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Transient reprogramming of crop plants for agronomic performance

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

Modern plant breeding relies on recombination to introduce useful genes/alleles into elite germplasm. Development of a new variety is time-consuming and expensive, even with use of most advanced technologies, such as genome editing. We sought to design a flexible, rapid and industrially scalable alternative platform to alter hormonal and other regulatory circuits within a plant, by rebuilding the known techniques of transient gene expression around gene delivery methods that can be performed on an industrial scale and that can be practiced with many crop plants. Our approach focused on two types of vectors commonly used in laboratory science: *Agrobacterium* as the primary DNA vector and RNA viral amplicons as secondary/primary vectors and amplifiers of information molecules. We and others have successfully used *Agrobacterium*-based transfection to design industrial-scale manufacturing processes for producing recombinant proteins in plants[1–4], including biopharmaceuticals, vaccines and biomaterials[5]. This earlier-generation transient reprogramming focused on a single plant species, *Nicotiana benthamiana*. The method required vacuum-assisted infiltration of bacteria into the intercellular leaf space and, by design, ignored the general agronomic performance of the plant other than the high-level expression of heterologous recombinant proteins that were mostly of non-plant origin. A few attempts to modify agronomic traits—viral induction of flowering—were also previously reported but were limited to research-scale experiments[6–11].

We report here that many economically important crop plants can be induced to exhibit desirable agronomic performance traits by simply spraying them with agrobacteria carrying viral replicons to express plant genes. Moreover, we also demonstrate that most of the agronomic traits can also be engineered by spraying plants with packaged RNA viral vectors thus eliminating DNA release into the environment altogether. In our studies, manipulation of traits was based on expression or silencing of specific plant regulatory genes that are components of hormonal circuits, such as flowering control, gibberellin, abscisic acid, ethylene pathways and several others. Using appropriate molecular techniques, we evaluated several viral vectors, tested a large set of plant genes and characterized molecular events linked to plant phenotypes. We show that proposed transfection generates a temporary cascade of new information in the plant and enables the controlled alteration of agronomic performance in many useful ways.

Results

***Agrobacterium* and packaged RNA viral vectors as tools for industrially scalable transfection.** On the basis of processes widely practiced in agronomy—spraying plants with solutions or suspensions to deliver agrochemicals—we evaluated delivery of agrobacteria and viral particles (VPs). These vectors were found effective when applied using standard conditions typical for industrial sprayers (1–3 bar pressure; 1–4-mm atomizer nozzles) and were unaffected by these spraying conditions. The delivery of agrobacteria required use of surfactants such as Silwet L-77, Silwet Gold, Triton-X100 or Tween 20 to induce 'stomatal flooding' and allow bacteria to enter the intercellular space of the leaf[4]. We also explored the use of abrasives such as silicon carbide F800 or diatomaceous earth as described in refs. [12,13], which allowed for improved transfection of several plant species. By applying a suspension of agrobacteria of ~1×10^6 colony-forming units (c.f.u.) per ml (1×10^3 dilution of the overnight culture, optical density $OD_{600}=1.5$) mixed with Silwet L-77 at a concentration of 0.1% to the test plant *N. benthamiana* (Fig. 1a), the frequency of transfection by agrobacteria was as high as 1×10^{-2} per leaf cell (Fig. 1b and ref. [4]). Agrobacteria were efficient in the delivery of viral vectors with either localized or systemic movement as well as movement-disabled ones (Fig. 1c,d).

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Subsequent studies revealed that for most traits to be efficiently delivered, the viral vector should be able to move locally or systemically through the phloem. In the proof-of-principle experiments using dipping of *N. benthamiana* leaves into the suspension of agrobacteria, we also showed that it is possible to achieve subsequent transfections by treating the same plant several times (Fig. 1e).

Using *Agrobacterium* carrying viral vectors with a *GFP* cargo (reporter) gene, we evaluated representatives of 28 plant species from six plant families (Supplementary Table 1) and found that leaves of many dicotyledonous plants of practical interest (tomato, potato, pepper, sugar beet, spinach and soybean) and monocotyledonous plants (maize, wheat and so on) can be efficiently transfected using our standard spray technique. Potato virus X (PVX)-based replicons performed efficiently in most crop plants we tested (Fig. 1f). In a few cases, we used other viral backbones (Supplementary Fig. 1) developed by us or by other laboratories, such as tobacco mosaic virus (TMV), tobacco rattle virus (TRV) or clover yellow vein virus (ClYVV)[14–17] (Fig. 1g). As an example of a monocotyledonous species, maize was transfected by maize streak virus (MSV) (Fig. 1g). It should be mentioned that the plant viral vectors used in our work had generally only mild effects on the plant phenotypes, such as slightly delayed growth and occasionally leaf mosaic.

We also tested the efficacy of spray-based delivery of RNA virus particles (Fig. 1h) and found that the process is less efficient, with a frequency of transfection in *N. benthamiana* of $<1\times10^{-4}$–1×10^{-5} per cell. Nevertheless, even at this low frequency, viral particle spraying was sufficient to achieve the results described below.

Transient manipulation of the flowering regulatory pathway. Underlying mechanisms of the transition from vegetative to flowering state in plants have been the subject of century-long studies, with the concept of a 'principle' or inducer moving from leaves to apical meristem and causing flowering (florigen) first postulated by Mikhail Chailakhyan in 1936[18]. The molecular basis of the process, however, has been clarified only during the last two decades[19]. In the core of the process is the so-called flowering locus T mobile protein (FT) whose expression is induced by external light intensity/day length via the phytochrome machinery; FT then moves from leaves through phloem to apical meristem and interacts with transcription factors that trigger the transition to flowering differentiation of the meristem[20,21]. Since the primary delivery site for our transfection treatment is the plant leaf, we decided to hijack the process by transiently producing additional FT protein in the leaves.

Most of these experiments relied on *Agrobacterium*-based delivery of PVX vectors harbouring one of the many genes known to be involved in flowering control (Supplementary Table 2). In agreement with their central and universal role in flowering control, expression of genes encoding mobile *Arabidopsis* FT or its orthologues from various species, such as tobacco, tomato, rice and others, promoted flowering, shortening floral transition in many plant species (*Arabidopsis thaliana*, tobacco, tomato, pepper and wheat) (Fig. 2a–j) whereas flowering repressors of the same family delayed flowering (Supplementary Fig. 3). The flowering induction results were most dramatically detectable in the tobacco variety 'Maryland Mammoth', which does not naturally flower under long-day field conditions (flowering occurs close to December in the northern hemisphere), leading to high accumulation of vegetative biomass. A few genes controlling upstream (*PhyB*) or downstream (*SOC1*) components of the flowering regulatory cascade that we tested did not result in any visible phenotypic changes (data not shown).

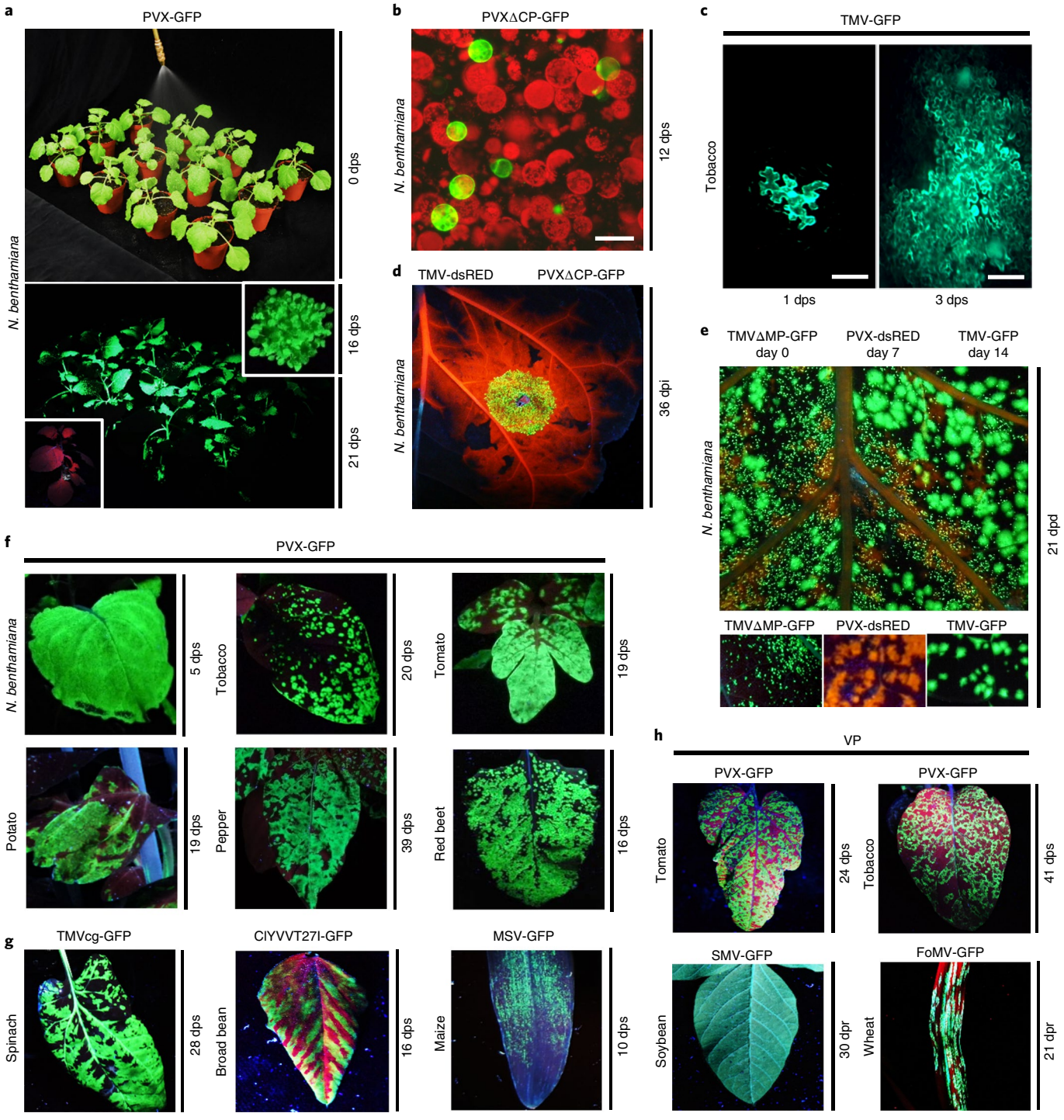
It would be practically useful to control vernalization, the requirement for prolonged seasonal cold (for example, winter) for successful flowering[22,23]. A tighter control of vernalization (to avoid 'bolting' in crops such as sugar beet), or an easy way of lifting the vernalization block, would be of importance for seed production and breeding of crops such as winter wheat, barley, rapeseed and others. In *Arabidopsis*, there are many biennial ecotypes that do not flower during the first year. We evaluated the effect of transient *FT* expression or antisense silencing of the flowering repressor *FLC* in proof-of-principle experiments. We found that both treatments were effective in promoting flowering in vernalization-dependent ecotypes such as Tul-0, Tamm-2, Lov-5 and others, without any vernalization treatment (Fig. 2j,k).

We subsequently evaluated the version of the transient delivery cascade that relies on spraying plants with PVX VPs produced in *N. benthamiana*, rather than *Agrobacterium* cells, thus obviating the need to release DNA-based vectors into the environment altogether. Transition to flowering was demonstrated with tobacco and tomato (Fig. 2l,m and Supplementary Fig. 3n,o). Due to practical considerations and performance, as well as compliance with environmental and regulatory constraints (no DNA release in the field; below and Discussion), we view the application to crops of self-limited RNA virus particles as the more promising version of the transient agronomic trait modification platform.

Transient manipulation of the gibberellin regulatory pathway for dwarfism. Traits such as dwarfism and semidwarfism were the basis of the 'green revolution' in plant breeding during the 1960s–70s and the underlying molecular mechanisms of these traits are well understood[24,25]; recently reviewed by Eshed and Lippman[26]. Many of the

Fig. 1 | Transfection of various crop plants with viral vectors delivered using *Agrobacterium* or as VPs. a, Spraying of *N. benthamiana* plants with *A. tumefaciens* strain ICF320 carrying PVX-GFP vector using industrial sprayer (top panel). All plants showed intensive green fluorescence at 21 dps (bottom panel). Inserts show an untreated *N. benthamiana* control plant under ultraviolet light (bottom left) as well as GFP fluorescence at 16 dps in seedlings sprayed with the same agrobacterial suspension (top right). **b**, Microscopic image of protoplasts isolated from plants sprayed with *Agrobacterium* harbouring cell-to-cell-movement-disabled PVXΔCP-GFP vector visualizes the efficiency of agrobacterial transfection based on GFP fluorescence. Scale bar, 100 µm. **c**, TMV vectors without own CP can still move cell-to-cell in tobacco using their own MP. Scale bar for both images, 50 µm. **d**, Co-infiltration of agrobacteria strain ICF320 carrying PVXΔCP-GFP and TMV-dsRED vectors, providing green and red fluorescence, respectively. Only the TMV construct shows systemic movement, as indicated by red fluorescent protein dsRED expression in leaf veins of *N. benthamiana* at 36 dpi. **e**, Transient delivery can be used repeatedly. *N. benthamiana* plants were dipped successively with 1-week interval in diluted agrobacterial cultures harbouring viral vectors TMVΔMP-GFP (day 0), PVX-dsRED (day 7) and TMV-GFP (day 14) (top panel). For controls (bottom panel), *N. benthamiana* plants were dipped in different *Agrobacterium* suspensions separately. Photos were taken at 21 d post-dipping (dpd). Dipping was used here for proof of principle to get more uniform distribution of the transfection spots for better visualization. Same concept applies for spray (see main text). **f**, GFP fluorescence in leaves of many plant species sprayed with agrobacteria carrying PVX-GFP: *N. benthamiana*, tobacco 'Samsun', tomato 'Balcony Red', potato 'Elfe', pepper 'Early California Wonder' and red beet 'Moulin Rouge'. **g**, GFP fluorescence in leaves of plant species sprayed with agrobacteria harbouring TMVcg-GFP, ClYVVT27I-GFP and MSV-GFP viral vectors: spinach 'Frühes Riesenblatt', broad bean 'Dreifach Weiße' and maize 'Sturdi Z'. **h**, GFP fluorescence in leaves of tomato 'Tamina', tobacco 'Samsun', soybean 'Blyskavytsya' and wheat 'Cadenza' plants sprayed and rubbed, respectively, with VPs containing PVX-GFP, SMV-GFP and FoMV-GFP constructs. VPs were previously isolated from *N. benthamiana* plants infected with PVX-GFP, SMV-GFP and FoMV-GFP vectors using agro-infiltration. Detailed description on the spraying and rubbing procedures is given in Methods (days post-rubbing, dpr). Photos of GFP-expressing transfected leaves and corresponding untreated controls are shown side-by-side in Supplementary Fig. 2.

agronomically important 'semidwarfism' genes belong to the gibberellin regulatory pathway, the best characterized of which are gibberellin (GA) oxidases; the others are dehydration-responsive element binding (DREB) proteins. To effect dwarfism using transient regulatory interference, we evaluated many GA oxidases as well as relevant DREB and DELLA-motif proteins[27–29] transiently expressed in some important model and crop plants including tobacco, tomato, pepper, pea, broad beans and wheat. Being delivered by *Agrobacterium*, PVX vectors harbouring *GA2-oxidase* or *DREB1-type* genes consistently suppressed stem elongation and plant height in tobacco, tomato, pepper and *N. benthamiana* (Fig. 3a–d and Supplementary Fig. 4c–f), while PVX delivery of GA20-oxidase enhanced stem length (Fig. 3g,h and Supplementary Fig. 4c–f). Similarly, *Agrobacterium*-delivered ClYVV vectors carrying *GA2-oxidase* gene significantly reduced the stem length in pea and broad bean (Fig. 3e and Supplementary Fig. 4). Notably, plant height responded to the dose of *Agrobacterium* (Supplementary Fig. 4a) and was in reversal to external GA application (Supplementary Fig. 4b). VP-based transfection with GA2-oxidase or GA20-oxidase was similarly effective in modulating plant height in wheat and tomato (Fig. 3i–l). Both *Agrobacterium*-based as well as VP-based transfection allowed the control of plant height; the effects of the various genes on plant height are in line with the current understanding of the role of those genes in gibberellin metabolism[30]. Various genes



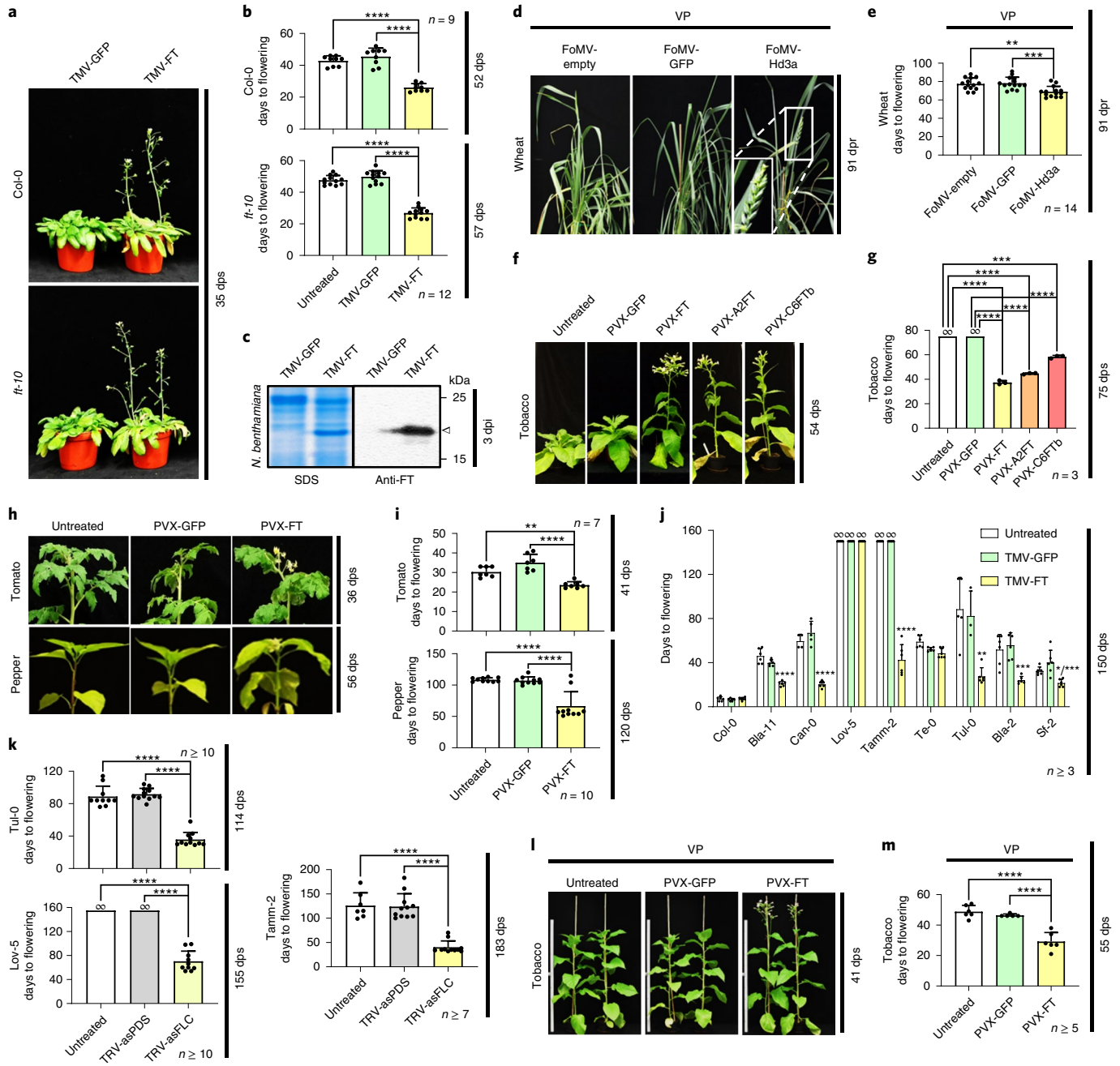


Fig. 2 | Induction and repression of flowering with viral vectors in several plant species. a,**b**, *A. thaliana* wild-type Col-0 and a mutant containing the null allele *ft-10* are induced to flower in response to the infection with TMV carrying the *Arabidopsis FT* gene: plant phenotypes (**a**) and flowering times (**b**). Plants were sprayed with agrobacteria carrying viral vectors. For each plant, days to flowering were counted from the day of spray until the day of bolting as days post-spray (dps). **c**, TMV vector-driven expression of the FT protein in *N. benthamiana* leaves detected by SDS–PAGE with Coomassie staining and immunoblotting with FT-specific antibodies. The arrowhead indicates the protein band corresponding to FT. **d**,**e**, Induced flowering in wheat 'Cadenza' by FoMV-based overexpression of Hd3a (*O. sativa*) using VP delivery: plant phenotypes (**d**) and flowering times (**e**). VPs were previously isolated from *N. benthamiana* plants infected with FoMV constructs by agro-infiltration. **f**,**g**, Five heterologous FT family genes from *Brassica napus* were expressed in tobacco 'Maryland Mammoth' using PVX vectors: plant phenotypes (**f**) and flowering times (**g**). Only two FT family members induced flowering, in both plant species. In **g**, the symbol '∞' indicates no flowering by the end of experiment. **h**,**i**, Tomato and pepper were induced to flowering by PVX-based vectors expressing FT: plant phenotypes (**h**) and flowering times (**i**). **j**, Vernalization-dependent *A. 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 *A. 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 VPs: plant phenotypes (**l**) and flowering times (**m**). VPs were previously isolated from *N. benthamiana* plants infected with PVX-FT using agro-infiltration. In the graphs, mean values with s.d. 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. For **j**, $n = 3$–6; for **k**, $n = 10$–12 and $n = 7$–11; for **m**, $n = 5$ or 6.

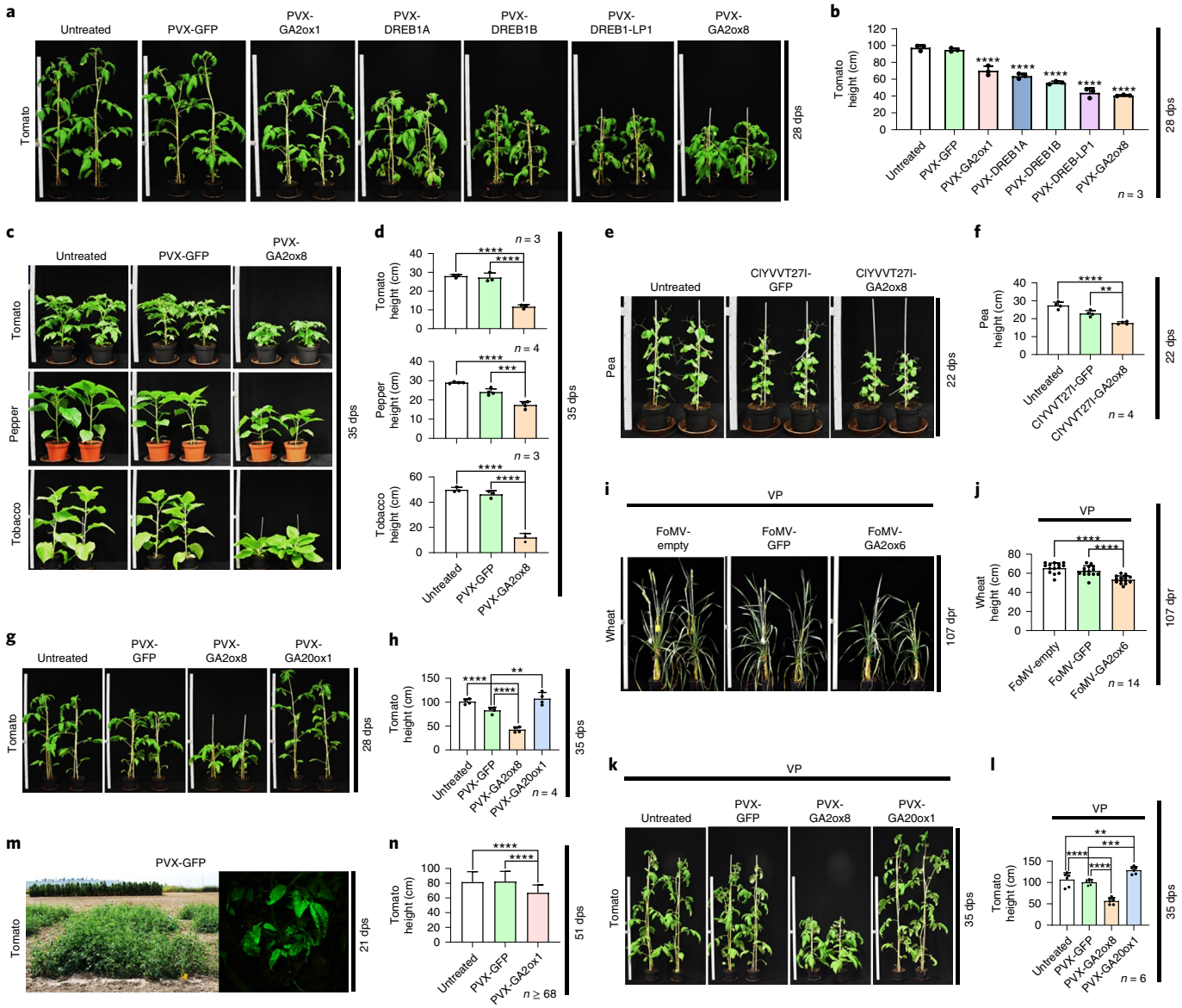


Fig. 3 | Modification of plant stature in several crop species via modulation of gibberellin metabolic pathway by viral vectors. a,**b**, Plant height reduction in tomato caused by PVX-based overexpression of gibberellin pathway genes: plant phenotypes (**a**) and plant height measurements (**b**). Tomato 'Tamina' plants were sprayed with agrobacteria harbouring 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: plant phenotypes (**c**) and plant height measurements (**d**). Viral vectors were delivered to plants using spraying with agrobacteria. **e**,**f**, Height reduction in pea 'Dinga' plants sprayed with *Agrobacterium* harbouring ClYVVT27I-GA2ox8 vector: plant phenotypes (**e**) and plant height measurements (**f**). **g**,**h**, Stem shortening and elongation in tomato 'Tamina' due to PVX-mediated overexpression of GA2ox8 (*G. max*) or GA20ox1 (*A. thaliana*) genes, respectively: plant phenotypes (**g**) and plant height measurements (**h**). Viral vectors were delivered to plants using spraying with agrobacteria. Plant height was measured at 35 dps. **i**,**j**, Height reduction in wheat 'Cadenza' by FoMV-based overexpression of GA2ox6 (*O. sativa*) using VP delivery: plant phenotypes (**i**) and plant height measurements (**j**). VPs were previously isolated from *N. benthamiana* plants infected with FoMV constructs by agro-infiltration. **k**,**l**, Stem shortening and reduction in tomato by modulating gibberellin metabolism using VP delivery: plant phenotypes (**k**) and plant height measurements (**l**). VPs were previously isolated from *N. benthamiana* plants infected with PVX constructs by agro-infiltration. **m**,**n**, Spraying of tomato 'Tamina' with agrobacteria carrying PVX-GA2ox1 vectors in the field resulted in stem shortening. **m**, Plants grown in the field (left panel), and GFP fluorescence in field-grown plants (right panel). **n**, Plant height measurements. In the graphs, mean values with s.d. are indicated; statistical significance was evaluated by one-way ANOVA test using GraphPad Prism v.8.0.2. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *n* = number of plants for each column. For **n**, *n* = number of plants for each column, consists of 68–88 plants.

and their homologues from different species yielded different levels of dwarfism. At least 40% reduction in height was obtained with *GA2ox8* from soybean and *DREB1A* from *Arabidopsis*. Detailed analyses of individual gibberellins in leaves and stems of transfected plants confirmed significant changes in active and inactive gibberellins in *N. benthamiana* and tomato which were in line with the phenotypes observed (Supplementary Figs. 4 and 5). We also overexpressed DELLA proteins which contained mutations known to

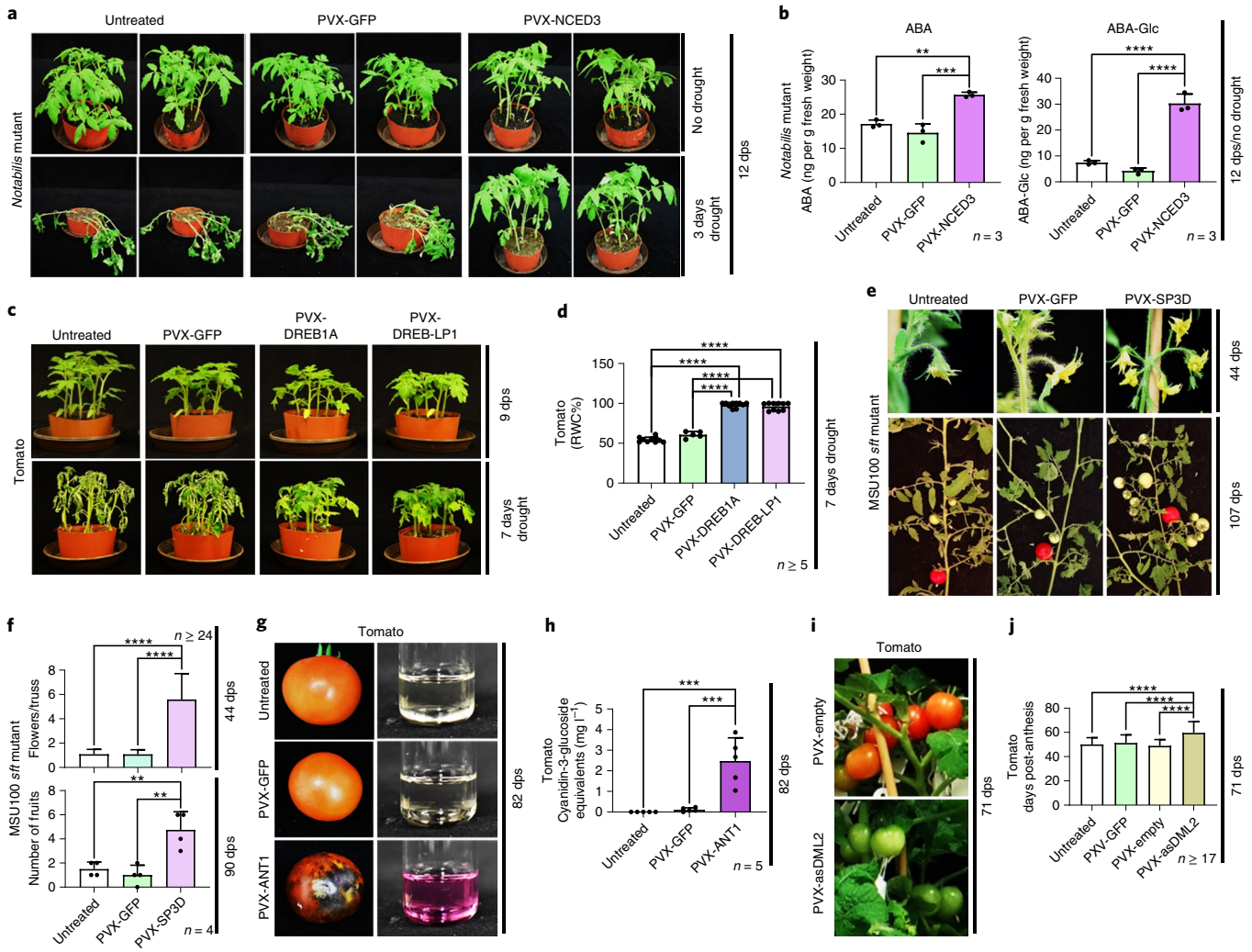


Fig. 4 | Transient reprogramming of other agronomic traits in tomato with PVX vectors delivered by *Agrobacterium* using spraying. a,**b**, PVX-mediated NCED3 complementation increases drought stress tolerance in ABA-deficient tomato mutant *notabilis*. Upon drought, only the tomato mutant plants ('Ailsa Craig' *notabilis*) which were transfected with NCED3 (*A. thaliana*) showed a tolerant phenotype (**a**). The mutant plants not subjected to drought were used t o determine the content of ABA and its derivative ABA-Glc using HPLC (**b**). **c**,**d**, PVX-mediated overexpression of *DREB1A* (*A. thaliana*) and *DREB-LP1* (*C. annuum*) augments drought stress tolerance in wild-type tomato 'Tamina' plants. **c**, Plant phenotypes. **d**, Increased RWC found after the drought correlated with tolerant phenotype (**d**). **e**,**f**, PVX-driven overexpression of *SP3D* (*S. lycopersicum*) gene converted *sft* mutant phenotype to wild-type inflorescence. **e**, Plant phenotypes. Number of flowers and fruits per truss were counted on untreated and sprayed tomato plants 'Ailsa Craig' MSU100 *sft* at 44 and 90 dps, respectively (**f**). **g**,**h**, Overexpression of *ANT1* (*S. lycopersicum*) gene induces anthocyanin production in tomato fruits. Visual changes in the fruit colour of tomato 'Balcony Red' (**g**) reflect the difference in anthocyanin concentrations measured in extracts (**h**). **i**,**j**, Delay in fruit ripening of tomato 'Balcony Red' plants by silencing of the *DML2* gene using PVX vector with a fragment of DML2 coding sequence in antisense orientation. **i**, Plant phenotypes. **j**, Duration of fruit ripening. The *y* axis (**j**) shows the duration of fruit ripening calculated as a time post-anthesis until complete fruit maturation. In the graphs, mean values with s.d. are indicated; statistical significance was evaluated by one-way ANOVA test using GraphPad Prism v.8.0.2. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *n*, number of plants for each column (**b**,**d**,**f**,**h**). For **d**, $n = 5$–10. For **f**, all the flowers of each plant were considered and counted (range 24–26). For **h**, all the fruits of each plant were pooled and measured. For **j**, *n* = number of flowers/fruits, which was in the range 17–135 (taken from all plants of each treatment). Number of plants was seven for each column, except for untreated and PVX-GFP, where the number of plants was two.

impair GA-promoted protein degradation and plant growth[30] but no relevant phenotypic effect was detected.

We also performed a limited set of open field experiments under permit no. 15-041-101r from the US Department of Agriculture (USDA). In those studies, tomato plants were sprayed with *Agrobacterium* carrying the PVX-based gene *GA2ox1*. As anticipated, the transfected plants exhibited significantly reduced height (Fig. 3m,n).

Transient reprogramming of other agronomic traits. Drought tolerance is among the most economically important agronomic traits and a potential to manipulate those responses rapidly and only when the stress factor is present ('trait on demand' concept) would be very useful. We therefore evaluated transient expression as a rapid-response intervention to induce drought tolerance by using the well-characterized *notabilis* mutant of tomato, which is deficient in 9-*cis*-epoxycarotenoid dioxygenase (NCED3), a central component of the abscisic acid (ABA) biosynthetic pathway[31]. Due to the lack of ABA, this mutant is highly sensitive to drought but we show that the sensitivity is dramatically reduced as a result of transfection with a functional *NCED3* gene (Fig. 4a). The transfected plants also showed increased water retention ability as well as significantly elevated concentrations of ABA and of its biologically inactive but

reversible glucose conjugate (Fig. 4b) and phaseic acid and dihydrophaseic acid (ABA catabolites)[32] (Supplementary Fig. 6). In another experiment, wild-type tomato plants were transfected with DREB transcription factors that are mediating stress tolerance[33]; again, higher drought tolerance along with higher water retention was demonstrated (Fig. 4c,d).

In an attempt to effect transiently the trait of insect resistance that has been the basis of modern transgenic insect-resistant crops (corn, soybean and cotton)[34], we expressed the *Bacillus thuringiensis* gene *cry2Ab* in tobacco using a PVX vector with subsequent infestation of the plants with tobacco-adapted hornworm *Manduca sexta*. Cry2Ab-transfected plants demonstrated high toxicity to hornworms, concomitant with the presence of relevant levels of Bt toxin in plant leaves (Supplementary Fig. 6).

One important feature of the transient reprogramming concept would be the ability to spray plant leaves to manipulate traits in distal organs such as flowers, seeds and roots. Efficient development of fruits and overall productivity are of special interest in fruit-bearing crops such as tomato. In a proof-of-principle study, we conducted expression experiments with a tomato *sft* (*single flower truss*) mutant deficient in flower organ formation, which results in development of a single flower per flower truss[35]. *SP3D*, the corresponding gene controlling the fate of meristem in inflorescence, was expressed using *Agrobacterium* delivery of PVX viral vectors. The treatment effectively restored multiflower truss structure of inflorescence and restored the number of flowers/fruits per truss (Fig. 4e,f).

In yet another demonstration of the usefulness of the proposed technology, we delayed fruit ripening in tomato by spraying plants with *Agrobacterium* carrying PVX vector containing antisense fragment of the *DML2* gene for DEMETER-like DNA demethylase regulating the transcription of genes involved in fruit ripening[36] (Fig. 4i,j).

Similarly, to demonstrate the ability of transient delivery to modulate another useful agronomic trait, production of a pigment with reported human health benefits, we expressed in tomato plants the *ANT1* gene encoding a transcription factor controlling anthocyanin synthesis[37,38]. Although fruits of the resultant transfected plants were only sectorially transfected (Fig. 4g), the fruits nevertheless accumulated high levels of anthocyanin in the pericarp (Fig. 4h).

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

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

Another way of controlling trait expression is to design vectors that provide higher expression of the cargo gene. A solution that we applied in these studies was to place the gene of interest in the distal 3'-end of the RNA genome, which is where the most highly expressed gene (viral coat protein) typically resides[40]. This solution somewhat compromises overall efficacy of the viral vector but provides for higher expression of the gene of interest. Among the empirical approaches, we chose to engineer our PVX backbone by inserting known modulators of viral pathogenicity[41,42]. We found that small viral genes of cysteine-rich proteins (CRP), which are believed to interfere with the plant gene-silencing machinery[42], inserted between the STOP codon of the gene of interest and 3' untranslated region of PVX, can dramatically increase gene-of-interest expression (Fig. 5b,g and Supplementary Fig. 7). This in turn provides for broader expression range of the specific agronomic traits described above (Fig. 5h,k). The effect was particularly pronounced for floral repression; it was stably achieved only with the new vector containing CRP.

Even limited levels of viral vector replication within the plant may impose some penalties on overall crop performance. In our experiments, we typically included two negative controls: plants that were not transfected and plants that were transfected with the GFP gene. The latter sometimes resulted in statistically measurable, although very limited in terms of phenotype and practical consequences, effects on the parameters under investigation: for example, somewhat lower height, altered time to flowering and so on (Figs. 2–4). An additional control consisting of empty viral vectors devoid of heterologous genes was found to be less practical because these vectors were in some cases phytotoxic, probably due to higher aggressiveness of the vector, and therefore confounding interpretation of results.

Safety and regulation-compliance aspects of transient expression technologies. We evaluated the stability of gene inserts upon systemic movement of RNA vectors using PVX-based constructs and found that the heterologous inserts are invariably, and relatively rapidly, lost during systemic movement of the vector in the plant (Fig. 6a). The loss is more rapid if the inserts are large genes and if the GC content of the insert is lower[38]. This sensitivity of the vectors to the GC content of the insert allowed us to engineer vectors having either higher stability, resulting in higher expression of the trait due to the longer time that the vector is intact (discussed above; Fig. 5h,k), or lower stability, leading to more rapid loss of the cargo gene from the RNA virus backbone. To illustrate, we rewrote the GFP gene (61.4% GC) by designing a sequence with lower GC content (40.3%) by altering the codon usage. The viral vector carrying this synthetic gene demonstrated lower overall GFP fluorescence in infected leaves and a more rapid loss of the insert (Fig. 6b,c).

Plant viruses fall into two general categories: (1) transmissible viruses that can be inherited by the progeny upon sexual reproduction of the plant, albeit with low frequency and (2) non-transmissible ones that cannot be transmitted to the progeny. The main viruses used as vector backbones in this study, PVX and TMV, are known to be non-transmissible, and our own studies confirmed this for PVX-based viral vectors (Fig. 6d).

In a limited set of experiments under permit no. 13-323-101r from the USDA, we released disarmed non-auxotrophic *Agrobacterium* strain NMX021 carrying a binary vector encoding a PVX-driven GFP gene into an open field environment by spraying tobacco (Fig. 6e) and tomato plants, resulting in a release of $\sim 1 \times 10^{11}$ bacterial c.f.u. We then followed the fate of released agrobacteria in transfected plants and in the soil surrounding the plant roots, and found that within 90 d after spraying the number of detectable bacteria in a plant drops by four logs, from $>1 \times 10^5$ to <10 cells per g of plant biomass (Fig. 6g). Similarly, the number of detectable agrobacteria in the soil falls from initially 1×10^4 cells per g of soil to undetectable levels 11 months later (Fig. 6f). It should be mentioned than during spring and summer time, the natural agrobacterial population in a rich soil can be as high as 1×10^7 cells per m^3 of soil[43].

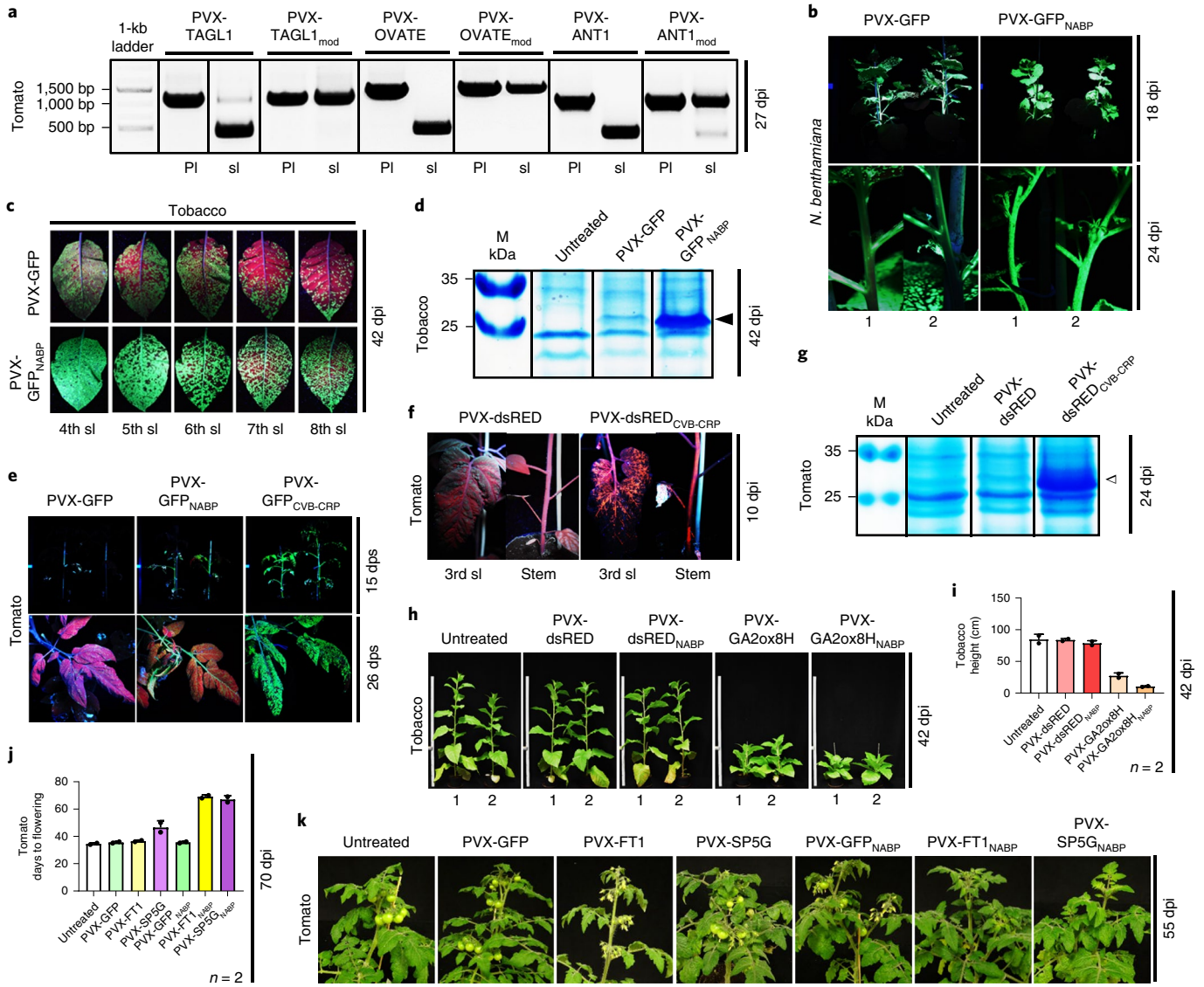


Fig. 5 | Enhanced virus spread, amplification and recombinant protein accumulation using modified PVX vectors. a, Stabilizing PVX constructs using codon-optimized inserts. Tomato 'Tamina' plants were inoculated using syringe with agrobacteria carrying PVX constructs containing wild-type or codon-optimized genes of TAGL1, OVATE and ANT1 from *S. lycopersicum*. Stability of the inserts in systemic leaves at 27 dpi was determined by RT–PCR with PVX specific primers using generated cDNA (sl) and plasmid control (PI) as templates. **b**, Enhancement of reporter gene expression in *N. benthamiana* by CPMMV NABP gene insertion in PVX backbone. Plants were syringe inoculated with agrobacteria carrying constructs PVX-GFP and PVX-GFP$_{NABP}$. **c**, Enhancement of reporter gene expression in tobacco 'Samsun'. Plants were syringe inoculated with agrobacteria carrying the constructs PVX-GFP and PVX-GFP$_{NABP}$. GFP fluorescence is shown for several systemic leaves. **d**, SDS–PAGE analysis of plant samples showing the enhancement of GFP expression in presence of NABP in the viral vector backbone. The arrowhead indicates the protein band corresponding to GFP. **e**, GFP expression enhancement in tomato 'Tamina'. Plants were transfected with *Agrobacterium* harbouring different GFP-expressing PVX constructs using spraying, resulting in a further increase with CVB-CRP in the backbone. **f**, dsRED expression enhancement in tomato 'Tamina': dsRED fluorescence is clearly visible only when CVB-CRP is added to the PVX backbone. Plants were transfected by agro-infiltration using syringe. **g**, SDS–PAGE analysis of samples obtained from PVX-dsRED and PVX-dsRED$_{CVB-CRP}$ treated plants showing the enhancement of dsRED accumulation. The arrowhead indicates the protein band corresponding to dsRED. **h**,**i**, Enhanced dwarfism effect by NABP insertion in PVX backbone. **h**, Plant phenotypes. **i**, Plant height measurements. Tobacco 'Samsun' plants were transfected with different PVX constructs by agro-infiltration using syringe. **j**,**k**, Enhanced flowering repression effect by NABP insertion in PVX backbone. **j**, Flowering times. **k**, Plant phenotypes. Tomato 'Balcony Red' plants were transfected with different PVX constructs by syringe agro-infiltration. Genes are: *FT1* (from tobacco) and *SP5G* (from tomato). In all these experiments, except for **e**, we used syringe inoculation of first true leaf (*N. benthamiana*) or both cotyledon and first true leaf together (tobacco and tomato) instead of spraying the whole plant so as to be able to track virus systemic movement and assess viral RNA stability and subtle quantitative differences in viral vector performance. In the graphs, mean values with s.d. are indicated; statistical significance was evaluated by one-way ANOVA test using GraphPad Prism v.8.0.2. *n*, number of plants for each column.

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

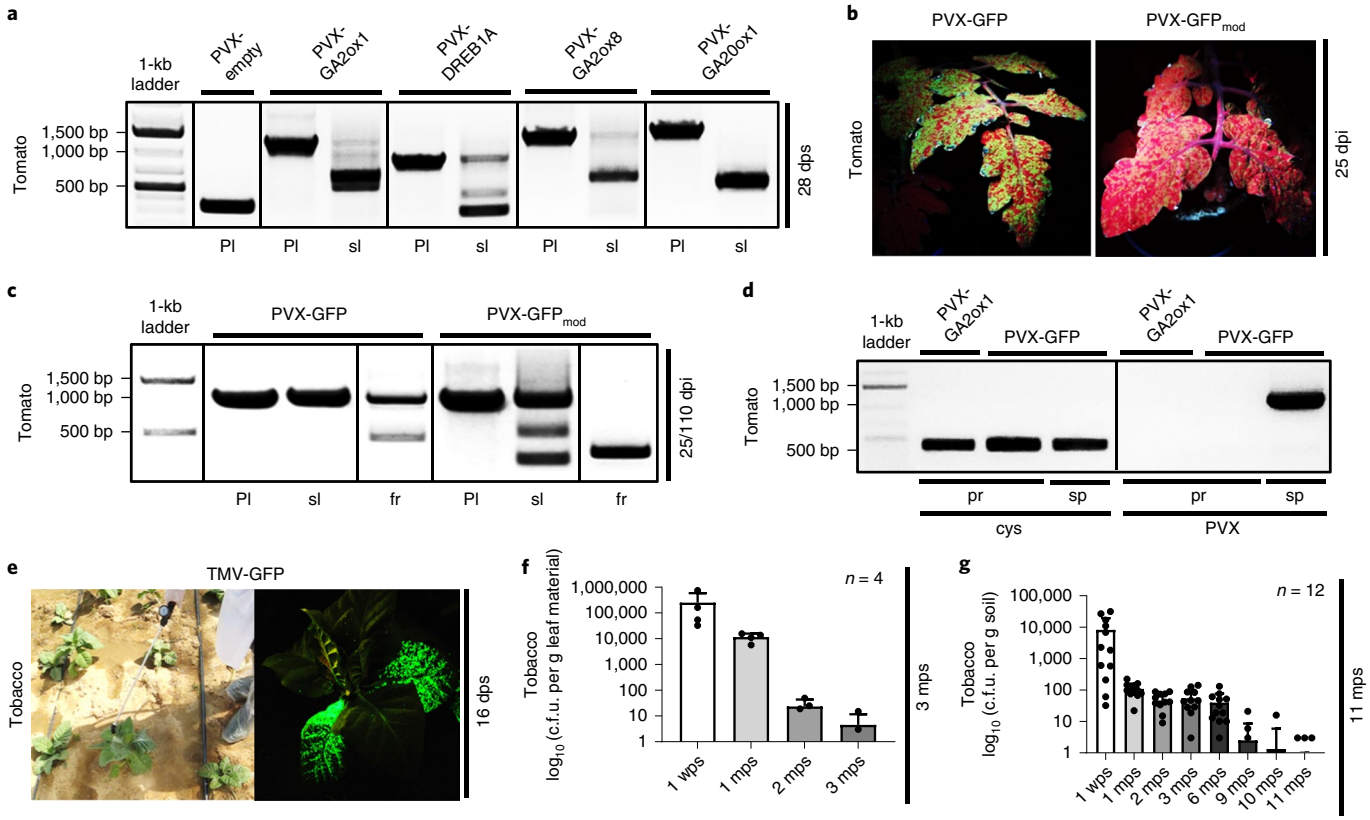


Fig. 6 | Fate of *Agrobacterium* and viral vectors in transfected plants and soil (greenhouse and open field). a, PVX vectors lose foreign gene inserts upon systemic movement in tomato. Plants were sprayed with agrobacteria harbouring PVX constructs with *GA2ox1* (*O. sativa*), *DREB1A* (*A. thaliana*), *GA2ox8* (*G. max*) and *GA20ox1* (*A. thaliana*) genes. Vector stability was determined in systemic leaves (sl) by RT–PCR using PVX specific oligos. PCR fragments generated using corresponding plasmid DNA as a template (Pl) were used as a positive control. **b**,**c**, Reducing the GC content destabilizes GFP gene inside the PVX vector. Tomato plants were agro-infiltrated by syringe with PVX-GFP constructs containing GFP sequences with original and reduced GC content. Stability of the inserts in systemic leaves (sl) at 25 dpi (**b**) and in fruits at 110 dpi (fr) was determined by RT–PCR (**c**) as described above. In this group of experiments, we used syringe inoculation of both cotyledon and first true leaf together instead of spraying the whole plant so as to be able to track the virus systemic movement and assess viral RNA stability. **d**, Absence of PVX in progeny of agrosprayed plants. The presence of PVX and cystatin housekeeping control gene was determined by RT–PCR using cDNAs generated from tomato 'Balcony Red' as templates and specific primers. Seedlings germinated from seeds collected on plants sprayed with the PVX constructs (pr) did not show the presence of PVX, while control leaves of sprayed plants (sp) generated a clear band. **e**–**g**, Transient presence of genetically modified agrobacteria in tobacco leaves and surrounding soil analysed in a field experiment. **e**, Tobacco 'Maryland Mammoth' plants were sprayed with auxotrophic *Agrobacterium* strain NMX021 harbouring TMV-GFP vector and monitored for GFP fluorescence. **f**, Time course after spraying, in which samples of leaves were collected and analysed for the presence of *Agrobacteria* by counting c.f.u. per g leaf material. **g**, Time course after spraying, in which samples of soil with a constant distance to the plants were collected and analysed for the presence of *Agrobacteria* by counting c.f.u. per g of soil. wps, weeks post-spraying; mps, months post-spraying. In the graphs, mean values with s.d. are indicated. For **f**, *n* = number of plants for each column. For **g**, *n* = number of soil samples collected for each column.

genetic modification of the crop. The fundamental differences between this technology and the current breeding methods (including those based on genetic transformation) are that our approach does not involve genetic modification of the plant genome, is broadly applicable, fast, tunable, versatile and limited to one plant generation, and can be used throughout most of the crop cultivation cycle.

The main differences between our approach and the other emerging transient technology—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 programme. Importantly, however, the trait development based on the transient approach is faster and allows for higher throughput.

We hope we have provided here substantial and broad evidence that practically useful phenotype changes can be generated through fast transient transfection of crops. This general proof-of-principle addressing many crops and many traits needs further development steps; those should include defining and testing most important traits/crop combinations, conducting field trials and generating data for regulatory approvals and scale up. Judging from our results, a single universal viral vector effective across all important crops is unrealistic; vectors' efficacy will be determined primarily by virus host spectrum: species-specific to multispecies-specific or even multifamily-specific. Currently, PVX vector backbone shows the broadest applicability but this backbone is not optimal for families such as beans or for monocotyledons.

Open field trials would need to be conducted using industrial-size equipment and an optimal control of the spraying under open field conditions (day time, wind and humidity) needs to be developed.

Early studies on the safety of proposed technology indicate that the initial vector organisms, *Agrobacterium* or packaged RNA virus particles, are self-limiting and it should be possible to improve their environmental containment further by introducing additional safety locks. For example, *Agrobacterium* can be made multiply auxotrophic, suicidal or otherwise disabled, or alternatively it can be re-coded[45] making it unable to interact with other bacteria or survive after release. The technology based on release of packaged RNA viral vectors is arguably safer than *Agrobacterium* vector, as no DNA is released into the environment, and remaining RNA degradation products are all already present in plants and in soil due to the ubiquitous nature of plant viruses. A precision spraying would probably allow more economical delivery with minimal undesired release to the environment.

Continuing emergence of multidrug- and pandrug-resistant bacteria and of novel viral pathogens repeatedly demonstrates power and technology potential inherent in the molecular machinery of microbes and viruses. The number of approved (and thus allowed to be released into the environment) recombinant bacteria (*Salmonella* as oral vaccine and *Agrobacterium* for control of its pathogenic species) and viruses (influenza virus vaccine and adeno-associated viruses for treatment of spinal muscular atrophy) illustrates the potency and safety of such technical solutions.

The introduction of new technologies is always a challenge. We expect that the technology described herein will initially gain regulatory approval and commercial recognition in certain niche areas before garnering attention for mainstream application on large-acreage major crops. One such application area is the production of commercial seed, where production can be made simpler and more efficient through acceleration of flowering time or control of sterility. The other would be a more rapid deployment of orphan crops (for example, millet, amaranth, buckwheat, cowpea, quinoa and cassava) for flowering control, drought tolerance improvement and so on. Current swift approval and adoption of RNA-based viral vaccines for human health shows that the speed of acceptance of RNA transfection-based agriculture will be greatly facilitated by its expected efficacy during unavoidable major plant pathogen outbreaks.

Methods

Bacterial strains and growth conditions. *Escherichia coli* DH10B cells were cultivated at 37 °C in LB medium. For ClYVV-based constructs, *E. coli* DH5α cells were used. *A. tumefaciens* ICF320 (auxotrophic derivative ($DcysK_a$, $DcysK_b$ and DthiG) of *A. tumefaciens* strain C58) cells[46] or NMX021 cells were cultivated at 28 °C in LBS medium (modified LB medium containing 1% soya peptone (Duchefa)). The NMX021 strain was a modified and fully disarmed version of CryX strain[47], wherein a Ti plasmid region was deleted (including the Amp resistance gene with its flanking left and right region—about 30 kilobases deletion, from nucleotide 30,499 to 60,264) and replaced with the *LacZ* gene to facilitate monitoring for the presence of this strain.

Plasmid constructs and viral vectors. TMV-based assembled vectors were described in ref. [2]. TMV vectors used in these studies lack a coat protein (CP) gene (ΔCP); those further modified by removal of the movement protein (MP) gene are indicated as TMVΔMP. The PVX viral vectors used here with the CP coding sequence placed between the polymerase and the triple gene block open reading frames (ORFs) were based on the ones first described in ref. [3]. PVX vectors lacking CP are indicated as PVXΔCP. ClYVV viral vectors were developed on the basis of refs. [16,17] with T27I mutation to obtain a less aggressive virus[48]. We received pClYVV-GFP as a gift from T. Matsumura (Hokkaido University, Japan) and modified it to be used with our transfer DNA binary vector for *Agrobacterium*. Bipartite TRV vectors[15] were obtained from the *Arabidopsis* Biological Resource Center, Ohio State University: pTRV1 (AF406990, stock no. CD3-1039) and pTRV2-MCS (AF406991, stock no. CD3-1040). We modified the pTRV2-MCS to be used with our T-DNA binary vector for *Agrobacterium*. SMV vector was designed on the basis of ref. [49]. TMVcg viral vectors were developed on the basis of sequence D38444 and modified similarly as in ref. [2]. MSV viral vectors were developed on the basis of a viral sequence deposited in NCBI as Y00514. Foxtail mosaic virus (FoMV) viral vectors are described in ref. [50].

Specifically modified PVX-based RNA amplicons including CRP from carlaviruses were created by insertion of one CRP from cowpea mild mottle virus (CPMMV) (called nucleic acid-binding protein, NABP)[41] or one CRP from chrysanthemum virus B (called CVB-CRP)[42]. Those proteins are pathogenicity determinants believed to be RNA silencing suppressors. They were inserted 2 base pairs (bp) downstream of the gene of interest in the PVX backbone, as is found in the cowpea mild mottle virus, downstream of the coat protein ORF. The presence of the specific CRP in the vector backbone is indicated as subscript (for example, $PVX\text{-}GFP_{NABP}$ and $PVX\text{-}GFP_{CVB\text{-}CRP}$).

Cloning of specific genes into the viral vectors was achieved either by inserting PCR products (PCR performed with KOD hot start DNA polymerase; Merck KGaA) or fragments synthesized by external providers (Eurofins Genomics). Synthesized fragments were for *BnA2FT*, *BnC6FTb*, *SlSP5G*, *OsGA2ox1*, *AtGA20ox1*, *AtDREB1B*, *AtNCED3*, *SlANT1*, *BtCry2Ab* and *IPT*.

Plant species and growth conditions. Plants and varieties used in the experiments described included: *N. benthamiana*; tobacco (*N. tabacum*) 'Samsun' and 'Maryland Mammoth'; tomato (*Solanum lycopersicum*) 'Balcony Red', 'Tamina' and 'Ailsa Craig', tomato 'Ailsa Craig' mutants *notabilis* (LA3614) and *sft* MSU100 (LA2460) (both obtained from Tomato Genetics Resource Center, University of California); pepper (*Capsicum annuum*) 'Early California Wonder' ('ECW'); *A. thaliana* ecotype Col-0 and the ecotypes responding to vernalization Bla-2, Bla-11, Can-0, Co-4, Lov-5, Sf-2, Tamm-2, Te-0 and Tul-0 (all obtained from M. Quint, Martin Luther University Halle-Wittenberg, Germany), *Arabidopsis* mutant *ft-10* (obtained from Nottingham *Arabidopsis* Stock Centre (NASC stock), as GABI-Kat T-DNA insertion library code 290E08 (https://www.gabi-kat.de). In *ft-10*, the T-DNA is inserted into the first intron: broad bean (*Vicia faba*) 'Dreifach Weiße'; pea (*Pisum sativum*) 'Dinga'; potato (*S. tuberosum*) 'Elfe'; spinach (*Spinacia oleracea*) 'Frühes Riesenblatt'; red beet (*Beta vulgaris*) 'Moulin Rouge'; soybean (*Glycine max*) 'Blyskavytsya'; maize (*Zea mays*) 'Sturdi Z'; wheat (*Triticum aestivum*) 'Cadenza'. Other plants were tested only for transfection and they are included in Supplementary Table 1.

Plants were grown in the greenhouse (day and night temperatures of 19–23 °C and 17–20 °C, respectively, with long-day condition as 12 h light/12 h dark and 35–70% humidity). *A. thaliana* plants in short-day conditions (8 h light/16 h dark) were grown in growth chambers (Model AR-22L, Percival Scientific) equipped with fluorescent lamps (four lamps, 100–130 μmol m^{-2} s). Wheat was grown in controlled-environment rooms with day/night temperatures of 26.7 °C/21.1 °C at around 65% relative humidity and a 16-h photoperiod with light intensity of ~220 μmol m^{-2} s.

***Agrobacterium*-mediated transfection of plants.** Plants were inoculated with diluted cultures of *A. tumefaciens* using one of three procedures: (1) infiltration of plant leaves using a needleless syringe or a vacuum (agro-infiltration), (2) spraying of aerial parts of plants using a sprayer (agrospray) and (3) dipping of aerial parts of plants into an agrobacterial suspension (agrodip).

Agro-infiltration procedure. Saturated *Agrobacterium* overnight cultures were adjusted to $OD_{600} = 1.5$ (~1×10^9 c.f.u. ml^{-1}) with *Agrobacterium* inoculation buffer (AIB: 10 mM MES pH 5.5, 10 mM $MgSO_4$) and further diluted with same solution to reach the desired dilution of the *Agrobacterium* suspension. Inoculation of individual leaf sectors was performed using a syringe (syringe infiltration). For inoculation of entire plants, a vessel containing the infiltration solution was placed in a vacuum chamber with the aerial parts of a plant dipped into the solution. A vacuum was applied for 5 min using a ME 8 NT pump (Vacuubrand), with pressure ranging from 0.1 to 0.2 bar (vacuum infiltration).

Agrospray procedure. Saturated *Agrobacterium* overnight cultures were adjusted to $OD_{600} = 1.5$ with AIB and further diluted with same solution supplemented with a surfactant to $OD_{600} = 0.015$ (1:100 dilution). In some cases, carborundum (silicon carbide SiC) F800 (Mineraliengrosshandel Hausen) used as an abrasive was added to agrobacterial suspensions. The surfactants used were: Silwet L-77 (Kurt Obermeier), Silwet Gold (Arysta LifeScience), Tween 20 (Carl Roth) and Triton-X100 (AppliChem). Plants were sprayed using high-performance sprayer 405 TK Profiline (Gloria Haus- & Gartengeraete), with 3 bar, and plastic hand sprayers for routine use (Carl Roth). Some test experiments were performed with the compressor sprayer Einhell BT-AC 200/24 OF (Einhell). Spraying solution composition depended on plants species. AIB supplemented with 0.1% (v/v) Silwet L-77 was used for spraying transfection of *Nicotiana* species, tomato, pepper, potato, red beet and spinach. AIB containing 0.05% (v/v) Tween 20 was used for *A. thaliana* and broad bean. For soybean, AIB supplemented with 0.05% (v/v) Tween 20, 1 mM DTT and 0.3% (w/v)) silicon carbide F800 was used. Maize plants were sprayed using AIB supplemented with 0.1% (v/v) Silwet Gold, 0.3% (w/v) silicon carbide F800 and 5% (w/v) sucrose.

Agrodip procedure. Saturated *A. tumefaciens* ICF320 overnight cultures of $OD_{600} = 4$ were diluted with AIB supplemented with 0.1% (v/v) Silwet L-77 to $OD_{600} = 0.004$

(dilution 1:1000). Aerial parts of *N. benthamiana* plants were dipped upside down into the agrobacterial suspension for 20 s.

Generation of VPs and spraying solution. To obtain VPs for plant infection, we inoculated *N. benthamiana* plants with a suspension of agrobacteria carrying a PVX construct using either syringe or vacuum infiltration. If performed with a syringe, systemic leaves of inoculated plants were collected 2 weeks post-infiltration; if performed with a vacuum, all leaves were collected 1 week post-infiltration. To recover sap containing VPs, plant material was further ground in liquid nitrogen and extracted using PBS buffer in a 5:1 (v/w) buffer:biomass ratio. The extract was filtered using Miracloth followed by centrifugation of the filtrate for 15 min at 4,500 r.p.m. at 4 °C. For syringe infiltration, this supernatant solution was used directly for spraying. For vacuum-infiltrated plants, the supernatant solution was first filtered to remove agrobacterial cells using sequential filtrations with filters of 8–12-µm and 0.22-µm pore size. Spraying solution included 0.3% (w/v) silicon carbide F800. Plant species sprayed were *N. benthamiana*, tobacco and tomato.

Protoplast isolation. Protoplasts were isolated as described in ref. [3].

Immunocytochemistry. Tomato (*S. lycopersicum*) 'Tamina' plants were grown for 24 d and then agro-infiltrated. Agrobacteria carried several PVX constructs (PVX-empty vector, PVX-GFP and PVXΔCP-GFP). Small leaf discs were collected at 10 days post-infiltration (dpi) for immune-staining. Segments of leaves were fixed with 3% paraformaldehyde/0.05 % Triton-X100 in PBS for 3 h at room temperature and subsequently embedded in PEG 1500 as described in ref. [51]. GFP was labelled in 3-µm sections with a polyclonal antibody from goat (no. 600-101-215; Rockland; diluted 1:500 in PBS containing 5% bovine serum albumin) detected with a donkey-anti-goat-Alexa 488 secondary antibody (no. A-11055, Thermo Fisher Scientific; diluted 1:500 in PBS containing 5% bovine serum albumin).

Protein analysis. About 100 mg of fresh weight plant leaf material was ground in liquid nitrogen and crude protein extracts were prepared with five volumes of 2× Laemmli buffer. Total soluble protein was extracted from approximately 100 mg of fresh weight plant material ground in liquid nitrogen and dissolved in 500 µl of 1× PBS and incubated for 30 min at room temperature. After centrifugation 40 µl of SDS sample buffer were added to 10 µl of supernatant solution. Sample aliquots (15 µl) were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (12% polyacrylamide gel) and Coomassie-stained using PageBlue Protein Staining Solution (Thermo Fisher Scientific). Protein extracts were denatured at 95 °C for 5 min before loading.

For immunoblot analysis, sample aliquots (15 µl) were resolved by SDS–PAGE (12% polyacrylamide gel) and subsequently blotted on a PVDF membrane. FT protein was detected using FT-specific antibodies (Agrisera; diluted 1/1,000); GA2ox8H or GA20ox1H (*H*: His_6-tag) were detected using Tetra His (Qiagen) mouse monoclonal IgG1 anti-His as the primary antibody (diluted 1/2500). GFP was detected using anti-GFP rabbit polyclonal antibody (Thermo Fisher Scientific; diluted 1/5,000). Secondary antibodies were IgG (whole molecule) peroxidase affinity isolated antibody (Sigma–Aldrich), anti-mouse (diluted 1/5,000) or anti-rabbit (diluted 1/10,000).

Genes used in the studies described. A list of genes with their GenBank accession numbers is provided in Supplementary Table 3.

Experiments to manipulate flowering. *A. thaliana* plants of wild-type ecotypes and the null mutant allele *ft-10* were induced to flower by spraying with agrobacteria carrying TMV vectors with genes inducing flowering (from *FT* gene family) or TRV vectors to silence the *FLC* gene. Plants were sprayed at ~4 weeks of age. For Col-0 ecotype, plants were kept under short-day conditions (see above) to avoid induction of flowering by day length. For the other ecotypes, plants were kept under long-day conditions in a greenhouse (see above). For each plant, days to flowering were counted from the day of spray until the day of bolting (around 1 cm of bolt appearing from the rosette leaves) as days post-spraying (dps).

Tobacco (*N. tabacum*) plants were sprayed with agrobacteria harbouring TMV and PVX vectors (expressing genes of the FT family, either inducers or repressors) when they were 3–4 weeks old. The cultivar 'Maryland Mammoth' was kept under long-day conditions (see above) in the greenhouse, which does not induce flowering. The cultivar 'Samsun' is unresponsive to day length; therefore, it flowers normally under the standard greenhouse conditions described. For each plant, days to flowering were counted from the day of spray until the day of bolting (floral buds visible arising from the plant) as dps.

Tomato (*S. lycopersicum*) 'Balcony Red' and pepper (*C. annuum*) 'ECW' plants 3–4 weeks old were sprayed with agrobacteria carrying PXV vectors harbouring genes of the FT family. Both plant species flower independently of day length (day-neutral plant species) and the constructs described were used to modulate their time to flowering.

Wheat (*T. aestivum*) 'Cadenza' plants at 2 weeks old were rub-inoculated using FoMV vectors virus particles prepared from infected *N. benthamiana* plants as described in ref. [50].

Experiments to manipulate gibberellin content. For all sets of experiments, *N. benthamiana*, tobacco (*N. tabacum*) 'Samsun', tomato (*S. lycopersicum*) 'Tamina' and pepper (*C. annuum*) 'ECW' plants were used for agrospray when they were 3–4 weeks old. Agrobacteria were applied to plants to introduce PVX constructs expressing genes that affect gibberellin metabolism. Plant height was scored at the day of spray (day 0) to ensure that no significant difference was present at the beginning of the experiment and later at several time points during a time course. Broad bean (*V. faba*) 'Dreifach Weiße' plants were used for agrospray when they were 2–3 weeks old. For each time point (days post-spraying, dps), plant height was scored as length between the soil level and the last apical inflorescence (end of the stem). The same conditions were used when spraying the plants with VPs.

Wheat (*T. aestivum*) cultivar 'Cadenza' plants at 2 weeks old were rub-inoculated as described above.

For the field trials, tomato (*S. lycopersicum*) 'Tamina' plants (28 days old) were sprayed with suspensions of the *Agrobacterium* strain NMX021 harbouring PVX constructs (single spray ~1×10^7 bacteria per ml or ~$2–3\times10^8$ bacteria per ft^2).

External application of active gibberellins via spray. The gibberellins GA_3 and GA_4 (Sigma–Aldrich) were dissolved in ethanol to 1 mM concentration and diluted 100 times with water supplemented with 0.02% (v/v) Silwet L-77 for spray application. *N. benthamiana* plants, previously inoculated with PVX constructs PVX-GFP and PVX-GA2ox8, were sprayed at two different time points (13 and 27 dps) either with a 1% ethanolic solution supplemented with 0.02% (v/v) Silwet L-77 with 10 µM of GA_3, GA_4 or no gibberellins (mock).

Gibberellin analysis. Analysis of gibberellins content in *N. benthamiana* and tomato 'Tamina' plants was performed as follows. Plants were sampled at the indicated dps and separated into leaf and stem material. Individual gibberellins were identified and quantified using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MSMS) as described in ref. [52]. The mass spectrometry data of individual gibberellins were processed by using TargetLynx v.4.1 SCN 904 (Waters Corporation).

Experiments on drought tolerance. *Experiment with mutant tomato variety.* The tomato (*S. lycopersicum*) 'Ailsa Craig' mutant *notabilis* variety was used for these experiments. Six pots, each with five 19-day-old plants, were sprayed with agrobacteria harbouring PVX vectors expressing either GFP or the NCED gene. At 9 dps, each three pots per treatment were not watered for 3 d. Watered (no drought) and stressed (drought) plants were photodocumented after 12 dps. At this time point, watered plants were harvested and ground and samples were used for high-performance liquid chromatography (HPLC) analysis to determine the content of ABA, its derivative abscisic acid glucose ester (ABA-Glc), phaseic acid (PA) and dihydrophaseic acid (DHPA), in relation to the treatments.

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

Experiments on anthocyanin production. To induce anthocyanin production in tomato fruits, 6-week-old tomato (*S. lycopersicum*) 'Balcony Red' plants were sprayed with agrobacteria carrying PVX-ANT1 constructs.

At fruit maturation, fruits were ground to a fine powder in liquid nitrogen. To extract anthocyanins, 1.5-g samples of ground fruit material were mixed with 3 ml of methanol supplemented with 1% HCl and further incubated overnight at 4 °C in the dark. On the next day, first 2 ml of sterile water were added and the samples intensively mixed, and subsequently 5 ml of chloroform were added and again the samples were intensively mixed. The samples were then centrifuged at 4,500 r.p.m. for 10 min at room temperature. Lastly, the supernatant solution was collected and each sample was supplemented with 4 ml of a 60% methanol solution plus 1% HCl. The concentration of anthocyanin pigment (in cyanidin-3-glucoside equivalents) in the extracts was subsequently determined by measuring of OD_{520} and OD_{700} in a 1:2 dilution of a KCl buffer (0.025 mM KCl, pH 1.0) and a Na-acetate buffer (0.4 M Na-acetate, pH 4.5) relative to a standard curve[53].

Experiments on fruit ripening. Tomato (*S. lycopersicum*) 'Balcony Red' plants (31 days old) were sprayed with agrobacteria harbouring PVX vectors with an antisense sequence (fragment 1–480 bp) of the gene *DEMETER-like DNA demethylase 2* from tomato (*DML2*). Because the absolute time of fruit maturation can be masked by changes in several parameters (principally the flowering time), the duration of fruit ripening procedure was measured, for each developing fruit, as the number of days to anthesis: the time between the appearance of a flower and

the maturation of the fruit developed from this specific flower. We score the fruit as mature once it becomes completely red.

Experiments on insect resistance. Tobacco (*N. tabacum*) 'Samsun' plants were sprayed with agrobacteria carrying PVX-Cry2Ab vectors. To determine the concentration of expressed Cry2Ab toxin, leaf material from each plant was collected at 7 dps, extracted in PBST buffer and analysed using a Cry2Ab specific ELISA Kit (Cry2Ab no. KBA010-10, KRISHGEN Biosystems) following the manufacturer´s instructions. At 7 dps, three larvae of the hornworm (*M. sexta*) were placed on each plant for feeding. Plant shapes were photodocumented at the beginning of insect feeding and 28 d later. In a parallel experiment, tobacco plants were syringe-infiltrated with agrobacteria harbouring PVX-Cry2Ab vectors. At 5 dpi, infiltrated leaves were detached and one hornworm larva was placed on each detached leaf. Individual leaves were photographed at the beginning of insect feeding and 5 d later.

Experiments on plant senescence. Tobacco (*N. tabacum*) 'Samsun' plants (62 days old) were sprayed with agrobacteria harbouring PVX-IPT vectors. At 39 d post-spraying, the four oldest leaves of sprayed and control plants were collected and photodocumented.

Detection of PVX in the progeny of agrosprayed plants. Seeds were collected from tomato (*S. lycopersicum*) 'Balcony Red' plants sprayed with the constructs PVX-GFP and PVX-GA2ox1 (*Oryza sativa*) and germinated on filter paper (pr). Total RNA was isolated from pooled seedlings as well as from leaves of PVX-GFP sprayed plant used as a positive control (sp) and used to generate complementary DNA. The presence of PVX and cystatin control was determined by PCR.

Detection of *Agrobacterium* in leaves and soil. *N. tabacum* 'Maryland Mammoth' plants (63 days old) were sprayed with a suspension of *A. tumefaciens* (strain NMX021) cells harbouring PVX-GFP (single spray, ~1×10^6 bacteria per ml or ~$2–3 \times 10^7$ bacteria per ft^2). At different time points after spraying, 12 samples of soil within a constant distance from the plants were collected and analysed for the presence of agrobacteria. For this purpose, 1 g of soil per sample was suspended in 5 ml of sterile SCP broth (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 ~15 min on an orbital shaker (120 r.p.m.), filtered through two layers of Miracloth and washed with two volumes of water. A total 250 µl of soil extract were plated on LB (rif, cyc, X-gal), incubated for 3 d at 28 °C and used for enumeration of colonies. Colony-forming units (c.f.u.) per g of soil were calculated. At different time points after spraying, 12 samples of leaves were collected and analysed for the presence of agrobacteria. For this purpose, 1 g of leaf material per sample was ground in liquid nitrogen, admixed with five volumes of sterile SCP broth, shaken for ~15 min on an orbital shaker (120 r.p.m.) and filtered through two layers of Miracloth. A volume of 250 µl of leaf extract was plated on LB (rif, cyc, X-gal), incubated for 3 d at 28 °C for colony enumeration. Colony-forming units per g of leaf material were calculated.

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

Field trials. Experiments with field release of agrobacteria were performed at Kentucky Bioprocessing facilities (Owensboro, USA) in 2014 and 2015. These studies were conducted under permits from the USDA no. 13-323-101r and no. 15-041-101r. Publicly available information can be accessed at: https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/sa_permits/status-update/release-permits.

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

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

Statistics and reproducibility. Statistical parameters are reported in the figures and corresponding figure legends. Statistical significance was evaluated by one-way analysis of variance (ANOVA) test using GraphPad Prism v.8.0.2. The *n* value corresponds to the number of samples for each column, where the type of sample is indicated in the figure legends. In cases where *n* is not the same for each column, it is indicated in the legends. Full datasets are reported in the Source Data. Experiments shown in the graphs were repeated at least twice, with equivalent results. Experiments showing expression of a reporter gene in different plant species and generated with different methods were repeated at least three times with equivalent results. Experiments performed on field trials were performed once for each type of experiment. Analysis of gibberellin content was performed once for each plant species tested.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

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

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Author contributions

Y.G. conceptualized and supervised the research. Y.G. and A.G. directed the research. Y.G., A.G., S.T., R.S., A.T., P.R., S.W. and K.K. designed the research. S.T., R.S., A.T., D.B., P.R., B.K., S.W., V.P. and G.H. performed the 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. analysed the data. Y.G., A.G., S.T. and R.S. wrote the paper. All authors read and approved the final 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. A.G., D.B., P.R. and Y.G. are inventors on the patent application entitled 'Process of transfecting plants' (European patent no. EP2601295 B1); P.R., D.B., A.G. and Y.G. are inventors on the patent application '*Agrobacterium* for transient transfection of whole plant' (European patent no. EP2834362 B1); Y.G. is an inventor on the patent application 'Potexvirus-derived replicon' (European patent no. EP2061890 B1); A.T., D.B., A.G. and Y.G. are inventors on the patent application 'Process of providing plants with abiotic stress resistance' (European patent no. EP2999790 B1); and S.T, R.S., A.G. and Y.G. are inventors on the patent application 'Method of improving potexviral vector stability' (European patent no. EP3456829 A1). The ownership of the patents resides with Nomad Bioscience. The authors have no other competing interests.

Additional information

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The mass spectrometry data of individual gibberellins were processed by using TargetLynx V4.1 SCN 904 (Waters Corporation, Milford, MA, USA).
Data analysis	Mean values, standard deviations, and significances (P-values) of analyzed differences were calculated for each experimental approach with one-way ANOVA using the program GraphPad Prism 8.0.2 (GraphPad Software, San Diego, USA). For some genes the sequences were changed by means of a different codon usage based on the algorithm from GENEius software (Eurofins Genomics Germany GmbH, Ebersberg, Germany). This is a software developed by Eurofins Genomics in close cooperation with BioLink GmbH (no version numbers are indicated).

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All data generated or analysed during this study are included in this published article (and its supplementary information files). All materials are available for research purpose upon request from the corresponding author under a material transfer agreement with Nomad Bioscience.
The following sequences of codon optimized genes have been deposited in NCBI as GenBank accession numbers:

MT877076 SlTAGL1, codon-optimized for rice
MT877077 SlOVATE, codon-optimized for rice
MT877078 SlANT1, codon-optimized for Bifidobacterium longum
MT877079 GFP, codon-optimized for tobacco

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No explicit calculations were performed to determine sample size. Sample size was demonstrated in pilot experiments to be sufficient to achieve desired outcomes. Sample sizes are indicated in Figure legends and Source data.
Data exclusions	No data were excluded from the study.
Replication	The exact number of replicates is stated in the relevant legend and in the section "Statistics and reproducibility". All attempts at replicating particular results were successful.
Randomization	In our study, for each experiment on a specific plant species, we used plants which were genetically homogeneous (same genome for each individual) and of the same age. Therefore, we did not need to rely on a real strict randomization process for each treatment, because the population was already homogeneous. Rather, we took plants "randomly", without any bias, and used them for our experiments. We may refer to this approach as haphazard.
Blinding	Blinding was not relevant to this study since all analyses were performed on homogeneous populations of plants (see previous paragraph). Upon treatments in individual experiments, we checked and made sure that the initial phenotype was equivalent for each treatment group. As an example: for genes causing dwarfism, we always measured the plants before treatment, and we therefore were sure that there was no significant difference in plant height between different groups at day 0 (we simply did not show these control data in the paper).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	anti-GFP goat polyclonal antibody (primary): supplied by Rockland (Catalog# 600-101-215) donkey-anti-goat-Alexa 488 antibody (secondary): supplied by Thermo Fisher Scientific (Catalog # A-11055) anti-GFP rabbit polyclonal antibody (primary): supplied by Thermo Fisher Scientific (Catalog # A-6455) anti-FT rabbit polyclonal antibody (primary): supplied by Agrisera (Catalog # A-S06-198) anti-His mouse monoclonal antibody (primary): supplied by Qiagen (Catalog # 34670) anti-rabbit-HRP polyclonal antibody (secondary): supplied by Sigma-Aldrich (Catalog # A-4416) anti-mouse-HRP polyclonal antibody (secondary): supplied by Sigma-Aldrich (Catalog # A-6154)
Validation	All antibodies were validated by the suppliers by staining and western blot analysis. Specific details are listed below for each antibody. https://rockland-inc.com/store/Antibodies-to-GFP-and-Antibodies-to-RFP-600-101-215-O4L_24115.aspx Shows reactivity to wt, rGFP, and eGFP

https://www.thermofisher.com/antibody/product/GFP-Antibody-Polyclonal/A-6455
Shows reactivity to GFP isolated from the jellyfish Aequorea victoria
https://www.agrisera.com/en/artiklar/ft_tsf-flowering-locus-t-and-twin-sister-of-ft.html
Shows reactivity to arabidopsis FT
https://www.qiagen.com/de/products/discovery-and-translational-research/protein-purification/tagged-protein-expression-purification-detection/tetra-his-antibody-bsa-free/?clear=true#orderinginformation
Shows reactivity to C-terminal, N-terminal, and internal His tags

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Newly hatched larvae of hornworm Manduca sexta were used for experiments on insect resistance.
Wild animals	No wild animals were used in this study.
Field-collected samples	No field collected samples were used in this study.
Ethics oversight	No ethical approval was required in this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.