Autophagy at Crossroads: Modulating Responses to Combined Stresses, Unveiling Metabolic Shifts and Bacterial Dependencies

Short title: Autophagy: Autophagy at the Nexus of Combined Stresses

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Abstract

Plants face diverse stresses in natural environments, necessitating complex responses for survival. Abiotic and biotic stress responses are typically counteractive, posing challenges for breeding crops resilient to multiple stresses. Autophagy, a cellular transport process, plays a vital role in plant stress response, facilitating the degradation of cellular components and enabling nutrient recycling. Here, we asked what the role of autophagy is in combined abiotic (heat) and biotic (bacterial infection) stress. We introduce a conceptual framework based on assays monitoring autophagy activation, bacterial infection, and metabolic profiling.

Our findings reveal the engagement of members of the autophagy-related 8 (ATG8) protein family in stress-specific activation. Notably, we observed that heat stress facilitates bacterial growth in an autophagy-dependent manner. Metabolic profiling highlights effector-dependent shifts in nutrient availability during stress, influencing bacterial performance. Our study challenges the assumption that combined stresses are simply the sum of individual responses as exemplified by activation of the autophagic pathway. Instead, it establishes autophagy as a pivotal link connecting environmental factors and plant-microbe interactions. Insights for our study can present a novel perspective for designing strategies to enhance crop resilience in the face of multifaceted challenges.

Introduction

Field-grown plants face many stresses, both abiotic (e.g., drought, temperature) and biotic (e.g., pathogens), requiring intricate physiological and molecular strategies for survival. Understanding these strategies has become more urgent due to climate change and the spread of new pests and diseases linked with these changes (Luck et al., 2011; Cavicchioli et al., 2019). Moreover, the nature and severity of stresses encountered by crops are predicted to worsen (Bebber, 2015; Hey et al., 2010). Abiotic and biotic stress responses are typically perceived to be counteractive, thus posing a significant challenge for breeding crops resilient to multiple stresses (Zhang and Sonnewald, 2017). Despite their distinct nature, there are intriguing similarities in the signaling patterns of biotic and abiotic stress responses, such as changes in phytohormone levels, calcium influx, reactive oxygen species (ROS) production, and the involvement of specific transcription factors for stress-induced gene expression (Atkinson and Urwin, 2012; Fraire-Velazquez et al., 2011; Jones and Dangl, 2006; Glazebrook, 2005; Dodds and Rathjen, 2010; Islam et al., 2018; Ohama et al., 2017; Zhu,

2016). Another common feature of these stresses is the redirection of energy resources from growth to stress responses (Signorelli et al., 2019).

Autophagy, a conserved eukaryotic mechanism, is vital for cellular component recycling and energy sourcing (Yang and Klionsky, 2009; Feng et al., 2014; Masclaux-Daubresse et al., 2020; Liu and Bassham, 2012). In plants, three types of autophagy have been described: Micro-, Macro-, and Megautophagy (Liu and Bassham, 2012; van Doorn and Papini, 2013; Marshall and Vierstra, 2018). Macroautophagy (hereafter called autophagy) is extensively studied and involves the formation of cytosolic double-membrane vesicles called autophagosomes. Autophagosomes deliver cytosolic cargo, which can be randomly (bulk autophagy) or selectively acquired (selective autophagy), to the vacuole for degradation (Liu and Bassham, 2012). Though autophagy functions in both plant biotic and abiotic stress response (Signorelli et al., 2019), little is known regarding its role in combined stresses. Here, we focused on the role of autophagy in the combination of heat stress (abiotic stress) and bacterial infection (biotic stress).

Autophagy progression is divided into distinct steps and, in plants, involves more than 40 autophagy-related (ATG) proteins (Liu and Bassham, 2012; Yang and Klionsky, 2010; Chen and Klionsky, 2011; Chung, 2019; Wang et al., 2021). *ATG* genes are regulated on a transcriptional level, and *ATG* transcript availability and timing are critical for an appropriate stress response (Magen et al., 2022). Plant transcription factors were shown to confer abiotic stress resistance by inducing *ATG* gene expression and, consequently, autophagy (Wang et al., 2015; Zhu et al., 2018). The WRKY transcription factor family is particularly interesting regarding combined stress. WRKY33 induces autophagy in response to both abiotic and biotic stresses by activating the transcription of *ATG5* and *ATG7*. It is involved in heat tolerance in different plant species and, in addition to *ATG* genes, induces the expression of defense-related genes in *Arabidopsis thaliana* (Arabidopsis) (Lai et al., 2011; Schmidt et al., 2020; Mao et al., 2011; Lippok et al., 2007; Zhou et al., 2014; Zheng et al., 2006). Arabidopsis knockout mutants of *ATG* genes (*atg* mutants) are hypersensitive to different stresses, while overexpression of *ATG* genes improves plant fitness and performance (Liu and Bassham, 2012; Slavikova et al., 2008; Minina et al., 2018; Magen et al., 2022; Zhang et al., 2021a). ATG proteins critical for stress response include ATG5, ATG7, and the selective autophagy receptor NBR1.

Heat can have cytotoxic effects resulting in cellular metabolism and homeostasis perturbations due to protein denaturation and aggregation. Plant autophagy can remove heat-induced protein aggregates (Baxter et al., 2014; Zhou et al., 2013; Zhou et al., 2014). In

addition, NBR1-mediated selective autophagy was shown to play a crucial role in the recovery from heat stress by regulating heat stress memory (Thirumalaikumar et al., 2021). NBR1 recruits cargo to the autophagosome by interaction with ATG8, a small ubiquitin-like protein, which undergoes lipidation by Phosphatidylethanolamine upon autophagy induction (Svenning et al., 2011; Zhou et al., 2013). The inner autophagosomal membrane, associated proteins (ATG8, NBR1), and cargo undergo degradation within the vacuole. The number of ATG8 isoforms varies among plant species, expanding from one isoform in algae to multiple isoforms in vascular plants (Zess et al., 2019). Notably, ATG8 exhibits a non-canonical function in response to heat stress, aiding the Golgi apparatus reassembly in Arabidopsis after acute heat stress (Zhou et al., 2023).

The role of autophagy in biotic stress response is more complicated and depends on the pathogen's lifestyle (Sertsuvalkul et al., 2022). Bacterial plant pathogens, residing extracellularly, employ a type 3 secretion system (T3SS) to deliver effectors into the plant cell, manipulating cellular processes, including autophagy, for their benefit (Üstün et al., 2016; Lal et al., 2020; Leong et al., 2022b). NBR1-mediated selective autophagy can antagonize the effector-driven virulence (Leong et al., 2022b; Üstün et al., 2018). Besides bacterial pathogens, the oomycete *Phytophthora infestans* inhibits autophagy through effectors, countered by NBR1-mediated selective autophagy (Pandey et al., 2021; Dagdas et al., 2016; Dagdas et al., 2018). In addition, overexpression of *ATG5* or *ATG7* increased resistance against the necrotrophic fungal pathogen *Alternaria brassicicola*, and other examples support a positive role for autophagy in defense responses against necrotrophic plant pathogens (Minina et al., 2018; Lai et al., 2011; Lenz et al., 2011; Patel and Dinesh-Kumar, 2008). In line with these findings, autophagy plays a negative role in defense responses against obligate biotrophic pathogens (Wang et al., 2011). In contrast to oomycetes and bacterial pathogens, plant viruses, present intracellularly, are removed directly through NBR1-dependent selective autophagy (Hafrén et al., 2017; Hafrén et al., 2018).

Despite the significance of combined stresses in real-world natural and agricultural settings, research on their impact, particularly involving autophagy, remains limited. Understanding how plants respond to combined stresses is crucial for ensuring crop fitness and productivity in the face of climate change. The interaction between plant stress responses can be synergistic. Still, in most cases, responses to combined stresses differ significantly from those to single stresses (Rasmussen et al., 2013; Pandey et al., 2015; Ramegowda and Senthil-Kumar, 2015; Zhang and Sonnewald, 2017). Plant autophagy as a pathway involved in various stress responses has an exciting potential to increase plant tolerance towards

combined stress scenarios with broad agricultural implications. Here, we investigate the pivotal role of autophagy in orchestrating the response to combined stresses. We chose a dual stress paradigm involving elevated temperature followed by infection with the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease on pepper and tomato. Autophagy was shown to contribute to plant defense against *Xanthomonas* pathogens, while *Xcv* targets autophagy to inhibit appropriate defense responses (Yan et al., 2017; Leong et al., 2022b).

Results

Stress-specific activation of ATG8 isoforms

Plants face diverse stresses requiring specific responses, with autophagy playing a crucial role (Avin-Wittenberg, 2019; Sertsuvalkul et al., 2022). Understanding stress-specific activation of autophagy remains largely unclear. We focused on the ATG8 protein family, which functions in target recognition and cargo binding. We speculated that ATG8 isoforms participate in stress-specific autophagy, which is yet to be determined (Kellner et al., 2017). Degradation of GFP-ATG8 fusion proteins in the vacuole and release of free GFP was investigated as a proxy of autophagic flux (Klionsky et al., 2021). We investigated the seven *St*ATG8 isoforms in potato (*Solanum tuberosum*), exploring their roles in stress-induced autophagy.

GFP-tagged versions of all seven *St*ATG8 isoforms were transiently expressed in *Nicotiana benthamiana*. The plants were then subjected to different autophagy-inducing stress conditions (darkness, heat, *Xcv* infection) (Avin-Wittenberg et al., 2015; Leong et al., 2022b; Zhou et al., 2014). Prolonged darkness leads to carbon starvation, widely known to induce autophagy, presumably in a non-selective manner (Barros et al., 2017). In contrast, pathogen infection or extreme temperatures require more specific plant responses. Carbon starvation was induced by three days of darkness, leading to increased degradation of all GFP-*St*ATG8 isoforms and high levels of free GFP (Fig1A). Heat stress reduced protein levels of all GFP-*St*ATG8 isoforms after 6 h and 8 h of 40°C (Fig1B). However, ATG8 degradation differed in timing and intensity. While some isoforms responded quickly (after 6 h), others showed a slower reduction upon stress (after 8 h). GFP-*St*ATG8-3.1 and GFP-*St*ATG8-4 showed a quick, strong, and lasting decrease in protein levels.

We next tested the effect of infection with *Xcv*. Although *N. benthamiana* is not a natural host of *Xcv*, the *Nicotiana benthamiana* mutant line Δ*roq1* (Grützner et al., 2021) can be infected by the bacteria. Only a subset of GFP-*St*ATG8 isoforms was degraded upon *Xcv* challenge

(Fig1C). The GFP-*St*ATG8-2 isoforms, GFP-*St*ATG8-3.1, and in some but not all experimental replicates, GFP-*St*ATG8-4 displayed increased degradation upon *Xcv* infection. GFP-*St*ATG8-1 isoforms showed the opposite effect. Interestingly, the *Xcv*-specific activation of GFP-*St*ATG8 isoforms was independent of bacterial effectors. Infection with the T3SS mutant *Xcv* Δ*hrcV* (hereafter called *hrcV*), lacking the effector secretion system, resulted in a GFP-ATG8 degradation pattern similar to that of *Xcv*. Surprisingly, the detected degradation band was not free GFP, unlike observed for carbon starvation. These results indicate a specific autophagic response to stress, particularly to *Xcv*/bacterial infection, unlike the non-specific reaction to general stressors like carbon starvation.

Differential autophagy activation under combined stress compared to single stresses

As *St*ATG8-3.1 was degraded upon heat stress as well as after inoculation with *Xcv*, we were interested in the degradation of this isoform in response to a combined heat-*Xcv* challenge. As performed in the single-stress experiments, GFP-*St*ATG8-3.1 was transiently expressed in *N. benthamiana* Δ*roq1* plants. The plants were then subjected to 6 h heat stress or 6 h *Xcv* infection. In addition, 6 h heat stress was followed by subsequent 6 h *Xcv* or a 6 h recovery period without *Xcv* inoculation (Fig2A). Although *Xcv* inoculation was the second and, therefore, more recent treatment, GFP-*St*ATG8-3.1 degradation after combined stress treatment resembled heat stress instead of *Xcv* infection (Fig2B).

ATG genes are transcriptionally regulated upon stress. We tested native transcript levels in tobacco plants subjected to single and combined stresses. *NbATG7* mRNA levels were reduced after 6 h heat stress and 6 h *Xcv*, respectively. They were further reduced after combined heat-*Xcv* stress. Unfortunately, this trend, though visibly striking, was not statistically significant (FigS1). In contrast, *NbJOKA2* (the *NBR1* homolog in solanaceous species) showed no transcriptional effect on the single or combined stress. These findings affirm that combined stresses surpass the sum of individual stress effects, highlighting autophagy's role in responding to such combined stress.

Heat stress facilitates bacterial growth in an autophagy-dependent manner

Since the degradation pattern of GFP-*St*ATG8-3.1 after combined heat-*Xcv* stress showed no signs of response to the bacterial infection, we next asked how a preceding heat stress affects bacterial infection. We syringe-infiltrated *Xcv* into *N. benthamiana* Δ*roq1* as well as its natural host tomato (*Solanum lycopersicum*) M82 plants. Bacterial titers were checked 3 days post-

185 infection (dpi) (Fig3A). *Xcv* grew better in heat-stressed plants in both species (Fig3B and C).
186 This beneficial effect of preceding heat stress on bacterial growth was dependent on
187 autophagy. In the presence of the autophagy inhibitor 3-Methyadenine (3-MA), preceding
188 heat stress did not affect bacterial growth. In addition, the T3SS mutant *hrcV* did not benefit
189 from previous heat stress in plants (Fig3D), making the increase in bacterial growth after heat
190 stress dependent on bacterial effector delivery. Since *NbATG7* showed a transcriptional
191 reduction after combined heat-*Xcv* stress (FigS1), we tested bacterial performance with and
192 without preceding heat stress in plants transiently expressing *AtATG7*. In plants transiently
193 overexpressing *AtATG7*, *Xcv* grew similarly in heat-stressed and unstressed plants (Fig3E).
194 These results confirm our previously observed reduced immune response to bacterial infection
195 in heat-stressed plants. The results also confirm that autophagy is involved in the response to
196 combined stress.
197

198 **Differential *At*ATG protein degradation upon combined stress is autophagy-dependent**
199 To test how changes in autophagic activity affect the response to combined stresses, we
200 changed the plant system and used available *ATG* overexpression and knockout Arabidopsis
201 lines (Minina et al., 2018). Since Arabidopsis is not a host for *Xcv*, we used the bacterial
202 elicitor flg22, a 22 amino acid peptide of the bacterial protein Flagellin, known to induce
203 autophagy (Rodriguez et al., 2020). Transiently expressed *St*ATG8-3.1 showed a reduced
204 response to the biotic stress (*Xcv*) when preceded by heat stress (Fig2B). To test the
205 conservation of this response, we checked GFP-*At*ATG8a degradation in stable
206 overexpression lines treated with heat stress, biotic stress (flg22), and a stress combination
207 (Fig4A). As seen for GFP-*St*ATG8-3.1 in *N. benthamiana*, *At*ATG8a was degraded in
208 Arabidopsis to a similar level in the combined stress scenario and the single heat stress
209 (Fig4B).
210 We next examined the response of other ATG proteins following combined stress.
211 Overexpressed *At*NBR1-TAP was degraded differentially after heat stress and flg22 (Fig4C).
212 While heat stress led to an accumulation of *At*NBR1, flg22 treatment reduced *At*NBR1 protein
213 levels. After combined stress, *At*NBR1-TAP band intensities were mid-range between heat
214 and flg22 stress (Fig4C). In wildtype plants, protein levels of native *At*ATG7 were lowest
215 under combined stress (Fig4D). This effect was more pronounced in *AtATG5* and *AtNBR1*-
216 TAP overexpression lines and absent in *atg5-1* plants. The results suggest that *At*ATG7 is
217 degraded in an autophagy-dependent manner upon combined stress. Taken together, our
218 results suggest a dynamic involvement of different components of the autophagy pathway in

219 response to combined stress. Depending on the component, combined stress either mimics or
220 intensifies the reaction to one of the individual stresses or shows an intermediate response
221 between the single stresses.

222

223 **Autophagy affects *FRK1* gene expression under single and combined stresses**

224 Exploring the impact of autophagic activity on stress response transcription, we assessed the
225 expression of stress marker genes in stable Arabidopsis *ATG* overexpression and knockout
226 lines under single and combined stress conditions. Despite highly consistent results across two
227 independent experiments, the lack of significance may be attributed to substantial biological
228 replicate variation. In wildtype plants, the flg22 marker gene *AtFRK1* (At2g19190) increased
229 1 h after flg22 treatment, as expected (Winter et al., 2007)(http://bar.utoronto.ca/efp/cgi-
230 bin/efpWeb.cgi) and showed a pronounced increase in samples that received the combined
231 stress (FigS2B). *AtFRK1* expression increased strongly upon flg22 treatment in lines
232 overexpressing GFP-*At*ATG8a, *At*ATG5, and *At*NBR1-TAP. Expression did not increase
233 further or was even reduced in samples treated with the heat-flg22 combination (FigS2B).
234 Overexpression of *At*ATG7, however, did not lead to changes in *AtFRK1* gene expression
235 compared to the wildtype. All knockout lines in this experiment displayed a wildtype-like
236 *AtFRK1* gene expression pattern (FigS2C). Hence, the biotic stress marker gene expression
237 proved to be autophagy-independent but responsive to increased autophagic activity. The heat
238 stress marker gene *AtHSP70* (At3g12580) was equally expressed in wildtype samples that
239 received heat stress and samples treated with the heat-flg22 combination (FigS2D). In
240 contrast, all overexpression lines responded to heat stress with highly increased *HSP70*
241 expression, while combined heat-flg22 stress did not lead to an increase in *HSP70* expression
242 (FigS2D). This effect was, however, also observed in *atg5-1* and *nbr1* lines (FigS2E),
243 highlighting the importance of autophagy in maintaining *HSP70* expression under combined
244 stress.

245

246 **Heat stress recovery fails to halt bacterial growth**

247 The Arabidopsis experiments underscored autophagy's pivotal role in combating combined
248 stress, suggesting benefits from heightened autophagic activity in ATG overexpression lines.
249 Given the limitations of Arabidopsis for *Xcv* experiments, we reverted to the *N. benthamiana*
250 Δ*roq1* system. We hypothesized that increased bacterial growth in previously heat-stressed
251 plants (see Fig3B) resulted from the exhaustion of autophagic resources for defense against

Xcv. The autophagic capacity, diverted to handle heat-induced damage, could not defend against bacterial effector proteins. To test our hypothesis, we repeated bacterial growth experiments with heat-stressed plants undergoing a 24-h recovery phase (Fig5A). We expected restored autophagic capacity after a 24-h recovery following a mild 6-h heat stress. However, comparable to results without a recovery period, previous heat treatment resulted in increased bacterial growth even after the recovery period (unpaired t-test p value 0.0067), although not significantly different in the ANOVA (Fig5B). As seen without the recovery period, this effect was again autophagy-dependent. These results suggest that the cause for increased bacterial growth was not pre-occupation of the autophagy machinery, or dependent on effects lasting over 24 h.

Metabolic shift caused by *Xcv* is treatment-resilient

Recognizing autophagy's role in plant nutrient availability (Signorelli et al., 2019), we theorized that its activation during heat stress might reshape metabolic content to favor bacterial growth. We performed metabolic profiling via GC-MS on previously *N. benthamiana* heat-stressed leaf tissue infected with bacteria. We concentrated on primary metabolites, representing nutrients potentially utilized by the bacteria. Principal component analysis (PCA) showed a major shift between non-treated plants (red) and samples from heat-treated plants (yellow) (Fig5C). *Xcv*-inoculation led to another shift. All samples that received *Xcv*-treatment clustered together independent of additional treatments. The complete analysis also included samples from *hrcV*-infected plants. Interestingly, some *hrcV*-treated samples clustered away from the *Xcv*-treated samples in the PCA, pointing to an effector-dependent shift of the metabolome (FigS3).

Generally, heat-treated plants as well as *Xcv*-infected plants, showed strong differences from control plants for most identified compounds (Fig5D). In particular, sugars and amino acids were reduced under heat stress and after infection, respectively. In contrast, the sugar alcohol Myo-inositol increased, specifically after heat challenge (see below). The phytohormone Abscisic acid (ABA), which is involved in signaling after many abiotic stresses, including heat (Larkindale and Knight, 2002; Zhu, 2016), increased especially under heat-*Xcv* combinations. Collectively, our findings indicate significant stress-induced metabolic shifts, with stress-specific patterns yet common alterations in energy sources. Notably, the response to *Xcv* infection exhibited remarkable resilience to additional factors or stresses.

285 **Changes in organic acids are independent of autophagy**

286 We next focused on specific metabolic changes induced by stress combinations and their
287 dependence on autophagy. Two compounds of the TCA cycle, succinic acid and malic acid,
288 were reduced only upon heat treatment but not infection (FigS4E,F). However, while levels of
289 malic acid stayed low in all plants that previously received heat treatment, *Xcv* but not *hrcV*
290 infection restored control levels of succinic acid in heat-stressed plants in an autophagy-
291 independent manner. The levels of a third detected member of the TCA cycle, citric acid, did
292 not change in response to any stress treatment (FigS4G). No changes in shikimic acid levels
293 were detected in response to heat stress, but levels were reduced significantly in response to
294 infection or a combined heat-pathogen stress (FigS4A). This response to infection was
295 independent of autophagy and similar in *Xcv* and *hrcV*. Nicotinic acid showed a mild
296 reduction under combined stress compared to the single stresses, which was significantly
297 lower than in control plants (FigS4B). Again, the response to combined stress was
298 independent of autophagy. Together, our results suggest that changes in organic acids in
299 response to stress combinations are not dependent on autophagy.

300

301 **Shift in amino acid levels is independent of bacterial effectors**

302 We also investigated metabolites functioning as energy sources. Amino acids are a major
303 energy source that can be mobilized under stress (Signorelli et al., 2019). We detected three
304 amino acids, which were generally reduced upon bacterial infection as well as heat stress,
305 especially in the case of glycine (Fig6A,B). *Xcv* and *hrcV* showed a similar effect. Asparagine
306 was reduced upon bacterial infection and further reduced in the presence of 3-MA under
307 control conditions (Fig6C). This effect was also observed in heat-stressed plants but to a
308 lesser extent. Thus, the shifts in amino acid levels are not a result of bacterial manipulation
309 but rather a general response of the plant to stress conditions. Urea levels, a metabolite
310 directly linked to amino acid catabolism (Witte, 2011), stayed stable upon heat challenge but
311 were reduced upon *Xcv* infection, independent of temperature (Fig6D). Interestingly, *hrcV*
312 treatment significantly increased urea levels in an autophagy-dependent and heat-sensitive
313 manner.

314

315 **Effector-dependent reduction of sugars is heat-sensitive and modulated by autophagy**

316 Sugars are a main energy source under stress conditions (Signorelli et al., 2019). Most of the
317 mono- and disaccharides were globally decreasing upon heat- and *Xcv*-treatment compared to

318 control samples, with sucrose showing only a decrease upon infection, not heat (Fig7A-C, E-
319 G). This effect was not influenced by 3-MA treatment. Samples treated with *hrcV* displayed a
320 more minor sugar reduction than *Xcv* infection (significant only for Glucose). However, when
321 autophagy was inhibited by 3-MA treatment, *hrcV* infection reduced sugar levels to a similar
322 extent as after *Xcv* infection. The effect of *Xcv* treatment was not further intensified by 3-MA
323 treatment, as seen for *hrcV*. We, therefore, suggest that the decrease in sugar levels upon
324 infection is partially dependent on effector(s) targeting autophagy. Interestingly, the effect
325 observed for *hrcV* was only present in control conditions and absent in heat-stressed plants
326 where *Xcv* and *hrcV* infection had similar, autophagy-independent effects.
327 The only detected Monosaccharide that did not fit into the described pattern is Xylose/Lyxose
328 (Fig7D). Levels of this sugar were not reduced upon *Xcv* infection or heat treatment but only
329 upon inhibition of autophagy, pointing to a role of autophagy in maintaining Xylose/Lyxose
330 levels under stress conditions.
331 Myo-inositol did not change upon *Xcv* infection (Fig7H). However, levels increased
332 significantly upon *hrcV*-treatment in an autophagy-dependent manner. Heat stress led to a
333 general increase in Myo-inositol levels, a well-documented response to heat and other abiotic
334 stresses in different plant species (Khurana et al., 2017; Sharma et al., 2020; Boominathan et
335 al., 2004). Combined heat-*Xcv* but not heat-*hrcV* stress reduced these levels significantly in an
336 autophagy-dependent manner (ANOVA performed in the absence of control *hrcV* samples).
337 The massive shifting of sugars is, at least partially, the result of an effector-mediated, heat-
338 sensitive inhibition of autophagy by *Xcv*. Taken together, our detailed metabolic profiling
339 showed a strong effect of the applied stress (combinations) on metabolites mainly involved in
340 primary (energy) metabolism. For most sugars and urea, the impact of *Xcv* infection was
341 effector-dependent and absent in *hrcV*. This effector-dependent effect was mimicked in *hrcV*
342 by inhibition of autophagy.

343

344 **Discussion**

345 The simultaneous occurrence of environmental stresses, such as heat, with pathogen infection
346 poses a widespread threat to plant health. It is crucial to comprehend how plants navigate
347 these dual challenges to enhance crop breeding initiatives aimed at creating robust varieties.
348 In this study, we highlight autophagy as a fundamental mechanism that bestows stress-specific
349 capabilities upon plants. Bacterial pathogens target autophagy to promote disease, and this
350 influence is evident in metabolic patterns during combined heat-pathogen stress.

351

352 **ATG8 confers specificity to combined plant stress responses**

353 Autophagy can selectively remove damaged or unwanted cellular components. The selectivity
354 is conferred by target binding to autophagy receptors, which, in turn, bind ATG8 (Stephani
355 and Dagdas, 2020). Thus, although many conditions induce autophagy, the autophagic cargo
356 and the resulting degradome are expected to be unique to each condition. Since plants have
357 multiple ATG8 isoforms, it has been proposed that these isoforms play a crucial role in
358 shaping the stress-specific response and selectivity of autophagy (Kellner et al., 2017; Lin et
359 al., 2023; Zess et al., 2019). There are two main methods to study autophagy activation using
360 GFP-ATG8. The GFP tag enables autophagosome visualization by confocal microscopy. In
361 addition, the ratio between GFP-ATG8 and free GFP by Western blot allows us to estimate
362 ongoing autophagic flux (Klionsky et al., 2021). We could not detect free GFP in all our *N.*
363 *benthamiana* Western blots. In fact, we often and repeatedly only detected an unknown but
364 GFP-specific degradation band. Similar bands were reported in other plant species (Zhou et
365 al., 2023), yet their existence was not explained. This novel band is clearly connected to the
366 degradation of GFP-*St*ATG8, and is absent without GFP-*St*ATG8 protein. Therefore, we
367 decided to use this degradation band instead of free GFP to calculate the ratio between its
368 intensity and GFP-*St*ATG8.

369 In our study, the seven StATG8 isoforms exhibited stress-specific responses based on the type
370 of stress (Fig1). Carbon starvation triggered a uniform activation of all isoforms, whereas *Xcv*
371 infection or heat differed in ATG8 activation pattern. Bacterial infection is hampered by
372 removing effector proteins via autophagy (Leong et al., 2022b). The process requires selective
373 autophagy receptors e.g., NBR1, which exhibits a preference for specific ATG8 isoforms
374 (Svenning et al., 2011). Accordingly, only selected *St*ATG8 isoforms were activated by *Xcv*
375 infection. By contrast, under heat stress, *St*ATG8 isoforms differed in activation timing. Heat
376 stress generally causes protein aggregation, but proteins vary in their ability to withstand
377 elevated temperatures. We suggest *St*ATG8 isoforms are activated at different times during
378 heat stress to account for the variability in protein heat sensitivity. One of the unique
379 structural features of ATG8 proteins is an N-terminal helix domain (Bu et al., 2020). This
380 domain determines the binding specificity to ATG8-interacting proteins and its sequence is
381 not conserved in plants (Zess et al., 2019). Thus, preferential binding of ATG8 isoforms to
382 different targets and selective autophagy receptors seems only logical (Kellner et al., 2017).
383 Besides differential activation of ATG8 proteins, expression of *AtATG8* genes differed when

exposed to starvation or (a)biotic stress (Wang et al., 2020; Chiu et al., 2023; Leong et al., 2022b), further confirming our finding of stress-specific involvement of *St*ATG8 isoforms.

Response to combined abiotic-biotic stress is very complex, involving the interaction of two living organisms (the plant and the pathogen) as well as the additional level of abiotic stress (Kissoudis et al., 2014). Subjecting the plants to heat while concurrently exposing them to *Xcv* implies that both the plants and the bacteria are experiencing above-optimum temperatures. We tried to reduce the level of complexity by applying the combined heat-*Xcv* stress as a consecutive stress where the bacteria are not experiencing direct heat stress. Studies investigating combined stresses usually focus on a single time point; however, we believe that our experimental system of a consecutive stress setup reflects a natural situation more accurately where stress may also occur sequentially rather than simultaneously.

GFP-*St*ATG8-3.1 was involved in the response to heat stress and *Xcv* infection (Fig1). Yet, when exposed to a combined heat-*Xcv* challenge, the activation did not mimic a combination of both stresses (Fig2). Hence, the assumption that combined stresses equal the sum of the involved single stresses falls short of the complexity of natural systems. There is a possibility that combined stress activates another suite of *St*ATG8 isoforms than the single stresses. However, degradation of GFP-*At*ATG8a after a combined heat-flg22 treatment showed similar behavior with degradation mimicking heat treatment, not flg22 treatment (Fig4). Infection assays with *Xcv* further confirmed the observation made for GFP-*St*ATG8-3.1 after combined heat-*Xcv* stress. The bacteria grew better in previously heat-stressed plants (Fig3). The effect was absent in *hrcV* infections and when autophagy was inhibited, confirming the results of GFP-*St*ATG8-3.1 activation during the heat-*Xcv* stress combination. Enhanced growth of another bacterial phytopathogen, *Pseudomonas Pst* DC300, at moderately elevated temperature during infection has been described before (Huot et al., 2017). As in our case, the increased growth depended on the delivery of bacterial effector proteins. We can now link this enhanced growth to bacterial manipulation of autophagy.

Autophagy components other than ATG8 fine-tune the response to combined stress

While ATG8 contributes to stress-specificity, monitoring other ATGs can give a more detailed insight into the activation of autophagy during combined stress. The degradation of *At*NBR1 and *At*ATG7 proteins in response to single and combined stresses gives a more complex picture than the degradation of ATG8 proteins. In contrast to GFP-*St*ATG8s in tobacco, *At*NBR1-TAP degradation in Arabidopsis in response to combined heat-flg22 stress

showed an intermediate response compared to the two single stresses (Fig4). Although a role for NBR1 was documented in immunity against various pathogens, including bacteria (Dagdas et al., 2016; Üstün et al., 2018; Hafrén et al., 2017), it is possibly not involved in the autophagic response activated by flg22 treatment. flg22 is recognized by the immune receptor kinase FLAGELLIN-SENSING 2 (FLS2) (Chinchilla et al., 2006). FLS2 is known to be recycled, and in Arabidopsis, orosomucoid (ORM) proteins were described as selective autophagy receptors specifically targeting FLS2, but not other immune receptors (Beck et al., 2012; Yang et al., 2019). Nevertheless, *At*NBR1-TAP was reduced upon flg22 challenge compared to the control.

In contrast, overexpressed *At*NBR1-TAP accumulated upon heat treatment. Although to a lesser extent than in *atg* mutants, accumulation of overexpressed *At*NBR1-TAP proteins has been documented after 6 to 9 h of severe heat stress in Arabidopsis (Zhou et al., 2013), similar to what we observed. This accumulation vanished when the heat stress was followed by flg22 challenge, contradicting the results from GFP-*St*ATG8-3.1 where combined stress mimicked the heat stress (Fig2). Interestingly, in the same *At*NBR1-TAP overexpression samples, both flg22 and combined heat-flg22 treatment led to an autophagy-dependent reduction of *At*ATG7 protein levels, which was not observed after heat treatment.

A similar pattern of *At*ATG7 protein was found in wildtype plants and an *At*ATG5 overexpression line (Fig4). The reduction of *At*ATG7 protein levels after flg22 and combined stress matches the results obtained for *NbATG7* mRNA levels (FigS1). However, we did not see an accumulation of *NbATG7* mRNA after heat stress as we did for *At*ATG7 protein, making it unlikely that the effects observed on the protein level result from transcriptional changes. We postulate that the changes in *At*ATG7 protein were caused by modulated protein degradation. While this degradation depends on autophagy, it is possible that *At*ATG7 was degraded via other pathways (e.g. the proteasome) as shown for SH3P2 upon *Xcv* infection. Here, the pathogen blocks autophagy by increasing SH3P2 degradation to enhance its pathogenicity (Leong et al., 2022b). Though our results aline with enhanced degradation of ATG proteins by bacterial manipulation, it is important to note that the response we observed was for heat and a bacterial elicitor. We cannot exclude that combining heat stress and infection with an actual pathogen might yield a different response. Still, our observations underline the importance of monitoring not only one but several components of the autophagic pathway to get a clearer picture of the highly dynamic involvement of autophagy in combined stress response.

Besides the degradation of ATG proteins, transcriptional induction of *ATG* genes and selective autophagy receptors plays a key role in the regulation of autophagy (Magen et al., 2022; Hachez et al., 2014; Stephani and Dagdas, 2020). *NbATG7* mRNA levels were downregulated in response to both moderate heat stress of 40 °C and *Xcv* infection alone and the combination (FigS1). Regulation of *ATG*s can differ drastically depending on the plant species and severity of the heat stress treatment (Zhou et al., 2014; Zhou et al., 2013). In contrast, *NbJOKA2* mRNA levels remained relatively stable (FigS1), although some instances of responsiveness to heat stress have been reported for *NBR1* (Zhou et al., 2013; Zhou et al., 2014). Studies on autophagy and pathogen defense also show contrasting effects. While *AtATGs* are induced by necrotrophic fungus *B. cinerea* and contribute to resistance, autophagy has a negative role in defense against biotrophic pathogens (Lai et al., 2011; Minina et al., 2018; Wang et al., 2011; Üstün et al., 2018; Leong et al., 2022a). Inhibition of *ATG* gene expression could be an essential strategy for bacterial pathogens to avoid autophagy-dependent immune responses. However, it remains unclear why heat also leads to the downregulation of *NbATG7* transcription, emphasizing caution when comparing heat stress of varying intensities across plant species with differing tolerance to high temperatures. Besides the plant species, timing is important when studying stress-related transcriptional regulation. Studies, including ours, often focus on one or a few time points, missing the dynamic regulation of transcription.

We also examined the expression of stress marker genes like *HSP70* for heat stress and *FRK1* for biotic stress upon combined stress (FigS2). *HSP70* was upregulated in wildtype upon single and combined heat-pathogen stress. In lines with altered autophagic activity, this response shifted towards a stronger response to the single stress and a sharply reduced response to combined heat-pathogen stress, underlining autophagy's role in *HSP70* transcriptional regulation during combined stress. In contrast, *FRK1* mRNA levels were induced by autophagy. Notably, *FRK1* gene expression does not depend on autophagy, as evidenced by wildtype-like induction upon flg22 perception in *atg* mutant plants. Similar results were observed for the Arabidopsis defensin gene, *AtPDF1.2* (Lai et al., 2011). This robust response is essential as pathogens evolved to undermine components of the immune response network (Tsuda et al., 2013). It is also of special interest when it comes to combined stress scenarios.

Despite reproducibility, the lack of significant differences was disappointing in the large-scale RT-qPCR experiment involving up to eight lines, five treatments, and a minimum of five biological replicates per treatment. The procedure was carefully controlled, including chamber transfers for temperature treatment and manual syringe infiltration for flg22 treatment.

However, potential changes in light quality and humidity between the experimental chamber and the cultivation room might have influenced marker gene expression. Additionally, the time-consuming tasks of infiltrating numerous plants and harvesting two leaves per plant made precise 1-hour (60 min) sample collection impractical, resulting in a more realistic time range of 55 to 75 mins. Considering the dynamic nature of *FRK1* gene expression (Winter et al., 2007)(http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), with a 44-fold increase at 1 hour and more than a 90-fold increase at 4 hours after flg22 treatment, variations in marker gene expression may be attributed, at least partially, to these factors.

Changes in metabolites as an underestimated effect of autophagy influencing bacterial performance

Nutrient availability plays a major role in pathogen growth. We thus asked whether previous heat stress affected nutrient availability for the invading pathogen. Nutrients in the apoplast available for bacterial nutrition are mainly sugars and amino acids (Rico and Preston, 2008; Fatima and Senthil-Kumar, 2015). Sugars and amino acids were generally reduced in the tested leaf tissue as a result of heat stress and also after infection. This observation is not surprising, given that stress responses are energetically costly (Signorelli et al., 2019). While we see this reduction in amino acids upon *Xcv* infection (Fig6), we cannot confirm it results from bacterial manipulation of autophagy or bacterial consumption of these metabolites. Urea, linked to amino acid catabolism (Witte, 2011), displayed an opposite response to stress. While amino acid levels decreased upon infection, heat stress alone had no major effect on urea levels, and *Xcv* infection or combined stress even slightly reduced urea levels (Fig6). Interestingly, this reduction resulted from bacterial manipulation as levels increased significantly following *hrcV* infection. More importantly, this increase was absent when *hrcV* infection was combined with autophagy inhibition. Amino acids are recycled from proteins degraded via autophagy.

In addition, components targeted by bacterial effectors to manipulate metabolite recycling via autophagy might be heat-sensitive. When *hrcV* infection followed heat stress, urea levels were reduced, resembling *Xcv* infection. The absence of increased urea in combined *hrcV*-heat stress can also be explained on a more general immune response level. Salicylic acid (SA) is the dominant phytohormone involved in the plant's defense against (hemi)biotrophic pathogens. This SA response is heat sensitive in Arabidopsis and tobacco (Wang et al., 2009). Loss of pathogen-induced SA was described as a central mechanism underlying enhanced

517 bacterial growth of *Pseudomonas Pst* DC300 at elevated temperatures (Huot et al., 2017).
518 Without an energy-intensive immune response, amino acids would not be channeled into
519 energy production, failing to increase urea levels.
520 *Xcv* infection at least partially manipulates sugar levels in an autophagy-dependent manner,
521 which seems to be mimicked by heat stress (Fig7). During infection, phytopathogens can
522 adjust their metabolic machinery towards the most abundant nutrients in the host. *Xcv* was
523 shown to modulate its manner of active nutrient acquisition (Fatima and Senthil-Kumar,
524 2015). So-called TBDTs (TonB-dependent transporters) are involved in uptake of
525 carbohydrates, with genomes of several *Xanthomonas* species having a high copy number of
526 TBDT genes (Blanvillain et al., 2007; Dugé de Bernonville et al., 2014; Pieretti et al., 2012).
527 Moreover, pathogens like *Xcv*, colonizing the apoplast, release effector proteins to manipulate
528 plant cells and optimize nutrient availability, e.g. by facilitating sugar efflux through induction
529 of gene expression of sugar transporters (Chen et al., 2010; Eom et al., 2015; Zhou et al.,
530 2015).
531 In addition to effectors influencing nutrient efflux, pathogens target autophagy directly (Üstün
532 et al., 2016; Lal et al., 2020; Leong et al., 2022a). The reduction of sugar levels is partially
533 dependent on effector proteins, as the *hrcV* mutant was unable to reduce sugar levels
534 comparable to *Xcv*. Inhibition of autophagy by 3-MA led to *Xcv*-like sugar levels after *hrcV*
535 infection, suggesting that the reduced sugar levels after Xcv infection resulted from autophagy
536 inhibition by bacterial effectors. The autophagy-dependent manipulation of sugar levels is no
537 longer observable when *hrcV* infection follows heat stress. Since heat stress affects autophagic
538 activity (Zhou et al., 2014) and thereby also metabolite recycling, manipulation of autophagy
539 might be obsolete under these conditions. Another interpretation is that, as mentioned above,
540 the effector targets are heat sensitive, and this manipulation is no longer needed when the
541 infection follows a heat stress. However, the effect of *Xcv* on sugar levels does not solely
542 depend on autophagy, as autophagy inhibition by 3-MA did not alter the *Xcv* sugar phenotype.
543 We thus postulate that manipulation of autophagy is not the only mechanism underlying
544 pathogen success. This observation also explains why *Xcv* growth benefits from preceding
545 heat stress whereas *hrcV* growth does not (Fig3). While the *hrcV* sugar phenotype can be
546 compensated by preceding heat stress, the *hrcV* growth phenotype is unaffected, presumably
547 because there are other effectors contributing to pathogen success than those targeting
548 autophagy. As *hrcV* can not secrete any effectors to the plant, future studies using more
549 specific effector mutants might be able to discern the particular effects of bacterial effectors in
550 autophagy-dependent nutrient availability.

551

552 Our findings underscore the specificity inherent in the autophagic response, particularly
553 regarding ATG8 isoforms. We demonstrate that combined stresses do not equal the sum of
554 their counterparts, specifically focusing on autophagy at the nexus of simultaneous
555 challenges. It will be interesting to see if the specificity of the autophagic response, especially
556 in the case of stress combinations, also translates to the autophagic cargo level. The influence
557 of heat stress on autophagy, seen in its facilitation of bacterial growth, establishes it as a key
558 link between environmental factors and plant-microbial interactions. This connection
559 underscores the broader ecological implications of autophagy in plant defense and unveils
560 potential targets for enhancing stress resilience. As autophagy functions in many aspects of
561 plant life, it is not possible to distinguish its roles in nutrient supply, abiotic stress, and
562 pathogen defense. As our results show, it is possible that other, still unknown factors
563 contribute to autophagy-dependent bacterial growth following heat stress.

564

565 **Materials and Methods**

566 **Biological material and growth conditions**

567 All *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype (Col-0). *A.*
568 *thaliana* lines overexpressing GFP-*AtATG8a* in Col-0, *atg5-1*, *atg7-2*, *AtATG5* OE and
569 *AtATG7* OE backgrounds were kindly provided by Elena Minina (Swedish University of
570 Agricultural Sciences, Sweden; (Minina et al., 2018)). *At*NBR1-TAP OE and *nbr1* CRISPR
571 lines were kindly provided by Agnieszka Sirko (Polish Academy of Sciences, Poland;
572 (Tarnowski et al., 2020)). *Nicotiana benthamiana* mutant line Δ*roq1* was kindly provided by
573 Johannes Stuttmann (CEA, France; (Grützner et al., 2021)). Transient expression constructs
574 were kindly provided by Yasin Dagdas (Gregor Mendel Institute, Austria; GFP-*StATG8*
575 isoforms) and Elena Minina (Swedish University of Agricultural Sciences, Sweden; *AtATG7*).
576 *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strains 85-10 and 85-10 Δ*hrcV* were kindly
577 provided by Guido Sessa (Tel Aviv University, Israel).
578 *Solanum lycopersicum* M82 and *N. benthamiana* were grown under a 16h light/8h darkness
579 cycle (long day, LD), 22 °C. Tomato plants were used for stress experiments at an age of five
580 weeks, tobacco plants were transformed and subsequently stressed at an age of approximately
581 four weeks. *A. thaliana* plants were grown under 8h light/16h darkness cycle (short day, SD),
582 22°C. Six-week-old plants were used stress experiments. All plant were grown in a mixture of
583 commercial plant soil, perlit, and vermiculite (2:1:1).

584

585 **Transient expression in tobacco**

586 To study the stress specificity of ATG8 isoforms, *Agrobacterium tumefaciens* strain
587 GV3101::pMP90 was transformed by electroporation with constructs of N-terminally GFP-
588 tagged versions of all seven *St*ATG8 isoforms (Zess et al., 2019), respectively. For transient
589 expression in *N. benthamiana* Δ*roq1* plants, bacteria were grown in LB medium at 28 °C
590 overnight, resuspended in infiltration buffer (10 mM MES, pH 5.7; 10 mM $MgCl_2$; 150 µM
591 Acetosyringone), adjusted to OD_{600} 0.5 and incubated for 2 hours at room temperature. The
592 bacteria were subsequently pressure-infiltrated into *N. benthamiana* leaf tissue using a needle-
593 less syringe. After an initial phase of 18 h under LD conditions for expression of constructs,
594 the plants were subjected to the respective stress conditions.

595 **Stress treatments**

596 To induce carbon starvation, plant trays were covered with aluminum foil for three days but
597 kept in the same climate chamber as control plants without carbon starvation. For biotic stress
598 treatments, *Xcv* suspensions of OD_{600} 0.2 in 10 mM $MgCl_2$ were pressure-infiltrated into leaf
599 tissue of *N. benthamiana* using a needle-less syringe. Alternatively, 200 nM flg22 peptide was
600 pressure-infiltrated into leaf tissue of *A. thaliana*. Plants were placed into a Percival chamber
601 at 22 °C (control temperature) for 6 h (tobacco) or 1 h (Arabidopsis), respectively. For heat
602 stress treatments, tobacco and tomato plants were placed into a second Percival chamber and
603 subjected to 40 °C for 6 h while Arabidopsis were incubated at 38 °C for 3 h.

604 **Protein extraction, SDS-PAGE, and Western blot**

605 Equal amounts of plant leaf tissue were ground in liquid nitrogen using metal beads. Total
606 proteins were extracted using a protein extraction buffer (50 mM Tris, pH 8; 20 mM NaCl;
607 10 % glycerol; 0.5 % Triton X-100; 1x Protease Inhibitor). Samples were mixed thoroughly,
608 incubated on ice for 10 min, and centrifuged (14 000 rpm, 10 min, 4 °C). The supernatant was
609 collected, mixed with SDS-PAGE sample buffer, and incubated at 80 °C for 5 min. Total
610 proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride
611 membrane. Equal loading was validated using Coomassie Brilliant Blue staining of the
612 membrane before the remaining unspecific protein binding sites were blocked with blocking
613 solution (3 % milk + 2 % BSA in 1x PBST). For immunodetection, primary antibodies (anti-
614 GFP: Abcam ab290; anti-NBR1: Agrisera AS142805; anti-ATG7: Agrisera AS194277) from
615 rabbit were used in combination with a secondary HRP-conjugated anti-rabbit antibody from
616 goat. For repeated detection of proteins from one membrane, membranes were first dried and
617 then re-wetted with 1x PBST before Coomassie Brilliant Blue staining. The membranes were

618 stripped by incubation in stripping buffer (200 mM glycine; 3.5 mM SDS; 1 % Tween 20;
619 pH 2.2) for 20 min and subsequent washing with 1x PBST (3x for 5 min). Membranes were
620 re-blocked with blocking solution prior to re-probing with the new antibody.

621 **Bacterial growth assay**

622 *Xcv* was grown in NYGB medium at 28 °C overnight. For measuring bacterial growth, leaves
623 of five-week old tomato plants or four-week old tobacco plants were pressure-infiltrated with
624 *Xcv* OD_{600} 0.0004 in 10 mM $MgCl_2$ with or without 5 mM 3-Methyladenine (3-MA) using a
625 needle-less syringe. Leaf disk samples were taken immediately after infiltration to ensure
626 equal infiltration densities. To check bacterial performance, leaf disk samples were taken three
627 days after inoculation. Bacterial growth was monitored by serial dilution plating of ground
628 leaf discs.

629 **Gene expression analysis by quantitative reverse transcription polymerase chain**
630 **reaction (RT-qPCR)**

631 To analyze transcript abundance, total RNA was extracted from leaf tissue using the TRIzol
632 method. Total RNA was treated with TURBO DNA-free kit (Invitrogen). The integrity of the
633 RNA was checked on 1% (w/v) agarose gels and the concentration was measured using a
634 Nanodrop spectrophotometer. Intron-specific primers (see Table S1) were used to exclude
635 contamination with genomic DNA. Total RNA was reverse transcribed with SensiFAST
636 cDNA Synthesis Kit (Bioline) according to the manufacturer's recommendations. Real-time
637 quantitative PCR analysis was performed in a final volume of 10 µl in 96-well plates using
638 the CFXDuet system (Bio-Rad). Amplification specificity was evaluated by post-
639 amplification dissociation curves. *ACTIN* was used as the internal control for quantification of
640 gene expression. Primer sequences are listed in Table S1.

641

642 **GC-MS analysis**

643 Plant metabolites from *N. benthamiana* were extracted following an established protocol for
644 GC-MS-based metabolite profiling (Lisec et al., 2006), with minor modifications. In short, the
645 samples (25 mg leaf tissue per sample with six biological replicates per treatment) were
646 extracted in methanol containing an internal standard (Pentaerythritol, stock concentration
647 0.6 mg/ml in DDW, final concentration 0.016 mg/ml), shaken for 15 min at 70 °C, and
648 centrifuged at 14 000 rpm for 10 min. The supernatant was transferred to new tubes, and
649 chloroform and double-distilled water were added. The mixture was centrifuged at
650 14 000 rpm for 15 min, and aliquots of 200 µL of the upper phase of each sample were

transferred to new tubes and dried by Speedvac, overnight. Derivatization was carried out as previously described (Lisec et al., 2006). Polar metabolites were measured by the Agilent 7200B GC/Q-TOF. The injection and separation procedures were performed according to Dahtt using the DB-35MS column (Dhatt et al., 2019). Metabolite detection and annotation were performed by Quant software (Agilent, Santa Clara, CA, USA), according to an accurate mass library of known markers generated by our group and run in the same system. Following blank subtraction, the peak area of each metabolite was normalized to the internal standard (i.e., Pentaerythritol) in each sample, as well as to the fresh weight of the respective sample. For more information see Table S2.

Statistical analysis and data visualization

Data were statistically examined using analysis of variance (Ordinary one-way ANOVA) and tested for significant differences using unpaired *t* tests using GraphPad Prism 8.0.1 software. Graphs and heatmaps were generated using GraphPad Prism 8.0.1 while Principal component analysis (PCA) of the metabolome was performed using Metaboanalyst, an open-source, web-based tool for metabolomics data analysis (https://www.metaboanalyst.ca/). Western blots were processed to maximize readers accessibility to the data. Original blots can be found in the supplemental data (FigS5).

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Author Contributions

HS and TAW designed the research; HS, EK, YS and TAW performed research experiments; HS, YS and TAW analyzed data; HS and TAW wrote the manuscript with input from all authors.

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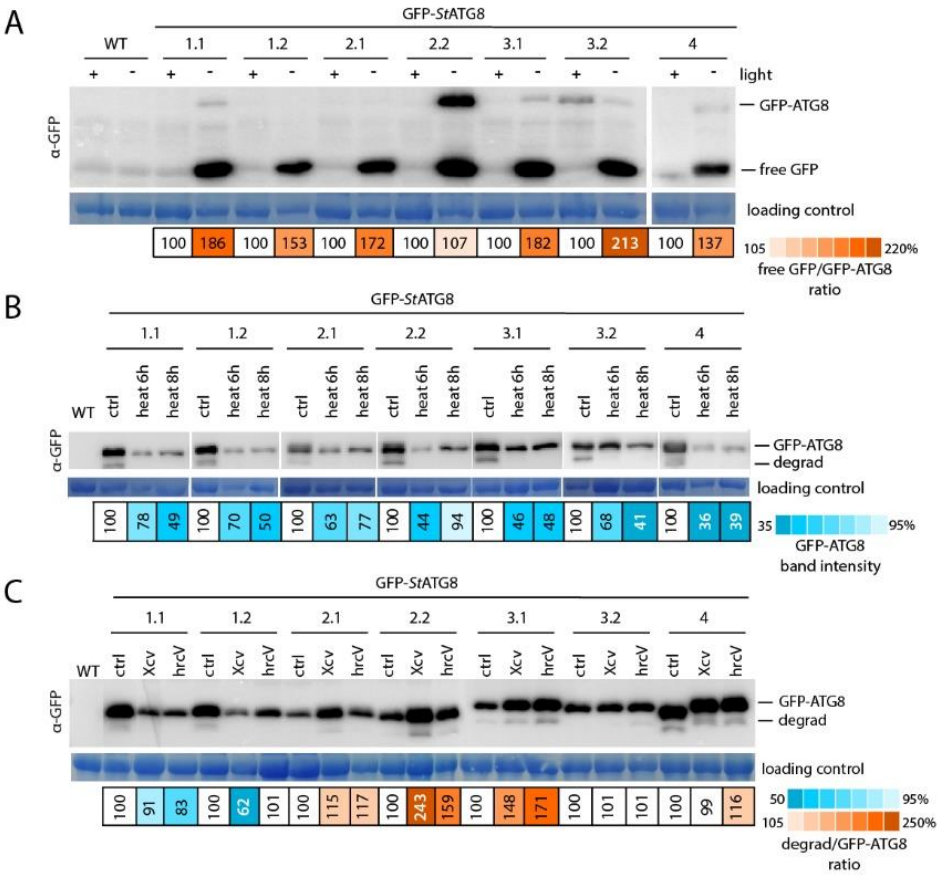

Figure 1: GFP-*St*ATG8 isoforms show a stress-specific degradation pattern. All seven GFP-*St*ATG8 isoforms were transiently expressed in *N. benthamiana*. The plants were subjected to different stresses 1 day after syringe-infiltration of GFP-*St*ATG8 constructs. Western blots show the degradation pattern after 3 days of darkness (**A**), 6/8 h of 40 °C (**B**) and 6 h after *Xcv/hrcV* infection (**C**). Degradation was quantified via measurement of band intensities of GFP-*St*ATG8 (**B**) or GFP-*St*ATG8 and free GFP (**A**) or a specific degradation band (**C**), respectively. Band intensities were normalized to the protein loading control and ratios of GFP-*St*ATG8 and a degradation band (if applicable) were calculated. Original blots can be found in the supplemental data. The experiments were carried out independently three times (B, C)/twice (A) with similar results.

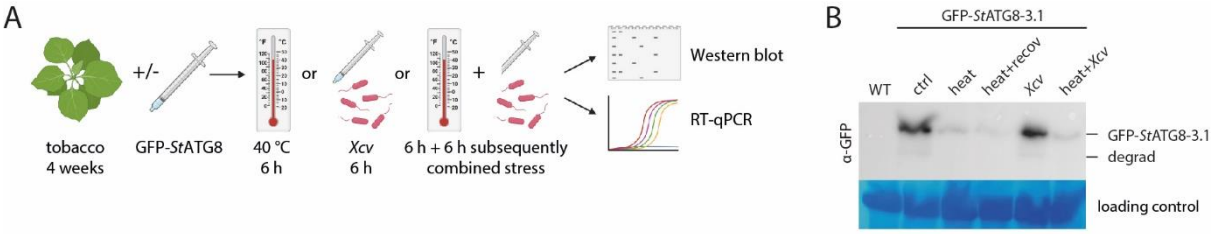

Figure 2: Autophagy is differentially activated under combined stress. (**A**) Schematic representation of the experimental setup to test the effect of combined heat-*Xcv* stress of GFP-*St*ATG8-3.1 degradation and gene expression. (**B**) Degradation pattern of transiently expressed GFP-*St*ATG8-3.1 upon heat, *Xcv,* and a combined heat-*Xcv* challenge. The original blot can be found in the supplemental data. The experiment was carried out independently three times with similar results.

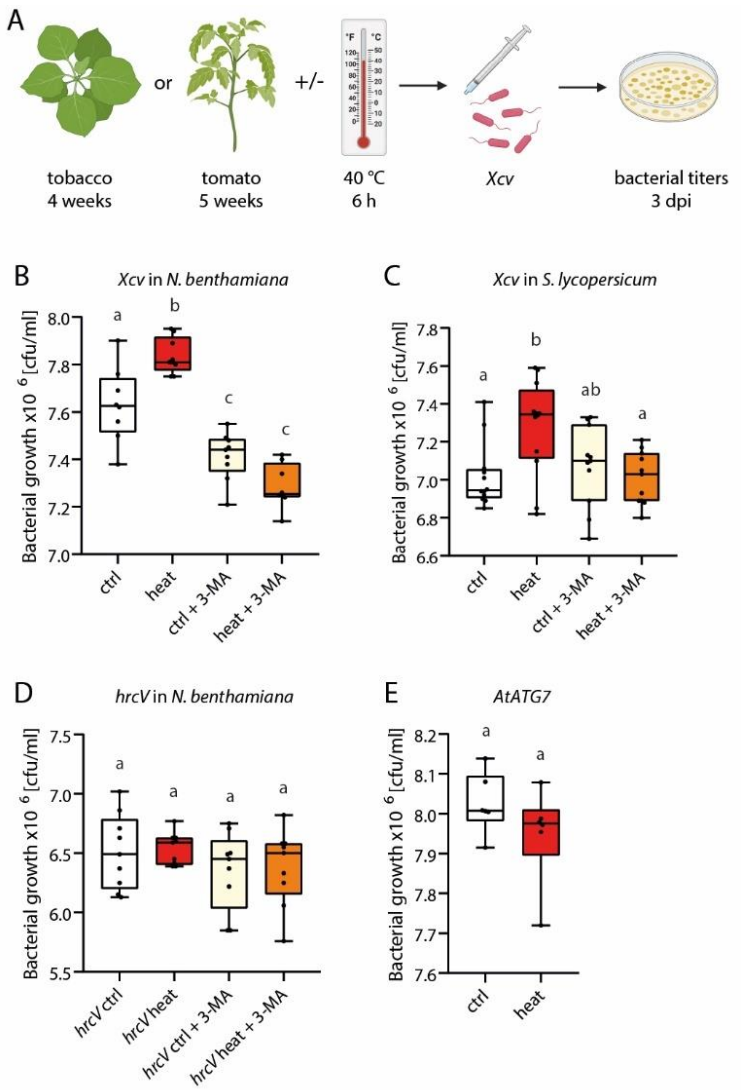

Figure 3: *Xcv* growth is benefited by preceding heat stress in an autophagy-dependent manner. (**A**) Schematic representation of the experimental setup to test bacterial growth in previously heat-stressed plants. (**B-D**) Bacterial growth (*Xcv* (**B**, **C**) or *hrcV* (**D**)) was tested in tobacco (**B**, **D**) and tomato (**C**) plants that were previously subjected to heat or control temperature and co-infiltrated with the autophagy inhibitor 3-MA. (**E**) Bacterial growth (*Xcv*) in heat- and control-treated tobacco plants transiently expressing *AtATG7*. Experiments were carried out independently at least three times (B, D)/twice (C, E) with similar results. Number of biologically independent replicates: *Xcv* in tobacco ($n = 9$), *Xcv* in tomato ($n = 12$), *hrcV* ($n = 9$), *At*ATG7 ($n = 6$). Statistical analysis was performed by One-way ANOVA and Turkey's multiple comparison test.

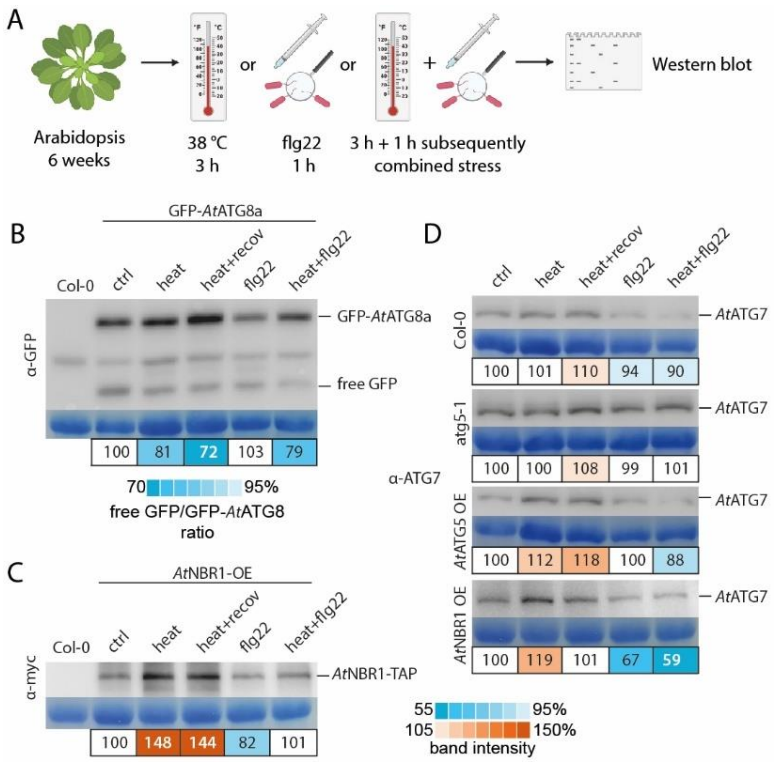

Figure 4: *At*ATG protein degradation upon combined stress is dependent on autophagy. (**A**) Schematic representation of the experimental setup to test protein levels and degradation of overexpressed and native *At*ATGs in response to single and combined stress. (**B-C**) Band intensities and degradation pattern of GFP-*At*ATG8a (**B**) and *At*NBR1-TAP (**C**) in stable overexpression lines. (**D**) Comparison of *At*ATG7 band intensities in different Arabidopsis overexpression and knockout lines. All band intensities were normalized to the protein loading controls. Original blots can be found in the supplemental data. Experiments were carried out independently three times with similar results.

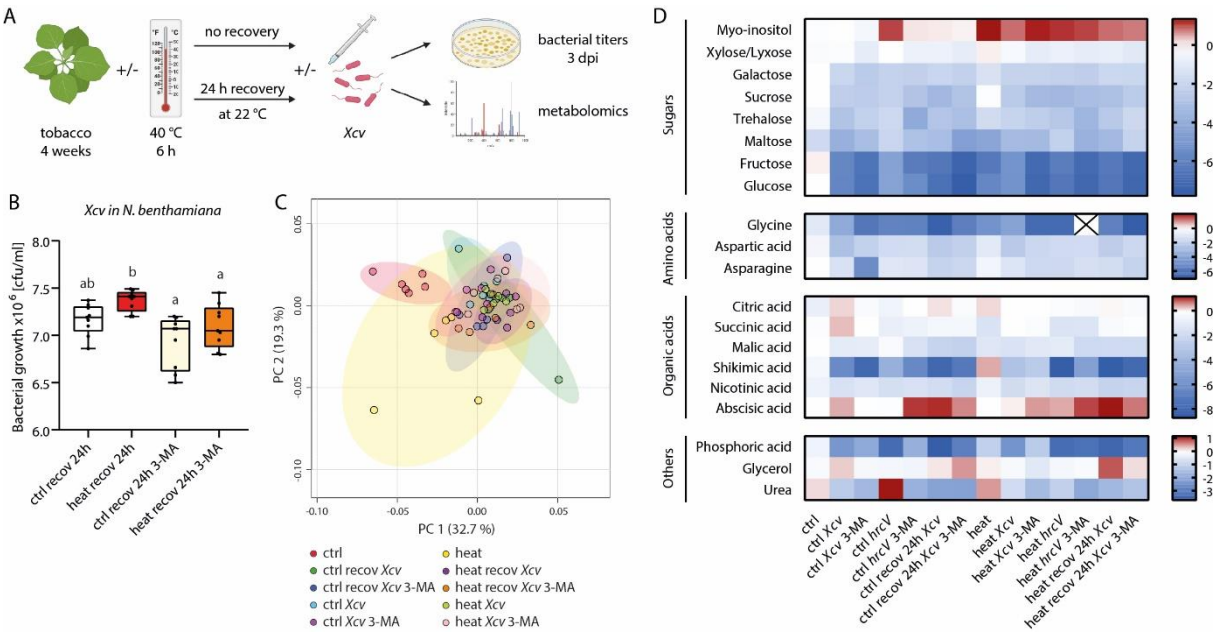

Figure 5: A recovery phase does not affect bacterial growth or the plant metabolome. (**A**) Schematic representation of the experimental setup to test the effect of a recovery phase on bacterial growth and the metabolome. (**B**) Bacterial growth of *Xcv* was tested in tobacco plants previously subjected to heat or control temperature followed by a 24 h recovery period at control temperature and co-infiltrated with the autophagy inhibitor 3-MA (n = 9). Statistical analysis was performed by One-way ANOVA and Turkey's multiple comparison test. (**C**) Principal component analysis of the metabolome in response to heat treatment or bacterial infection with and without a recovery phase and 3-MA treatment. (**D**) Heat maps displaying median values in log2 scale of the response of 4 different groups of metabolites to stress treatment as in (A). Red color refers to upregulation compared to the control; blue color refers to downregulation compared to the control.

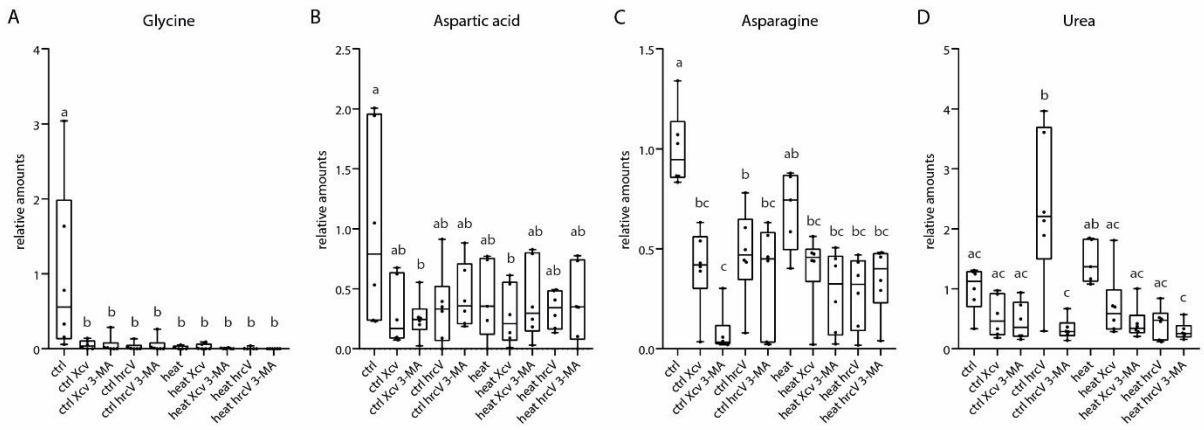

Figure 6: Detailed overview on effects of *Xcv* infection on the levels of three amino acids and urea. Boxplots showing levels of Glycine (**A**), Aspartic acid (**B**), Asparagine (**C**), and urea (**D**) in response to *Xcv*, *hrcV*, and heat treatment, respectively (n = 6). Statistical analysis was performed by One-way ANOVA and Turkey's multiple comparison test.

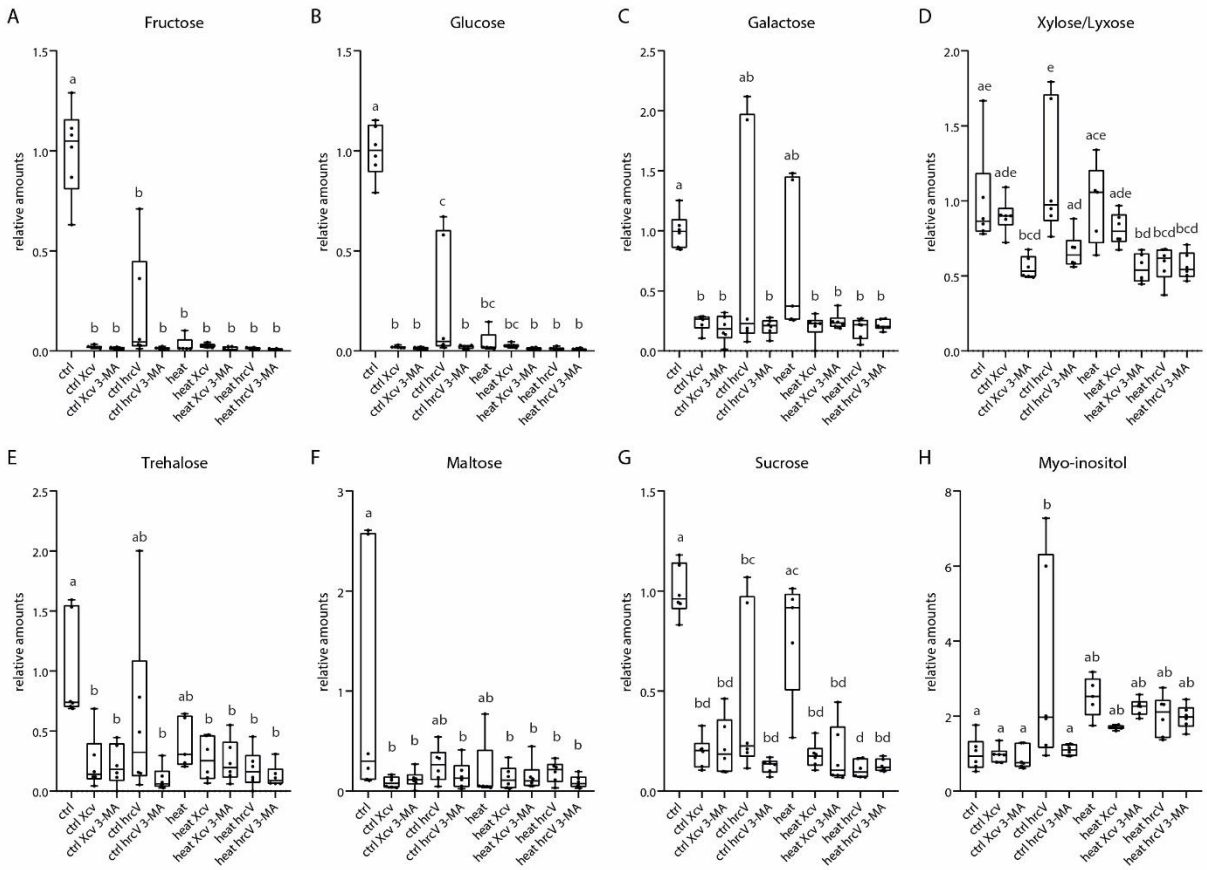

Figure 7: Detailed overview on effector-dependent effects of *Xcv* infection on sugar levels. Boxplots showing levels of (**A-D**) Mono-, (**E-G**) Disaccharides and (**H**) sugar alcohol in response to *Xcv*, *hrcV*, and heat treatment, respectively (n = 6). Statistical analysis was performed by One-way ANOVA and Turkey's multiple comparison test.

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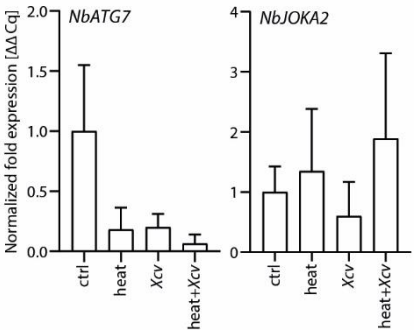

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1069 **Supplemental Figure 1:** Transcript levels of native *NbATG7* and *NbJOKA2* upon single and
1070 combined stress. Error bars, SEM (n = 4-6); Statistical analysis was performed by One-way
1071 ANOVA, Turkey's multiple comparisons test.

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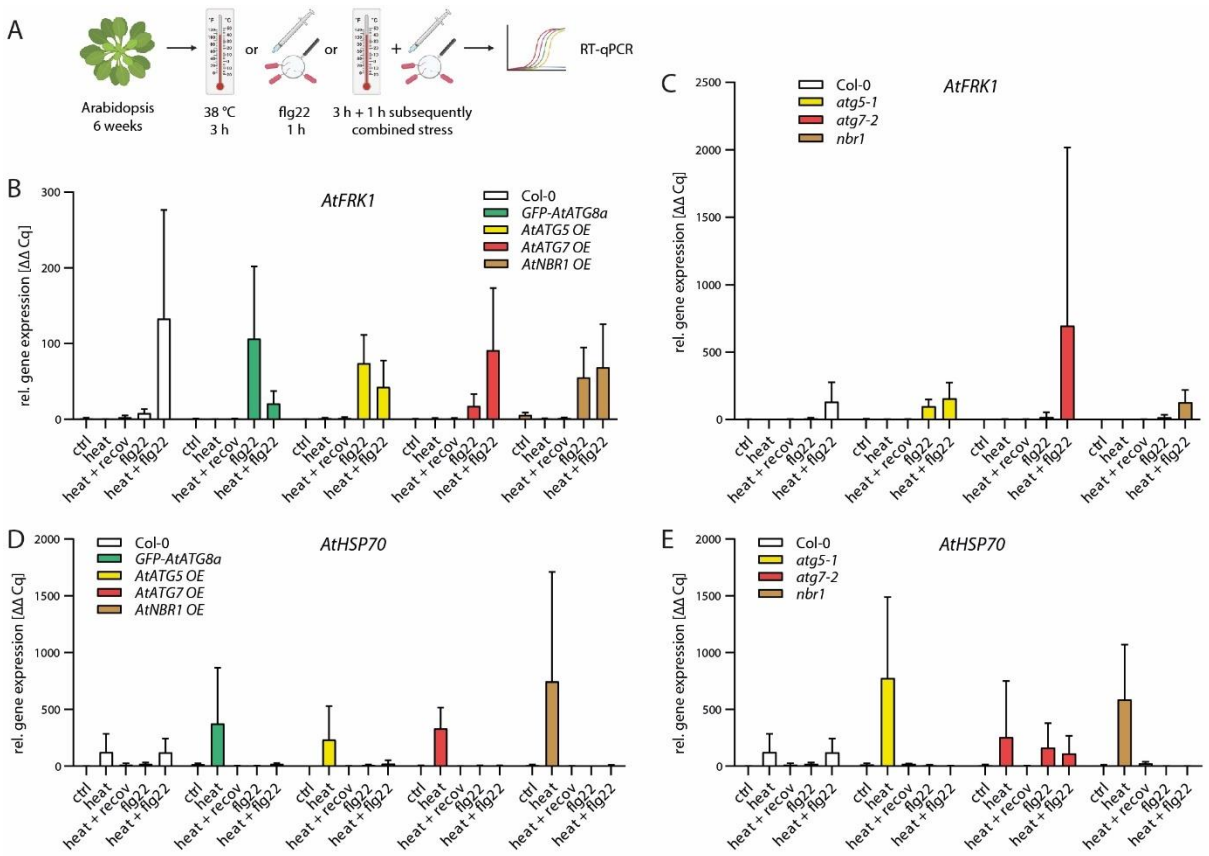
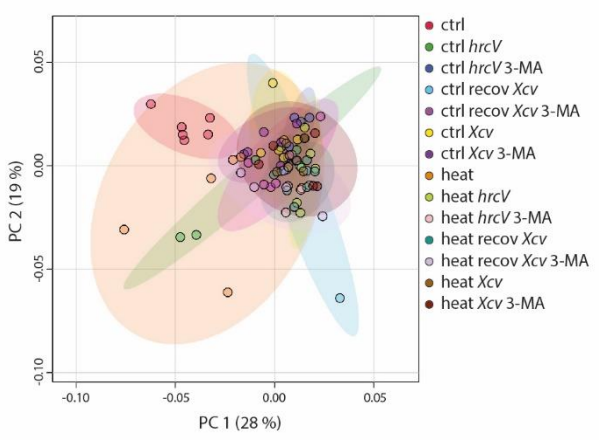
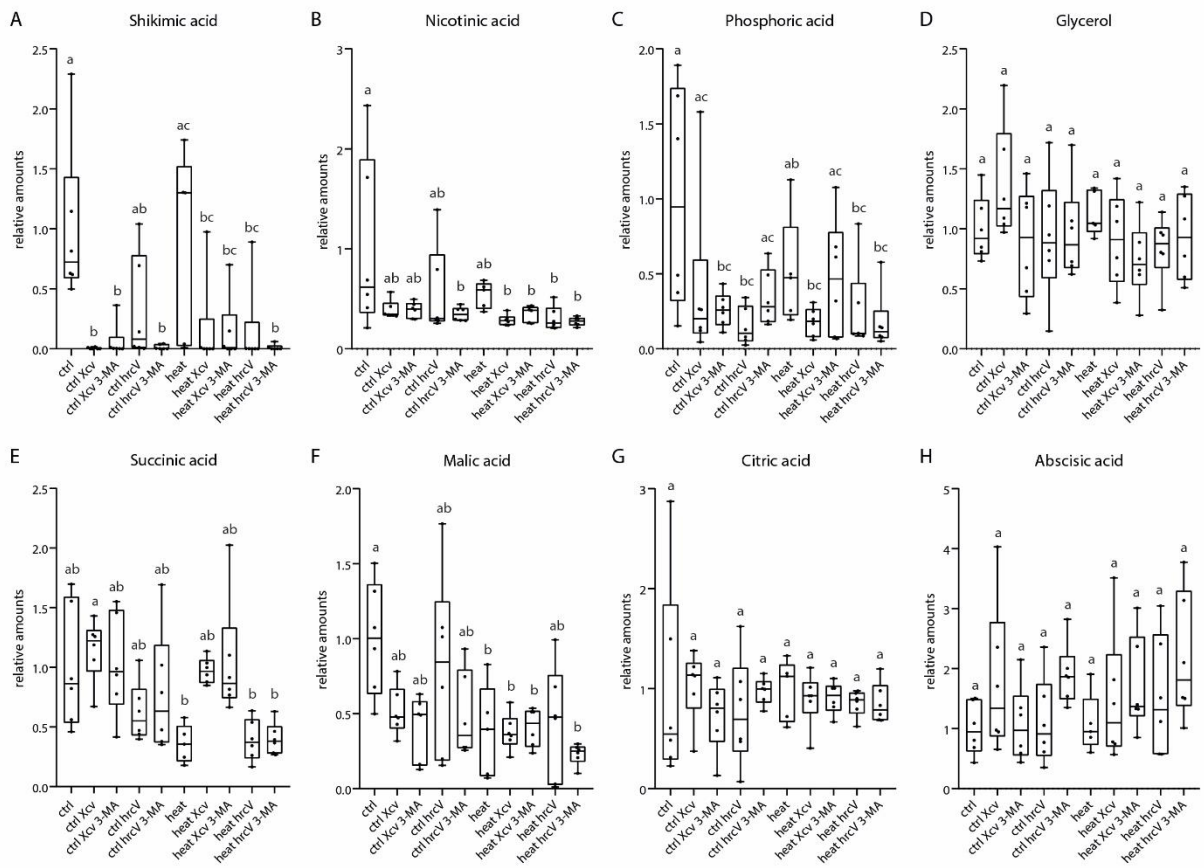


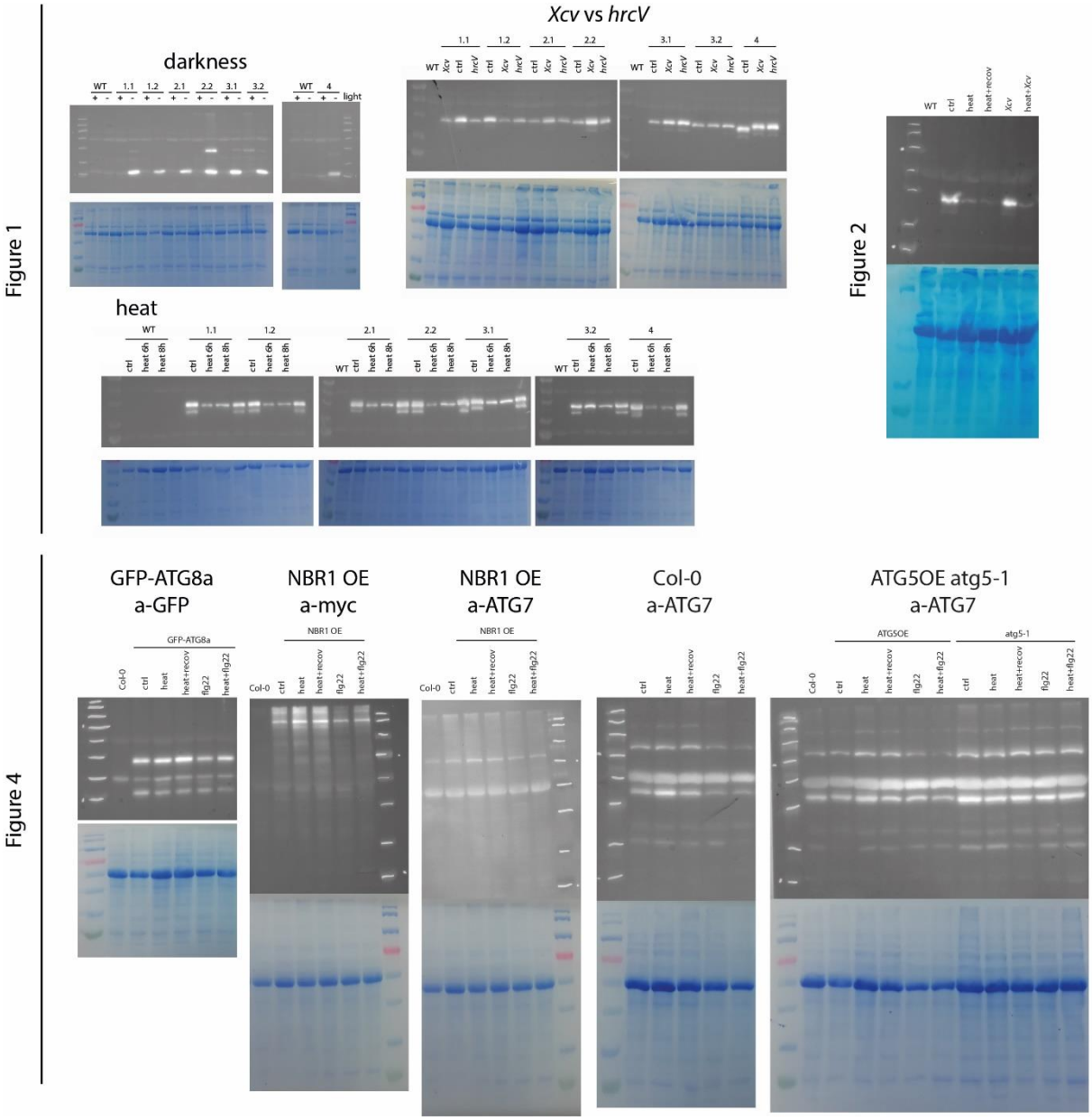
Supplemental Figure 2: Transcriptional response to biotic stress is independent of autophagy but can be induces by increased autophagic activity. Schematic representation of the experimental setup to test the effect of autophagy on stress marker gene transcript levels (**A**). Transcript levels of the biotic stress marker gene *AtFRK1* (**B**, **C**) and the heat stress marker gene *AtHSP70* (**D**, **E**) in wildtype and transgenic overexpression and knockout lines of Arabidopsis. Error bars, SEM (n = 3-4); Statistical analysis was performed by One-way ANOVA, Turkey's multiple comparisons test. The experiment was carried out independently twice with similar results.



Supplemental Figure 3: Principal component analysis of the metabolome as in Figure 5C including *hrcV* samples



Supplemental Figure 4: Detailed overview on effects of *Xcv* infection and heat on the levels of organic compounds. Boxplots showing levels of Shikimic acid (**A**), Nicotinic acid (**B**), Phosphoric acid (**C**), Glycerol (**D**), Succinic acid (**E**), Malic acid (**F**) Citric acid (**G**), and Abscisic acid (**H**) in response to *Xcv*, *hrcV*, and heat treatment, respectively (n = 6). Statistical analysis was performed by One-way ANOVA and Turkey's multiple comparison test.



Supplemental Figure 5: Original blots

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Primer	Sequence	Organism	Gene ID	Reference
qAtActin2_fwd	TCCCTCAGCACATTCCAGCAGAT	A. thaliana	At3g18780	(Dubiella et al., 2013)
qAtActin2_rev	AACGATTCCTGGACCTGCCTCATC			
qIntron_fwd	TTTTTTGCCCCCTTCGAATC	A. thaliana	At5g65080	(Guerra et al., 2020)
qIntron_rev	ATCTTCCGCCACCACATTGTAC			
qAtHsp70_fwd	TCAAGCGGATAAGAGTCACT	A. thaliana	At3g12580	(Sung et al., 2001; Zhang et al., 2021b)
qAtHsp70_rev	CTCGTCCGGGTTAATGCT			
qAtFRK1_fwd	CGGTCAGATTTCAACAGTTGTC	A. thaliana	At2g19190	(Dubiella et al., 2013)
qAtFRK1_rev	AATAGCAGGTTGGCCTGTAATC			
qNbActin_fwd	AAAGACCAGCTCATCCGTGGAGAA	N. benthamiana		(Leong et al., 2022b)
qNbActin_rev	TGTGGTTTCATGAATGCCAGCAGC			
qNbJoka2_fwd	CGTTGTGATGGTTGTGGTGT	N. benthamiana		(Leong et al., 2022b)
qNbJoka2_rev	AGGGACGCCGGTAAGTTAAA			
qNbATG7_fwd	ATGGCGGATAGTGGAAGAGG	N. benthamiana		(Leong et al., 2022b)
qNbATG7_rev	CCTGACTAGCTAGCCGAGAC			

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