Title: Transient reprogramming of crop plants for agronomic performance

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Dedicated to Dmitri Ivanovsky (1864-1920), the discoverer of viruses.

16 **Abstract:** The development of a new crop variety is a time-consuming and costly process due to
17 plant breeding's reliance on gene shuffling to introduce desired genes into elite germplasm
18 followed by backcrossing. We propose alternative technology that transiently targets various
19 regulatory circuits within a plant, leading to operator-specified alterations of agronomic traits,
20 such as time of flowering, vernalization requirement, plant height or drought tolerance. We
21 redesigned techniques of gene delivery, amplification and expression around RNA viral
22 transfection methods that can be implemented on an industrial scale and with multiple crop
23 plants. The process does not involve genetic modification of the plant genome and is thus limited
24 to a single plant generation, is broadly applicable, fast, tunable, versatile, and can be used
25 throughout much of the crop cultivation cycle. The RNA-based reprogramming may be
26 especially useful in case of major plant pathogen pandemics, but also for commercial seed
27 production and for rapid adaptation of orphan crops.

28 Modern plant breeding relies on recombination to introduce novel useful genes/alleles into elite
29 germplasm. Development of a new variety is time-consuming and expensive, even with a use of
30 most advanced technologies such as genome editing. We sought to design a flexible, rapid and
31 industrially scalable alternative platform to alter hormonal and other regulatory circuits within a
32 plant, by rebuilding the known techniques of transient gene expression around gene delivery
33 methods that can be performed on an industrial scale, and that can be practiced with multiple
34 crop plants. Our approach focused on two types of vectors commonly used in laboratory science;
35 namely, *Agrobacterium* as the primary DNA vector, and RNA viral amplicons as
36 secondary/primary vectors and amplifiers of information molecules. We and others have
37 successfully used *Agrobacterium*-based transfection to design industrial-scale manufacturing
38 processes for producing recombinant proteins in plants[1-4], including biopharmaceuticals,
39 vaccines and biomaterials[5]. This earlier-generation transient reprogramming focused on a single
40 plant species, *Nicotiana benthamiana*. The method required vacuum-assisted infiltration of
41 bacteria into the intercellular leaf space and, by design, ignored the general agronomic
42 performance of the plant other than the high-level expression of heterologous recombinant
43 proteins that were almost exclusively of non-plant origin. A few attempts to modify agronomic
44 traits, namely viral induction of flowering, were also previously reported, but were limited to
45 research-scale experiments[6-11].
46 We report here that multiple economically important crop plants can be induced to exhibit
47 desirable agronomic performance traits, by simply spraying them with agrobacteria carrying viral
48 replicons to express plant genes. Moreover, we also demonstrate that most of the agronomic
49 traits can also be engineered by spraying plants with packaged RNA viral vectors thus

50 eliminating DNA release into environment altogether. In our studies, manipulation of traits was
51 based on expression or silencing of specific plant regulatory genes that are components of
52 hormonal circuits, such as flowering control, gibberellin, abscisic acid, ethylene pathways and
53 several others. Using appropriate molecular techniques, we evaluated several viral vectors, tested
54 a large set of plant genes and characterized molecular events linked to plant phenotypes. We
55 show that proposed transfection generates a temporary cascade of new information in the plant
56 and enables the controlled alteration of agronomic performance in multiple useful ways.

57 ## Results

58 ***Agrobacterium* and packaged RNA viral vectors as tools for industrially scalable**
59 **transfection**

60 Based on processes widely practiced in agronomy, namely spraying plants with solutions or
61 suspensions to deliver agrochemicals, we evaluated delivery of agrobacteria and viral particles.
62 These vectors were found effective when applied using standard conditions typical for industrial
63 sprayers (1-3 bar pressure; 1-4 mm atomizer nozzles) and were unaffected by these spraying
64 conditions. The delivery of agrobacteria required use of surfactants such as Silwet® L-77,
65 Silwet® Gold, Triton™ X-100 or Tween® 20 to induce 'stomatal flooding' and allow bacteria to
66 enter the intercellular space of the leaf[4]. We also explored the use of abrasives such as silicon
67 carbide F800 or diatomaceous earth as described in ref. [12,13], which allowed for improved
68 transfection of several plant species. By applying a suspension of agrobacteria of approximately
69 10^6 cfu per ml (10^3 dilution of the overnight culture $OD_{600} = 1.5$) mixed with Silwet® L-77 at a
70 concentration of 0.1% to the test plant *N. benthamiana* (**Fig. 1a**), the frequency of transfection by
71 agrobacteria was as high as 10^{-2} per leaf cell (**Fig. 1b**, and ref. [4]). Agrobacteria were efficient in

72 the delivery of viral vectors with either localized or systemic movement as well as movement-
73 disabled ones (**Fig.1c,d**). Subsequent studies revealed that for most traits to be efficiently
74 delivered, the viral vector should be able to move locally or systemically through the phloem. In
75 the proof-of-principle experiments using dipping of *Nicotiana benthamiana* leaves into the
76 suspension of agrobacteria, we also showed that it is possible to achieve subsequent transfections
77 by treating the same plant several times (**Fig. 1e**).
78 Using *Agrobacterium* carrying viral vectors with a *GFP* cargo (reporter) gene, we evaluated
79 representatives of 28 plant species from 6 plant families (**Supplementary Table 1**) and found
80 that leaves of many dicotyledonous plants of practical interest such as tomato, potato, pepper,
81 sugar beet, spinach, soybean, and monocotyledonous plants maize, wheat, etc., can be efficiently
82 transfected using our standard spray technique. *Potato virus X* (PVX)-based replicons performed
83 efficiently in most crop plants we tested (**Fig. 1f**). In a few cases, we used other viral backbones
84 (**Supplementary Fig. 1**) developed by us or by other laboratories, such as *Tobacco mosaic virus*
85 (TMV), *Tobacco rattle virus* (TRV), or *Clover yellow vein virus* (ClYVV)[14-17] (**Fig. 1g**). As an
86 example of a monocotyledonous species, maize was transfected by *Maize streak virus* (MSV)
87 (**Fig. 1g**). It should be mentioned that the plant viral vectors used in our work had generally only
88 mild effects on the plant phenotypes such as slightly delayed growth and occasionally leaf
89 mosaic.
90 We also tested the efficacy of spray-based delivery of RNA virus particles (**Fig. 1h**) and found
91 that the process is less efficient, with a frequency of transfection in *N. benthamiana* of less than
92 10^{-4}-10^{-5} per cell. Nevertheless, even at this low frequency, viral particle spraying was sufficient
93 to achieve the results described below.

94 **Transient manipulation of the flowering regulatory pathway**

95 Underlying mechanisms of the transition from vegetative to flowering state in plants have been
96 the subject of century-long studies, with the concept of a 'principle' or inducer moving from
97 leaves to apical meristem and causing flowering ('florigen') first postulated by Mikhail
98 Chailakhyan in 1936[18]. The molecular basis of the process, however, has been clarified only
99 during the last two decades[19]. In the core of the process is the so called Flowering Locus T
100 mobile protein (FT) whose expression is induced by external light intensity/day length via the
101 phytochrome machinery; FT then moves from leaves through phloem to apical meristem and
102 interacts with transcription factors that trigger the transition to flowering differentiation of the
103 meristem[20,21]. Since the primary delivery site for our transfection treatment is the plant leaf, we
104 decided to hijack the process by transiently producing additional FT protein in the leaves.
105 Most of these experiments relied on *Agrobacterium*-based delivery of PVX vectors harboring
106 one of the multiple genes known to be involved in flowering control (**Supplementary Table 2**).
107 In agreement with their central and universal role in flowering control, expression of genes
108 encoding mobile *Arabidopsis* FT or its orthologs from various species such as tobacco, tomato,
109 rice, and others promoted flowering, shortening floral transition in multiple plant species
110 (*Arabidopsis thaliana*, tobacco, tomato, pepper, and wheat) (**Fig. 2a-j**) whereas flowering
111 repressors of the same family delayed flowering (**Supplementary Fig. 3**). The flowering
112 induction results were most dramatically detectable in the tobacco variety 'Maryland Mammoth',
113 which does not naturally flower under long-day field conditions (flowering occurs close to
114 December in the Northern hemisphere), leading to high accumulation of vegetative biomass. A
115 limited number of genes controlling upstream (*PhyB*) or downstream (*SOC1*) components of the

116 flowering regulatory cascade that we tested didn't result in any visible phenotypic changes (data
117 not shown).
118 It would be practically useful to control vernalization, the requirement for prolonged seasonal
119 cold (e.g., winter) for successful flowering[22,23]. A tighter control of vernalization (to avoid
120 'bolting' in crops such as sugar beet), or an easy way of lifting the vernalization block, would be
121 of importance for seed production and breeding of crops such as winter wheat, barley, rapeseed
122 and others. In *Arabidopsis*, there are numerous biennial ecotypes that do not flower during the
123 first year. We evaluated the effect of transient *FT* expression or antisense silencing of the
124 flowering repressor *FLC* in proof-of-principle experiments. We found that both of those
125 treatments were effective in promoting flowering in vernalization-dependent ecotypes such as
126 Tul-0, Tamm-2, Lov-5 and others, without any vernalization treatment (**Fig. 2j, k**).
127 We subsequently evaluated the version of the transient delivery cascade that relies on spraying
128 plants with PVX viral particles produced in *N. benthamiana*, rather than *Agrobacterium* cells,
129 thus obviating the need to release DNA-based vectors into the environment altogether. Transition
130 to flowering was demonstrated with tobacco and tomato (**Fig. 2l,m** and **Supplementary Fig.**
131 **3n,o**). Due to practical considerations and performance, as well as compliance with
132 environmental and regulatory constraints (no DNA release in the field; see also below and
133 Discussion), we view the application to crops of self-limited RNA virus particles as the more
134 promising version of the transient agronomic trait modification platform.

135 **Transient manipulation of the gibberellin regulatory pathway: dwarfism**

136 Traits such as dwarfism and semi-dwarfism have been the basis of the 'green revolution' in plant
137 breeding during 1960s-70s, and the underlying molecular mechanisms of these traits are well

138 understood[24,25]; recently reviewed by Eshed and Lippman[26]. Many of the agronomically
139 important ‘semi-dwarfism’ genes belong to the gibberellin regulatory pathway, the best
140 characterized of which being gibberellic acid (GA) oxidases; the others are dehydration-
141 responsive element binding (DREB) proteins. To effect dwarfism using transient regulatory
142 interference, we evaluated multiple GA oxidases as well as relevant DREB and DELLA-motif
143 proteins[27-29] transiently expressed in a number of important model and crop plants including
144 tobacco, tomato, pepper, pea, broad beans and wheat. Being delivered by *Agrobacterium*, PVX
145 vectors harboring *GA2-oxidase* or *DREB1-type* genes consistently suppressed stem elongation
146 and plant height in tobacco, tomato, pepper and *Nicotiana benthamiana* (**Fig. 3a-d** and
147 **Supplementary Fig. 4c-f**), while PVX-delivery of GA20-oxidase enhanced stem length (**Fig.
148 3g,h** and **Supplementary Fig. 4c-f**). Similarly, *Agrobacterium*-delivered ClYVV vectors
149 carrying *GA2-oxidase* gene significantly reduced the stem length in pea and broad bean (**Fig. 3e**
150 and **Supplementary Fig. 4**). Notably, plant height responded to the dose of *Agrobacterium*
151 (**Supplementary Fig. 4a**) and was reversal to external GA application (**Supplementary Fig.
152 4b**). Viral particle-based transfection with GA2-oxidase or GA20-oxidase was similarly effective
153 in modulating plant height in wheat and tomato (**Fig. 3i-l**). Both *Agrobacterium*-based as well as
154 viral particle-based transfection allowed the control of plant height, and the effects of the various
155 genes on plant height are in line with the current understanding of the role of those genes in
156 gibberellin metabolism[30]. Various genes and their homologues from different species yielded
157 different levels of dwarfism. Up to 40% reduction in height was obtained with *GA2ox8* from
158 soybean and *DREB1A* from *Arabidopsis*. Detailed analyses of individual gibberellins in leaves
159 and stems of transfected plants confirmed significant changes in active and inactive gibberellins
160 in *Nicotiana benthamiana* and tomato which were in line with the phenotypes observed

161 (**Supplementary Figs. 4 and 5**). We also overexpressed DELLA proteins which contained
162 mutations known to impair GA-promoted protein degradation and plant growth[30], but no
163 significant phenotypic effect was detected.
164 We also performed a limited set of open field experiments under permit No. 15-041-101r from
165 the US Department of Agriculture (USDA). In those studies, tomato plants were sprayed with
166 *Agrobacterium* carrying the PVX-based gene *GA2ox1*. As anticipated, the transfected plants
167 exhibited significantly reduced height (**Fig. 3m,n**).

168 **Transient reprogramming of other agronomic traits**

169 Drought tolerance is among the most economically important agronomic traits, and a potential to
170 manipulate those responses rapidly and only when the stress factor is present ('trait on demand'
171 concept) would be very useful. We therefore evaluated transient expression as a rapid-response
172 intervention to induce drought tolerance by using the well-characterized *notabilis* mutant of
173 tomato, which is deficient in 9-cis-epoxycarotenoid dioxygenase (NCED3), a central component
174 of the abscisic acid (ABA) biosynthetic pathway[31]. Due to the lack of ABA, this mutant is
175 highly sensitive to drought, but we show that the sensitivity is significantly reduced as a result of
176 transfection with a functional *NCED3* gene (**Fig. 4a**). The transfected plants also showed
177 increased water retention ability as well as significantly elevated concentrations of abscisic acid
178 and of its biologically inactive but reversible glucose conjugate (**Fig. 4b**), and phaseic acid and
179 dihydrophaseic acid (abscisic acid catabolites)[32] (**Supplementary Fig. 6**). In another experiment,
180 wild-type tomato plants were transfected with DREB transcription factors that are mediating
181 stress tolerance[33]; again, higher drought tolerance along with higher water retention was
182 demonstrated (**Fig. 4c,d**).

183 In an attempt to effect transiently the trait of insect resistance that has been the basis of modern
184 transgenic insect-resistant crops (corn, soybean, cotton)[34], we expressed the *Bacillus*
185 *thuringiensis* gene *cry2Ab* in tobacco using a PVX vector with subsequent infestation of the
186 plants with tobacco-adapted hornworm *Manduca sexta*. Cry2Ab-transfected plants demonstrated
187 high toxicity to hornworms, concomitant with the presence of relevant levels of Bt toxin in plant
188 leaves (**Supplementary Fig. 6**).
189 One important feature of the transient reprogramming concept would be the ability to spray plant
190 leaves to manipulate traits in distal organs such as flowers, seeds and roots. Efficient
191 development of fruits and overall productivity are of special interest in fruit-bearing crops such
192 as tomato. In a proof-of-principle study, we conducted expression experiments with a tomato *sft*
193 ('*single flower truss*') mutant deficient in flower organ formation, which results in development
194 of a single flower per flower truss[35]. *SP3D*, the corresponding gene controlling the fate of
195 meristem in inflorescence, was expressed using *Agrobacterium* delivery of PVX viral vectors.
196 The treatment effectively restored multi-flower truss structure of inflorescence and restored the
197 number of flowers/fruits per truss (**Fig. 4e,f**).
198 In yet another demonstration of the usefulness of the proposed technology, we delayed fruit
199 ripening in tomato by spraying plants with *Agrobacterium* carrying PVX vector containing
200 antisense fragment of the *DML2* gene for DEMETER-like DNA demethylase regulating the
201 transcription of genes involved in fruit ripening[36] (**Fig. 4i,j**).
202 Similarly, to demonstrate the ability of transient delivery to modulate another useful agronomic
203 trait, production of a pigment with reported human health benefits, we expressed in tomato plants
204 the *ANT1* gene encoding a transcription factor controlling anthocyanin synthesis[37,38]. Although

205 fruits of the resultant transfected plants were only sectorially transfected (**Fig. 4g**), the fruits
206 nevertheless accumulated high levels of anthocyanin in the pericarp (**Fig. 4h**).

207 **Tunable control of agronomic trait expression**

208 Results of our experiments demonstrate that the amplitude of expression of multiple agronomic
209 traits can be modulated by more than one mechanism; for example, by inducing expression of
210 different proteins participating in the same specific regulatory circuit, or by using protein
211 orthologs from various plant species. We also explored additional approaches to vector
212 optimization with the ultimate goal of achieving flexible control of trait expression. These
213 studies were done using PVX, because in our hands it represents the more flexible and broadly
214 effective viral platform.

215 PVX-based vectors used in these studies achieved systemic movement within the plant and
216 exhibited the ability to move and transfect organs other than primary transfected leaves. The
217 latter feature was dependent on various factors, the most obvious one being the length of the
218 heterologous gene insert. We also found that the GC content of the insert is another important
219 factor in this process. Genes with higher GC content were more stable and were not eliminated
220 as quickly from the vector relative to genes with lower GC content[39]. Consequently, using inserts
221 optimized for GC content resulted in much more stable vectors that better delivered the genes of
222 interest to distal parts of the plant (**Fig. 5a**).

223 Another way of controlling trait expression is to design vectors that provide higher expression of
224 the cargo gene. A solution that we applied in these studies was to place the gene of interest
225 (GOI) in the distal 3' end of the RNA genome, which is where the most highly expressed gene
226 (viral coat protein) typically resides[40]. This solution somewhat compromises overall efficacy of

227 the viral vector but provides for higher expression of the gene of interest. Among the new

228 empirical approaches, we chose to engineer our PVX backbone by inserting known modulators

229 of viral pathogenicity[41,42]. We found that small viral genes of cysteine rich proteins (CRP), which

230 are believed to interfere with the plant gene-silencing machinery[42], inserted between the STOP-

231 codon of gene of interest and 3'UTR of PVX, can dramatically increase GOI expression (**Fig.**

232 **5b,g** and **Supplementary Fig. 7**). This in turn provides for broader expression range of the

233 specific agronomic traits described above (**Fig. 5h,k**). The effect was particularly pronounced for

234 floral repression; it was stably achieved only with the new vector containing CRP.

235 Even limited levels of viral vector replication within the plant may impose some penalties on

236 overall crop performance. In our experiments, we typically included two negative controls:

237 plants that were not transfected and plants that were transfected with the GFP gene. The latter

238 sometimes resulted in statistically measurable although very limited in terms of phenotype and

239 practical consequences effects on the parameters under investigation, for example somewhat

240 lower height, altered time to flowering, etc. (e.g. **Figs. 2-4**). An additional control consisting of

241 empty viral vectors devoid of heterologous genes was found to be less practical because these

242 vectors were in some cases phytotoxic, likely due to higher aggressiveness of the vector, and

243 therefore confounding interpretation of results.

244 **Safety and regulation-compliance aspects of transient expression technologies**

245 We evaluated the stability of gene inserts upon systemic movement of RNA vectors using PVX-

246 based constructs, and found that the heterologous inserts are invariably and relatively rapidly lost

247 during systemic movement of the vector in the plant (**Fig. 6a**). The loss is more rapid if the

248 inserts are large genes and if the GC content of the insert is lower[38]. This sensitivity of the

249 vectors to the GC content of the insert allowed us to engineer vectors having either higher
250 stability, resulting in higher expression of the trait due to the longer time that the vector is intact
251 (discussed above, **Fig. 5h,k**), or lower stability, leading to more rapid loss of the cargo gene from
252 the RNA virus backbone. To illustrate, we rewrote the GFP gene (61.4% GC) by designing a
253 sequence with lower GC content (40.3%) by altering the codon usage. The viral vector carrying
254 this synthetic gene demonstrated lower overall GFP fluorescence in infected leaves and a more
255 rapid loss of the insert (**Fig. 6b,c**).
256 Plant viruses fall into two general categories: (a) transmissible viruses that can be inherited by
257 the progeny upon sexual reproduction of the plant, albeit with low frequency, and (b) non-
258 transmissible ones that cannot be transmitted to the progeny. The main viruses used as vector
259 backbones in this study, namely PVX and TMV, are known to be non-transmissible, and our own
260 studies confirmed this for PVX-based viral vectors (**Fig. 6d**).
261 In a limited set of experiments under permit No. 13-323-101r from the USDA, we released
262 disarmed non-auxotrophic *Agrobacterium* strain NMX021 carrying a binary vector encoding a
263 PVX-driven GFP gene into an open field environment by spraying tobacco (**Fig. 6e**) and tomato
264 plants, resulting in a release of approximately 10^{11} bacterial CFU. We then followed the fate of
265 released agrobacteria in transfected plants and in the soil surrounding the plant roots, and found
266 that within 90 days after spraying the number of detectable bacteria in a plant drops by four logs,
267 from $>10^5$ to less than 10 cells/gram of plant biomass (**Fig. 6g**). Similarly, the number of
268 detectable agrobacteria in the soil falls from initially 10^4 cells per gram of soil to undetectable
269 levels 11 months later (**Fig. 6f**). It should be mentioned than during spring and summer time, the
270 natural agrobacterial population in a rich soil can be as high as 10^7 cells per cm^3 of soil[43].

Discussion

We demonstrate here in a range of various crop species a transient and practically useful alteration of the major regulatory circuits that have been the basis of crop domestication and past agricultural revolutions. These include vegetative/reproductive changes and short/tall stature control[27] and several others. The procedure results in industrially scalable delivery of genetic information in the form of self-replicating RNA vectors. The practice does not involve permanent genetic modification of the crop. The fundamental differences between this technology on the one hand, and the current breeding methods including those based on genetic transformation on the other, are that our approach does not involve genetic modification of the plant genome, is broadly applicable, fast, tunable, versatile, limited to one plant generation, and can be used throughout most of the crop cultivation cycle.

The major differences between our approach and the other emerging transient technology, namely treatment of plants with short double-stranded RNAs (in its present form, limited to RNA interference-based control of plant insects[44]) are that the vectors described here are capable of limited self-replication and movement within a plant, thus providing virtually endless applicability and, additionally, the vector products can be made at very low manufacturing cost. The described interventions involving replication of viral constructs within a host can result in certain penalties, but those can be minimized through further tuning and weighted against the obvious (and demonstrated) benefits. Plant viral vectors used in this study had generally only mild phenotypic effects such as slightly delayed growth and occasionally leaf mosaic. In this regard, it should be mentioned that selection of the optimal final result is also a requirement of any conventional crop breeding program. Importantly, however, the trait development based on transient approach is faster and allows for higher throughput.

294 We hope we have provided here substantial and broad evidence that practically useful phenotype
295 changes can be generated through fast transient transfection of crops. This general proof of
296 principle addressing multiple crops and multiple traits needs further development steps in future;
297 those should include defining and testing most important traits/crop combinations, conducting
298 field trials and generating data for regulatory approvals, and scale up. Judging from our results, a
299 single universal viral vector effective across all important crops is unrealistic; vectors' efficacy
300 will be determined primarily by virus host spectrum: species-specific to multi-species or even
301 multi-family specific. Currently, PVX vector backbone shows the broadest applicability, but this
302 backbone is not optimal for families such as beans, or for monocots.
303 Open field trials would need to be conducted using industrial-size equipment and an optimal
304 control of the spraying under open field conditions (day time, wind, humidity) needs to be
305 developed.
306 Early studies on the safety of proposed technology indicate that the initial vector organisms,
307 *Agrobacterium* or packaged RNA virus particles, are self-limiting, and it should be possible to
308 improve their environmental containment further by introducing additional safety locks. For
309 example, *Agrobacterium* can be made multiply auxotrophic, suicidal or otherwise disabled, or
310 alternatively it can be re-coded[45] making it unable to interact with other bacteria or survive after
311 release. The technology based on release of packaged RNA viral vectors is arguably safer than
312 *Agrobacterium* vector, as no DNA is released into environment, and remaining RNA degradation
313 products are all already present in plants and in soil due to the ubiquitous nature of plant viruses.
314 A precision spraying would probably allow more economical delivery with minimal undesired
315 release to the environment.

316 Continuing emergence of multidrug- and pandrug-resistant bacteria and of novel viral pathogens
317 repeatedly demonstrates power and technology potential inherent in the molecular machinery of
318 microbes and viruses. The number of approved (and thus allowed to be released into
319 environment) recombinant bacteria (*Salmonella* as oral vaccine, *Agrobacterium* for control of its
320 pathogenic species) and viruses (influenza virus vaccine, adeno-associated viruses for treatment
321 of spinal muscular atrophy) illustrates the potency and safety of such technical solutions.
322 The introduction of new technologies is always a challenge. We expect that the technology
323 described herein will initially gain regulatory approval and commercial recognition in certain
324 niche areas before garnering attention for mainstream application on large-acreage major crops.
325 One such application area is the production of commercial seed, where production can be made
326 simpler and more efficient through acceleration of flowering time or control of sterility. The
327 other would be a more rapid deployment of orphan crops (e.g., millet, amaranth, buckwheat,
328 cowpea, quinoa, cassava, etc.) for flowering control, drought tolerance improvement etc. Current
329 swift approval and adoption of RNA-based viral vaccines for human health shows that the speed
330 of acceptance of RNA transfection-based agriculture will be greatly facilitated by its expected
331 efficacy during unavoidable major plant pathogen outbreaks.

332 # Methods

333 ***Bacterial strains and growth conditions***

334 *Escherichia coli* DH10B cells were cultivated at 37 °C in LB medium. For ClYVV-based
335 constructs, *Escherichia coli* DH5α cells were used. *Agrobacterium tumefaciens* ICF320
336 (auxotrophic derivative (DcysK_a, DcysK_b, DthiG) of *Agrobacterium tumefaciens* strain C58)
337 cells[46] or NMX021 cells were cultivated at 28 °C in LBS medium (modified LB medium

338 containing 1% soya peptone (Duchefa)). The NMX021 strain was a modified and fully disarmed
339 version of CryX strain[47], wherein a Ti plasmid region was deleted (including the Amp resistance
340 gene with its flanking left and right region - about 30 Kb deleted, from nucleotide 30499 to
341 60264) and replaced with the *LacZ* gene to facilitate monitoring for the presence of this strain.

342 ***Plasmid constructs and viral vectors***

343 TMV-based assembled vectors were described in ref. [2]. TMV vectors used in these studies lack a
344 coat protein (CP) gene (ΔCP); those further modified by removal of the movement protein (MP)
345 gene are indicated as TMVΔMP. The PVX viral vectors used here with the CP coding sequence
346 placed between the polymerase and the triple gene block ORFs were based on the ones first
347 described in ref. [3]. PVX vectors lacking CP are indicated as PVXΔCP. ClYVV viral vectors were
348 developed based on ref. [16,17] with T27I mutation to obtain a less aggressive virus[48]. We received
349 pClYVV-GFP as a gift from Dr. Takeshi Matsumura (Hokkaido University, Japan) and modified
350 it to be used with our T-DNA binary vector for *Agrobacterium*. Bipartite TRV vectors[15] were
351 obtained from Arabidopsis Biological Resource Center (ABRC), Ohio State University,
352 Columbus, OH, USA): pTRV1 (AF406990, stock #CD3-1039) and pTRV2-MCS (AF406991,
353 stock #CD3-1040). We modified the pTRV2-MCS to be used with our T-DNA binary vector for
354 *Agrobacterium*. SMV vector was designed based on ref. [49]. TMVcg viral vectors were developed
355 based on sequence D38444 and modified similarly as in ref. [2]. MSV viral vectors were
356 developed based on a viral sequence deposited in NCBI as Y00514. *Foxtail mosaic virus*
357 (FoMV) viral vectors are described in ref. [50].

358 Specifically modified PVX-based RNA amplicons including cysteine rich proteins (CRP) from
359 Carlaviruses were created by insertion of one CRP from *Cowpea mild mottle virus* (called
360 NABP)[41] or one CRP from *Chrysanthemum virus B* (called CVB-CRP)[42]. Those proteins are

361 pathogenicity determinants believed to be RNA silencing suppressors. They were inserted 2 bp
362 downstream of the gene of interest in the PVX backbone, similarly as is found in the *Cowpea*
363 *mild mottle virus*, downstream of the coat protein ORF. The presence of the specific CRP in the
364 vector backbone is indicated as subscript (e.g. PVX-GFP_{NABP} , PVX-$GFP_{CVB\text{-}CRP}$).
365 Cloning of specific genes into the viral vectors was achieved either by inserting PCR products
366 (PCR performed with KOD hot start DNA polymerase, Merck KGaA) or fragments synthesized
367 by external providers (Eurofins Genomics). Synthesized fragments were for *BnA2FT, BnC6FTb,*
368 *SlSP5G, OsGA2ox1, AtGA20ox1, AtDREB1B, AtNCED3, SlANT1, BtCry2Ab, IPT*.

369 ***Plant species and growth conditions***

370 Plants and varieties used in the experiments described included: *Nicotiana benthamiana*; tobacco
371 (*Nicotiana tabacum L.*) 'Samsun' and 'Maryland Mammoth'; tomato (*Solanum lycopersicum*)
372 'Balcony Red', 'Tamina' and 'Ailsa Craig', tomato 'Ailsa Craig' mutants *notabilis* (LA3614) and
373 *sft* MSU100 (LA2460) (both obtained from TGRC - Tomato Genetics Resource Center at
374 University of California); pepper (*Capsicum annuum*) 'Early California Wonder' ('ECW');
375 *Arabidopsis thaliana* ecotype Col-0, and the ecotypes responding to vernalization Bla-2, Bla-11,
376 Can-0, Co-4, Lov-5, Sf-2, Tamm-2, Te-0, Tul-0 (all obtained from Prof. Marcel Quint, Martin
377 Luther University Halle-Wittenberg, Germany), *Arabidopsis* mutant *ft-10* (obtained from
378 Nottingham Arabidopsis Stock Centre (NASC stock), as GABI-Kat T-DNA insertion library
379 code 290E08; http://www.gabi-kat.de/). In *ft-10*, the T-DNA is inserted into the first intron;
380 broad bean (*Vicia faba*) 'Dreifach Weiße'; pea (*Pisum sativum*) 'Dinga'; potato (*Solanum*
381 *tuberosum*) 'Elfe'; spinach (*Spinacia oleracea*) 'Frühes Riesenblatt'; red beet (*Beta vulgaris*)
382 'Moulin Rouge'; soybean (*Glycine max*) 'Blyskavytsya'; maize (*Zea mays*) 'Sturdi Z'; wheat

383 (*Triticum aestivum*) cultivar 'Cadenza'. Other plants were tested only for transfection and they
384 are included in **Supplementary Table 1**.
385 Plants were grown in the greenhouse (day and night temperatures of 19-23 °C and 17-20 °C,
386 respectively, with long day condition as 12 h light / 12 h dark, and 35-70% humidity).
387 *Arabidopsis thaliana* plants in short day conditions (8 h light / 16 h dark) were grown in growth
388 chambers (Model AR-22L, Percival Scientific), equipped with fluorescent lamps (4 lamps, 100 –
389 130 µmoles / m^2 x s). Wheat was grown in controlled-environment rooms with day/night
390 temperatures of 26.7°C/21.1°C at around 65% relative humidity and a 16-h photoperiod with
391 light intensity of approximately 220 µmoles / m^2 x s.

392 ***Agrobacterium-mediated transfection of plants***

393 Plants were inoculated with diluted cultures of *Agrobacterium tumefaciens* using one of three
394 procedures: 1) infiltration of plant leaves using a needleless syringe (agroinfiltration), 2)
395 spraying of aerial parts of plants using a sprayer (agrospray), and 3) dipping of aerial parts of
396 plants into an agrobacterial suspension (agrodip).
397 Agroinfiltration procedure. Saturated *Agrobacterium* overnight cultures were adjusted to OD_{600} =
398 1.5 (approximately 10^9 cfu/mL) with *Agrobacterium* inoculation buffer (AIB: 10 mM MES pH
399 5.5, 10 mM $MgSO_4$), and further diluted with same solution to reach the desired dilution of the
400 *Agrobacterium* suspension. Inoculation of individual leaf sectors was performed using a syringe
401 (syringe infiltration). For inoculation of entire plants, a vessel containing the infiltration solution
402 was placed in a vacuum chamber with the aerial parts of a plant dipped into the solution. A
403 vacuum was applied for 5 min using a ME 8 NT pump (Vacuubrand), with pressure ranging
404 from 0.1 to 0.2 bar (vacuum infiltration).

405 Agrospray procedure. Saturated *Agrobacterium* overnight cultures were adjusted to OD_{600} = 1.5
406 with AIB, and further diluted with same solution supplemented with a surfactant to OD_{600} =
407 0.015 (1:100 dilution). In some cases, carborundum (silicon carbide SiC) F800
408 (Mineraliengrosshandel Hausen) used as an abrasive was added to agrobacterial suspensions.
409 The surfactants used were: Silwet® L-77 (Kurt Obermeier), Silwet® Gold (Arysta LifeScience),
410 Tween® 20 (Carl Roth) and Triton™ X-100 (AppliChem). Plants were sprayed using High-
411 performance sprayer 405 TK Profiline (Gloria Haus- & Gartengeraete), with 3 bar pressure, and
412 plastic hand sprayers for routine use (Carl Roth). Some test experiments were performed with the
413 compressor sprayer Einhell® BT-AC 200/24 OF (Einhell). Spraying solution composition
414 depended on plants species. AIB supplemented with 0.1% (v/v) Silwet® L-77 was used for
415 spraying transfection of *Nicotiana* species, tomato, pepper, potato, red beet, and spinach. AIB
416 containing 0.05% (v/v) Tween® 20 was used for *Arabidopsis thaliana* and broad bean. For
417 soybean, AIB supplemented with 0.05% (v/v) Tween® 20, 1 mM DTT, and 0.3% (w/v)) silicon
418 carbide F800 was used. Maize plants were sprayed using AIB supplemented with 0.1% (v/v)
419 Silwet® Gold, 0.3% (w/v) silicon carbide F800 and 5% (w/v) sucrose.
420 Agrodip procedure. Saturated *Agrobacterium tumefaciens* ICF320 overnight cultures of OD_{600}=
421 4 were diluted with AIB supplemented with 0.1% (v/v) Silwet® L-77 to OD_{600} = 0.004 (dilution
422 1:1000). Aerial parts of *Nicotiana benthamiana* plants were dipped upside down into the
423 agrobacterial suspension for 20 seconds.

424 ***Generation of viral particles and spraying solution***

425 In order to obtain viral particles (VPs) for plant infection, we inoculated *Nicotiana benthamiana*
426 plants with a suspension of agrobacteria carrying a PVX construct using either syringe or
427 vacuum infiltration. If performed with a syringe, systemic leaves of inoculated plants were

428 collected two weeks post infiltration; if performed with a vacuum, all leaves were collected one
429 week post infiltration. To recover sap containing VPs, plant material was further ground in liquid
430 nitrogen and extracted using PBS buffer in a 5:1 (v/w) buffer:biomass ratio. The extract was
431 filtered using Miracloth followed by centrifugation of the filtrate for 15 min at 4500 rpm at 4°C.
432 For syringe infiltration, this supernatant solution was used directly for spraying. For vacuum-
433 infiltrated plants, the supernatant solution was first filtered to remove agrobacterial cells using
434 sequential filtrations with filters of 8-12 µm and 0.22 µm pore size. Spraying solution included
435 0.3% (w/v) silicon carbide F800. Plant species sprayed were *Nicotiana benthamiana*, tobacco
436 and tomato.

437 ***Protoplast isolation***

438 Protoplasts were isolated as described in ref. [3].

439 ***Immunocytochemistry***

440 Tomato (*Solanum lycopersicum*) 'Tamina' plants were grown for 24 days and then Agro-
441 infiltrated. Agrobacteria carried several PVX constructs (PVX-empty vector, PVX-GFP and
442 PVXΔCP-GFP). Small leaf discs were collected at 10 dpi for immune-staining. Segments of
443 leaves were fixed with 3 % para-formaldehyde/0.05 % Triton™ X-100 in PBS for 3 hours at
444 room temperature and subsequently embedded in PEG 1500 as described in (*51*). GFP was
445 labelled in 3 µm sections with a polyclonal antibody from goat (# 600-101-215; Rockland;
446 diluted 1:500 in PBS containing 5% bovine serum albumin) detected with a donkey-anti-goat-
447 Alexa 488 secondary antibody (# A-11055, Thermo Fisher Scientific; diluted 1:500 in PBS
448 containing 5% bovine serum albumin).

449 ***Protein analysis***

450 About 100 mg fresh weight plant leaf material was ground in liquid nitrogen, and crude protein
451 extracts were prepared with 5 volumes of 2 x Laemmli buffer. Total soluble protein (TSP) was
452 extracted from approximately 100 mg fresh weight plant material ground in liquid nitrogen and
453 dissolved in 500 µl 1 x PBS and incubated for 30 min at room temperature. After centrifugation
454 40 µl SDS sample buffer were added to 10 µl supernatant solution. Sample aliquots (15 µl) were
455 resolved by SDS-PAGE (12% polyacrylamide gel) and Coomassie-stained using PageBlue^{TM}
456 Protein Staining Solution (Thermo Fisher Scientific). Protein extracts were denatured at 95°C for
457 5 minutes before loading.
458 For immunoblot analysis, sample aliquots (15 µl) were resolved by SDS-PAGE (12%
459 polyacrylamide gel) and subsequently blotted on a PVDF membrane. FT protein was detected
460 using FT-specific antibodies (Agrisera; diluted 1/1000); GA2ox8H or GA20ox1H (*H*: His_6-tag)
461 were detected using Tetra His^{TM} (Qiagen) mouse monoclonal IgG1 anti-His antibody as the
462 primary antibody (diluted 1/2500). GFP was detected using anti-GFP rabbit polyclonal antibody
463 (Thermo Fisher Scientific; diluted 1/5000). Secondary antibodies were IgG (whole molecule)
464 peroxidase affinity isolated antibody (Sigma-Aldrich), anti-mouse (diluted 1/5000) or anti-rabbit
465 (diluted 1/10000).
466 ***Genes used in the studies described***
467 A list of genes with their GenBank accession numbers is provided in **Supplementary Table 3**.
468 ***Experiments to manipulate flowering***
469 *Arabidopsis thaliana* plants of wild-type ecotypes and the null mutant allele *ft-10* were induced
470 to flower by spraying with agrobacteria carrying TMV vectors with genes inducing flowering
471 (from *FT* gene family) or TRV vectors to silence the *FLC* gene. Plants were sprayed at
472 approximately 4 weeks of age. For Col-0 ecotype, plants were kept under short day conditions

473 (see above) to avoid induction of flowering by day length. For the other ecotypes, plants were
474 kept under long day conditions in a greenhouse (see above). For each plant, days to flowering
475 were counted from the day of spray until the day of bolting (around 1 cm of bolt appearing from
476 the rosette leaves) as days post spraying (dps).
477 Tobacco (*Nicotiana tabacum*) plants were sprayed with agrobacteria harboring TMV and PVX
478 vectors (expressing genes of the FT-family, either inducers or repressors) when they were 3 to 4
479 weeks of age. The cultivar 'Maryland Mammoth' was kept under long day conditions (see above)
480 in the greenhouse, which does not induce flowering. The cultivar 'Samsun' is unresponsive to day
481 length; therefore, it flowers normally under the standard greenhouse conditions described. For
482 each plant, days to flowering were counted from the day of spray until the day of bolting (floral
483 buds visible arising from the plant) as days post spraying (dps).
484 Tomato (*Solanum lycopersicum*) 'Balcony Red' and pepper (*Capsicum annuum* 'ECW') plants 3
485 to 4 weeks of age were sprayed with agrobacteria carrying PXV vectors harboring genes of the
486 FT-family. Both plant species flower independently of day length (day-neutral plant species) and
487 the constructs described were used to modulate their time to flowering.
488 Wheat (*Triticum aestivum*) cultivar 'Cadenza' plants at 2 weeks of age were rub-inoculated using
489 FoMV vectors virus particles prepared from infected *Nicotiana benthamiana* plants as described
490 in ref. [50].

491 ***Experiments to manipulate gibberellin content***

492 For all sets of experiments, *Nicotiana benthamiana*, tobacco (*Nicotiana tabacum*) 'Samsun',
493 tomato (*Solanum lycopersicum*) 'Tamina' and pepper (*Capsicum annuum*) 'ECW' plants were
494 used for agrospray when they were in the range of 3 to 4 weeks of age. Agrobacteria were
495 applied to plants to introduce PVX constructs expressing genes that affect gibberellin

496 metabolism. Plant height was scored at the day of spray (day 0) to ensure that no significant
497 difference was present at the beginning of the experiment, and later at several time points during
498 a time course. Broad bean (*Vicia faba*) 'Dreifach Weiße' plants were used for agrospray when
499 they were 2 to 3 weeks of age. For each time point (days post spraying; dps), plant height was
500 scored as length between the soil level and the last apical inflorescence (end of the stem). The
501 same conditions were used when spraying the plants with viral particles (VPs).
502 Wheat (*Triticum aestivum*) cultivar 'Cadenza' plants at 2 weeks of age were rub-inoculated as
503 described above.
504 For the field trials, tomato (*Solanum lycopersicum*) 'Tamina' plants (28 days-old) were sprayed
505 with suspensions of the *Agrobacterium* strain NMX021 harboring PVX constructs (single spray
506 ca. 10^7 bacteria/ml or ca. 2-3 x 10^8 bacteria per sq ft).
507 ***External application of active gibberellins via spray***
508 The gibberellins GA_3 and GA_4 (Sigma-Aldrich) were dissolved in ethanol to 1 mM concentration
509 and diluted 100 times with water supplemented with 0.02% (v/v) Silwet® L-77 for spray
510 application. *Nicotiana benthamiana* plants, previously inoculated with PVX constructs PVX-
511 GFP and PVX-GA2ox8, were sprayed at two different time points (13 dps and 27 dps) either
512 with a 1 % ethanolic solution supplemented with 0.02% (v/v) Silwet® L-77 with 10 µM of GA_3,
513 GA_4 or no gibberellins (mock).
514 ***Gibberellin analysis***
515 Analysis of gibberellins content in *Nicotiana benthamiana* and tomato 'Tamina' plants was
516 performed as follows. Plants were sampled at the indicated dps and separated into leaf and stem
517 material. Individual gibberellins were identified and quantified using UPLC-MSMS as described

518 in ref. [52]. The mass spectrometry data of individual gibberellins were processed by using
519 TargetLynx V4.1 SCN 904 (Waters Corporation).

520 ***Experiments on drought tolerance***

521 *Experiment with mutant tomato variety.* The tomato (*Solanum lycopersicum*) 'Ailsa Craig' mutant
522 *notabilis* variety was used for these experiments. Six pots, each with five 19 days-old plants,
523 were sprayed with agrobacteria harboring PVX vectors expressing either GFP or the NCED
524 gene. At 9 dps, each three pots per treatment were not watered for three days. Watered (*no*
525 *drought*) and stressed (*drought*) plants were photo documented after 12 dps. At this time point
526 watered plants were harvested and ground and samples were used for HPLC analysis to
527 determine the content of abscisic acid (*ABA*), its derivative abscisic acid-glucose ester *(ABA-*
528 *Glc*), phaseic acid (*PA*) and dihydrophaseic acid (*DHPA*), in relation to the treatments.

529 *Experiment with wild-type tomato plants.* Three pots, each with five 19 days-old tomato
530 (*Solanum lycopersicum*) 'Tamina' plants, were sprayed with agrobacteria carrying PVX
531 expressing GFP, DREB1 or DREB-LP1. After 9 dps, plants were not watered for 7 days and
532 subsequently analyzed regarding the relative water content (RWC). To determine the relative
533 water contents (RWC %), immediately after drought stress all plants were cut directly over the
534 soil, weighted separately and watered overnight in glasses filled with 100 ml tap water. After
535 weighting the plants again, the plant material was dried separately at 60°C for 24h to determine
536 the dry weight per plant. The relative water content per plant was calculated using the formula
537 RWC% = ((desiccated weight-dry weight) / (fresh weight-dry weight)) x 100.

538 ***Experiments on anthocyanin production***

539 In order to induce anthocyanin production in tomato fruits, 6-week-old tomato (*Solanum*
540 *lycopersicum*) 'Balcony Red' plants were sprayed with agrobacteria carrying PVX-ANT1
541 constructs.
542 At fruit maturation, fruits were ground to a fine powder in liquid nitrogen. To extract
543 anthocyanins, 1.5 g samples of ground fruit material were mixed with 3 ml methanol
544 supplemented with 1% HCl and further incubated overnight at 4°C in the dark. At the next day,
545 first 2 ml sterile water were added and the samples intensively mixed, and subsequently 5 ml
546 chloroform were added and again the samples were intensively mixed. The samples were then
547 centrifuged at 4500 rpm for 10 min at room temperature. Lastly, the supernatant solution was
548 collected and each sample was supplemented with 4 ml of a 60% methanol solution plus 1%
549 HCl. The concentration of anthocyanin pigment (in cyanidin-3-glucoside equivalents) in the
550 extracts was subsequently determined by measuring of OD_{520} and OD_{700} in a 1:2 dilution of a
551 KCl buffer (0.025 mM KCl, pH 1.0) and a Na-acetate buffer (0.4 M Na-acetate, pH 4.5) relative
552 to a standard curve[53].
553 ***Experiments on fruit ripening***
554 Tomato (*Solanum lycopersicum*) 'Balcony Red' plants (31 days-old) were sprayed with
555 agrobacteria harboring PVX vectors with an antisense sequence (Fragment 1-480 bp) of the gene
556 *DEMETER-like DNA demethylase 2* from tomato (*DML2*). Because the absolute time of fruit
557 maturation can be masked by changes in several parameters (principally the flowering time), the
558 duration of fruit ripening procedure was measured, for each developing fruit, as the number of
559 days to anthesis: the time between the appearance of a flower and the maturation of the fruit
560 developed from this specific flower. We scored the fruit as mature once it becomes completely
561 red.

562 ***Experiments on insect resistance***

563 Tobacco (*Nicotiana tabacum*) 'Samsun' plants were sprayed with agrobacteria carrying PVX-
564 Cry2Ab vectors. To determine the concentration of expressed Cry2Ab toxin, leaf material from
565 each plant was collected at 7 dps, extracted in PBST buffer and analyzed using a Cry2Ab
566 specific ELISA Kit (Cry2Ab #KBA010-10, KRISHGEN Biosystems) following the
567 manufacturer´s instructions. At 7 dps, 3 larvae of the hornworm (*Manduca sexta*) were placed on
568 each plant for feeding. Plant shapes were photo documented at the beginning of insect feeding
569 and 28 days later. In a parallel experiment, tobacco plants were syringe-infiltrated with
570 agrobacteria harboring PVX-Cry2Ab vectors. At 5 dpi, infiltrated leaves were detached, and one
571 hornworm larva was placed on each detached leaf. Individual leaves were photographed at the
572 beginning of insect feeding and 5 days later.

573 ***Experiments on plant senescence***

574 Tobacco (*Nicotiana tabacum*) 'Samsun' plants (62 days-old) were sprayed with agrobacteria
575 harboring PVX-IPT vectors. At 39 days post spraying, the four oldest leaves of sprayed and
576 control plants were collected and photo documented.

577 ***Detection of PVX in the progeny of agrosprayed plants***

578 Seeds were collected from tomato (*Solanum lycopersicum*) 'Balcony Red' plants sprayed with the
579 constructs PVX-GFP and PVX-GA2ox1 (*Oryza sativa*) and germinated on filter paper (*pr*). Total
580 RNA was isolated from pooled seedlings as well as from leaves of PVX-GFP sprayed plant used
581 as a positive control (*sp*), and used to generate cDNA. The presence of PVX and cystatin control
582 was determined by PCR.

583 ***Detection of Agrobacterium in leaves and soil***

584 *Nicotiana tabacum* 'Maryland Mammoth' plants (63 days-old) were sprayed with a suspension of
585 *Agrobacterium tumefaciens* (strain NMX021) cells harboring PVX-GFP (single spray, ca. 10^6
586 bacteria/ml or ca. 2-3 x 10^7 bacteria per sq ft). At different time points after spraying, 12 samples
587 of soil within a constant distance from the plants were collected and analyzed for the presence of
588 agrobacteria. For this purpose 1 g soil per sample was suspended in 5 ml of sterile SCP broth
589 (0.43% (w/v) NaCl, 0.1% (w/v) peptone, 0.36% KH_2PO_4, 0.58% Na_2HPO_4, pH 7.0), shaken for
590 ca. 15 min on an orbital shaker (120 rpm), filtered through 2 layers of Miracloth and washed
591 with 2 volumes water. 250 µl of soil extract were plated on LB (rif, cyc, X-gal), incubated for 3
592 days at 28°C and used for enumeration of colonies. Colony forming unites (cfu) per g soil were
593 calculated. At different time points after spraying, 12 samples of leaves were collected and
594 analyzed for the presence of agrobacteria. For this purpose 1 g leaf material per sample was
595 ground in liquid nitrogen, admixed with 5 volumes of sterile SCP broth, shaken for ca. 15 min on
596 an orbital shaker (120 rpm), and filtered through 2 layers of Miracloth. A volume of 250 µl of
597 leaf extract was plated on LB (rif, cyc, X-gal), incubated for 3 days at 28°C for colony
598 enumeration. Colony forming unites (cfu) per g leaf material were calculated.

599 ***Detection of T-DNA in plants transfected with Agrobacterium***

600 Seeds were collected from untreated *Nicotiana benthamiana* plants and from those sprayed with
601 agrobacteria carrying PVX-GFP vectors. Genomic DNA (gDNA, 100 ng) was isolated using
602 NucleoSpin RNA Plant II kit (Macherey-Nagel) from 100 mg seeds. The PVX-GFP plasmid
603 DNA spike-in (0.2 pg) was used as a positive control. The presence of PVX and NbSO
604 (housekeeping gene control) was determined by using KAPA3G-PCR Kit (Sigma-Aldrich) with
605 primers specific for PVX, GFP and NbSO. For PCR analysis, 10^{-1}, 10^{-2} and 10^{-3} dilutions of
606 gDNA were used.

607 ***Field trials***

608 Experiments with field release of agrobacteria were performed at Kentucky Bioprocessing, Inc.
609 facilities (Owensboro, KY, USA) in 2014 and 2015. These studies were conducted under permits
610 from the US Department of Agriculture (USDA) No. 13-323-101r and No. 15-041-101r. Publicly
611 available information can be accessed at URL:
612 https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-
613 petitions/sa_permits/status-update/release-permits.

614 ***RT-PCR***

615 RNA was isolated from plant material using NucleoSpin RNA Plant kit (Macherey-Nagel) and
616 used to generate cDNA by reverse transcriptase reaction with PrimeScript RT Reagent kit
617 (Takara Bio). PCR was performed using Taq-polymerase (Thermo Fisher Scientific) and target-
618 specific oligos (synthesized by Thermo Fisher Scientific).

619 ***Codon optimization and relative sequences***

620 Various ORFs from plant genes were inserted into PVX constructs for expression of specific
621 protein products. For some of these genes, the protein sequence was kept as the original but the
622 sequence was changed by means of a different codon-usage (defined here as codon
623 optimization). We used the algorithm from GENEius software designed and developed for
624 Eurofins Genomics and let the company synthesize the ORFs (Eurofins Genomics). With this
625 program, it is possible to choose for codon-optimization based on specific organisms. For the
626 selected genes, PVX-constructs with the wild-type and the codon optimized sequence of the gene
627 were compared for their stability to keep the insert in the vector with time (as in ref. [39]). The
628 ORFs shown are relative to *SlTAGL1*, *SlOVATE*, *SlANT1* (all from *Solanum lycopersicum*) and
629 GFP. All the sequences are reported in **Supplementary Table 4**.

630 ***Statistics and reproducibility***

631 Statistical parameters are reported in the figures and corresponding figure legends. Statistical
632 significance was evaluated by one-way ANOVA test using GraphPad Prism v. 8.0.2. 'n' value
633 corresponds to the number of samples for each column, where the type of sample is indicated in
634 the figure legends. In cases where 'n' value is not the same for each column, it is indicated in the
635 legends. Full datasets are reported in the **Source data**. Experiments shown in the graphs were
636 repeated at least two times, with equivalent results. Experiments showing expression of a
637 reporter gene in different plant species and generated with different methods were repeated at
638 least three times with equivalent results. Experiments performed on field trials were performed
639 one time for each type of experiment. Analysis of gibberellin content was performed one time for
640 each plant species tested.

641 Data availability

642 All data generated or analysed during this study are included in this published article (and its
643 supplementary information files). All materials are available for research purpose upon request
644 from the corresponding author under a material transfer agreement with Nomad Bioscience. The
645 following sequences of codon optimized genes have been deposited in NCBI as GenBank
646 accession numbers: MT877076 (SlTAGL1, codon-optimized for rice), MT877077 (SlOVATE,
647 codon-optimized for rice), MT877078 (SlANT1, codon-optimized for Bifidobacterium longum),
648 MT877079 (GFP, codon-optimized for tobacco).

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865 ## Acknowledgments

866 We thank Eva-Maria Franken (Bayer CropScience) for encouragement and valuable advices on
867 experiments and Hugh Haydon (Kentucky BioProcessing) for support in conducting field trials.
868 We would also like to thank our colleagues at Nomad Bioscience and Icon Genetics Carola
869 Engler, Katharina Havranek, Tim-Martin Ehnert, Victor Klimyuk, Frank Thieme, Romy
870 Kandzia, Anja Nickstadt, Yuri Symonenko, Elsbeth Stegemann-Oelerich for their valuable help.
871 We are grateful to Ernie Hiatt, Jennifer Poole, John W. Shepherd, Ethan Blandford (Kentucky
872 BioProcessing) for their help in conducting field trials. We would like to thank Dr. Takeshi
873 Matsumura and Dr. Ichiro Uyeda (Hokkaido University) for the plasmid pClYVV-GFP received
874 as a gift, Prof. Marcel Quint (Martin Luther University Halle-Wittenberg) for providing several
875 *Arabidopsis* ecotypes, Dr. Margret Köck (Martin Luther University Halle-Wittenberg) for
876 providing of climatic cabinets for plant growth. We thank Kai Eggert and Barbara Kettig (IPK-
877 Gatersleben) for phytohormone analysis and Anke Steppuhn (Free University Berlin) for
878 providing hornworm eggs. We thank Daniel Tusé (DT/Consulting Group) and Nikolaus Amrhein
879 (ETH Zürich) for critical reading of manuscript. **Funding:** Part of this work has been financially
880 supported by Bayer CropScience. KK and VP would like to acknowledge financial support by
881 the Institute Strategic Program Grant 'Designing Future Wheat' (BB/P016855/1) from the
882 Biotechnology and Biological Sciences Research Council of the UK (BBSRC).

883 ## Author contributions

884 Y.G. conceptualized and supervised research. Y.G. and A.G. directed the research. Y.G., A.G.,
885 S.T., R.S., A.T., P.R., S.W., and K.K. designed research. S.T., R.S., A.T., D.B., P.R., B.K., S.W.,

V.P., and G.H. performed research. Y.G., A.G., S.T., R.S., A.T., P.R., B.K., S.W., V.P., K.K., J.D.G.J., N.v.W., and G.H. analyzed data. Y.G., A.G., S.T., R.S. wrote the paper. All authors read and approved the manuscript.

Competing interests

Y.G. has shares in Nomad Bioscience. S.T., R.S., A.T., D.B., P.R., B.K., A.G., and Y.G. are employed by Nomad Bioscience. S.W. has been employed by Nomad Bioscience. V.P. and K.K. are affiliated with Rothamsted Research, Harpenden, United Kingdom; N.v.W. is affiliated with Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany; J.D.G.J. is affiliated with The Sainsbury Laboratory, University of East Anglia, Norwich, United Kingdom; and G.H. is affiliated with Martin Luther University of Halle-Wittenberg, Halle, Germany. A.G., D.B., P.R., and Y.G. are inventors on the patent application entitled “Process of transfecting plants”; P.R., D.B., A.G., and Y.G. are inventors on the patent application “Agrobacterium for transient transfection of whole plant”; Y.G. is an inventor on the patent application “Potexvirus-derived replicon”; A.T., D.B., A.G., and Y.G. are inventors on the patent application “Process of providing plants with abiotic stress resistance”; and S.T, R.S., A.G., and Y.G. are inventors on the patent application “Method of improving potexviral vector stability”. The ownership of the patents resides with Nomad Bioscience. The authors declare that they have no other competing interests.

904 # Figure legends:

905 **Fig. 1: Transfection of various crop plants with viral vectors delivered using *Agrobacterium***
906 **or as viral particles.** (**a**) Spraying of *N. benthamiana* plants with *Agrobacterium tumefaciens*
907 strain ICF320 carrying PVX-GFP vector using industrial sprayer (top panel). All plants showed
908 intensive green fluorescence at 21 days post spraying (dps) (bottom panel). Inserts show an
909 untreated *N. benthamiana* control plant under UV-light (bottom left) as well as GFP fluorescence
910 at 16 dps in seedlings sprayed with the same agrobacterial suspension (top right). (**b**)
911 Microscopic image of protoplasts isolated from plants sprayed with *Agrobacterium* harboring
912 cell-to-cell-movement-disabled PVXΔCP-GFP vector visualizes the efficiency of agrobacterial
913 transfection based on GFP fluorescence (CP: coat protein). Scale bar, 100 µm. (**c**) TMV vectors
914 without own CP can still move cell-to-cell in tobacco using own MP (movement protein). Scale
915 bar for both images, 50 µm. (**d**) Co-infiltration of agrobacteria strain ICF320 carrying PVXΔCP-
916 GFP and TMV-dsRED vectors, providing green and red fluorescence, respectively. Only the
917 TMV construct shows systemic movement, as indicated by dsRED expression in leaf veins of *N.*
918 *benthamiana* at 36 days post infiltration (dpi). (**e**) Transient delivery can be used repeatedly. *N.*
919 *benthamiana* plants were dipped successively with one week interval in diluted agrobacterial
920 cultures harboring viral vectors TMVΔMP-GFP (day 0), PVX-dsRED (day 7), and TMV-GFP
921 (day 14) (top panel). For controls (bottom panel) *N. benthamiana* plants were dipped in different
922 *Agrobacterium* suspensions separately. Photos were taken at 21 days post dipping (dpd). Dipping
923 was used here for proof-of-principle in order to get more uniform distribution of the transfection
924 spots for better visualization. Same concept applies for spray (see main text). (**f**) GFP
925 fluorescence in leaves of multiple plant species sprayed with agrobacteria carrying PVX-GFP: *N.*
926 *benthamiana*, tobacco 'Samsun', tomato 'Balcony Red', potato 'Elfe', pepper 'Early California

927 Wonder‘, red beet 'Moulin Rouge'. (**g**) GFP fluorescence in leaves of plant species sprayed with
928 *agrobacteria* harboring TMVcg-GFP, ClYVVT27I-GFP, and MSV-GFP viral vectors: spinach
929 'Frühes Riesenblatt', broad bean 'Dreifach Weiße', maize 'Sturdi Z'. (**h**) GFP fluorescence in
930 leaves of tomato 'Tamina‘, tobacco 'Samsun‘, soybean 'Blyskavytsya', and wheat ‘Cadenza‘
931 plants sprayed and rubbed, respectively, with viral particles (VPs) containing PVX-GFP, SMV-
932 GFP, and FoMV-GFP constructs. VPs were previously isolated from *N. benthamiana* plants
933 infected with PVX-GFP, SMV-GFP, and FoMV-GFP vectors using agroinfiltration. Detailed
934 description on the spraying and rubbing procedures is given in **Methods** (dpr: days post
935 rubbing). Photos of GFP-expressing transfected leaves and corresponding untreated controls are
936 shown side-by-side in **Supplementary Fig. 2**.

937 **Fig. 2: Induction and repression of flowering with viral vectors in several plant species.**
938 (**a**,**b**) *Arabidopsis thaliana* wild-type Col-0 and a mutant containing the null allele *ft-10* are
939 induced to flower in response to the infection with TMV carrying the *Arabidopsis FT* gene (FT).
940 Plants were sprayed with agrobacteria carrying viral vectors. For each plant, days to flowering
941 were counted from the day of spray until the day of bolting as days post spray (dps). (**c**) TMV
942 vector-driven expression of the FT protein in *N. benthamiana* leaves detected by SDS-PAGE
943 with Coomassie staining and immunoblotting with FT-specific antibodies. (**d**,**e**) Induced
944 flowering in wheat ‘Cadenza' by FoMV-based overexpression of Hd3a (*O. sativa*) using viral
945 particle (VP) delivery. VPs were previously isolated from *N. benthamiana* plants infected with
946 FoMV constructs by agroinfiltration. (**f,g**) Five heterologous FT family genes from *Brassica*
947 *napus* were expressed in tobacco 'Maryland Mammoth' using PVX vectors. Only two FT family
948 members induced flowering, in both plant species. “∞” indicates no flowering by the end of
949 experiment. (**h,i**) Tomato and pepper were induced to flowering by PVX-based vectors

expressing FT. (**j**) Vernalization-dependent *Arabidopsis thaliana* ecotypes were induced to flowering in non-vernalizing condition using TMV vectors expressing FT. TMV-FT vectors had a weak or no effect (depending on the experiment) on the ecotype Lov-5 only. (**k**) Silencing of *FLC* gene by TRV vectors resulted in robust induction of flowering in *Arabidopsis thaliana*. Also ecotypes with stricter vernalization requirements including Lov-5 were induced to flower with this alternative approach. (**l,m**) Tobacco was induced to flowering by PVX-FT vector delivered as viral particles. VPs were previously isolated from *N. benthamiana* plants infected with PVX-FT using agroinfiltration. In the graphs mean values with standard deviation are indicated; statistical significance was evaluated by one-way ANOVA test using GraphPad Prism v. 8.0.2. * = $p<0.05$; **= $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$. n=number of plants for each column. (**j**) n=3-6; (**k**) n=10-12, and n= 7-11; (**m**) n= 5-6.

Fig. 3: Modification of plant stature in several crop species via modulation of gibberellin metabolic pathway by viral vectors. (**a**,**b**) Plant height reduction in tomato caused by PVX-based overexpression of gibberellin pathway genes. Tomato 'Tamina' plants were sprayed with agrobacteria harboring PVX vectors with genes for GA2ox1 (*O. sativa*), DREB1A (*A. thaliana*), DREB1B (*A. thaliana*), DREB-LP1 (*C. annuum*), and GA2ox8 (*G. max*). Controls consisted of untreated plants and plants treated with PVX-GFP. Plant height was measured at 28 dps. (**c**,**d**) PVX-mediated GA2ox8 overexpression caused height reduction in tomato 'Balcony Red', pepper 'ECW', and tobacco 'Samsun' plants. Viral vectors were delivered to plants using spraying with agrobacteria. (**e**,**f**) Height reduction in pea 'Dinga' plants sprayed with *Agrobacterium* harboring ClYVVT27I-GA2ox8 vector. **(g**,**h)** Stem shortening and elongation in tomato 'Tamina' due to PVX-mediated overexpression of GA2ox8 (*G. max*) or GA20ox1 (*A. thaliana*) genes, respectively. Viral vectors were delivered to plants using spraying with agrobacteria. Plant height

973 was measured at 35 dps. (**i**,**j**). Height reduction in wheat 'Cadenza' by FoMV-based

974 overexpression of GA2ox6 (*O. sativa*) using viral particle delivery. VPs were previously isolated

975 from *N. benthamiana* plants infected with FoMV constructs by agroinfiltration. (**k**,**l**) Stem

976 shortening and reduction in tomato by modulating gibberellin metabolism using viral particle

977 delivery. VPs were previously isolated from *N. benthamiana* plants infected with PVX constructs

978 by agroinfiltration. (**m**,**n**) Spraying of tomato 'Tamina' with agrobacteria carrying PVX-GA2ox1

979 vectors in the field resulted in stem shortening. In the graphs mean values with standard

980 deviation are indicated; statistical significance was evaluated by one-way ANOVA test using

981 GraphPad Prism v. 8.0.2. **= $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$. n = number of plants

982 for each column. (**n**) n = number of plants for each column, consisting of 68-88 plants.

983 **Fig. 4: Transient reprogramming of other agronomic traits in tomato with PVX vectors**

984 **delivered by *Agrobacterium* using spraying.** (**a**,**b**) PVX-mediated NCED3 complementation

985 increases drought stress tolerance in ABA deficient tomato mutant *notabilis.* Upon drought, only

986 the tomato mutant plants ('Ailsa Craig' *notabilis*) which were transfected with NCED3

987 (*A. thaliana)* showed a tolerant phenotype. The mutant plants not subjected to drought were used

988 to determine the content of abscisic acid (ABA) and its derivative abscisic acid-glucose ester

989 *(*ABA-Glc) using HPLC. (**c**,**d**) PVX-mediated overexpression of *DREB1A* (*A. thaliana*) and

990 *DREB-LP1 (C. annuum*) augments drought stress tolerance in wild-type tomato 'Tamina' plants.

991 Increased relative water content (RWC) found after the drought correlated with tolerant

992 phenotype. (**e**,**f**) PVX-driven overexpression of *SP3D* (*S. lycopersicum*) gene converted *single*

993 *flower truss* (*sft*) mutant phenotype to wild-type inflorescence. Number of flowers and fruits per

994 truss were counted on untreated and sprayed tomato plants 'Ailsa Craig' MSU100 *sft* at 44 and 90

995 dps, respectively. (**g**,**h**) Overexpression of *ANT1* (*S. lycopersicum*) gene induces anthocyanin

996 production in tomato fruits. Visual changes in the fruit color of tomato 'Balcony Red' reflect the
997 difference in anthocyanin concentrations measured in extracts. (**i,j**) Delay in fruit ripening of
998 tomato 'Balcony Red' plants by silencing of the *DML2* gene using PVX vector with a fragment of
999 DML2 coding sequence in antisense orientation. Y axis shows the duration of fruit ripening
1000 calculated as a time post anthesis till complete fruit maturation. In the graphs, mean values with
1001 standard deviation are indicated; statistical significance was evaluated by one-way ANOVA test
1002 using GraphPad Prism v. 8.0.2. ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$. n = number of
1003 plants for each column (**b**,**d**,**f**,**h**). (**d**) n = 5-10. For (**f**), all the flowers of each plant were
1004 considered and counted (range 24-26). For (**h**), all the fruits of each plant were pooled and
1005 measured. For (**j**), n = number of flowers/fruits, which was in the range 17-135 (taken from all
1006 plants of each treatment. Number of plants was 7 for each column, except for untreated and
1007 PVX-GFP, where the number of plants was 2.

1008 **Fig. 5: Enhanced virus spread, amplification and recombinant protein accumulation using**
1009 **modified PVX vectors.** (**a**) Stabilizing PVX constructs using codon-optimized inserts. Tomato
1010 'Tamina' plants were inoculated using syringe with agrobacteria carrying PVX constructs
1011 containing wild-type or codon-optimized genes of TAGL1, OVATE, and ANT1 from *S.*
1012 *lycopersicum*. Stability of the inserts in systemic leaves at 27 days post infiltration (dpi) was
1013 determined by RT-PCR with PVX specific primers using generated cDNA (sl) and plasmid
1014 control (Pl) as templates. (**b**) Enhancement of reporter gene expression in *N. benthamiana* by
1015 CPMMV nucleic acid-binding protein (NABP) gene insertion in PVX backbone. Plants were
1016 syringe inoculated with agrobacteria carrying constructs PVX-GFP and PVX-GFP_{NABP}. (**c**)
1017 Enhancement of reporter gene expression in tobacco 'Samsun'. Plants were syringe inoculated
1018 with agrobacteria carrying the constructs PVX-GFP and PVX-GFP_{NABP}. GFP fluorescence is

1019 shown for several systemic leaves. (**d**) SDS-PAGE analysis of plant samples showing the
1020 enhancement of GFP expression in presence of NABP in the viral vector backbone. (**e**) GFP
1021 expression enhancement in tomato 'Tamina'. Plants were transfected with *Agrobacterium*
1022 harboring different GFP-expressing PVX constructs using spraying, resulting in a further
1023 increase with CVB-CRP in the backbone. (**f**) dsRED expression enhancement in tomato
1024 'Tamina‘: dsRED fluorescence is clearly visible only when CVB-CRP is added to the PVX
1025 backbone. Plants were transfected by agroinfiltration using syringe. (**g**) SDS-PAGE analysis of
1026 samples obtained from PVX-dsRED and PVX-dsRED$_{\text{CVB-CRP}}$ treated plants showing the
1027 enhancement of dsRED accumulation. (**h**,**i**) Enhanced dwarfism effect by NABP insertion in
1028 PVX backbone. Tobacco 'Samsun' plants were transfected with different PVX constructs by
1029 agroinfiltration using syringe. (**j**,**k**) Enhanced flowering repression effect by NABP insertion in
1030 PVX backbone. Tomato 'Balcony Red' plants were transfected with different PVX constructs by
1031 syringe agronfiltration. Genes are: *FT1* (from tobacco), and *SP5G* (from tomato). In all these
1032 experiments except for (**e**), we used syringe inoculation of first true leaf (*Nicotiana*
1033 *benthamiana*) or both cotyledon and first true leaf together (tobacco and tomato) instead of
1034 spraying the whole plant so as to be able to track virus systemic movement and assess viral RNA
1035 stability and subtle quantitative differences in viral vector performance. In the graphs, mean
1036 values with standard deviation are indicated; statistical significance was evaluated by one-way
1037 ANOVA test using GraphPad Prism v. 8.0.2. n = number of plants for each column.

1038 **Fig. 6: Fate of *Agrobacterium* and viral vectors in transfected plants and soil (greenhouse**
1039 **and open field).** (**a**) PVX vectors loose foreign gene inserts upon systemic movement in tomato.
1040 Plants were sprayed with agrobacteria harboring PVX constructs with *GA2ox1* (*O. sativa*),
1041 *DREB1A* (*A. thaliana*), *GA2ox8* (*G. max*), and *GA20ox1* (*A. thaliana*) genes. Vector stability was

1042 determined in systemic leaves (sl) by RT-PCR using PVX specific oligos. PCR fragments
1043 generated using corresponding plasmid DNA as a template (Pl) were used as a positive control.
1044 (**b**,**c**) Reducing the GC-content destabilizes GFP gene inside the PVX vector. Tomato plants
1045 were agroinfiltrated by syringe with PVX-GFP constructs containing GFP sequences with
1046 original and reduced GC-content. Stability of the inserts in systemic leaves (sl) at 25 dpi and in
1047 fruits at 110 dpi (fr) was determined by RT-PCR as described above. In this group of
1048 experiments, we used syringe inoculation of both cotyledon and first true leaf together instead of
1049 spraying the whole plant so as to be able to track the virus systemic movement and assess viral
1050 RNA stability. (**d**) Absence of PVX in progeny of agrosprayed plants. The presence of PVX and
1051 cystatin housekeeping control gene was determined by RT-PCR using cDNAs generated from
1052 tomato 'Balcony Red' as templates and specific primers. Seedlings germinated from seeds
1053 collected on plants sprayed with the PVX constructs (pr) did not show the presence of PVX,
1054 while control leaves of sprayed plants (sp) generated a clear band. (**e**,**g**) Transient presence of
1055 genetically modified agrobacteria in tobacco leaves and surrounding soil analyzed in a field
1056 experiment. (**e**) Tobacco 'Maryland Mammoth' plants were sprayed with auxotrophic
1057 *Agrobacterium* strain NMX021 harboring TMV-GFP vector and monitored for GFP
1058 fluorescence. (**f**) Time course after spraying, in which samples of leaves were collected and
1059 analyzed for the presence of *Agrobacteria* by counting cfu per g leaf material (wps: weeks post
1060 spraying, mps: months post spraying). (**g**) Time course after spraying, in which samples of soil
1061 with a constant distance to the plants were collected and analyzed for the presence of
1062 *Agrobacteria* by counting colony forming units (cfu) per g of soil. In the graphs, mean values
1063 with standard deviation are indicated. (**f**) n = number of plants for each column. (**g**) n = number
1064 of soil samples collected for each column.

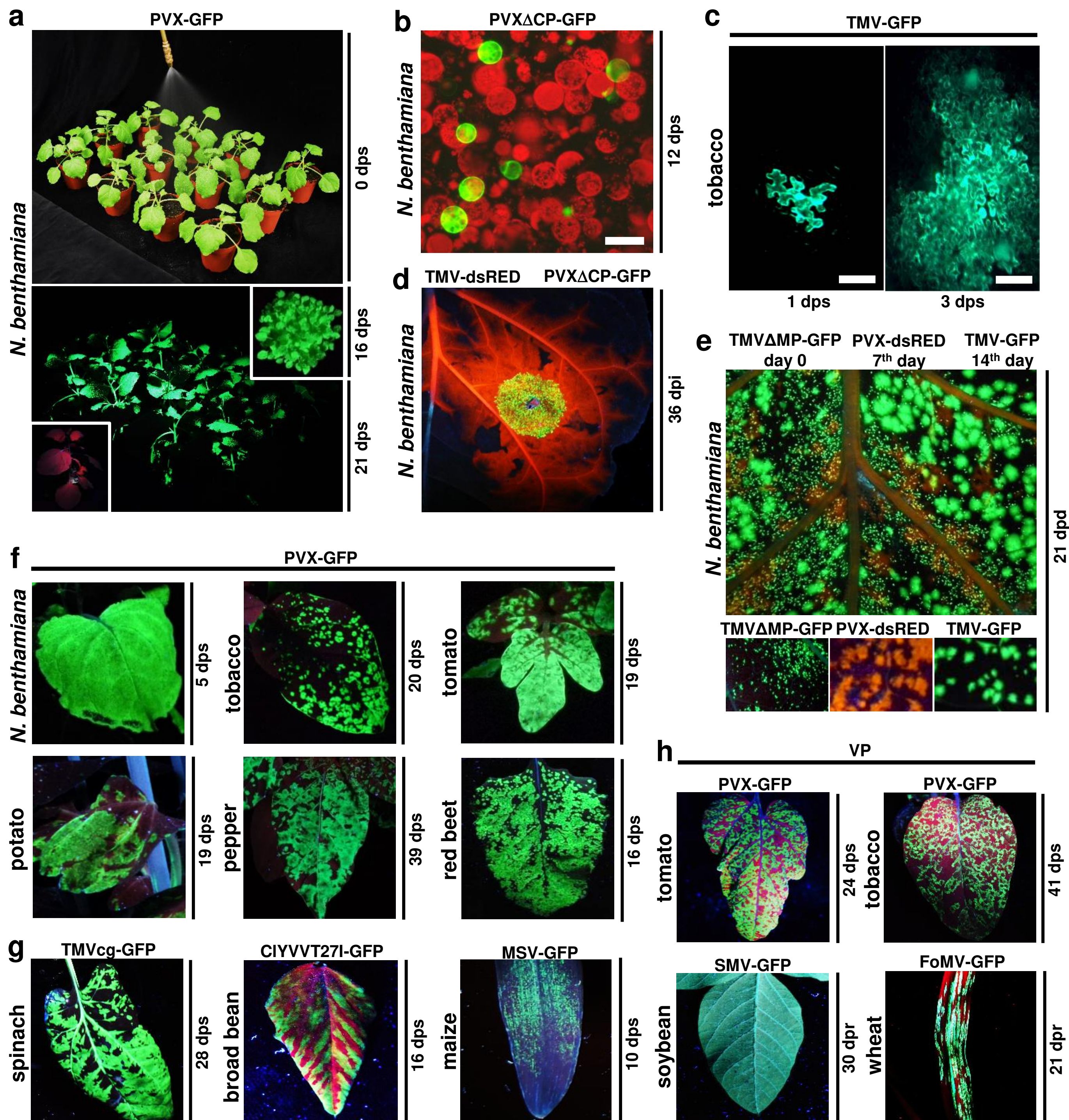


Figure 1

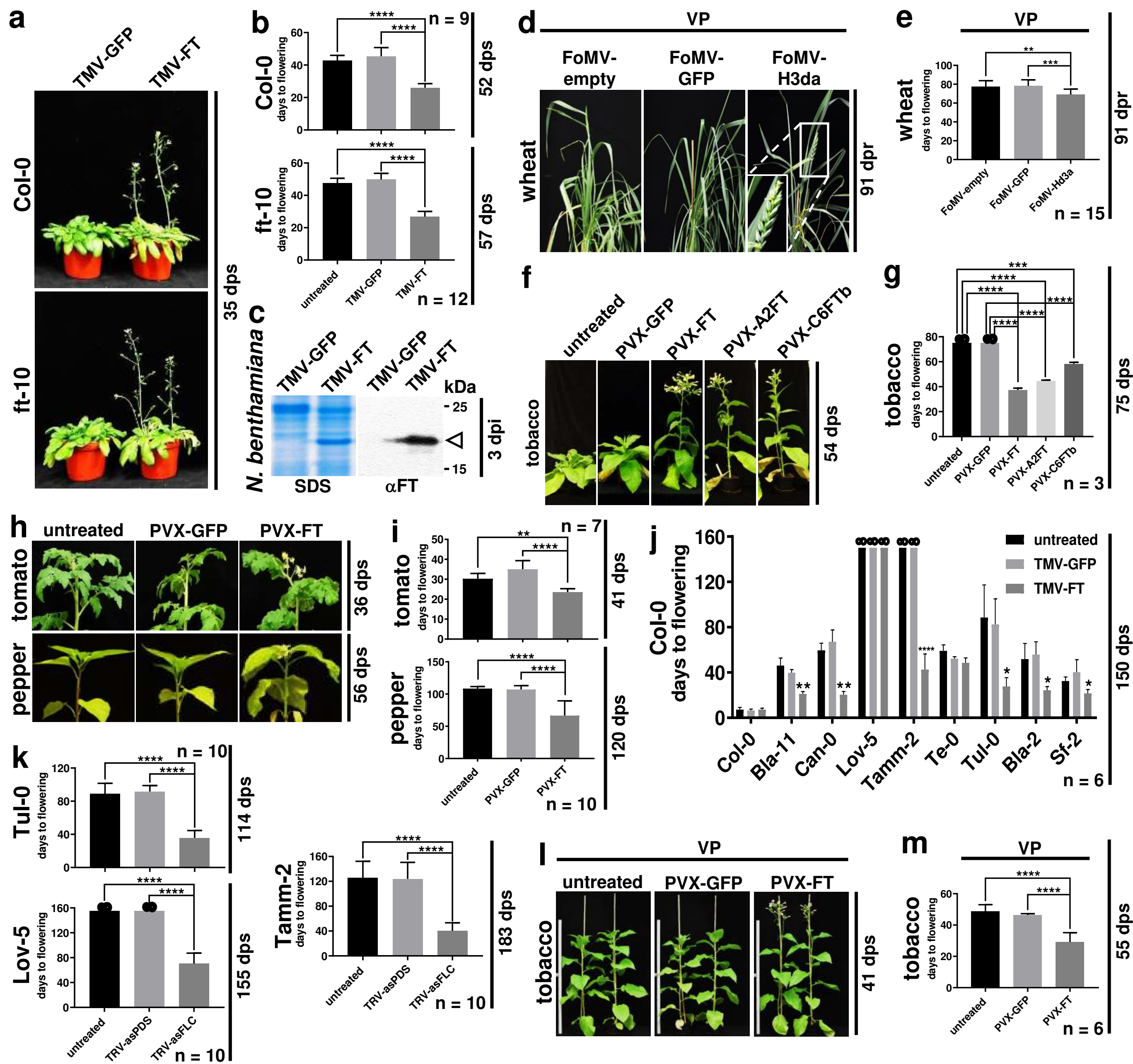
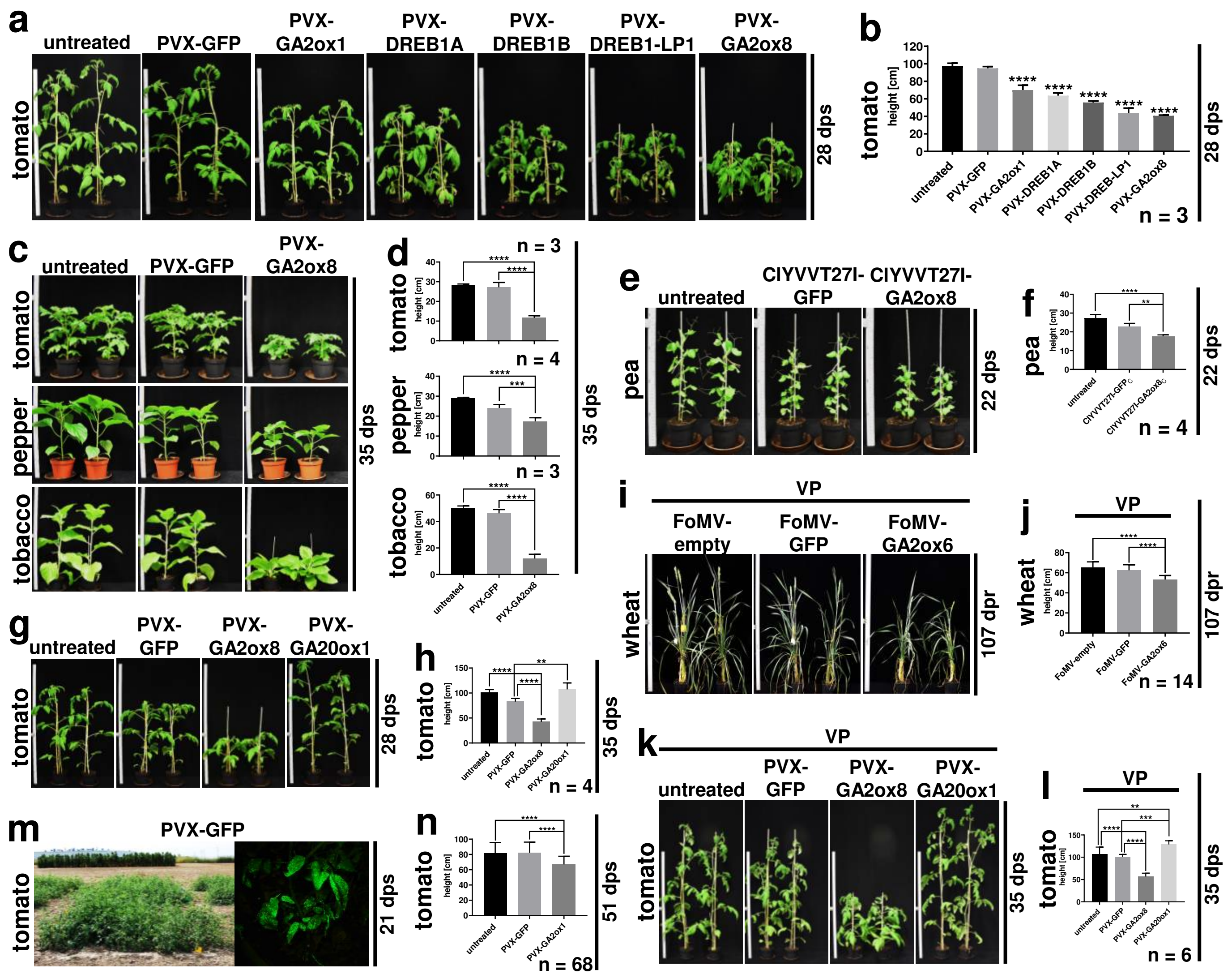


Figure 2



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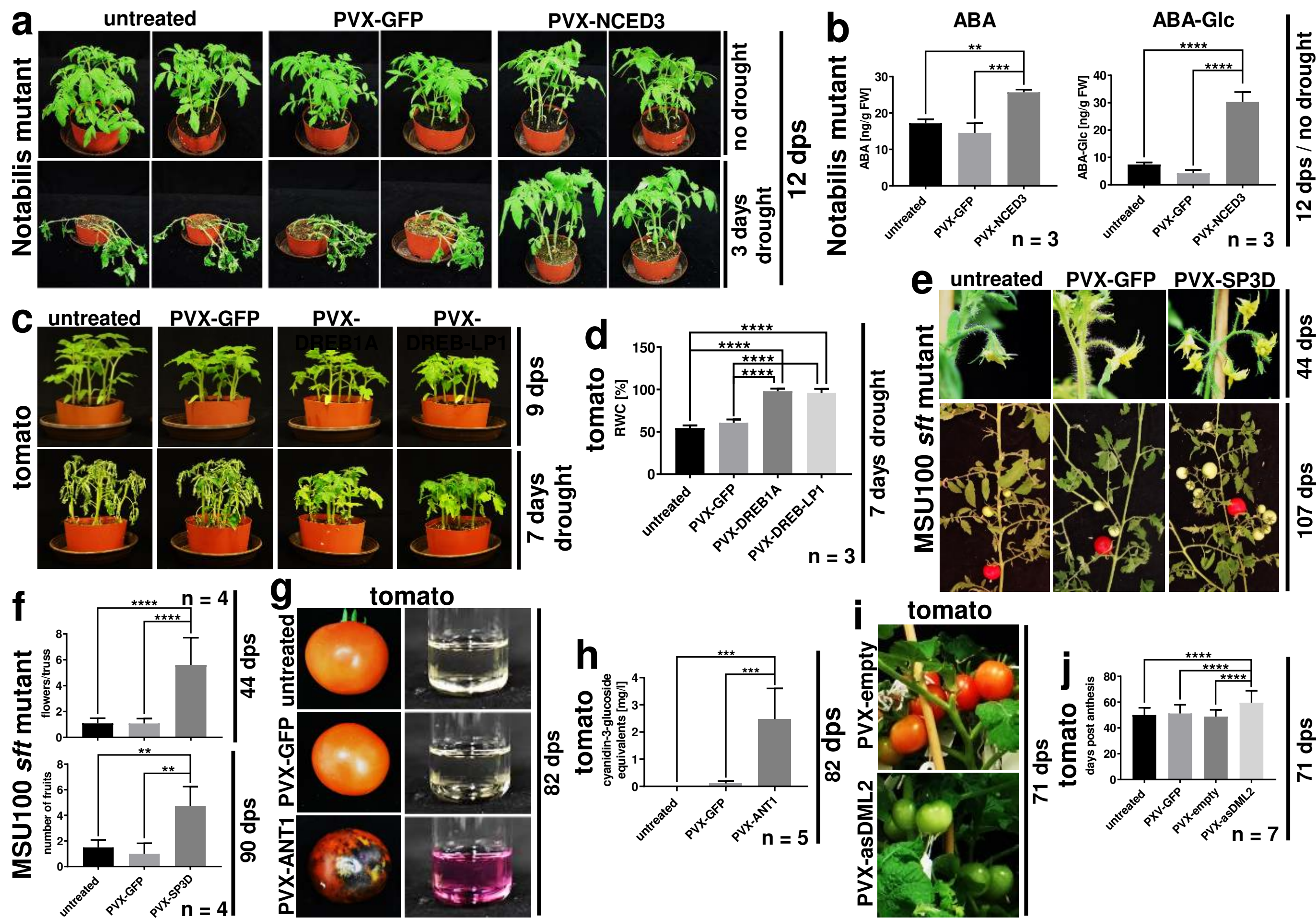

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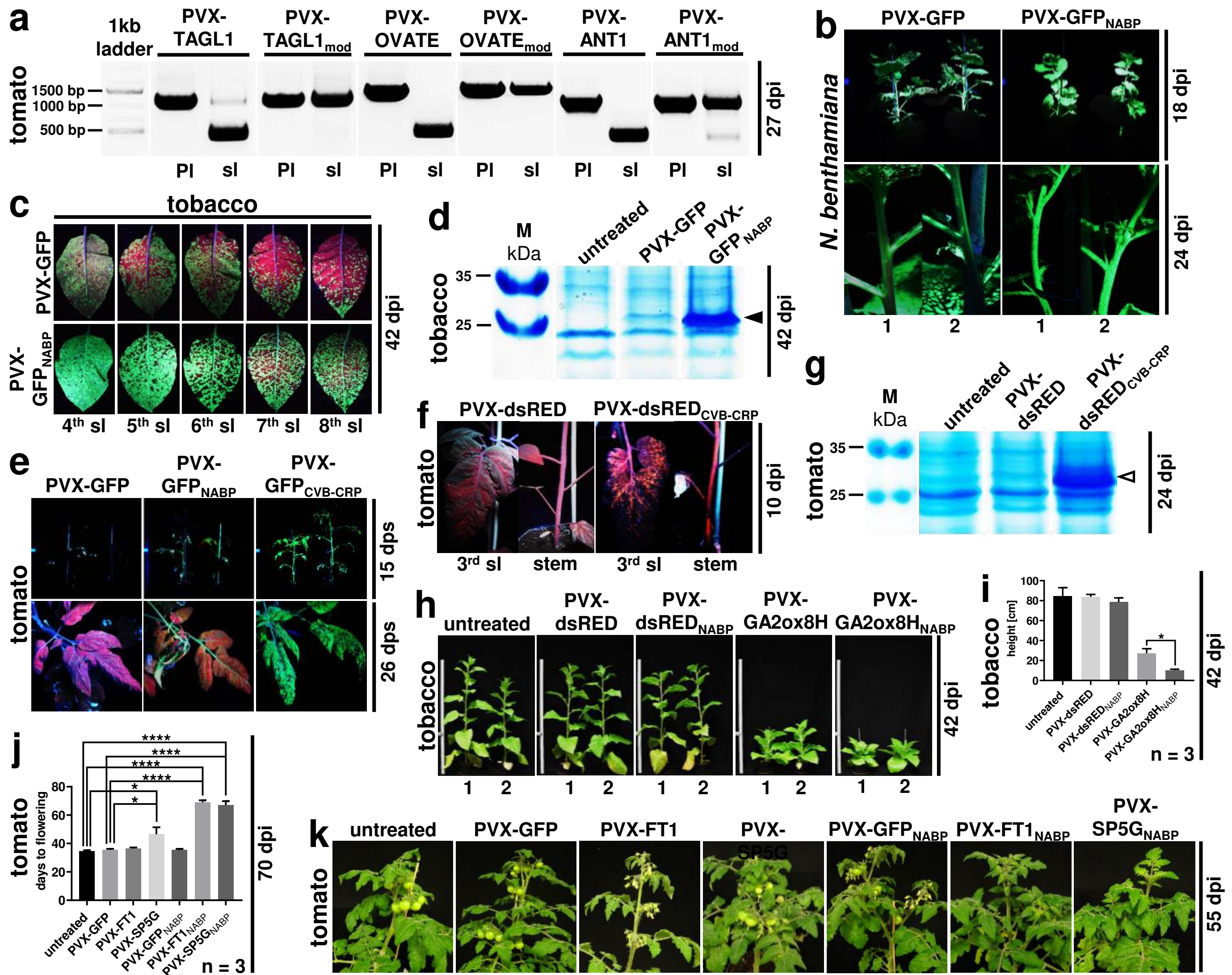

Figure 5

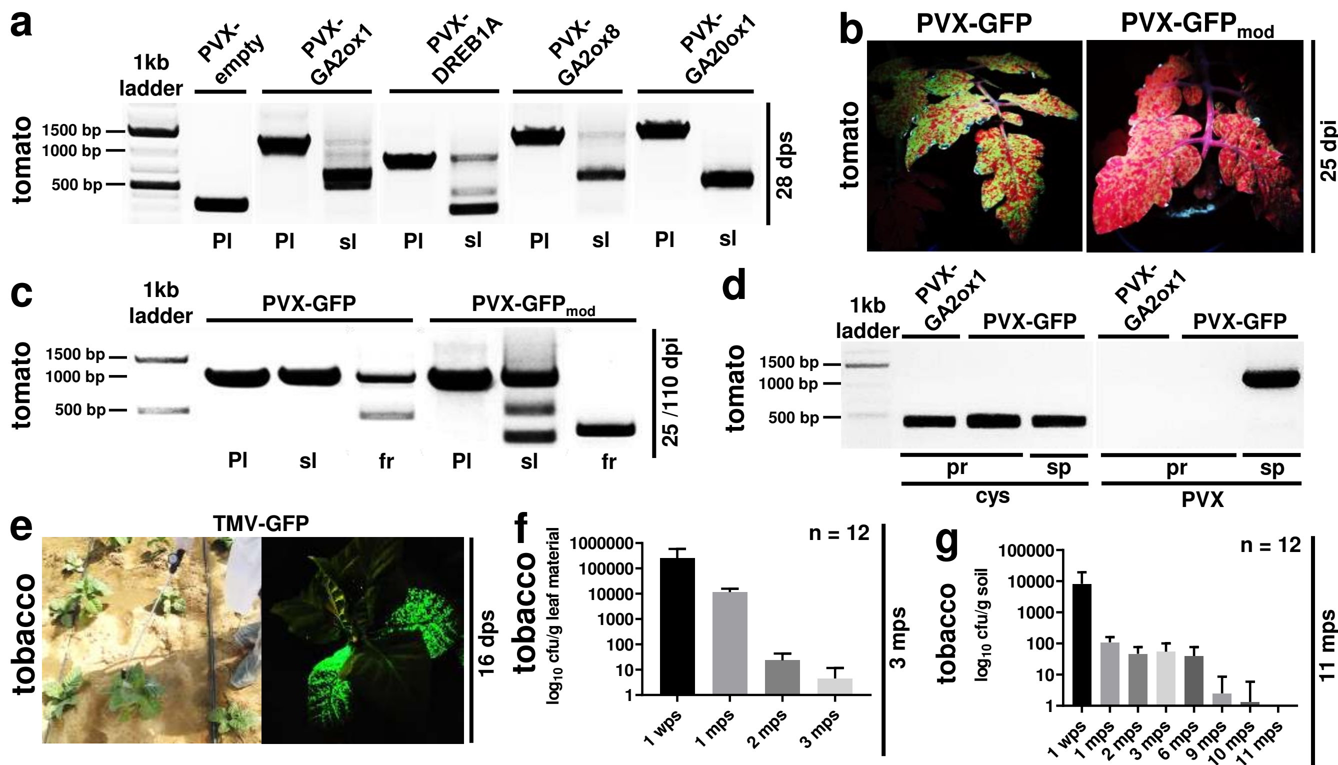

Figure 6