CRISPR/Cas9 genome editing of potato *St*DMR6-1 results in plants less affected by different stress conditions

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Running title: *Stdmr6-1* mutant plants less affected by different stress conditions

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Abstract

Potato is the third most important food crop, but cultivation is challenged by numerous diseases and adverse abiotic conditions. To combat diseases, frequent fungicide application is common. Knocking out susceptibility genes by genome editing could be a durable option to increase resistance. *DMR6* has been described as a susceptibility gene in several crops, based on data that indicates increased resistance upon interruption of the gene function. In potato, *Stdmr6-1* mutants have been described to have increased resistance against the late blight pathogen *Phytophthora infestans* in controlled conditions. Here, we present field evaluations of CRISPR/Cas9 mutants, in a location with a complex population of *P. infestans*, during four consecutive years that indicate increased resistance to late blight without any trade-off in terms of yield penalty or tuber quality. Furthermore, studies from potato tubers from the field trials indicated increased resistance to common scab, and the mutant lines exhibit increased resistance to early blight pathogen *Alternaria solani* in controlled conditions. Early blight and common scab are problematic targets in potato resistance breeding, as resistance genes are very scarce. The described broad-spectrum resistance of *Stdmr6-1* mutants may further extend to some abiotic stress conditions. In controlled experiments of either drought simulation or salinity, *Stdmr6-1* mutant plants are less affected than the background cultivar. Together, these results demonstrate the prospect of the *Stdmr6-1* mutants as a useful tool in future sustainable potato cultivation without any apparent trade-offs.

Introduction

Current challenges for agri- and horticultural production systems include disease management, the transition towards sustainable practices, and adaptation to the effects of climate change, which likely will include increased drought and salinity of soils (Dai 2012; Velmurugan *et al.* 2019). At the same time, global population is projected to continue rising throughout this century and increasing food demands need to be fulfilled (Gerland *et al.* 2014).

Potato (*Solanum tuberosum L.*) is a widespread staple crop and the third most important food crop in the world (https://www.fao.org/faostat/). Potato tubers are a good source of nutrients such as carbohydrates, proteins, minerals and vitamin C (Raigond *et al.* 2020; Devaux *et al.* 2021), and produce a higher yield per hectare than any of the other top food crops wheat, rice or maize (https://www.fao.org/faostat/), which makes it a candidate for providing calories and nutrients where there are deficiencies (Raigond *et al.* 2020; Devaux *et al.* 2021; FAO 2023). However, potato cultivation is challenged by a number of diseases and pests such as blights, viral diseases, scabs, and cyst nematodes (https://cipotato.org/potato/potato-pests-diseases/). The late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, is a main target in resistance breeding but is still largely controlled by repeated fungicide application, which is not regarded as a sustainable practice. Resistance genes (R-genes) are generally scarce and are frequently overcome by pathogen evolution, rendering them ineffective when deployed individually (Fry 2008). Single or no R-genes are currently available for other important diseases, such as the foliar

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84 disease early blight caused by *Alternaria solani* that impacts yield and currently also requires
85 fungicide application, and tuber skin scabs that affect marketability.

86 Susceptibility genes (S-genes) that are exploited by pathogens to facilitate their survival
87 and proliferation in the host, could be functionally excluded from the genome to increase
88 resistance (van Schie & Takken 2014). In recent years, there has been growing interest in the
89 use of S-gene knock-outs in potato research, particularly for their potential to reduce
90 susceptibility to late blight (Sun *et al.* 2016; Chen *et al.* 2022; Moon *et al.* 2022; Sun *et al.*
91 2022; Bi *et al.* 2024). However, an S-gene knock-out may also confer broad-spectrum
92 resistance, as these types of genes often suppress general defence responses (van Schie &
93 Takken 2014). In *Arabidopsis thaliana*, the S-gene *DOWNY MILDEW RESISTANT 6*
94 (*AtDMR6)* was shown to encode a salicylic acid 5-hydroxylase (S5H) in a major catabolising
95 pathway of salicylic acid (SA), a phytohormone involved in defence responses (Zhang *et al.*
96 2017; Peng *et al.* 2021). Recently, the potential of deleting *DMR6* to increase resistance has
97 been explored in several crops, and increased resistance has been found e.g. to hemibiotrophic
98 bacteria and biotrophic fungi in the close potato relative tomato, hemibiotrophic bacteria in
99 banana, a biotrophic oomycete in sweet basil, hemibiotrophic bacteria in citrus, a biotrophic
100 oomycete in grapevine, and to hemibiotrophic bacteria and fungi in rice (de Toledo
101 Thomazella *et al.* 2021; Hasley *et al.* 2021; Tripathi *et al.* 2021; Parajuli *et al.* 2022; Pirrello
102 *et al.* 2022; Zhang *et al.* 2022). Most commonly, the DMR6 (S-gene) system has
103 demonstrated effectiveness in reducing downy mildew disease, which originally earned it the
104 name (Van Damme *et al.* 2005). Until now, no resistance has been indicated towards
105 necrotrophic pathogens.

106 In potato, the function of *St*DMR6-1 has been distinguished from *St*DMR6-2, as the
107 *Stdmr6-1* knock-out showed increased resistance to *P. infestans*, while *Stdmr6-2* did not (Kieu
108 *et al.* 2021). However, increased late blight resistance of the *Stdmr6-1* potatoes has only been
109 evaluated in controlled conditions.

110 SA is involved in regulation of a diverse range of physiological processes that include
111 regulation of growth and development but it is mainly associated with defence responses (An
112 & Mou 2011). The expression of SA-induced genes increase in *Stdmr6*-silenced potato plants
113 exposed to *P. infestans* infection (Sun *et al.* 2022). Silencing of the corresponding *S5H* genes
114 in rice was shown to increase the intrinsic level of SA in the plant tissue, and increased broad-
115 spectrum disease resistance in the crop (Zhang *et al.* 2022). Similarly, broad-spectrum
116 resistance was shown in *Sldmr6-1* tomatoes (*Solanum lycopersicum*) (de Toledo Thomazella
117 *et al.* 2021). However, broad-spectrum biotic resistance by *dmr6* mutation has not been
118 described before in potato or any other tuber or root crop.

119 In addition to biotic stress regulation, SA has been associated with abiotic stress
120 responses, e.g. in conditions such as salinity or drought (Kang *et al.* 2014; Koo *et al.* 2020;
121 Ma *et al.* 2020; Chen *et al.* 2023). Foliar application of SA on potato can alleviate stress
122 caused by salinity by increasing antioxidant activity and osmolytes, improving water
123 relations, gaseous exchange, morphological parameters, tuber yield, and K^+ contents, although
124 the SA concentration could be an important factor (Faried *et al.* 2022). However, increased
125 resistance to abiotic stress has not been indicated in any *dmr6* plants.

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126 Because of the broad involvement of SA in defence responses, we hypothesized that
127 the *Stdmr6-1* knock-out could aid plant vigor both under infection by diverse pathogens and
128 under abiotic stress conditions. Furthermore, we conducted four years of field trials where
129 data has been collected regarding field resistance to *P. infestans.* Tuber quality and yield were
130 also analysed.
131
132

133 # Results & discussion

134

135 The aim was to investigate the presence of broad-spectrum resistance, encompassing both
136 biotic and abiotic stressors, in the *Stdmr6-1* mutant plants. Most of these experiments were
137 conducted under controlled conditions, which are presented in the first section of the results.
138 However, to assess resistance to late blight, which had been previously documented only
139 under controlled conditions with a single strain of *P. infestans*, spontaneous infection was
140 monitored during four years of field trials. Additionally, a series of quality tests were
141 performed on the tubers obtained from the field trials, the results of which are included in the
142 second section, detailing the analysis of field trial data.
143

144 ## Experiments under controlled conditions

145

146 **Early blight infection assay**
147 Previously, we described increased resistance to the hemi-biotrophic pathogen *P. infestans* in
148 the *Stdmr6-1* mutants (denoted as KD lines) (Kieu *et al.* 2021). It was of interest to
149 additionally test resistance against an agriculturally important necrotrophic pathogen.
150 *Alternaria solani*, the necrotrophic fungus responsible for the globally important foliar disease
151 early blight, was used for this purpose. The leaves of five weeks old plants were infected in a
152 whole plant assay, and subsequent lesion development was measured at five dpi as lesion
153 diameter (Fig. 1A). All *Stdmr6-1* lines exhibited smaller lesion sizes, indicating greater
154 resistance against the necrotrophic fungal pathogen *A. solani* and suppression of its growth.
155 This conclusion is also supported by qPCR measurement of pathogen DNA abundance
156 transformed to pathogen biomass (Fig. 1B). The visible symptoms and the pathogen biomass
157 ratio followed a similar trend, with significantly lower levels of early blight in all mutant lines
158 compared to the KE (King Edward) background.
159 In the model plant *Arabidopsis thaliana*, it has been suggested that plant defence
160 involving SA is primarily linked to defence against biotrophs and hemi-biotrophs, while
161 jasmonic acid (JA) is the dominant hormone regulating defence against necrotrophs
162 (Glazebrook 2005). However, it has been shown that SA signalling is required in potato for
163 defence against the nectrotroph *A. solani*, indicating that the relationships between the
164 mentioned phytohormones and defence might be different in potato (Brouwer *et al.* 2020).
165 Now, we show that removal of an SA catabolic gene decreases symptoms of this pathogen.
166 More specifically, it is the first time a *dmr6* mutant of any crop is shown to have increased
167 resistance to a necrotrophic pathogen.
168

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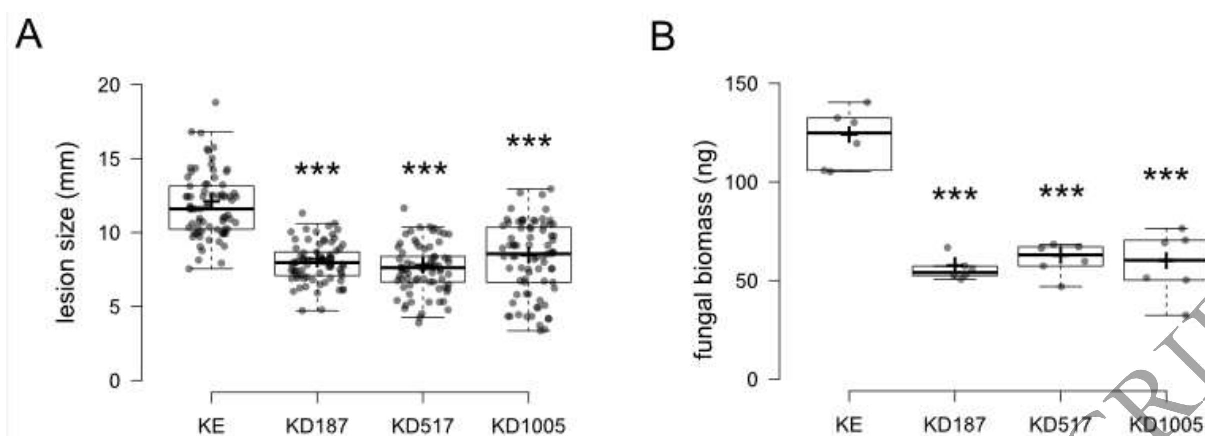
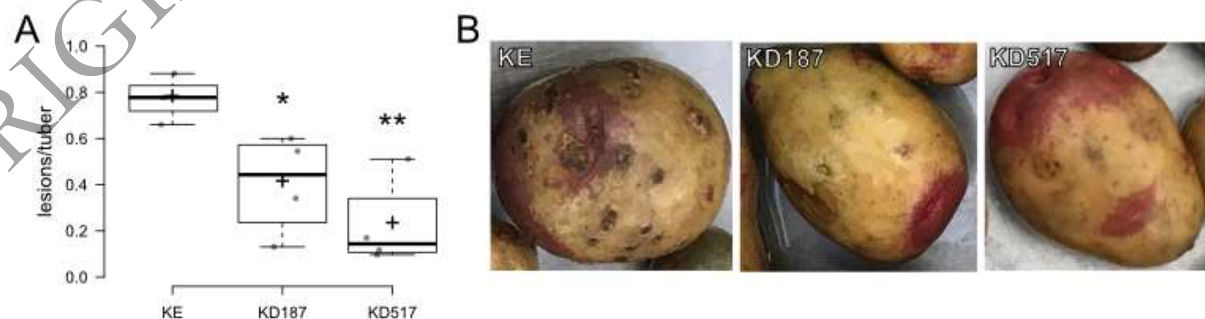


170 *Figure 1. Disease quantification five days post inoculation by A. solani on leaves in whole*
171 *plant assays. KE denotes the cultivar King Edward background, and KD denotes the Stdmr6-*
172 *1 mutant lines. Asterisks denote significant difference as compared to KE (***p<0.001). (A)*
173 *Lesion sizes, n=77. (B) Relative fungal biomass per mg sample, n=6.*
174

175 **Common scab resistance in stored potato tubers**

176 Bacterial scabs on potato are caused by species in the *Streptomyces* genus of Actinobacteria,
177 which often are present in soils (Bastas 2023). The pathogen was present at the site of our
178 multi-year field trial in southern Sweden, causing spontaneous infection and symptoms on
179 tubers. Quantification of scab lesions on harvested tubers that had been stored in a cold room
180 showed a significantly lower severity of the disease in each of the mutant lines as compared to
181 KE background (Fig. 2A). Representative tubers from KE and the mutant lines can be seen in
182 Fig. 2B.

183 While development of skin lesions can lead to secondary infections, potato skin scabs
184 such as common scab mostly affect the marketability of tubers and do not primarily affect
185 yield. Impact on marketability, however, leads to food waste. Prevention of common scab
186 disease is usually done by crop rotation and by the use of certified seed tubers, but is
187 complicated due to the commonplace presence of pathogens in soils (Bastas 2023). Resistance
188 has been suggested as the best practice of combating the disease, but resistant cultivars are
189 scarce (Bastas 2023). The broad-spectrum resistance in the *Stdmr6-1* potato could thereby aid
190 towards decreasing the severity of common scab in potato.
191
192



193
194 *Figure 2. Common scab on potato tubers. (A) The average number of lesions per tuber in a*
195 *plot represents one replicate. Asterisks denote significant difference as compared to KE*
196 *(*p<0.05, **p<0.01, n=3-4). (B) Pictures of tubers from the KE line, KD187 line, and*

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197 *KD517 line. The common scab lesions can be seen covering parts of the KE tuber, and a*
198 *lesser number of lesions are visible on the KD187 and KD517 tubers.*
199
200 **Salt stress**
201 Considering the indications of an increased broad-spectrum biotic stress resistance, and the
202 involvement of salicylic acid in both biotic and abiotic stress response, it was of interest to
203 investigate whether the increased resistance extended to plant tolerance of abiotic stress. Soil
204 salinization poses a common and detrimental abiotic challenge to agricultural areas, and crops
205 that are tolerant to growing in such conditions could be part of a solution for how to address
206 these areas.
207 Tolerance to a NaCl concentration was assessed in young *Stdmr6-1* plants using a
208 hydroponic system, and was quantified by measuring fresh weight after one week of growth
209 under the saline conditions. Each of the mutant lines had grown to significantly larger plants
210 than KE (background), as quantified by a heavier fresh weight (Fig. 3A). Representative
211 plants of each line can be seen in Fig. 3C. In growth experiments without salt, no weight
212 difference was seen among any of the lines or the background genotype (Fig. 3B).
213 Representative plants of each line from the control group can be seen in Fig. 3D.
214 Additionally, the abundance of the SA marker gene *PR1* in samples collected 12 hours after
215 initiation of the salt treatment was analysed (Fig. 3E). The function of *Stdmr6-1* was
216 confirmed by significantly higher abundance of PR1 in all mutant lines, while no difference
217 was seen in untreated samples (not shown). Resistance in *St*DMR6-silenced plants has
218 previously been associated with a super induction of SA-mediated signalling pathways during
219 infection by *P. infestans* (Sun *et al.* 2022), which is consistent with our findings. However,
220 the measured PR1 levels under salt stress are not as striking as during pathogen infection.
221 Growth of the mutant lines was impaired by the salt treatment compared to control
222 conditions without salt stress, but to a lesser extent than of the growth of KE. This suggests
223 that the *Stdmr6-1* mutants exhibit greater tolerance to saline conditions and maintain a higher
224 growth rate compared to the background genotype. This indication of improved growth could
225 be compared to the improved morphological parameters, and even yield, found after foliar
226 application of SA to potato plants under salt stress (Faried *et al.* 2022). Similar results of
227 improved growth have also been presented regarding other important crops, such as maize
228 and rice under salt stress (Jini & Joseph 2017; Tahjib-Ul-Arif *et al.* 2018). Various
229 horticultural crops have shown improved tolerance to stresses including salinity, osmotic
230 stress, heat- and cold-stress, heavy metals and radiation upon SA supplementation, as
231 reviewed by Chen *et al.* (2023). However, the intrinsic modification of SA regulation by
232 *dmr6-1* mutation would be easier to handle in large scale growth conditions, compared to the
233 more extensively studied method of extrinsic application of the hormone or derivates thereof.
234 However, to confirm our results and determine if *Stdmr6-1* could be a viable option for
235 resilient cultivation in saline soil, it is essential to conduct field trials in such circumstances.
236

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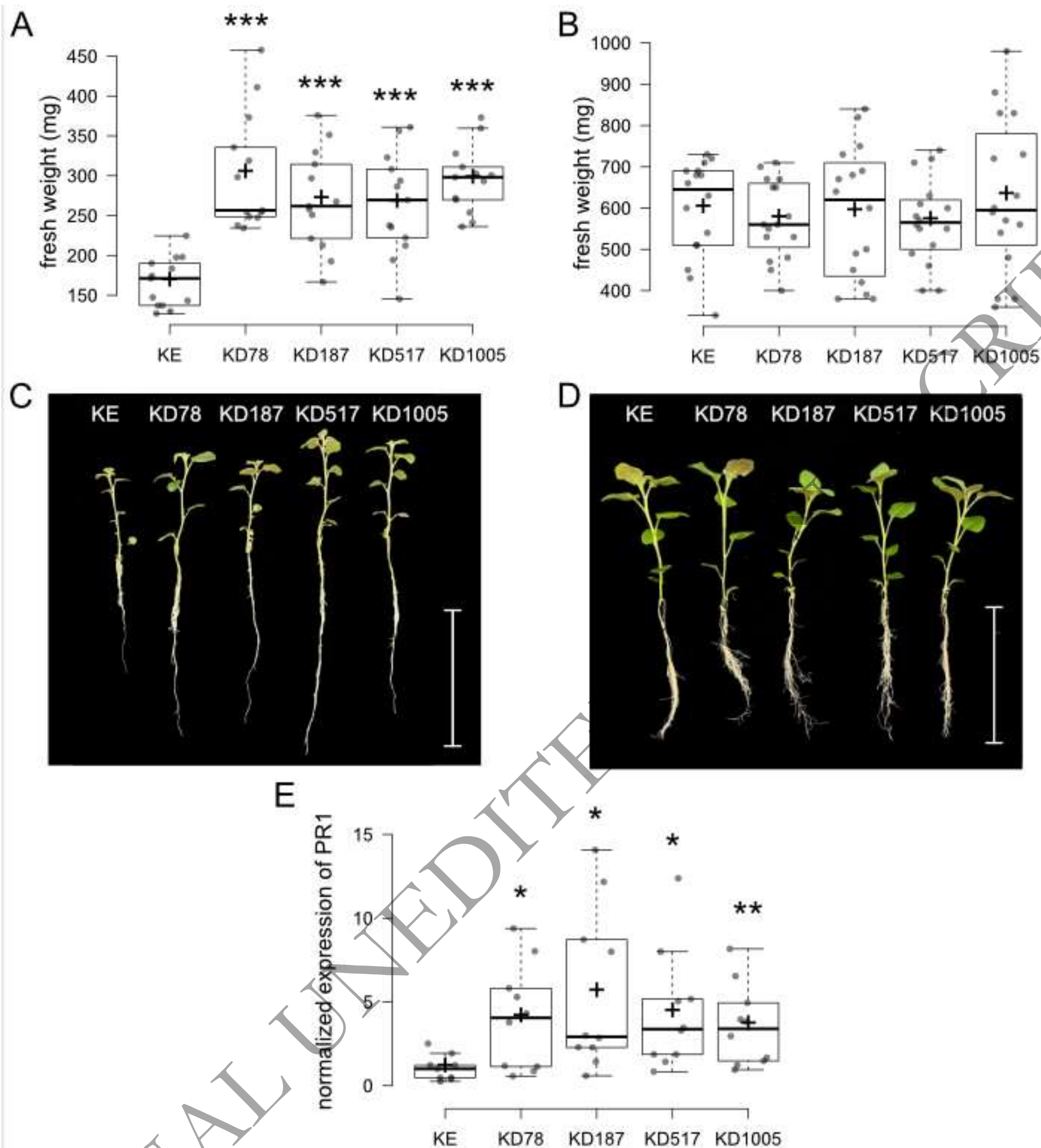

237
238 ***Figure 3****. Results of growth experiment in salinity. KE denotes the cultivar King Edward*
239 *background, and KD denotes the Stdmr6-1 mutant lines. Asterisks denote significant*
240 *difference as compared to KE (*p<0.05, **p<0.01, ***p<0.001). (A) Fresh weight in mg of*
241 *KE and four Stdmr6-1 mutant lines after exposure to 60 mM NaCl for seven days, n=13. (B)*
242 *Fresh weight in mg of plants grown in the control conditions without added NaCl, n=16. (C)*
243 *Relative size of one representative plant from each line after the experiment with 60 mM*
244 *NaCl. The scale bar represents 100 mm. (D) Relative size of one representative plant from*
245 *each line in the control experiment without added NaCl. The scale bar represents 50 mm. (E)*
246 *Normalized expression of SA marker gene PR1 in samples collected 12 hours after initiation*
247 *of the salt treatment.*
248
249

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250 **Drought simulation experiments**
251 Drought affecting agricultural land is a prevalent environmental concern. After indications of
252 abiotic stress tolerance in salt stress experiments, the *Stdmr6-1* plants were therefore further
253 subjected to experiments of mimicking controlled drought by imposing severe osmotic stress
254 in hydroponics, and in short-term drought experiments in soil.
255 In the initial experiments involving imposed osmotic stress, the plants were subjected to
256 a hydroponic system for two weeks before being deprived of water uptake by replacing the
257 growth medium with a PEG-6000 solution. After 24 hours, the solution was removed and
258 plants were re-cultured and left to recover and grow for two additional weeks. Fresh weight
259 was then measured of individual plants in each line and survival rate was noted (Fig. 4A-B).
260 The mutant lines had generally grown significantly larger during the recovery time, compared
261 to KE (background) (Fig 4C). The fraction of dead plants was 21 % for KE, 3 % for KD1005,
262 and 0 % for the other mutant lines. Results are from three iterations of the experiment
263 combined, each with similar results.
264 In the second set of experiments, plants were potted in soil and kept under well-watered
265 conditions, with soil moisture levels at 70-80 % of the water capacity, for a period of two
266 weeks. Subsequently, watering was withdrawn and the soil water content was monitored
267 daily, as well as stomatal conductance, and quantum yield of Photosystem II. A difference in
268 the area under curve (AUC) for stomatal conductance during days seven to ten, was observed
269 between KE and the mutant lines KD517 and KD1005, respectively (Fig. 4D). We could not
270 observe any difference in quantum yield during this time (Fig. 4E). Plants were allowed to
271 recover on day 21 post watering by immersing the pots in water. The recovery rate was
272 assessed two days later, and the fresh weight of above-ground tissues was measured. We
273 could not find any significant differences in the recovery rate or fresh weight under our
274 experimental conditions with relatively high humidity (Supplementary data 1).
275 In a study by Poor *et al.* (2011), tomato plants treated with exogenous SA exhibited
276 lower stomatal conductance during the first week, while it was restored in the longer term.
277 Similarly, we saw a slight decrease of stomatal conductance in the initial stages of drought,
278 starting while the soil water capacity reached around 40 % on day seven, down to around 20
279 % on day ten, levels at which a stress response could be expected to have been initiated. No
280 difference in stomatal conductance was seen during the later stages of drought (data not
281 shown). Stomatal closure-induced drought tolerance caused by endogenous build-up of SA
282 has also been shown in Arabidopsis (Miura *et al.* 2013). Hence, our data suggest that deletion
283 of *StDMR6-1* allows faster adaptation to the condition of initial drought by regulation of
284 stomata in the soil experiments. In the PEG-induced osmotic stress experiment, it is possible
285 the tolerance to the sudden and severe stress might also have been aided by additional
286 beneficial properties associated with elevated SA levels, such as osmolyte accumulation or
287 ROS scavenging (Ma *et al.* 2020).
288
289

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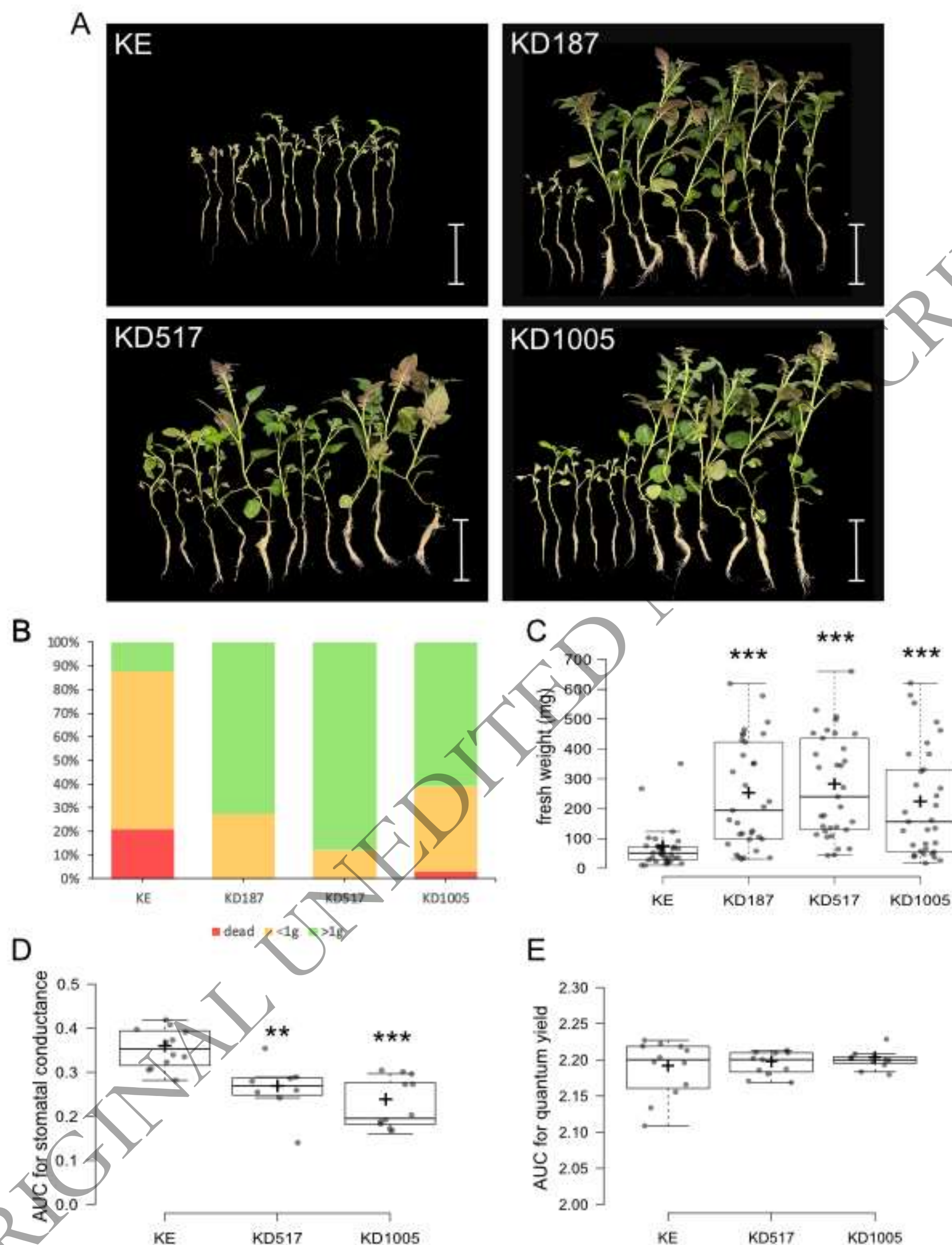

290
291 ***Figure 4***. *Drought simulation experiments by PEG treatments and in soil. (A) Pictures of*
292 *recovery phenotypes two weeks after exposure to PEG-6000 are given for lines KE (cultivar*
293 *King Edward background), KD187, KD517, and KD1005. The scale bars represent 100 mm.*
294 *(B) The distribution in percentage of plants that died (red color), recovered to a fresh weight*
295 *of below one gram (yellow color), or recovered to a fresh weight of above one gram (green*
296 *color) two weeks after exposure to PEG-6000, n=33. (C) Fresh weight of all plants after two*
297 *weeks recovery post exposure to PEG-6000, n=33. (D) Area under curve (AUC) for stomatal*

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298 *conductance (mol m^{-2} s^{-1}) days seven through ten of drought experiments in soil, n=8-12. (E)*
299 *AUC for quantum yield of Photosystem II (ΔF/Fm') days seven through ten of drought*
300 *experiments in soil, n=11-12.*
301
302 **Tuber quality**
303 Tuber characteristics are important for the marketability of a cultivar. Using tubers harvested
304 from the field season 2023, tuber hardness was measured with a penetrometer, on each half of
305 five halved raw tubers. Average force needed for KE was 109 N, 107 N for KD187, and 108
306 N for KD517. Two-tailed Student's t-tests against KE (background) showed no significant
307 difference (n=10). This indicates that no major texture differences are present in tubers from
308 the mutant lines.
309 Specific gravity was calculated for each of the genotypes, resulting in no significant
310 difference as calculated in two-tailed Student's t-test against KE (background) (n=5). Mean
311 values for specific gravity were 1.10 for KE, 1.09 for KD187, and 1.10 for KD517. This
312 indicated similar ratios of dry mass (Schippers 1976). Dry mass ratio was further confirmed
313 by weighing fresh and dried tubers. Average dry mass was 27.6 % for KE (background), 28.9
314 % for KD187, and 28.4 % for KD517, which indicates high quality tubers (Bewell 1937). No
315 significant difference was measured with two-tailed Student's t-tests (n=5). Tubers were also
316 used for a standard cooking quality test (Fig. 5). No obvious quality difference was observed
317 during this test, as all tubers remained similarly whole and non-soggy.
318

319
320 ***Figure 5****. Cooking quality test of five tubers of each line from the field trial in 2023. KE*
321 *denotes the cultivar King Edward background, and KD denotes the Stdmr6-1 mutant lines.*
322
323
324 ## Data from field trials
325
326 **Field resistance to late blight**
327 In our previous publication with the *Stdmr6-1* mutant lines, we showed increased resistance to
328 *P. infestans* in controlled conditions (Kieu *et al.* 2021). In the present study we have collected
329 four years of field trial data, quantifying the field resistance to complex natural *P. infestans*
330 infestation (Bubolz *et al.* 2022). The disease was manually scored as percentage of
331 symptomatic foliage twice a week, and the severity was then quantified by the area under the

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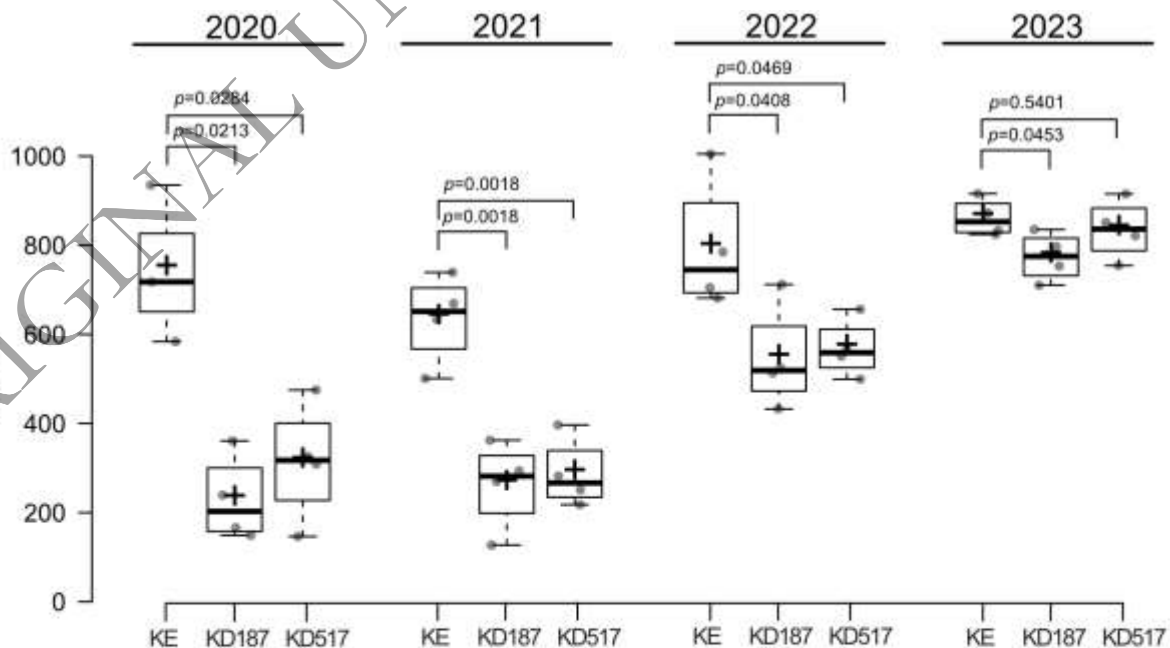
332 disease progression curve (AUDPC) (Fig. 6), as this is a standard method for pathogen
333 symptom scoring in the field and the method of quantification recommended by the
334 International Potato Center (CIP) when investigating field resistance to the polycyclic disease
335 late blight (Forbes *et al.* 2014). During the years 2020, 2021 and 2022, significantly lower
336 disease severity was observed in both mutant lines as compared to KE (background).
337 Generally, the disease progression curves followed a gradual increase starting mid- or end of
338 July during these years, and the mutant lines had a slower increase of disease. In 2023,
339 however, a significant disease reduction was observed only in the line KD187, while there
340 was no significant decrease in disease in the line KD517 (Fig. 6). We speculate that this
341 deviation from the trend of the previous years could be influenced by the special weather and
342 disease pattern of the 2023 growing season. The start of the season was exceptionally dry,
343 while the latter half was exceptionally humid. Disease onset started when the weather
344 changed, around a week into August, and progression was quicker than any of the previous
345 years, reaching complete infection in under two weeks. This resulted in few data points from
346 the disease start until complete infection, revealed by the high AUDPC values for all
347 genotypes.
348 Based on these results, knock-out of *StDMR6-1* emerges as a potential aid in the
349 reduction of fungicide use, or for supporting prolonged functionality of R-genes, by
350 mitigating infection. Similar findings of disease suppression in field conditions have been
351 described in tomato, where severity of bacterial spot disease caused by *Xanthomonas*
352 *perforans* was lower in *Sldmr6* mutants (de Toledo Thomazella *et al.* 2021). While a general
353 decrease in susceptibility was observed in our trials, these results might not be representative
354 of other geographical or climatic contexts.
355 Considering our four years of field trials, it is evident that solely targeting *StDMR6-1*
356 will not solve the potato late blight problem without the inclusion of other integrated pest
357 management techniques, which might involve additional knock-out targets or R-genes.
358



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360 ***Figure 6**. Late blight area under disease progression curve (AUDPC) shown for each*
361 *genotype in the field trials for each separate year. P-values are given for each year*
362 *separately, of each mutant line compared to KE (background) in two-tailed Student's t-tests.*
363 *KE denotes the cultivar King Edward background, and KD denotes the Stdmr6-1 mutant*
364 *lines. N=3-4.*
365
366 **Yield analysis from field experiments**
367 Yield was measured per plot and was normalized to average ton ha^{-1}, accounting for the
368 number of plants per plot each year. A major question was if the *Stdmr6-1* removal results in
369 any yield penalty. Measurements were taken separately for the untreated plots and the plots
370 treated with fungicide against late blight. No significant yield difference was observed in two-
371 tailed Student's t-tests between any of the mutant lines compared to KE (background) in
372 2020, 2022 or 2023 with or without fungicide treatment (Table 1). Only the mutant line
373 KD187 had significantly lower yield in the 2021 season (Table 1). Upon analysis of the yield,
374 no difference in tuber size distribution was observed during our potato field trials (data not
375 shown). In field trials of the potato relative tomato (*Solanum lycopersicum*), a decrease of
376 extra-large fruits was observed in *Sldmr6-1* lines, which however did not affect total
377 marketable yield (de Toledo Thomazella *et al.* 2021). The tomato field trial was done in the
378 humid, tropical climate of Florida, different from the temperate climate of southern Sweden.
379 The warmer climate might have induced breakdown of SA, leading to differences in SA
380 accumulation impacting growth, as SA has been suggested vulnerable to degradation at higher
381 temperatures (Kim *et al.* 2022). Nonetheless, tomato and potato are different crops in many
382 respects that might experience different effects upon the deletion of this gene.
383 The data suggesting no yield loss reduction of *Stdmr6*-1 lines in the unsprayed plots,
384 despite the lower disease severity, could be a cultivar-specific issue. In repeated field trials at
385 the same site, with the same best practice cultivation, other cultivars do have such yield
386 differences while King Edward repeatedly does not. One possible explanation is that the yield
387 of cultivars with an earlier tuber onset can be less affected by late blight, which is the reason
388 why earlier tuber initiation by pre-sprouted tubers has been used as a measure to decrease
389 yield losses (Karalus & Rauber 1997; Möller & Reents 2007). Hence, a question could be
390 raised about the general standard fungicide recommendations that were followed in these
391 experiments. The recommendations do not account for cultivar- or site differences, and
392 therefore it is possible that they result in excess use of fungicides without necessarily
393 enhancing yield for a particular cultivar, but merely alleviate general pathogen pressure.
394 Importantly in this context, after disrupting this S-gene in potato, yield of each mutant
395 line was generally comparable to that of the background cultivar, both in fungicide-treated
396 and untreated plots. The possibility of a yield penalty is an outstanding general question when
397 working with S-gene mutants, as the targeted genes often have broad functions with
398 pleiotropic effects (van Schie & Takken 2014). Targeting *StDMR6-1*, and thereby regulation
399 of the broadly active SA, certainly could be expected to be such a gene. No growth phenotype
400 was discovered by previous above-ground studies (Kieu *et al.* 2021), and no yield penalty was
401 present after four consecutive years of field trials. Passing these major hurdles greatly
402 enhances the potential for the *Stdmr6-1* genotype to be deployed in food production.

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403 ***Table 1***. *Yield generated from the field trial during the consecutive years 2020-2023, given as*
404 *average ton ha^{-1} for each of the genotypes in untreated and fungicide treated conditions,*
405 *separately. P-value is described for each mutant genotype, which should be interpreted as a*
406 *significant difference compared to KE (background) if p<0.05. Furthermore, SE and n*
407 *numbers (number of plots) are provided for all genotypes.*

Year	**Lines**	**Yield (ton ha $^{-1}$)**	***p* value**	**±SE & n number**	**Lines**	**Yield (ton ha $^{-1}$)**	***p* value**	**±SE & n number**
2020	Untreated				Fungicide treated			
	KE	36.2	NA	3.4 (n=3)	KE	39.0	NA	1.3 (n=4)
	KD187	30.7	*p*=0.286	3.1 (n=4)	KD187	30.4	*p*=0.137	4.3 (n=4)
	KD517	27.0	*p*=0.109	3.2 (n=4)	KD517	35.4	*p*=0.220	2.2 (n=4)
2021	Untreated				Fungicide treated			
	KE	10.7	NA	0.5 (n=4)	KE	15.4	NA	1.7 (n=4)
	KD187	7.3	*p*=0.011	0.7 (n=4)	KD187	10.3	*p*=0.053	1.2 (n=4)
	KD517	10.6	*p*=0.916	0.7 (n=4)	KD517	11.8	*p*=0.163	1.5 (n=4)
2022	Untreated				Fungicide treated			
	KE	31.2	NA	3.7 (n=4)	KE	83.0	NA	32.4 (n=2)
	KD187	40.0	*p*=0.088	1.5 (n=4)	KD187	91.2	*p*=0.849	17.2 (n=2)
	KD517	38.5	*p*=0.174	3.0 (n=4)	KD517	56.3	*p*=0.559	5.0 (n=4)
2023	Untreated				Fungicide treated			
	KE	30.5	NA	1.4 (n=4)	KE	46.1	NA	3.0 (n=4)
	KD187	30.8	*p*=0.879	1.2 (n=4)	KD187	45.8	*p*=0.958	4.6 (n=4)
	KD517	32.5	*p*=0.519	2.5 (n=4)	KD517	47.0	*p*=0.848	3.1 (n=4)

408
409
410
411 # Conclusion
412
413 A major challenge in potato cultivation is obtaining disease resistant cultivars, with the
414 scarcity of R-genes for numerous diseases presenting a specific obstacle. As an alternative or
415 possibly complementary strategy, the *DMR6* (S-gene) approach has been explored in various
416 crops. However, data from field trials and assessments of broad-spectrum stress resilience are
417 needed to fully evaluate its potential for practical use, as well as potential detrimental side
418 effects.
419 Potato *Stdmr6-1* CRISPR/Cas9 mutants have been previously described by our group
420 with focus on increased resistance to the late blight oomycete *P. infestans* in controlled
421 conditions (Kieu *et al.* 2021). Now, we show that increased resistance is generally present
422 also in field conditions in our multiyear field test with diverse *P. infestans* populations.
423 Interestingly, no consistent yield penalty was observed in the mutant lines. Based on the
424 disease resistance data, it is evident that the sole use of *StDMR6-1* as an S-gene in potato
425 breeding will not resolve the late blight problem. However, we envision that it could

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426 contribute to a reduction in fungicide application frequency and possibly extend the efficacy
427 of resistance genes. Furthermore, there could be potential in integrating other S-gene knock-
428 out targets to achieve an additive decrease in disease prevalence, such as the novel potato S-
429 gene *StPM1* which is suggested to work via vacuolar degradation in contrast to SA
430 accumulation, but also decreases susceptibility to *P. infestans* in controlled conditions, upon
431 knock-out (Bi *et al.* 2024).
432 Notably, we also describe increased resistance to necrotrophic fungi *A. solani*, and
433 reduced symptoms to the bacterial disease common scab. These are diseases for which no or
434 few strong R-genes are currently known. The observed resistance to these diverse types of
435 pathogens not only signifies a broad-spectrum resistance in the *Stdmr6-1* mutant potato but
436 also underscores the evolutionary conservation of this S-gene across various plant species, as
437 supported by resistance data observed in other plants. Furthermore, the increased broad
438 resistance or tolerance observed in *Stdmr6-1* lines may extend to abiotic stressors, although
439 this aspect requires further evaluation, especially in field conditions. Severe climate-related
440 challenges may lie ahead, making resilient crops essential in order to sustain a more robust
441 agricultural production system. Our field trial and other stress tolerance data further motivate
442 the potential usefulness of field trials in other plant systems where *DMR6* has been described
443 as an S-gene, to gain insight into the applicability across different agricultural contexts.
444 Lastly, various tuber quality aspects were analysed, with none indicating any differences in
445 the quality of the *Stdmr6-1* lines.
446 Together, these results demonstrate the prospect of *Stdmr6-1* mutants as valuable
447 assets in future sustainable potato cultivation, which come without any apparent trade-offs.
448
449
450 # Materials and methods
451
452 **Plant material and *in-vitro* propagation**
453 The tetraploid potato cultivar King Edward (KE), the background genotype, along with lines
454 of KE with *Stdmr6-1* knocked out using CRISPR/Cas9 described by Kieu et al (2021), were
455 maintained *in vitro* by sub-culturing stem internodes every three to four weeks. For
456 experimental use, apical shoots with two to three leaves were sub-cultured and left for seven
457 days to allow root development, before transference to experimental setups in a hydroponic
458 system or soil. All propagation was done onto 90 × 25 mm Petri dishes containing 40 mL of
459 Murashige and Skoog (MS) basal nutrients including vitamins (Duchefa Biochemie,
460 M0222.0050), with 10 g/L sucrose and 4 g/L Gelrite (Duchefa Biochemie). The dishes were
461 sealed with micropore medical sealing tape and kept in 20° C and 40–60 μmol/m^2/s in a 16 h
462 photoperiod.
463 The mutant lines denoted KD78, KD187, KD517, and KD1005 were utilized for the
464 *Alternaria solani* assay and salt stress experiments. For the PEG experiments, KD187,
465 KD517, and KD1005 were employed, while KD517 and KD1005 were used for the drought
466 simulation in soil. Lastly, only KD187 and KD517 were utilized in the field trial.
467
468 ***Alternaria solani* assay**

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469 A whole-plant drop inoculation assay was done using *Alternaria solani* strain 112, performed
470 as described previously by Brouwer *et al.* (2020), but using five weeks old plants that were
471 inoculated with 10 µl of 100 000 spores per ml. Results were recorded by manual measuring
472 each lesion diameter at five days post inoculation (dpi), and by measuring the relative
473 pathogen biomass by the qPCR method also described by Brouwer *et al.* (2020).
474
475 **Saline stress experiments**
476 A hydroponic culture system was applied. First, rooted shoots were washed with tap water to
477 remove away any residual agar, then they were put in shared boxes containing MS basal
478 nutrients solution (Duchefa Biochemie, 4.3 g in five litres tap water). The boxes were kept in
479 20° C with a 16 h photoperiod of 100–120 µmol/m^2/s and the liquid growth medium was
480 replaced every two days. After four days, when plants had adapted to the new environment,
481 the liquid growth medium was exchanged to a saline medium (MS basal nutrients solution
482 with 60 mM NaCl) to induce stress. The saline medium was renewed three times weekly.
483 After seven additional days, the fresh weight was measured of whole individual plants, patted
484 dry. In the control treatment plants were treated in an identical way with the exception of the
485 addition of NaCl to the liquid medium. Samples for qPCR analysis were taken 12 h after
486 introduction to the saline medium. Whole plants, briefly patted dry and immediately frozen in
487 liquid nitrogen, were sampled.
488
489 **Recovery phenotype study after osmotic stress**
490 Apical shoots that had been rooted in MS agar in petri dishes for one week were transferred to
491 a hydroponic system, which was set up in the same way as during experiments with salinity.
492 First, plants were grown two weeks in the MS basal nutrients solution, which was renewed
493 three times weekly. Then, the medium was replaced with a 20 % polyethylene glycol (PEG)-
494 6000 (Merck, 8074911000) solution, diluted in tap water, to induce osmotic stress for 24
495 hours. Afterwards, plants and boxes were thoroughly rinsed with water to remove the PEG-
496 6000 solution and re-cultured in the MS basal nutrients solution. After two additional weeks,
497 during which time plants were treated identically to before the drought treatment, the fresh
498 weight of whole plants, patted dry, was measured.
499
500 **Drought experiment in soil**
501 Apical shoots that had been rooted in MS agar in petri dishes for one week were transferred to
502 0.5L pots, each containing an equal weight of thoroughly mixed potting soil (Emmaljunga
503 Torvmull AB, S 28022 Vittsjö, Sweden), and allowed to grow in well-watered conditions (70-
504 80 % of soil water capacity, watered daily) for two weeks in a controlled environment
505 chamber. The chamber was kept at 20° C with 14/10 hour light/dark cycles, light at 160 µmol
506 m^{-2} s^{-1}, and humidity around 65 %. After two weeks, watering was withdrawn and the soil
507 water content was monitored daily. At the same time, measurements were taken using the LI-
508 600 (LI-COR), monitoring stomatal conductance (gsw, mol m^{-2} s^{-1}) and quantum yield of
509 Photosystem II quantified by fluorescence (PhiPS2, or ΔF/Fm'). Stomatal conductance was
510 measured at mid-day to early afternoon, at the peak of stomatal activity, and measurement of
511 quantum yield followed. Three mature leaves were measured on each of six plants of each

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512 genotype and the average of each plant was used for the analysis of stomatal conductance.
513 Two technical replicates were used for quantum yield. The area under the curve (AUC) was
514 measured according to Simko (2021) for the days number seven through ten post watering.
515 Water was continually withheld until day 21, at which point plants were re-watered to full
516 water capacity and allowed to recover. The rate of recovery was observed and fresh weight
517 was measured two days post recovery.
518

519 **Quantitative PCR**

520 Extraction of mRNA was conducted on each biological replicate using the RNeasy Plant Mini
521 Kit (Qiagen), following the manufacturer's recommendations. Subsequently, mRNA
522 concentration and sample quality were assessed using a NanoDrop spectrophotometer
523 (Thermo Scientific). For first-strand cDNA synthesis, 500 ng of mRNA was used. Prior to
524 cDNA synthesis, samples were treated with DNase I (Thermo Scientific) according to the
525 manufacturer's protocol, with slight modifications: 1 µl of Ribolock RNase inhibitor was
526 added to the reaction, and the termination heat treatment was adjusted to 75°C. The first-
527 strand cDNA synthesis was conducted using the SuperScript™ III First-Strand Synthesis
528 SuperMix for qRT-PCR by Invitrogen, following the manufacturer's protocol, which included
529 RNase treatment.

530 The qPCR template contained 10 µl SYBR Green PCR Master Mix, 0.4 µl of
531 respective forward and reverse primer (10 µM), 7.2 µl water and 2 µl cDNA, for a total
532 reaction volume of 20 µl. The cycling protocol started at 95° C for 3 minutes, then repeated
533 30 rounds of 95° C for 10 seconds followed by 60° C annealing temperature for 30 seconds.
534 Results from the qPCR were analysed by the $2^{-\Delta\Delta Ct}$ method, as described by Livak and
535 Schmittgen (2001). Primer sequences for the reference gene *StEF1α* were forward 5'
536 ATTGGAAACGGATATGCTCCA, and reverse 5' TCCTTACCTGAACGCCTGTCA.
537 Primer sequences for *StPR1* were forward 5' GGGAGAAGCCAAACTACAACTATG and
538 reverse 5' ACGAGCCCGACCACAACC.
539
540

541 **Field trials and analysis**

542 Field trials were conducted during the consecutive years of 2020, 2021, 2022 and 2023 in
543 Borgeby in southern Sweden (geographic position 55.75289, 13.04872), with a rotation
544 scheme of at least four years. The experimental design was as described in Bubolz *et al.*
545 (2022), with four randomized blocks. The only change was that the number of plants was
546 increased from 10 to 16 per row (plot) in 2023, while the planting distance remained the
547 same. Tubers were harvested by individual rows in the middle of September each year. Yield
548 was measured in kg per plot, and then normalized to ton ha^{-1} with consideration of number of
549 plants per plot each year. Late blight scoring was done as described by Bubolz *et al.* (2022),
550 from mid-June until the end of August. Late blight disease incidence based on area under the
551 disease progress curve (AUDPC) was calculated according to Simko (2021). The whole field
552 was sprayed against aphids with Fibro (paraffin oil) once a week, and one time with the
553 insecticide Teppiki. For the fungicide-treated part of the trial, treatment started in late June,
554 with application of fungicide once a week. The plots were treated with recommended doses of

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555 Revus (three times), followed by Ranman Top (two times), Infinito (two to four times) and
556 Ranman Top (two times). All treatments were according to the manufacturer's
557 recommendations. Fungicide treatment continued until the week before haulm killing.
558 The field trials were granted a permit by the Swedish Board of Agriculture (Dnr
559 4.6.18-01726/2020), and were performed in line with the 'Environmental Code' (1998;808),
560 and the Code of Regulations of the Swedish Board of Agriculture on deliberate release of
561 GMOs to the environment (2002:1086) and (SJVFS 2003:5).
562
563 **Common scab quantification on tubers**
564 Potato tubers had been harvested from the trial field in the middle of September 2022, and
565 kept in cold storage (5-8° C) in separate bags, corresponding to the separate plots in the field.
566 After three months of cold storage, in the middle of December 2022, the tubers were washed
567 and photographed. From the photographs, manual counting of scab lesions of each tuber
568 (between 58-179 tubers from each plot) was carried out, and each plot provided one replicate
569 given as number of lesions over total number of harvested tubers in that plot.
570
571 **Tuber quality measurements**
572 Five tubers of similar size (approximately 70-90 mm length) were selected from each line for
573 each quality measurement. Specific gravity was measured by a water displacement method.
574 Each tuber was weighed in air and then weighed suspended in 4° C de-ionized water (specific
575 gravity of 1), which revealed the tuber weight in water according to Archimedes' principle.
576 Specific gravity was calculated for each tuber by dividing the weight in air by the sum of the
577 weight in air minus the weight in water (Schippers 1976). Furthermore, the force needed to
578 pierce the raw tuber flesh was measured with the penetrometer Digital Fruit Hardness Tester 4
579 in 1 (STEP Systems GmbH), using an 8 mm diameter probe that was inserted to the halfway
580 marking in the centre of each tuber half, and the maximum force needed was noted. As is part
581 of standard screening in potato breeding programs, a cooking test was also performed to
582 indicate differences in cooking ability (Bewell 1937). Tubers were peeled, put in boiling
583 water, and cooked for 20 minutes until tender, after which they were allowed to cool, cut in
584 half, and subsequently photographed. The ratio of dry weight was measured by weighing
585 whole fresh tubers, cutting them into quarter pieces, drying them in 60° C in an oven for four
586 days, and subsequently weighing them again.
587
588 **Statistical analysis**
589 In each respective dataset, pairwise Student's t-tests were conducted comparing each mutant
590 genotype against the wild-type control (KE).
591
592
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598

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599 ## Author contributions

600 M.K, N.P.K., S.A., and E.A. jointly conceptualized the study. The investigation was carried
601 out by M.K., N.P.K., M.A.Z., M.L., and S.R. Funding acquisition was managed by E.A.,
602 N.P.K. and M.L. The original draft was written by M.K., and E.A. contributed to writing and
603 editing. All authors reviewed the manuscript before submission.

604

605 ## Data availability

606 Biological material are available upon request to Erik Andreasson (Erik.Andreasson@slu.se).
607 The numeric data used for presentation in this article are available in supplementary data 2.

608

609 ## Conflict of interest

610 The authors of this work declare that they have no competing interest.

611

612 ## Supplementary data

613 Supplementary data is available at Horticulture Research online. Supplementary data 1
614 contains data on drought experiments done in soil. The numeric data used for presentations in
615 this article are available in supplementary data 2.

616

617

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