Breakthrough Technologies

Switching toxic protein function in life cells

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Running title: Conditional toxin as cell growth arrest module

SUMMARY

Toxic proteins are prime targets for molecular farming and efficient tools for targeted cell ablation in genetics, developmental biology, and biotechnology. Achieving conditional activity of cytotoxins and their maintenance in form of stably transformed transgenes is challenging. We demonstrate here a switchable version of the highly cytotoxic bacterial ribonuclease barnase by using efficient temperature-dependent control of protein accumulation in living multicellular organisms. By tuning the levels of the protein, we were able to control the fate of a plant organ *in vivo*. The on-demand-formation of specialized epidermal cells (trichomes) through manipulating stabilization versus destabilization of barnase is a proof-of-concept for a robust and powerful tool for conditional switchable cell arrest. We present this tool both as a potential novel strategy for the manufacture and accumulation of cytotoxic proteins and toxic high-value products in plants or for conditional genetic cell ablation.

Keywords

cell ablation, cell death, conditional alleles, molecular farming, N-end rule, plant-made pharmaceuticals/biopharming, protein degradation, trichomes.

INTRODUCTION

The low-temperature degron cassette (lt-degron) fused to a protein of interest (POI) can efficiently control its accumulation or degradation in a temperature-dependent manner. The technique is based on altering the *in vivo* stability of the lt-degron:POI fusion protein by using a temperature stimulus and relies on the proteostatic machinery of the N-end rule pathway of protein degradation (Dissmeyer et al., 2018) for fast removal from the cell. The lt-degron:POI fusion can thus be efficiently accumulated at a permissive temperature or removed from the cell at a restrictive temperature generating an artificial temperature-sensitive variant of the POI (Faden et al., 2016; Dissmeyer, 2017). Opposed to many other systems for conditional protein expression and gene regulation, the lt-degron approach relies on posttranslational oinzerference by protein degradation of the entire fusion protein and is applicable in living multicellular organisms, in both plants and animals. By this, it offers tight control over the levels of active/functional protein which directly correlates to the temperature stimulus used to induce the system.

Molecular farming, the generation of pharmacologically active or biotechnologically usable compounds in plants, is an emerging topic especially for the production of peptides and proteins requiring special glycosylation patterns impossible to generate in classical fermenter-based expression systems using microorganisms like bacteria (Stoger et al., 2014). Various cytotoxic peptides are under investigation as highly potent cancer therapeutics (Boohaker et al., 2012). Some toxic proteins, such as the mistletoe lectins possess anti-cancer properties when administered orally (Pryme et al., 2007) and recombinantly produced immunotoxins are in clinical and pre-clinical evaluation for cancer treatments (Kelly et al., 2012; Madhumathi and Verma, 2012). So far, lectin proteins from plants as well as from animals have mainly been efficient-

ly produced in *E. coli* with moderate yields (Lam and Ng, 2011), however, the production of toxic peptides and proteins in plants is still challenging also due to difficult transgene maintenance. So far, production of phytotoxic peptides, such as e.g. antimicrobial peptides, has been difficult and needed either further modification of the peptide (Company et al., 2014) or was achieved through vigorous control of transgene expression through e.g. inducible promoters (Company et al., 2014). The lt-degron technique offers tremendous advantages over inducible promoters due to easier control and non-aversive effects of the stimulus of elevated ambient temperature versus chilling.

The bacterial ribonuclease barnase (BAR), a potent, non-specific RNAse secreted by the soil bacterium *Bacillus amyloliquefaciens* (Buckle and Fersht, 1994) possesses anti-cancer properties (Edelweiss et al., 2008) and has also been used as a potent tool for plant breeders to create male sterile mutants in tobacco, *Arabidopsis*, and wheat (Glimcher and Sparks, 1992; Burgess et al., 2002; Gils et al., 2008). Additionally, it has been exploited as a cell-ablation tool to destroy inflorescences to prevent spread of transgenes from birch, tobacco, and *Arabidopsis* (Lannanpaa et al., 2005). Other applications include a system to defend potato plants against the pathogen *Phytophthora infestans* (Strittmatter et al., 1995) and a conditional cell ablation tool in mammalian cell culture (Leuchtenberger et al., 2001). Due to the conserved mechanism of barnase activity as a non-specific RNase, it is considered to be highly cytotoxic in literally all organisms, highlighting the broad spectrum of possible applications for a controllable ribonuclease.

Cell ablation has been proven to be a powerful tool to study the effects of elimination of entire cell populations in a biological context. Conditional as well as constitutive

91 techniques have been extensively used in the animal field, e.g. to map cell popula-
92 tions in the murine nervous system where the natural resistance of some mouse cell
93 types against the highly cytotoxic *Diphtheria* toxin chain A (DT-A) has been exploited.
94 By controlling the expression of a DT-A receptor, cells were indirectly but efficiently
95 ablated upon exogenous administering of DT-A (Saito et al., 2001; Hermand, 2006).
96 DT-A was also used extensively to induce cell death in yeast, fruit flies, and plants
97 (Bellen et al., 1992; Guerineau et al., 2003). Another example is a regeneration study
98 in zebra fish through the guided, enzymatic conversation of a prodrug (precursor
99 drug) into an active, DNA-toxic compound by a bacterial nitroreductase (Curado et
100 al., 2007). Another system, albeit not applicable in plants, is the auxin-controlled 'sui-
101 cide module' based on rapid degradation of the inhibitory chaperone ICAD. Its deg-
102 radation enabled caspase activity inducing cell death (Samejima et al., 2014).
103 Cell ablation by toxic proteins needs to be tightly controlled in a conditional rather
104 than constitutive manner. Otherwise, the host organism or expressing tissue will be
105 irreversibly destroyed. However, most conditional, non-constitutive, cell ablation pro-
106 tocols rely on the addition of exogenous compounds such as hormones or other
107 small molecules triggering cell death. Depending on the experimental conditions,
108 these systems struggle with uneven induction of the system, low penetration or met-
109 abolic secondary effects of the inducing agents. Moreover, the systems have intrinsic
110 delays in response due to the nature of induction of transcription followed by transla-
111 tion of target protein. The protein accumulation is irreversible which makes control at
112 the level of acute toxicity namely the protein level, impossible.
113
114 Here we report a strategy to control barnase protein levels via temperature and
115 therefore turn on/off its activity. This allows generating a "*phenotype on demand"* and
116 results in conditional organ formation versus ablation in intact, living plants. We com-

117 bined the lt-degron with the cytotoxic barnase protein and expressed the resulting lt-
118 BAR construct in leaf hairs (trichomes), which are specialized single cells of the epi-
119 dermis, under control of the trichome-specific *TRIPTYCHON* (*TRY)* promoter (Pesch
120 and Hulskamp, 2011).
121 This setup allowed controlling trichome cellular fate in a temperature-dependent
122 manner and serves as a proof-of-concept for conditional cell ablation. This new tool
123 also paves the way towards a more efficient molecular pharming of cytotoxic proteins
124 *in planta*.
125
126
127 **MATERIALS AND METHODS**
128
129 **Cloning and DNA work**
130 DNA cloning was performed following standard procedures using *Escherichia coli*
131 strain DH5α (Invitrogen). The constructs were generated by fusion PCR (*Pfu* poly-
132 merase, fermentas) using the following primer combinations. DNA amplicons were
133 purified with ExoSap-IT (USB) preceding fusion PCRs. All fusions were flanked by
134 Gateway *attB1/attB2* sites and recombined by BP reactions into *pDONR201* (Invitro-
135 gen).
136 The lt-degron cassette (K2) was amplified from *pEN-L1-K2-L2* (Addgene ID: 80684)[1]
137 using primers TMV_att_frw (GGGGACAAGTTTGTACAAAAAA-
138 GCAGGCTTActcgagctgcagaattactatttacaattac) and K2barn_f1_rev (tgtgccatagcac-
139 cagcaccagcgtaa).
140 The lt-degron K2 starts with a 3' tobacco Omega (Ω) leader sequence for translation
141 enhancement from *pRTUB8*, a derivative of *pRTUB1* (Bachmair et al., 1990;
142 Bachmair et al., 1993). The leader contains the 20 nucleotides upstream of the start

143 codon of tobacco mosaic virus strain U1(Gallie et al., 1987) and is followed by a co-
144 don-optimized synthetic human *Ubiquitin* gene(Ecker et al., 1987), a triplet for the
145 destabilizing bulky and hydrophobic amino acid Phe (F), a short linker peptide (trans-
146 lates to HGSGI)(Bachmair and Varshavsky, 1989) to further expose the N-terminal
147 residue, a temperature-sensitive mouse dihydrofolate reductase sequences (*DHFR*^{ts})
148 triggering the protein unfolding response, a triple hemagglutinin (HA)-tag (HAT) for
149 immunodetection. Phe is used as destabilizing N-terminal residue, because it was
150 shown to be recognized by the *Arabidopsis* N-end rule pathway(Bachmair et al.,
151 1993; Potuschak et al., 1998; Stary et al., 2003; Faden et al., 2016; Mot et al., 2018).
152 HAT was amplified from *pSKTag3SUM6* (kind gift of Andreas Bachmair, Max F. Pe-
153 rutz Laboratories, Vienna, Austria). The DHFR moiety of K2 is derived from *pJH10*^{mut}
154 (*pJH23*), containing a mutated mouse *DHFR*^{T39A,E173D} (Gowda et al., 2013). An Entry
155 clone containing *K2:TTG1* was used as a template for *K2* (Faden et al., 2016).
156 BARNASE, including the artificial intron, was amplified from *pICH43601* (kind gift of
157 Sylvestre Marillonnet, IPB Halle, Germany) using primers K2barn_f2_frw (ctggtgc-
158 tatggcacaggttatcaacacgtt) and K2barn_f2_rev (GGGGACCACTTTGTACAAGAAA-
159 GCTGGGTAttatctgatttttgta). Primers K2barn_f1_rev and K2barn_f2_frw contained
160 each an eight basepair overhang to the respective other fragment. A fusion PCR was
161 carried out using primers TMV_att_frw and K2barn_f2_rev. The fusion product was
162 flanked with BP compatible Gateway recombination sites, subcloned into *pDONR201*
163 (Invitrogen) and the *pENTR::lt-BAR* Entry clones sequenced after a BP reaction. For
164 stable expression in transgenic plants, *pENTR::lt-BAR* was recombined in an LR re-
165 action into the *attR* site-containing binary Gateway destination vector *pAM-*
166 *PAT:ProTRY:GW* (Addgene ID: 79755 (Pesch and Hulskamp, 2011)) yielding *pAM-*
167 *PAT:ProTRY::lt-BAR*. These Gateway-compatible Destination vectors are derived
168 from *pAM-PAT-MCsS* (multiple cloning site; GenBank accession number AY436765),

169 derivative of *pPAM* (GenBank accession number AY027531) (Rademacher et al.,
170 2002). These backbones carry the *Streptomyces hygroscopicus bar* gene that trans-
171 lates to phosphinothricin-N-acetyltransferase (PAT) as plant selection marker confer-
172 ring resistance against PPT. This vector was used for Agrobacterium-mediated trans-
173 formation of Col-0 wildtype plants.
174
175 **Plant work and growth conditions**
176 *Arabidopsis thaliana* (L.) Heynh. plants were grown either on a steamed (sterilized for
177 3h at 90°C) soil mixture (Einheitserde Classic Kokos (45% (w/w) white peat, 20%
178 (w/w) clay, 15% (w/w) block peat, 20% (w/w) coco fibers; 10-00800-40, Einheitser-
179 dewerke Patzer); 25% (w/w) Vermiculite (grain size 2-3 mm; 29.060220, Gärt-
180 nereibedarf Kamlott); 300-400 g of Exemptor (100 g/kg thiacloprid, 802288, Hermann
181 Meyer)) per m³ soil mixture or aseptically *in vitro* on plastic Petri dishes containing ½
182 Murashige & Skoog (MS) (2.16 g/l MS salts, Duchefa Biochemicals, M0221; 0.5%
183 Glu sucrose; 8 g/l phytoagar, Duchefa Biochemicals, P10031; pH to 5.6 - 5.8 using
184 KOH). Aseptic culture was done or under long-day regime (16/8 hours light/dark) in
185 growth cabinets.
186 Seeds for soild-grown plants were germinated after stratification of four to five days
187 at 4°C in the dark and plants grown under standard long-day (16/8 hours light/dark)
188 or short day (8/16 hours light/dark) greenhouse conditions between 18 and 25°C. For
189 strictly controlled development such as during temperature shift experiments, plants
190 were grown either in growth cabinets (AR-66L2 and AR-66L3, Percival Scientific,
191 CLF PlantClimatics) or walk-in phyto chambers (Johnson Controls, equipped with
192 ESC 300 software interface) at a humidity of 60% depending on the requirements
193 and watered with prewarmed or precooled tap water.

194 Transgenic, Basta resistant plants were selected *in solium* in cotyledon stage by
195 spraying 150 mL per tray of a 1:1000 dilution of Basta (contains 200 g/L glufosinate-
196 ammonium; Bayer CropScience) in tap water which was repeated three times in a
197 two-days interval.
198 For aseptic culture, dry seeds were sterilized with chlorine dioxide gas produced from
199 75% *Eau de Javel* (FLOREAL Haagen) and 25% HCl. Selective MS media contained
200 10 mg/L DL-phosphinotricin (PPT, Basta, glufosinate-ammonium, sc-235254, Santa
201 Cruz Biotechnology), 50 mg/L kanamycin sulfate, 5.25 mg/L sulfadiazine sodium salt
202 (Sigma-Aldrich, S6387), or 20 mg/L hygromycine B (Duchefa Biochemicals, H0192).
203 Plants used in this study were all in the background of the Columbia-0 accession
204 (Col-0) and either wild-type plants or an ethyl methanesulfonate (EMS) mutant for
205 *PRT1* (*prt1* HygS or *prt1-1*, kind gift of Andreas Bachmair, Max F. Perutz Laborato-
206 ries, Vienna, Austria),(Bachmair et al., 1993; Potuschak et al., 1998; Stary et al.,
207 2003)
208 For controlled environment experiments plants were cultivated in growth cabinets
209 (AR-66L2 or AR-66L3, Percival Scientific, CLF PlantClimatics). For experiments
210 needing less stringent environmental conditions plants were cultivated in the green-
211 house.
212
213 **Stable plant transformation and selection of transformants**
214 The binary plant Expression vectors were retransformed into *Agrobacterium tumefa-*
215 *ciens GV3101-pMP90RK* (*C58C1 Rif^r Gm^r Km^r*)(Koncz and Schell, 1986) to obtain a
216 bacterial transformation suspension. The identity of the *Agrobacterium* strains was
217 verified by backtransformation of isolated plasmid into *E. coli* DH5α and at least three
218 independent analytical digestions. All constructs were transformed by a modified ver-
219 sion of the floral dip method (Dissmeyer and Schnittger, 2011).

220 Individual T1 (generation 1 after transformation) transgenic plant lines were pre-selected with Basta or
221 kanamycin as described above. To exclude lines showing position effects, e.g. by disrupting essential
222 genes by the construct T-DNA, the number of insertion loci was determined in a segregation analysis
223 in the T2 generation and only transgenic plants carrying one single insertion locus were further used.
224 Standard lines were established by isolating T3 plants homozygous for the transgene. Independent
225 representative reference lines displaying a typical conditional phenotype upon temperature up- and
226 downshifts were used in the final experiments. Standard lines were established by isolating T3 plants
227 homozygous for the transgene. In order to identify responsive transgenic lines, we prescreened by
228 developmental and histological phenotype.**Transient transfection of tobacco**

229 For transient transformation of ***Nicotiana benthamiana* (tobacco)**, leaves of five-
230 week-old plants were infiltrated with *Agrobacteria GV3101-pMP90RK*, carrying binary
231 plant expression vectors. *Agrobacteria* were grown to the stationary phase overnight
232 in 10 mL of YEB medium and pelleted at 5000 x g at RT. The pellet was washed
233 once in 10 mL of infiltration buffer (10 mM MES pH 5.6, 10 mM $MgSO_4$, 100 µM ace-
234 tosyringone (D134406, Sigma)) and subsequently resuspended in 10 mL of the same
235 buffer. *Agrobacteria* strains were always co-infiltrated together with the *Agrobacte-*
236 *rium* strain GV3101 expressing *p19* of tomato bushy stunt virus from the vector
237 *pBIN6Ip19* to suppress post-transcriptional gene silencing and increase ectopic gene
238 expression (Voinnet et al., 2003). Prior to infiltration, bacteria suspensions were ad-
239 justed to an OD_{600} of 0.5. Bacteria suspensions containing the lt-BAR transgene were
240 mixed with *p19* bacteria suspension in a 1:1 ratio (final OD_{600} = 0.5) and used for
241 transformation. Bacteria suspensions were then infiltrated into the epidermis on the
242 lower side of the tobacco leaf. Infiltrated areas were marked on the upper side of the
243 leaf with a permanent marker. For an easier infiltration procedure, plants were wa-
244 tered and transferred to standard greenhouse LD conditions the day before in order
245 to allow plant stomata to open. To allow efficient transformation and expression
246 plants were kept for 48 h in the greenhouse, before applying temperature shift exper-

247 iments by transferring them into a growth cabinet at either permissive or restrictive
248 growth conditions. For one data point, 15 - 20 leaf discs of 5 mm diameter were har-
249 vested from infiltrated areas and snap frozen in liquid nitrogen. Extraction was per-
250 formed using RIPA buffer as mentioned below.
251
252 **Microscopy and documentation of plants**
253 Photographs of *in vitro* cultures and histological stainings were taken with a stereo
254 microscope (Stemi 2000-C) equipped with a Zeiss CL 6000 LED illumination unit,
255 and a video adapter 60 C including an AxioCam ERc 5s digital camera (all from Carl
256 Zeiss MicroImaging). Trichome visualization through polarized light microscopy was
257 carried out as described previously (Gudesblat et al., 2012; Pomeranz et al., 2013)
258 with the only change that destaining was achieved with a saturated chloral hydrate
259 solution (dissolve 1 kg in 400 ml of H_2O). Trichomes were visualized using a Nikon
260 AZ100 zoom microscope equipped with a Nikon DS-Ri2 color camera. Microscopy
261 images of trichomes and DAPI stained nuclei were obtained using the AxioImager
262 system (Zeiss) equipped with two cameras (AxioCam MRm und AxioCam MRc5).
263
264 **Protein extraction and western blot analysis**
265 Plant tissue (*Arabidopsis*: leafs or seedlings, tobacco: five leaf discs) was collected in
266 a standard 2 mL reaction tube containing three Nirosta stainless steel beads (3.175
267 mm; 75306, Mühlmeier), snap frozen in liquid N_2 and stored at -80°C. Material was
268 ground frozen using a bead mill (Retsch, MM400; 45 s, 30 Hz) in collection microtube
269 blocks (adaptor set from TissueLyser II, 69984, Qiagen; (Faden et al., 2016;
270 Dissmeyer, 2017). Tissue was lysed using radioimmunoprecipitation assay (RIPA)
271 buffer (50 mM Tris-Cl pH 8, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 1
272 mM benzamidine hydrochloride, 15 mM $Na_4P_2O_7$, and 1% Nonidet P-40 supplement-

273 ed with freshly added EDTA-free Complete Protease Inhibitor cocktail, Roche). The
274 protein content of the samples was determined using a DirectDetect infrared spec-
275 trophotometer (MerckMillipore). Equal protein amounts were resolved by 12% SDS-
276 PAGE. Blotting was done onto polyvinylidene fluoride (PVDF) transfer membrane by
277 semi-dry blot using a Trans-Blot SD semi-dry electrophoretic transfer cell (170-3940,
278 Bio-Rad).
279 Equal loading and general protein abundance was confirmed by staining of the blot-
280 ted and probed membranes after immunostaining with Coomassie Brilliant Blue G250
281 (CBB G150). Immunodetection was done using mouse monoclonal anti-HA epitope
282 tag antibody (HA.11, clone 16B12: MMS-101R, Covance, 1:1000) and HRP conju-
283 gated secondary antibodies which were detected by enhanced chemiluminescence
284 (ECL) using ECL SuperSignal West Pico or Femto (34087 or 34096, Pierce) followed
285 by exposure on autoradiography film. Western data were confirmed by analysis of at
286 least three biological replicates.
287
288 **Transcript analysis**
289 Two-week-old seedlings were collected and snap-frozen. After milling, about 50 mg
290 of tissue was used for RNA extraction with RNeasy Plant Mini Kit (Qiagen). RNA was
291 measured with a spectrophotometer and quality assessed via agarose gel electro-
292 phoresis. For first-strand cDNA synthesis, 500 ng of total RNA were used with an
293 equimolar mixture of four oligo(dT) primers (CDSIII-NotIA/C/G/T (Faden et al., 2016))
294 and RevertAid H Minus Reverse Transcriptase (Thermo Scientific). 1 µL of cDNA
295 was used for PCR analysis using self-made *Taq* in two reactions per sample; one
296 with generic degron-specific primers (DHFR_frw/DHFR_rev) to test transcript levels
297 of the transgene and one with intron-spanning primers EF1ss/EF1as for *ELONGA-*

298 *TION-FACTOR1* (*EF1*) as a housekeeping gene (amplicon sizes: genomic 810 bp;
299 cDNA 709 bp). Both PCRs were run for 30 cycles.
300
301
302 **RESULTS**
303
304 **Conditional toxicity of ribonuclease leads to specific cell ablation in stable**
305 **transgenic lines**
306 The lt-degron cassette was fused to the coding sequence of barnase (BAR) that con-
307 tains an artificial intron efficiently suppressing expression of functional toxic protein in
308 bacteria due to possible promoter leakiness (Gils et al., 2008).
309 When expressed in trichomes of wildtype plants under the control of the *TRY* pro-
310 moter (*ProTRY*), lt-BAR resulted in efficient ablation of trichomes. When plants were
311 grown at the permissive temperature of 14°C, leaves appeared completely glabrous,
312 as known from plants with mutated transcriptional regulators needed for trichome
313 formation such as e.g. TRANSPARENT TESTA GLABRA1, GLABRA1 and 3, or CA-
314 PRICE (Hulskamp, 2004). Constitutive growth under the restrictive temperature of
315 28°C led to a destabilization of lt-BAR and allowed trichomes to form on newly
316 emerging leaves (**Figure 1a**). Next, we evaluated the efficiency of the organ ablation
317 through analysis of trichomes within the leaf epidermis. We used polarized light mi-
318 croscopy to analyze trichome spacing and number on true leafs. The wild type devel-
319 oped trichomes under both permissive and restrictive conditions whereas no tri-
320 chomes could be spotted on mature leaves of *ProTRY::lt-BAR* plants when grown at
321 the permissive temperature (**Figure 1b**). In contrast, these *lt-BAR* expressing plants
322 fully retained trichomes when grown under restrictive conditions. This demonstrates
323 the high efficiency and temperature-sensitivity of the function of the lt-BAR fusion

324 protein as well as its effect on cell fate and organ formation. At the restrictive temper-
325 ature, leaves of *ProTRY::lt-BAR* expressing plants were indistinguishable from wild
326 type plants demonstrating an efficient degradation of the lt-BAR fusion protein to an
327 extend where the barnase protein is unable to elicit a phenotype (**Figure 1b**).
328

329 **Transient expression of lt-barnase in tobacco leaves**

330 The lt-degron, as a conditional protein 'shut-off' technique, relies on a temperature-
331 sensitive switch between POI depletion and accumulation on protein level without
332 altering transcript levels (Faden et al., 2016; Dissmeyer, 2017). Therefore, we tested
333 conditional accumulation of lt-BAR upon shift of *ProTRY::lt-BAR* expressing *Ara-*
334 *bidopsis* plants to permissive temperature. Although the cell-ablation phenotype was
335 visible (**Figure 1a,b**), detection of lt-BAR protein with standard western blotting tech-
336 niques gave only variable and unsatisfactory results. Because also detection of *lt-*
337 *BAR* transcript of *ProTRY::lt-BAR* by semi-quantitative RT-PCR proved to be difficult,
338 which is possibly due to very low levels of transcript as an effect of the additional
339 RNAse activity from barnase, we transiently expressed the lt-BAR construct under
340 the control of the *UBIQUITIN10* promoter (*ProUBQ10*) in *Nicotiana benthamiana* (to-
341 bacco) leaves. Surprisingly, the expression led to an almost temperature-
342 independent phenotype with developing necrosis and chlorosis of the leaves at both
343 permissive and restrictive temperature. The phenotype even appeared to be stronger
344 at the restrictive temperature. This might be explained through an override of the
345 degradation system in regard to lt-BAR-dependent degradation, most likely being a
346 result of expression from the strong *ProUBQ10* combined with a possibly higher cata-
347 lytic activity of the lt-BAR protein at the higher temperature (**Figure 2a**). We tried
348 once more to demonstrate the connection between the phenotype and the abun-
349 dance of lt-BAR fusion protein by western blotting and semi-quantitative RT-PCR and

350 could obtain transcript data clearly linking the observed phenotype to the expression
351 of lt-BAR (**Figure 2b**).
352
353 **Conditional toxin function can be shifted and is active at ambient temperature**
354 We then elucidated the sensitivity of the lt-BAR module. For this, plants were grown
355 at permissive temperature, shifted after three weeks to restrictive temperatures for
356 nine days, and finally shifted back to permissive temperature. During this process,
357 trichomes on newly forming leaves were monitored. The trichome phenotype of shift-
358 ed plants followed the temperature stimulus, exhibiting glabrous leaves at the per-
359 missive temperature, developing wild type-like leaves containing trichomes when
360 grown under restrictive temperature, and again showing glabrous leaves when shift-
361 ed back to the permissive temperature (**Figure 3a**). This shows that also the function
362 of lt-BAR follows the temperature stimulus exhibiting efficient ON/OFF transitions as
363 previously described for the lt-degron for non-toxic proteins (Faden et al., 2016;
364 Dissmeyer, 2017). Next, we asked whether lt-BAR expressing plants could success-
365 fully elicit their glabrous leaf phenotype when grown at standard greenhouse condi-
366 tions. Stability of lt-BAR at ambient temperature, thus the possibility to evoke a phe-
367 notype, would greatly facilitate its application as only one controlled growth environ-
368 ment, used for restrictive conditions, would be necessary in order to be applied suc-
369 cessfully. Indeed, lt-BAR was able to robustly result in glabrous leaves when plants
370 were grown in the greenhouse, suggesting sufficient stability of the fusion protein
371 even at a temperature that is higher than the standard permissive temperature of
372 14°C (**Figure 3b**). Remarkably, the lt-BAR expressing plants maintain the glabrous
373 leaf phenotype under greenhouse conditions even though the greenhouse represents
374 a significantly less controlled environment with higher temperature fluctuations and

375 initially higher then permissive temperatures then the previously used growth cabi-
376 nets.
377
378 **Conditional toxin causes growth arrest rather than cell death**
379 Then, we further elucidated the effect of lt-BAR on trichome development. Trichome
380 spacing and therefore TRY activity begins in the leaf primordia (Larkin et al., 1996).
381 Since TRY is not important for formation of the trichome itself but crucial for its spac-
382 ing and patterning by suppressing trichome initiation in neighboring cells, ablation or
383 arrest of a TRY-expressing cell could allow its expression in the neighboring cell po-
384 tentially resulting in its own ablation and starting a chain reaction. To address this
385 hypothesis, we prepared agarose imprints of the leaf surface of plants grown at the
386 permissive temperature. Analysis revealed that the trichome forming cells went into
387 an early arrest and were not able to form the typical trichome structure (**Figure 3c**).
388 This could be explained through two different mechanisms. Either the *TRY* promoter
389 is only active for a limited period which allows to express only a level of lt-BAR that is
390 sufficient to arrest cell growth but which is not sufficient to kill the cell. Or an equilibri-
391 um is reached where synthesis of active lt-BAR and possible degradation due to a
392 slight leakiness of the degron is reached. Such degradation at the permissive tem-
393 perature has been demonstrated previously for a *lt-degron::TTG1* protein fusion
394 (Faden et al., 2016). Also, growth arrest of a single cell, including its functional dis-
395 ruption, does not seem to trigger trichome formation in neighboring cells indicating
396 that trichome spacing is a restricted process only happening for a defined period dur-
397 ing leaf formation. To test whether the cells underwent cell death or resided in a state
398 of growth arrest, nuclei were fluorescently stained with DAPI. All cells showed normal
399 nuclei indicating indeed cell arrest rather than cell death (**Figure 3d**).
400

401 In very rare cases, cells were able to partially overcome the toxic effect of lt-BAR.
402 This was indicated by started initiation and formation of a trichome in these cells
403 (**Figure 4a**). This observation indicates that indeed the TRY promoter activity was
404 reduced and that, potentially, cells are able to degrade the lt-BAR over an extended
405 period of time even at the permissive temperature. However, it is noteworthy that ma-
406 ture trichomes where never found on any lt-BAR expressing plant at the permissive
407 temperature. Surprisingly, the phenotype evoked by lt-BAR strikingly resembled a
408 mutant of the transcription factor GLABRA2, the *glabra2* (*gl2*) mutant (Rerie et al.,
409 1994; Schwab et al., 2000). The *ProTRY::lt-BAR*-expressing line shows similarly ar-
410 rested cells that may grow out into basic trichome precursors (**Figures 3c,d and 4a**)
411 as described for the *gl2* mutant. GL2 is thought to be mainly important for trichome
412 formation, however, also playing a role in trichome patterning was discussed (Rerie
413 et al., 1994; Hulskamp, 2004).
414 TRY is expressed in seeds (compare eFP-browser expression data;
415 http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and therefore, seed development might
416 be affected in *ProTRY::lt-BAR* expressing plants. When plants were grown under
417 permissive temperature, siliques appeared indistinguishable from wildtype plants
418 (**Figure 4b**) and seeds did not show any alterations in amount, shape, and germina-
419 tion rate.
420

421 **N-end rule E3 ubiquitin ligase PRT1 is required for modulating lt-BAR toxicity**

422 We recently showed that in *Arabidopsis*, lt-degron fusions, for technical reasons, are
423 mainly degraded by the N-end rule E3 ligase PROTEOLYSIS1 (PRT1) (Faden et al.,
424 2016) which has a high preference for aromatic N-terminal amino acids (Potuschak
425 et al., 1998; Stary et al., 2003; Naumann et al., 2016; Dong et al., 2017; Reichman
426 and Dissmeyer, 2017; Mot et al., 2018). To generate a stable genetic situation with

427 constitutively stabilized lt-BAR and to test whether the observed phenotype was a
428 result of the fusion to the lt-degron cassette or rather a response of barnase activity
429 to the changed temperature, we introgressed *ProTRY::lt-BAR* into the *prt1* mutant.
430 Indeed, crossing the lt-BAR into the *prt1* mutant background resulted in a complete
431 and temperature-independent stabilization of the phenotype evoked through lt-BAR
432 showing that presence of PRT1 and therefore altered activity of the N-end rule path-
433 way of targeted protein degradation are responsible for the temperature-responsive
434 phenotype (**Figure 4c**).
435
436
437 **DISCUSSION**
438 Conclusively, we have shown that lt-BAR is able to efficiently mediate cell arrest and
439 thereby influence organ fate in *Arabidopsis.* The lt-BAR is superior to many systems
440 used due to a few of its characteristics. First of all, the control over the lt-BAR protein
441 is easy to execute. Since it follows a temperature stimulus, stabilization as well as
442 degradation and tuning of active protein are easy to carry out. Additionally, exoge-
443 nous addition of compounds and/or other stimuli for induction of the system are not
444 needed hence eliminating issued connected to infiltration, uneven induction of the
445 system due to uneven perfusion of inducing agents as well as possible toxic side ef-
446 fects on the system by used compounds and solvents. Additionally, the temperature
447 stimulus is easily controllable enabling for easy tuning and regulation of the system
448 therefore rendering maintenance of the lt-BAR transgene simple and straightforward.
449 Summing up, here we present a highly versatile and easy system for conditional or-
450 gan formation.
451
452

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Author Contributions

ND initiated the project and designed the experiments; FF, SM, ND carried out the plant experiments. SM and FF performed molecular cloning; SM prepared stable plant lines and carried out transient expression tests. SM and FF did transcript analysis; FF performed protein analysis. FF and ND wrote the paper and drafted the figures.

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Competing Financial Interests

The authors declare no competing financial interest.

482 **FIGURES**

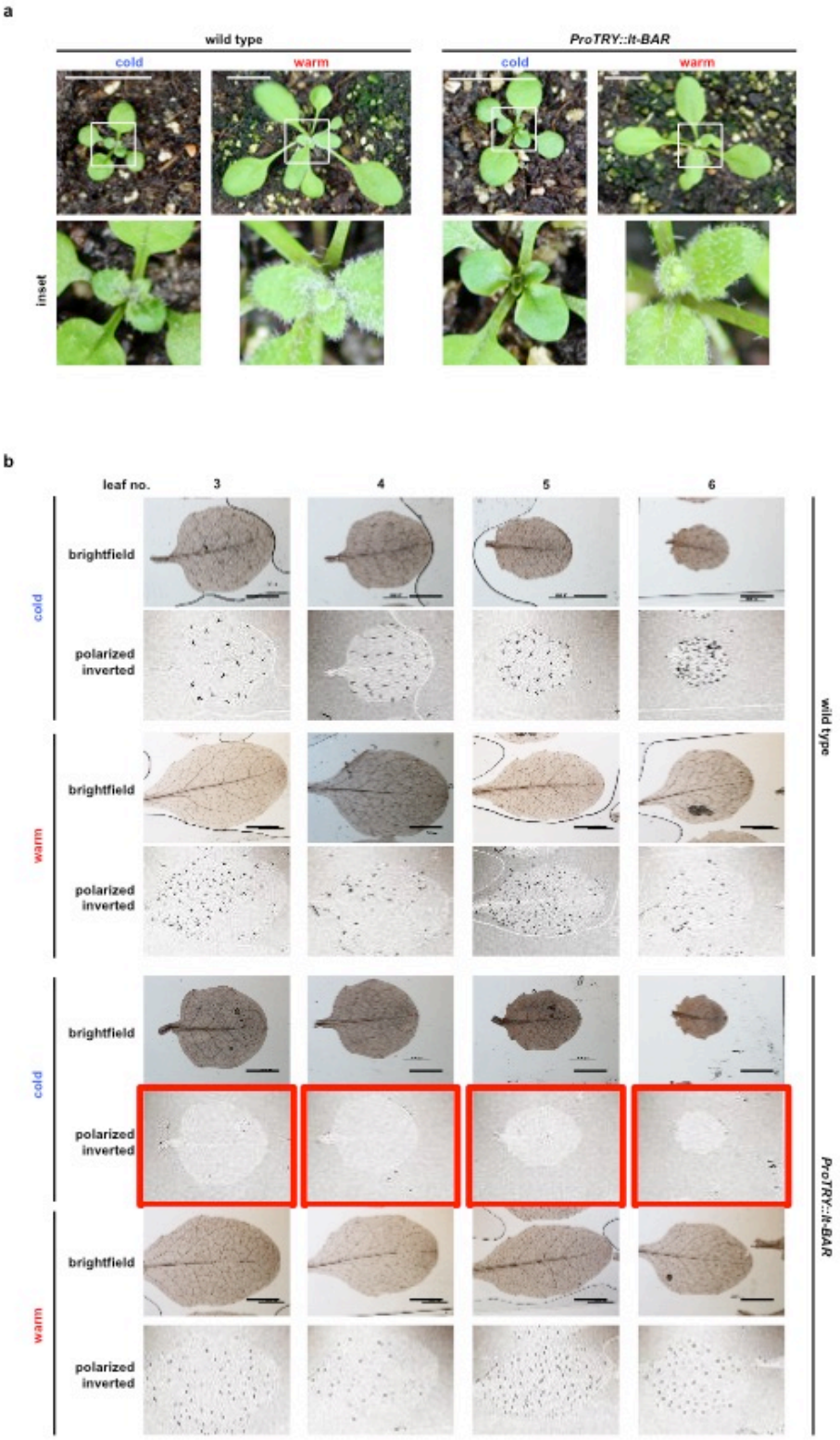

Figure 1 – Conditional function of lt-BAR mediates robust temperature-dependent ablation of specific cells. (a) Plants expressing *ProTRY::lt-BAR* and the wild type grown for two weeks at permissive (cold) and restrictive (warm) temperatures. Leaves of transgenic plants appear glabrous at the permissive temperature. Size bar, 1 cm. **(b)** In-depth analysis of true leaves of *ProTRY::lt-BAR* and wildtype plants using polarized light microscopy. Conditionally cold or warm grown plants three weeks after germination. Cold grown transgenic plants show glabrous leafs (red boxes). Size bar, 500 µm.

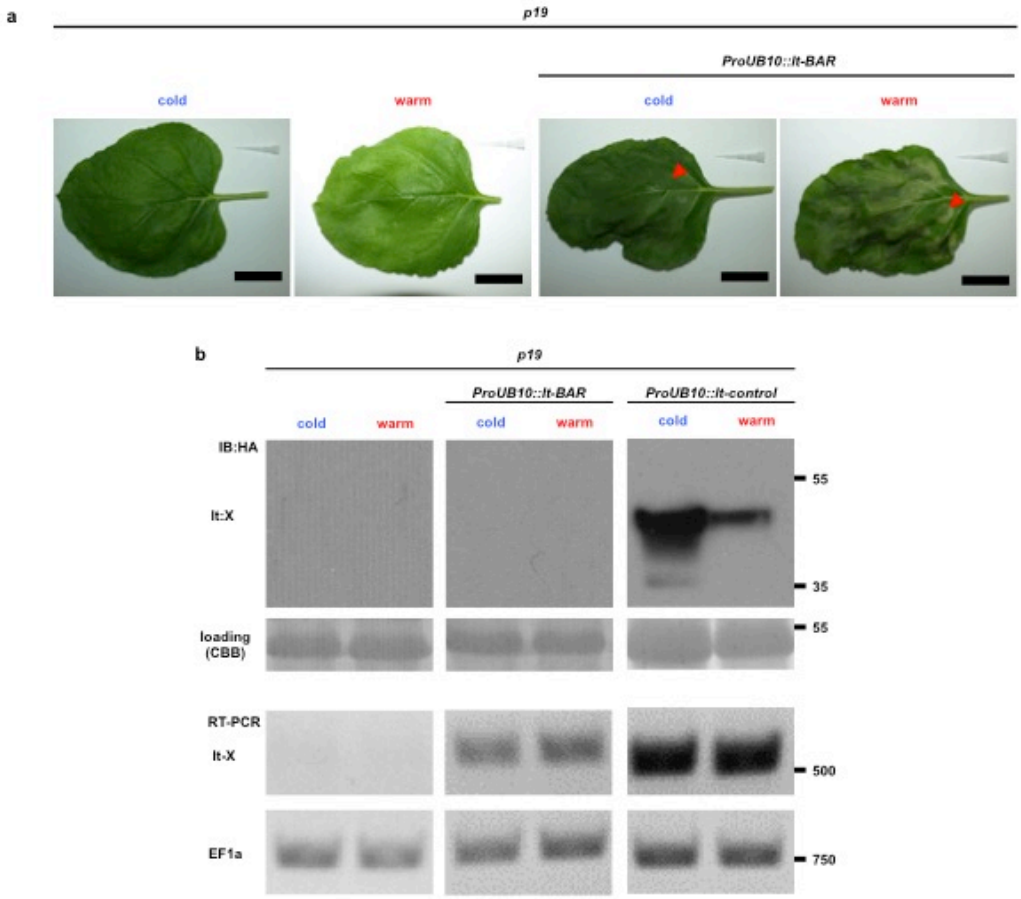

Figure 2 – Transient expression of lt-BAR and transcript analysis. (a) Transient expression of lt-BAR in leaves of *N. benthamiana* leads to a temperature-independent chlorosis/necrosis phenotype. Control plants were transformed with Agrobacteria carrying the p19 plasmid only. Size bar, 3 cm. **(b)** Analysis of lt-BAR transcript and protein levels demonstrates that it is expressed but protein amounts are below the detection limit. An lt-control protein expressed from the same promoter served as a positive control during western blot analysis. Control plants were transformed with Agrobacteria carrying the p19 plasmid only.

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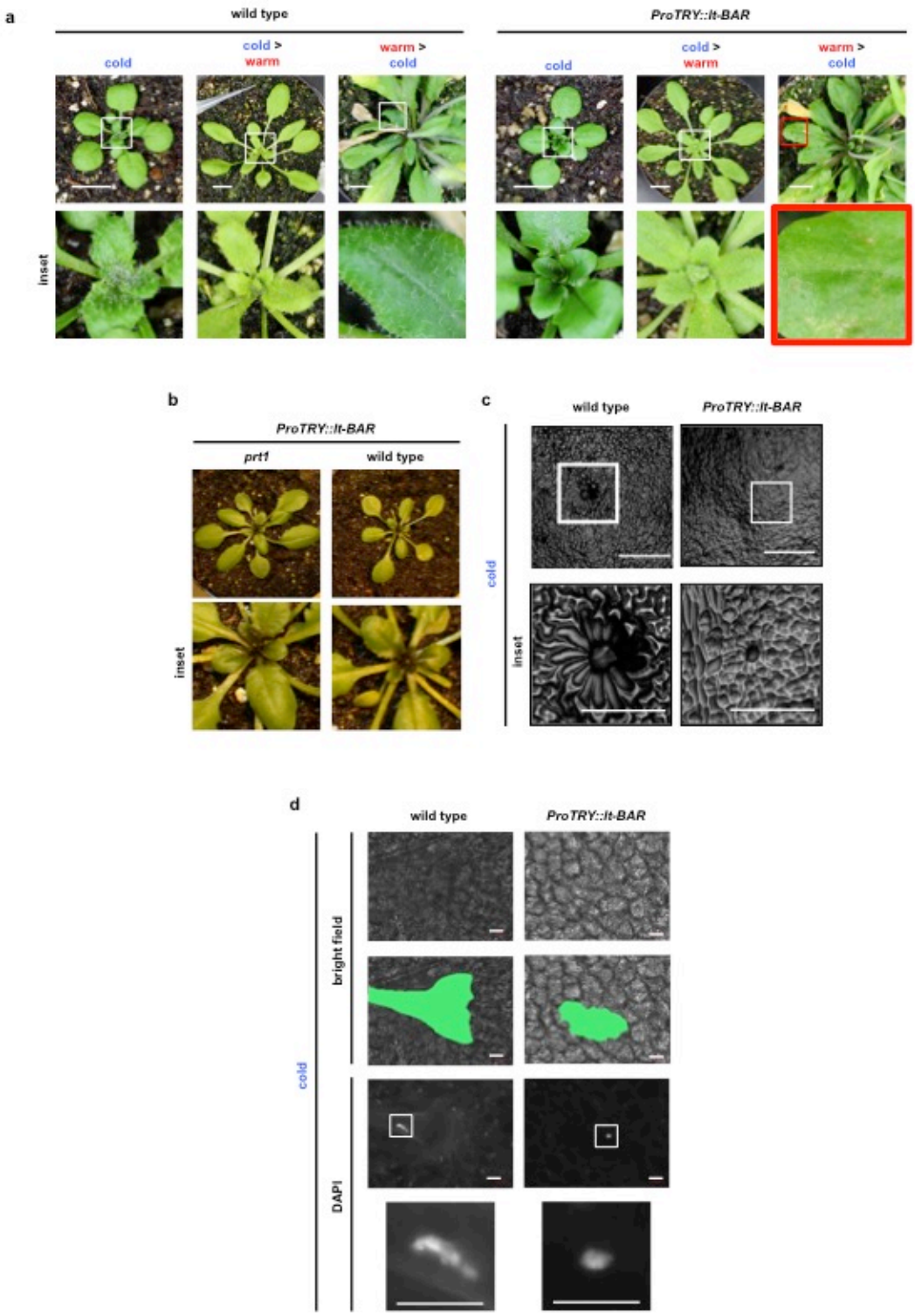

Figure 3 – The phenotype caused by lt-BAR follows the temperature stimulus but drives cells into growth arrest rather than cell death. (a) Plants grown at the permissive temperature where shifted to the restrictive temperature after two weeks and shifted back to the permissive temperature after another 9 days. The glabrous leaf phenotype occurs in relation to the temperature stimulus. Size bar = 1 cm. **(b)** *ProTRY::lt-BAR* elicits a reliable "ON"-phenotype under standard greenhouse conditions in 21-day-old plants showing glabrous leaves. **(c)** Agarose imprints of the leaves of wildtype and *ProTRY::lt-BAR* plants reveal that expression of lt-BAR impairs trichome initiating cells in developing the typical single-celled trichome structure within the plant tissue. Size bar, 500 µm. **(d)** DAPI staining of leaf epidermis. The green mark in the second lane shows the surrounding of the trichome-forming cell. The unexpected shape in the wild type results from the mature trichome being pushed on the surface of the leaf by the cover slide. Size bar, 50 µm.

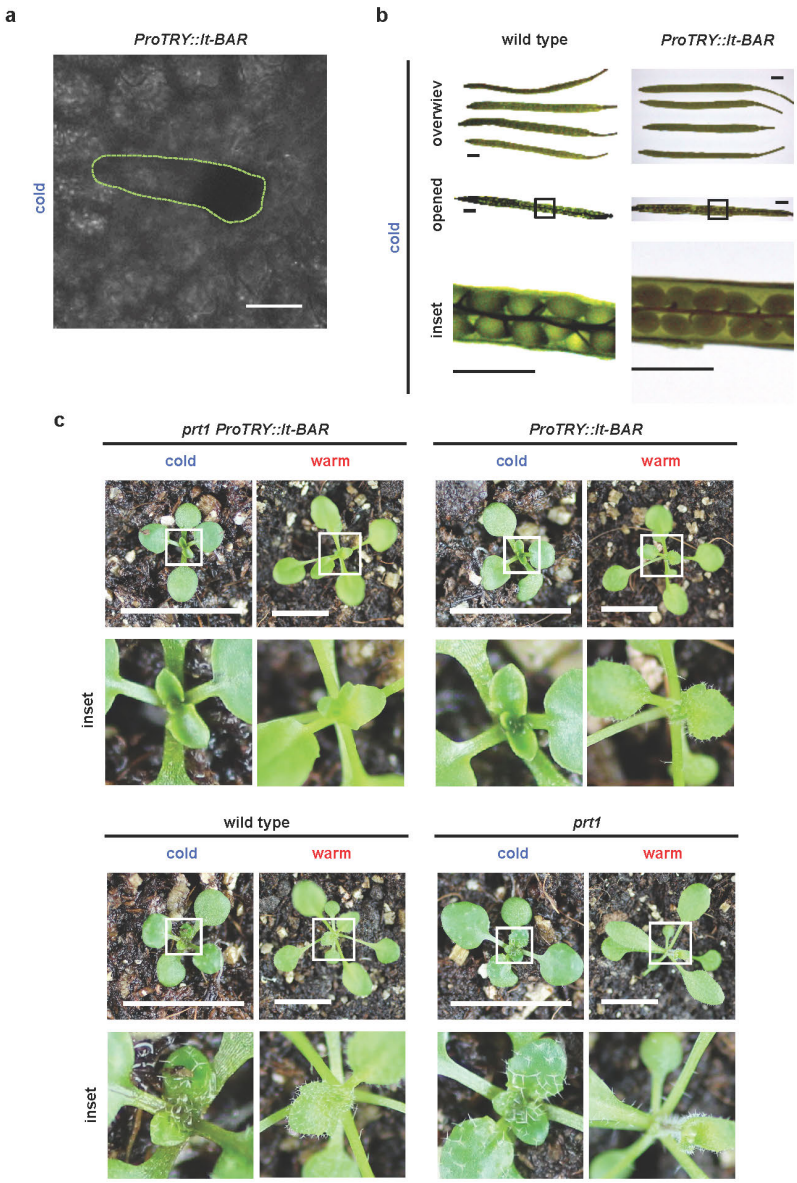

Figure 4 – Phenotypes of lt-BAR and requirement of E3 ligase PRT1 for functionality. (a) Example of a trichome precursor (green-dotted shape) forming on a late leaf of a five-week-old plant expressing ProTRY::lt-BAR and grown at permissive conditions. Size bar, 50 µm. **(b)** Expression of lt-BAR in the prt1 mutant background causes temperature-independent cytotoxic effects and constitutive phenotypes of trichome ablation. Size bar, 1 cm. **(c)** Siliques of wildtype and ProTRY::lt-BAR plants. Size bar, 1 mm.

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