAM of three seedlings was screened for each primed and unprimed (control) state.

4. Results

4.1. Autophagy-Related Genes Induction Upon Thermopriming in SAM of Arabidopsis Thaliana

Transcript analysis of high-quality cDNA of *ATG*s (data not shown) in shoot apices of 5-day old Arabidopsis seedlings indicated the presence of autophagy induction upon thermopriming in two-time points of 4h (first) and 24h (second). The results presented in **Figure 1** shows that thermopriming-induced autophagy doesn't have the same effect on the expression of all genes. Most of the *ATG*s expressions were increased compared to the control. That implies the effect of thermopriming on autophagy induction in SAM. Fast upregulation of *ATG*s after 4h indicated the impact of defensive autophagy mechanism subsequent to thermopriming as an inducer.

Figure 1 is the heat map of up-and down-regulation of *ATG*s expression relative to their phasic control, 4P to 4C and 24P to 24C. This represents the differentiated expression of *ATG1*, *ATG5*, *ATG8A*, *ATG8B*, *ATG8F*, *ATG8G*, *ATG8H*, *ATG13B*, *ATG18B*, also *rATG* including *ATI1* (ATG8-Interacting Protein 1), *ATI2*, *NBR1* (Neighbour of BRCA Gene1), and *TSPO* (Tryptophan-Rich Sensory Protein-related Outer Membrane).

The expression pattern of the genes would be described as following. *ATG8A*, *ATG8G*, *ATG8H*, *ATG13B*, *ATG18B*, and *ATI1* were induced upon thermopriming and their expression has been increased in the first phase but decreased in the second phase. So, those are introduced as early-short response *ATG*s (**ESRG**s). Induction of *ATG5* and *ATG8B* caused ascending expression in 4h after thermopriming and stayed constantly high till 24 h, and would be grouped as early-long response *ATG*s (**ELRG**s). While *ATG8F* and *ATG1C* induction didn't have a big fold change in 4h and increased expression was visible in 24 h, and would be grouped in late response *ATG*s (**LRG**s). *ATI2*, *NBR1*, and *TSPO* which have been strongly induced during two phases would be grouped as strong response genes (**SRG**s) which are more related to selective autophagy. Some genes like *ATG1A*, *ATG1B*, *ATG3*, *ATG4B*, and *ATG8I* had a (little) descending expression in the first phase but were ascending in the second phase, hence grouped as negative short response *ATG*s (**NSRG**s). *VPS15* (Vacuolar Protein Sorting 15) and *VPS30* expression have not been much notably changed during the first two phases, but their expression tends to follow a mild trend of NSRGs. Also, the expression of *ATG8D*, *ATG8E*, *ATG9*, *ATG16*, *ATG18G*, *ATG18A,* and *ATG20* has not been significantly changed after thermopriming phases.

4.2. Thermopriming-Associated Autophagy Genes

Autophagy induction upon thermopriming revealed several expression groups of genes. Hereby, core *ATG8*s which coexpressed r*ATG* would be introduced as thermopriming-associated autophagy-related genes and dominant *ATG*s in SAM. **Figure 2** (40-ΔCt expression level), shows the high expression level of these *ATG*s in SAM, that first of all presents the pivotal role of autophagy in SAM and emphasizes the assumed importance of choosing this target tissue. Core *ATG8*s had significant expression compared to their control in the first phase while this was observed for r*ATG* in the second phase

Genes	phase 1 4P	phase 2 24P	Genes	phase 1 4P	phase 2 24P
ATG1A	-0.61302	0.362	ATG12A	-0.239	0.333
ATG1B	-0.370	0.626	ATG12B	-0.323	0.154
ATG1C	0.194	1.618	ATG13A	0.365	0.888
ATG3	-0.556	-0.019	ATG13B	1.045	0.284
ATG4A	0.010	0.665	ATG16	0.440	0.598
ATG4B	-0.583	0.689	ATG18A	0.498	0.457
ATG5	1.000	1.182	ATG18B	1.092	0.305
ATG6	0.728	0.223	ATG18C	0.028	0.804
ATG7	0.572	0.153	ATG18D	-0.743	0.073
ATG8A	1.008	0.642	ATG18F	0.361	0.169
ATG8B	1.374	1.332	ATG18G	0.117	0.137
ATG8C	-0.068	0.768	ATG18H	0.503	0.164
ATG8D	0.292	0.496	ATG20	0.172	0.373
ATG8E	0.663	0.524	ATI1	1.122	0.713
ATG8F	0.131	1.029	ATI2	0.612	1.750
ATG8G	1.953	0.793	NBR1	1.726	1.952
ATG8H	1.600	1.231	VPS15	-0.199	0.317
ATG8I	-0.245	0.608	VPS34	-0.015	0.232
ATG9	0.348	0.245	TSPO	1.789	2.903
ATG10	-0.486	-0.036			

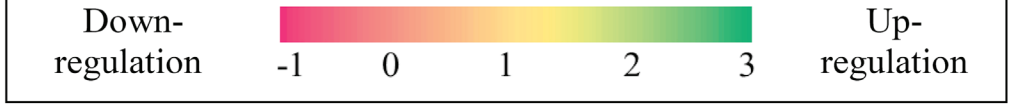

Figure 1. Relative expression of the ATG genes in shoot apices of 5-day old Arabidopsis thaliana (Col-0) seedlings, 4 h and 24 h after thermopriming. Data were normalized by *SAND* as a reference gene. Heat map showing the fold change (log2 basis) of the relative expression compared to their controls (4C for 4P in the first phase and 24C for 24P in the second phase). Values are means ± SE (n=3). Green: upregulated; red: downregulated; the scale bar shows the fold change value. Abbreviations: Control 4 h (4C); Primed 4 h (4P); Control 24 h (24C); Primed 24 h (24P).

and especially for *TSPO* in both phases.

ATG8G is the most elevated *ATG8* despite decreased expression during the recovery phase, so that remained still high in 24h. *ATG8* A, B, and H that had similar expression trends are headings of three classes in evolutionary Neighbour–Joining tree of *ATG8*s as published by (32). Out of nine *ATG8*s, Core *ATG8*s are differentially expressed in SAM. This implies that each *ATG*8 homolog may have a distinct function instead of redundancy. Also, this is declared by (32) because of different expressions of *ATG8*s in different regions of root apical meristem.

4.3. Autophagy Detection after Thermopriming in SAM of Arabidopsis Thaliana

In addition to transcript level, autophagy induction would be assayed *in vivo* at protein state in SAM. For this purpose, Col0 plants expressing AtATG8A-GFP protein were used to investigate the autophagosome formation in the shoot apices of Col-0 plants in both control and primed conditions under the confocal microscope. GFP fluorescence was observed in many ring-shaped

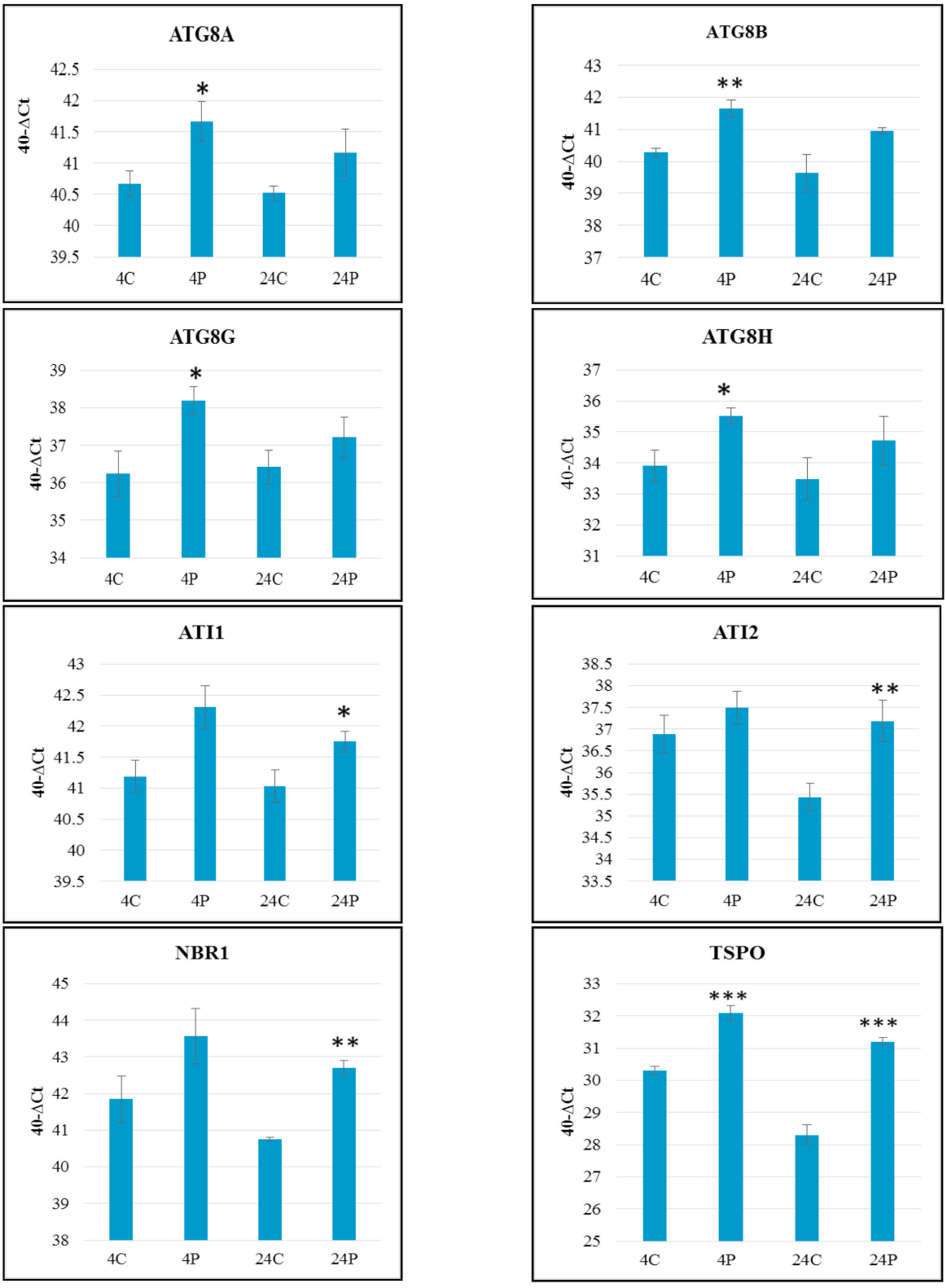

Figure 2. Relative expression level of the *ATG* selected genes in shoot apices of 5-day old Arabidopsis (Col-0), 4 h and 24 h after thermopriming presented as 40-ΔCt. Data were normalized by *SAND* as a reference gene. Values are means ± SE (n=3). Statistical significance was calculated using Student's T-test (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). Abbreviations: Control 4 h (4C); Primed 4 h (4P); Control 24 h (24C); Primed 24 h (24P).

and punctate structures, which corresponded to autophagosomes and their intermediates. Because of autophagy disruption and no autophagosome formation after thermopriming in transgenic *atg5/GFP-ATG8a* Arabidopsis plant, autophagic bodies structure in Col-0/*GFP-ATG8a* could determine autophagy induction (21). Accumulation of autophagic bodies in the SAM seems more abundant in primed condition compared to the control after 48 h of thermopriming (**Fig. 3**). It shows the induction of autophagy subsequent to thermopriming (in primed) and the presence of the basic level of autophagy as a housekeeping work (in control). These are in the proteomic level that confirms the measured transcription level in SAM explained in section 4.1.

5. Discussion

Defence signalling in SAM usually processes through reactive oxygen species, mitogen-activated protein kinases, and phytohormones to respond to environmental stresses while doing cell division (33). According to the described results, autophagy is recommended as a potential protection system for the homeostasis of SAM. Hence, more investigations are needed to identify the role of autophagy in the induction of immunity and defence response networks in SAM.

Induced autophagy following the priming in SAM (**Fig. 1**) showed that the expression level of each gene varies in different fold changes that confirm the presence of a regulating network in autophagy transcriptome level. Although, some key regulators in the human and yeast autophagy system are identified, regulating transcription factors of plant autophagy are still almost unknown. Thereby, more investigation of plant autophagy regulators and their regulating network of *ATG*s are proposed. Since transcription factors (TFs) of some *ATG8*s by gene ontology (GO) enrichment analysis have been indicated responsive to various stresses and hormones and involved in salicylic acid-mediated systemic acquired resistance, cellular response to glucose stimulus, and abscisic acid-activated signalling pathway (34), it would be suggested to unravel linking points between autophagy and phytohormone defensive pathway and even reactive oxygen species.

The expression pattern of *ATG*s described in several groups of genes considers the intensity and time of the induction of genes. The increasing expression level of several *ATG*s in the second phase shows that the induction still exists after 24 h. However, it is known

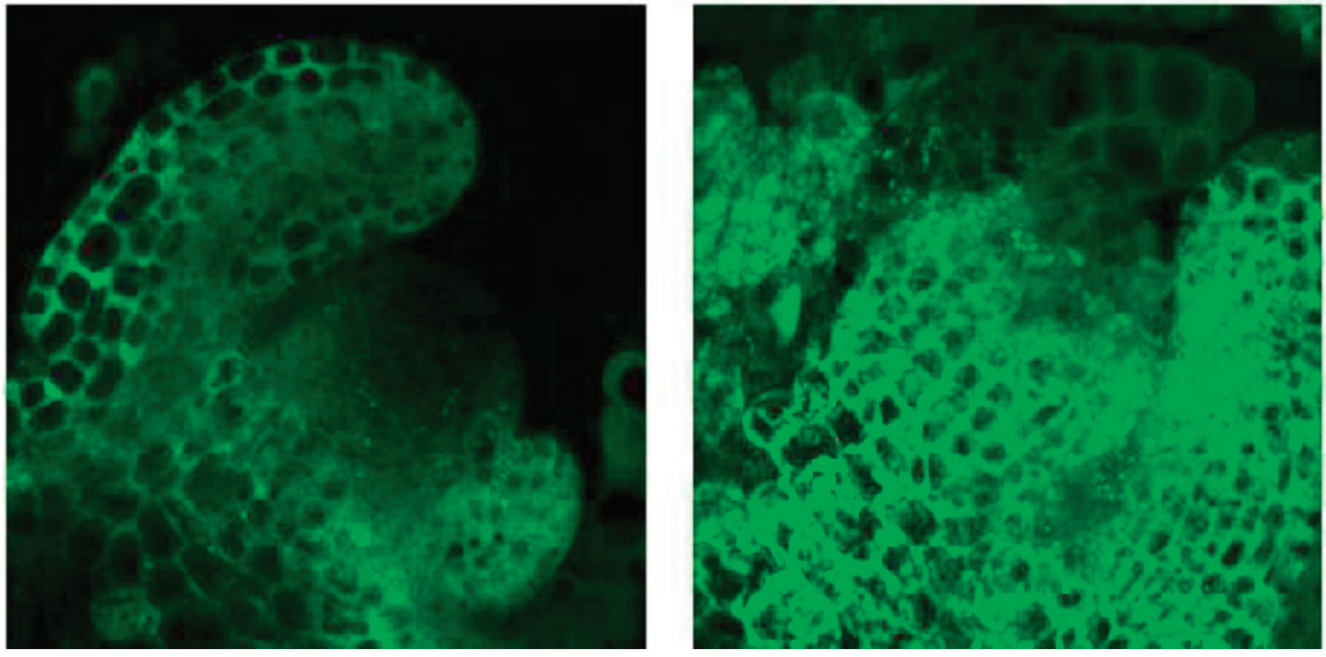

Figure 3. Autophagy induction 48h after thermopriming in shoot apical meristem of Arabidopsis thaliana (Col-0/*GFP -ATG8a*). Left: control; Right: primed.

that autophagy is not active for more than 2 days in transcriptomic or 3 days in proteomic levels upon thermopriming (21). Remarkably, the increasing trend of expression of NSRGs is so milder than SRGs. This would be related to the function of SRGs that are involved in selective autophagy.

ATI1, *ATI2*, *NBR1*, and *TSPO* are involved in selective autophagy as receptors incorporating with ATG8s response in biotic and abiotic stresses, through recognition of targeted cell cargos by specific interaction with special receptors in a delicate programmed pathway (35-43). Furthermore, it has been assessed that ATG8s are responsive to various abiotic stresses and have a distinct expression pattern in the parts of plants (32, 34, 44). However, their functional role was not studied in SAM.

Herein, core *ATG8*s are introduced as capital *ATG*s in SAM between 40 autophagy-related genes. Also, *ATG8 A*, *B*, *F*, *G*, and *H* are highlighted as the relevant thermopriming-expressed *ATG8*s corresponding with elevated r*ATG* expression implying the probable presence of selective autophagy in SAM. Therefore, more investigation on autophagy flux at the ultrastructural level of SAM is proposed to identify any evidence for the presence of selective autophagy in SAM at the protein level.

6. Conclusion

This work showed that by a mild stress (thermopriming), autophagy as one of its subsequent signalling networks has been activated in plant apical meristem and relevant thermopriming-induced autophagy genes were identified. Autophagy, bulk or selective, as a plant protective mechanism that may induce broad-spectrum or specific defence response could be considered a great solution for enhancing plant resistance against pathogens and abiotic stressors. Therefore, uncovering potential molecular links between the defence signalling pathways mediated by autophagy would be an interesting challenge in the field of plant stress co-interactions. Identification of key regulators of both biotic and abiotic stress response linking defence pathways provides opportunities to achieve stress-resistant crops with a wide range of stresses and leading to enhanced yield.

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