Manipulation of the Blue Light Photoreceptor Cryptochrome 2 in Tomato Affects Vegetative Development, Flowering Time, and Fruit Antioxidant Content[1]

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Cryptochromes are blue light photoreceptors found in plants, bacteria, and animals. In Arabidopsis, cryptochrome 2 (cry2) is involved primarily in the control of flowering time and in photomorphogenesis under low-fluence light. No data on the function of cry2 are available in plants, apart from Arabidopsis (*Arabidopsis thaliana*). Expression of the tomato (*Solanum lycopersicum*) *CRY2* gene was altered through a combination of transgenic overexpression and virus-induced gene silencing. Tomato *CRY2* overexpressors show phenotypes similar to but distinct from their Arabidopsis counterparts (hypocotyl and internode shortening under both low- and high-fluence blue light), but also several novel ones, including a high-pigment phenotype, resulting in overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits. The accumulation of lycopene in fruits is accompanied by the decreased expression of lycopene β-cyclase genes. *CRY2* overexpression causes an unexpected delay in flowering, observed under both short- and long-day conditions, and an increased outgrowth of axillary branches. Virus-induced gene silencing of *CRY2* results in a reversion of leaf anthocyanin accumulation, of internode shortening, and of late flowering in *CRY2*-overexpressing plants, whereas in wild-type plants it causes a minor internode elongation.

Cryptochromes are flavin-containing blue light photoreceptors, first discovered in plants. The first cryptochrome gene was isolated through the insertional cloning of an Arabidopsis (*Arabidopsis thaliana*) mutant allelic to *hy4* (Ahmad and Cashmore, 1993). Our knowledge of the function of higher plant cryptochromes relies almost exclusively on the study of a single plant, Arabidopsis. Arabidopsis contains at least three cryptochromes, two (cryptochrome 1 [cry1] and cry2) localized predominantly in the nucleus and the cytoplasm (Lin and Shalitin, 2003) and one (cry3) in the organelles (Kleine et al., 2003). Functional characterization of Arabidopsis mutants has shown that cry1 is mainly involved in the control of photomorphogenesis, including hypocotyl elongation and anthocyanin biosynthesis, while cry2 is mainly involved in the control of flowering time and of hypocotyl elongation (Guo et al., 1998; Lin et al., 1998; Lin and Shalitin, 2003). The role of cry2 in Arabidopsis is strictly dependent on light fluence and photoperiod; *cry2*$^-$ mutants and *CRY2* overexpressors have, respectively, long and short hypocotyls under low, but not high, blue light intensities. *cry2*$^-$ mutants flower later than the wild type in long-day but not short-day conditions, and *CRY2* overexpressors flower earlier than the wild type in short-day but not long-day conditions (Guo et al., 1998; Lin and Shalitin, 2003). A naturally found, gain-of-function allele of *CRY2* is responsible for the early flowering in short days of the Cape Verde ecotype of Arabidopsis, with respect to its northern European counterparts (El-Din El-Assal et al., 2001, 2003).

The developmental patterns of Arabidopsis and tomato (*Solanum lycopersicum*) are different. Arabidopsis shows a monopodial type of growth (Schmitz and Theres, 1999) in which the shoot apical meristem (SAM) gives rise to vegetative metamers, each composed of a very short internode, a rosette leaf, and a bud. Upon transition to flowering, the SAM is transformed into an inflorescence meristem (IM), giving rise to an inflorescence with cauline leaves, elongated internodes, and flowers. Axillary meristems are rec-

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ognizable in Arabidopsis only after the transition to flowering. Tomato, or at least its indeterminate varieties, such as the *Moneymaker* cultivar used in this study, shows a sympodial type of development. Early on, the SAM gives rise to vegetative metamers similar to Arabidopsis, the major differences being a much more elongated internode and a clearly recognizable axillary meristem, located at the axil of each leaf. After 7 to 11 metamers, the SAM is transformed into an IM, and the axillary meristem located immediately below it becomes the novel SAM. This novel shoot apex forms approximately three metamers and is then transformed into an IM, while a new axillary meristem becomes the SAM (and so on). The transition from a SAM to an IM is tightly regulated by daylength in Arabidopsis, with most Arabidopsis ecotypes showing a facultative long-day requirement for flowering, whereas no major effects of photoperiod on tomato flowering time have been reported (Koornneef et al., 1998).

Another fundamental difference between the two plants is the development of a fleshy fruit in tomato, whose function is to attract animals involved in seed dispersion. After a period of cell division and cell expansion, leading to the mature green stage, the fruit undergoes a series of changes, controlled by ethylene and collectively known as ripening, which involve the transformation of chloroplasts into chromoplasts (containing high levels of the linear carotene lycopene), the softening of the fruit, and the accumulation of a series of compounds increasing fruit palatability. At the gene expression level, this change involves the differential regulation of genes involved in the biosynthesis of ethylene, carotenoids, and the degradation of cell wall components (Giovannoni, 2001).

We have started the molecular and functional characterization of the tomato cryptochrome gene family. To date, two *CRY1*, one *CRY2*, and one *CRY3* gene have been isolated in tomato (Perrotta et al., 2000, 2001; G. Perrotta, unpublished data). The role of one of the *CRY1* genes, *CRY1a*, has been elucidated through the use of antisense (Ninu et al., 1999) and mutant (Weller et al., 2001) plants. cry1a controls seedling photomorphogenesis, anthocyanin accumulation, and adult plant development. No effects of cry1a on flowering time or fruit pigmentation have been observed. Little is known about the functional role of the remaining tomato cryptochromes, mainly due to the lack of mutants. In this article, we present the phenotypic characterization of tomato plants in which *CRY2* expression has been modified through a combination of transgenic overexpression and virus-induced gene silencing (VIGS).

RESULTS

Effects of *CRY2* Overexpression on Vegetative Growth

To alter the expression of *CRY2*, we transformed tomato (cv *Moneymaker*) with a *35S*::*CRY2* construct carrying the tomato *CRY2* cDNA (Perrotta et al., 2000) under the control of the cauliflower mosaic virus *35S* promoter. Several independent transformants were regenerated, selfed, and the T1 seeds collected. Since one of the phenotypes of Arabidopsis *CRY2* overexpressors is hypocotyl shortening under low-irradiance blue light (Lin et al., 1998), seeds were germinated under blue and red light (2 μmol m^{-2} s^{-1}). Seven out of 10 transgenic lines showed a short-hypocotyl phenotype under blue but not under red light, segregating in an approximately 3:1 ratio. The hypocotyl lengths for

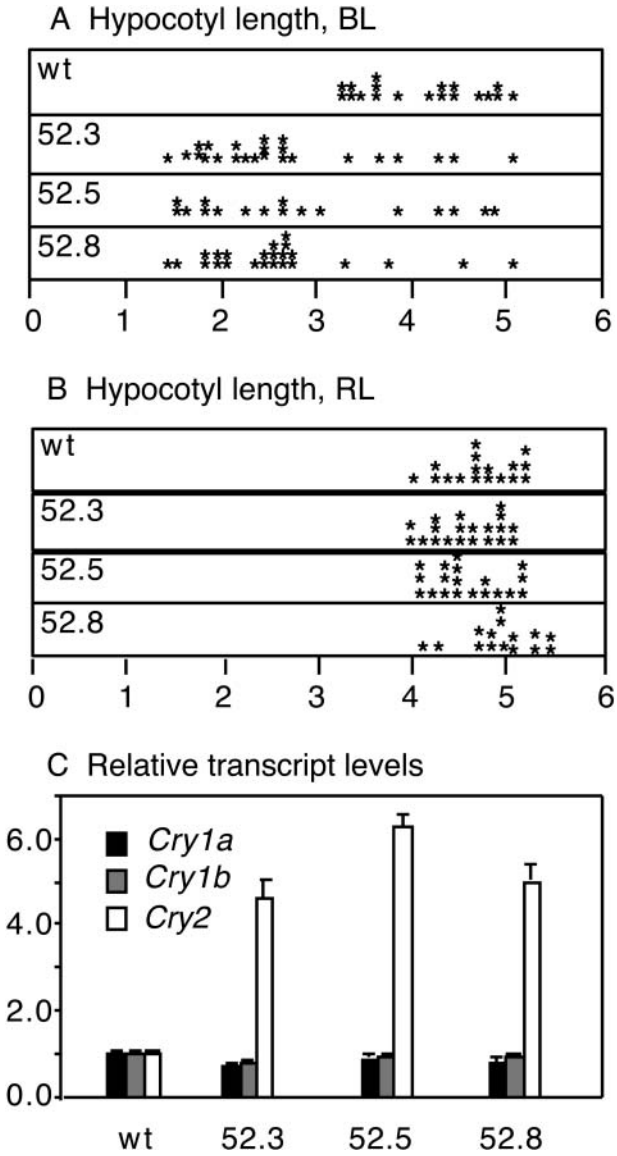

Figure 1. cry2 overexpression produces a short-hypocotyl phenotype under blue light. A and B, Hypocotyl lengths of the wild type and of three independent transgenic lines segregating for the *35S*::*CRY2* transgene. Seeds were germinated for 7 d under continuous low-irradiance (2 μmol m^{-2} s^{-1}) blue (A) or red (B) light. Each asterisk represents an individual seedling. C, Relative expression, normalized for the actin transcript, of *CRY* transcripts in "short" (2-cm) seedlings from A.

three representative lines are shown in Figure 1, A and B. In all three lines, the short-hypocotyl phenotype cosegregated with the presence of the *35S*::*Cry2* transgene (data not shown), confirming that the phenotype was indeed due to cry2 overexpression and not to somaclonal effects arising during the transformation procedure. We measured transcript levels for *CRY1a*, *CRY1b*, and *CRY2* via real-time quantitative reverse transcription (RT)-PCR (RT-QPCR) in transgenic seedlings (Fig. 1B); *CRY2* was overexpressed 5- to 6-fold in the seedlings showing the short-hypocotyl phenotype, whereas the other two cryptochromes showed essentially normal transcript levels.

We then grew *CRY2*-overexpressing (*CRY2-OX*) plants under high-irradiance white light (40 μmol m^{-2} s^{-1}) in a long-day photoperiod (16 h light/8 h dark). These plants showed several additional phenotypes. The seedlings accumulated high levels of anthocyanins in all organs, including roots (Fig. 2D). Adult plants were dwarfed (Fig. 2A), had darker green leaves with anthocyanin-accumulating veins (Fig. 2B), and showed an abnormal outgrowth of axillary meristems (Fig. 2C). Spectrophotometric measurements indicated a generalized pigment increase in leaves of the transgenic lines, very evident (up to 3-fold) for anthocyanins and less so for chlorophylls and carotenoids (Fig. 3, A–C). The internodes of adult plants were significantly shorter (Figs. 2C and 3D).

Homozygous transgenic seed populations were obtained from lines 52.3 and 52.8 and germinated un-

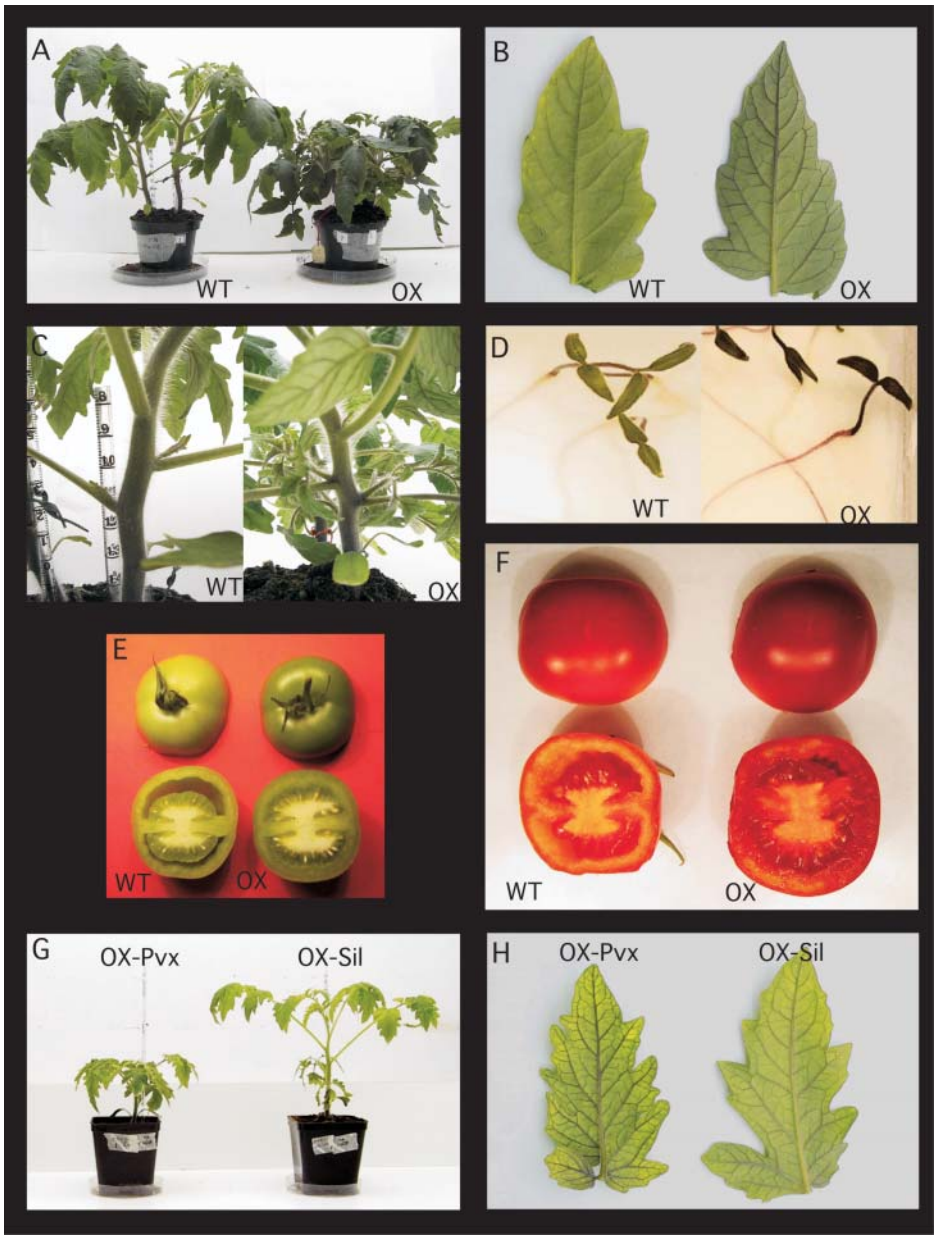

Figure 2. Visual phenotypes of cry2-overexpressing (OX) plants (line 52.3) before and after VIGS. A, Five-week-old plants. B, Leaves from plants shown in B. C, Detail showing short internodes and lateral branching of plants shown in B. D, Seven-day-old seedlings. E and F, Immature green and fully ripe (10 d after breaker) fruits from greenhouse-grown plants. G, Overexpressors infected with PVX (left) and *PVX*::*CRY2* (right), 3 weeks after infection. H, Leaves from plants shown in G. Plants shown in panels A to D, G, and H are grown in long-day conditions under high-irradiance (40 μmol m^{-2} s^{-1}) white light. For further details, see "Materials and Methods."

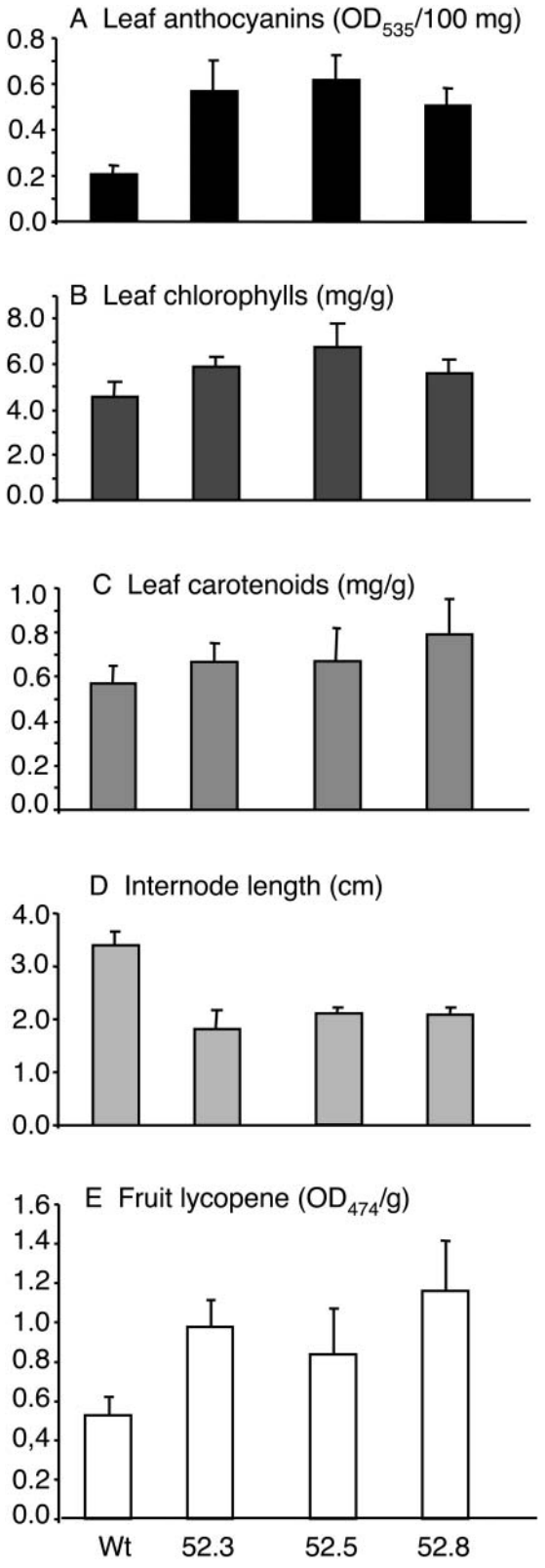

Figure 3. Pigmentation and internode length (average of three transgenic plants grown in the same conditions as in Fig. 2). A, Leaf anthocyanins. B, Leaf chlorophylls. C, Leaf carotenoids. D, Average length of the first five internodes. E, Lycopene content in ripe fruit pericarps from greenhouse-grown plants.

der different light qualities and intensities. *CRY2-OX* seedlings showed, with respect to their wild-type counterparts, reduced hypocotyl length and elevated anthocyanin content when grown under white and blue but not red light (Fig. 4, A and B). Increasing the fluence rate (from 2–16 μmol m^{-2} s^{-1} for blue and from 5–40 μmol m^{-2} s^{-1} for white) magnified both responses in *CRY2-OX* seedlings. The strongest responses, both for hypocotyl shortening and for anthocyanin accumulation, were observed under high-irradiance white light, suggesting that blue light alone is insufficient for complete responses. Under these conditions, ll organs of *CRY2-OX* seedlings, including roots, showed strong anthocyanin pigmentation (Fig. 2D).

Collectively, these results indicate that *CRY2* overexpression in tomato produces a series of alterations in

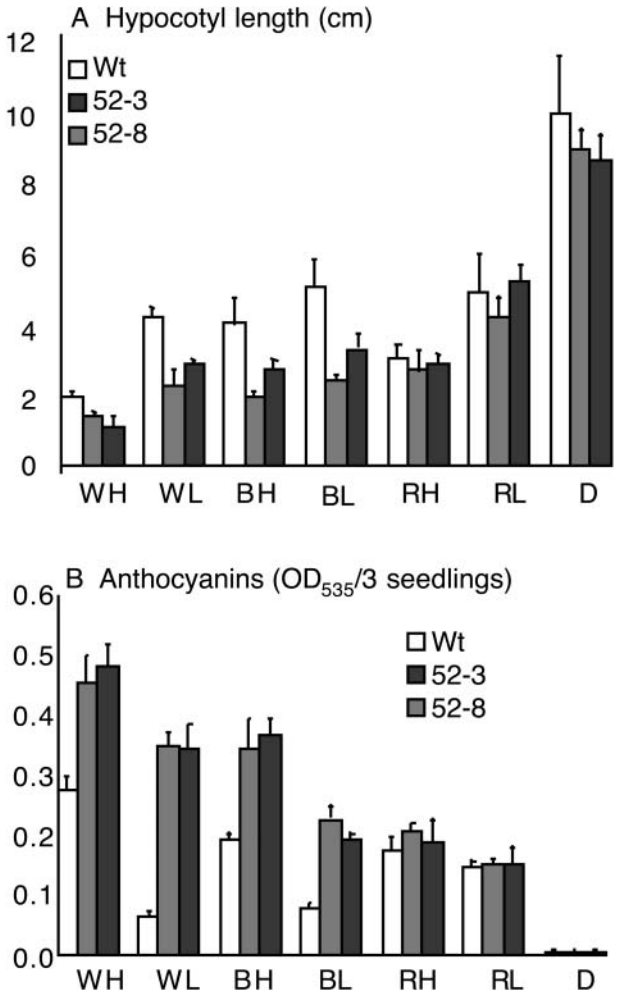

Figure 4. Seedling phenotypes. (Homozygous, 7-d-old transgenic seedlings of lines 52.3 and 52.8 were induced to germination with a 12-h pulse of high-irradiance white light and then grown for 7 d under different continuous light conditions.) A, Hypocotyl length. B, Anthocyanin content. W, White; B, blue; R, red; D, dark; H, high irradiance (40 μmol m^{-2} s^{-1} for W and 15 μmol m^{-2} s^{-1} for B and R); L, low irradiance (5 μmol m^{-2} s^{-1} for W and 2 μmol m^{-2} s^{-1} for B and R). For further details, see ''Materials and Methods.''

vegetative growth, some of which (hypocotyl shortening under high irradiances, anthocyanin accumulation, and dwarfism of adult plants) are more similar to those of Arabidopsis *CRY1*, rather than *CRY2*, overexpressors (Lin et al., 1996, 1998). Yet another phenotype (induction of axillary branch growth) has not been described before in Arabidopsis *CRY1* or *CRY2* overexpressors, whereas tomato *cry1*$^-$ mutants show decreased growth of axillary branches (Weller et al., 2001). These observations suggest that, in tomato, both cry1 and cry2 play stimulatory roles in the growth of axillary branches.

Effects on Flowering Time

In Arabidopsis, cry2 plays a major role in the control of flowering time; *cry2*$^-$ mutants flower later than the wild type under long but not short days, and *CRY2* overexpressors flower earlier than the wild-type under short but not long days (Guo et al., 1998). Although tomato has a different developmental pattern than Arabidopsis, it is possible to measure flowering time as the time elapsed between seed sowing and anthesis of the first flower.

Surprisingly, all three *CRY2-OX* lines show retardation, instead of anticipation, of flowering under both short and long days (Table I). This retardation in time, however, does not correspond to a change in the number of vegetative metamers that are formed before the first inflorescence appears (Table I).

Effects on Fruit Antioxidants

Tomato fruit pigmentation is influenced by light perceived from the environment. Fruit-localized phytochromes positively influence the accumulation of lycopene (Alba et al., 2000). Tomato orthologs of genes that in Arabidopsis are involved in light signal transduction, such as *LeHy5*, *Hp2/LeDet1*, *Hp1*, and *LeCop1-like*, have positive or negative effects on tomato fruit pigmentation, in agreement with their proposed positive or negative signaling roles in Arabidopsis photomorphogenesis (Mustilli et al., 1999; Liu et al., 2004). Regarding the role of specific photoreceptors, phytochrome B2 positively regulates the pigmentation of mature green fruits (Weller et al., 2001), whereas cry1a manipulation through antisense silencing, mutation, or overexpression has only minor effects on fruit pigmentation (Ninu et al., 1999; Weller et al., 2001).

Table I. *Flowering time of wild-type and CRY2-OX plants grown under high-irradiance (40 μmol m^{-2} s^{-1}) white light*

Line	Days to Flowering		Leaves before First Flower	
	Long Days	Short Days	Long Days	Short Days
Wild type	55 ± 6	75 ± 2	8 ± 0.5	8 ± 0.4
52.3	71 ± 2	90 ± 10	8 ± 0.5	8 ± 0.5
52.5	64 ± 9	n.d.[a]	8 ± 0.4	n.d.[a]
52.8	68 ± 5	n.d.[a]	8 ± 0.5	n.d.[a]

[a]Not determined.

Table II. *Carotenoid/flavonoid content in ripe fruit pericarps from greenhouse-grown plants*

	Wild Type	*CRY2-OX* 52.3	Fold Increase
Carotenoids (μg/g dry weight)			
Lycopene	775 ± 110	1,353 ± 84	1.7
β-Carotene	78 ± 8	101 ± 31	1.3
Lutein	23 ± 1	36 ± 4.2	1.5
Total	876 ± 113	1,490 ± 114	1.7
Flavonoids (μg/mg dry weight)			
p-Coumaric acid	0.14 ± 0.05	0.42 ± 0.10	3.1
Chlorogenic acid	0.06 ± 0.02	0.21 ± 0.05	3.5
Rutin	0.80 ± 0.29	3.48 ± 0.95	4.3
Ferulic acid	0.38 ± 0.10	0.38 ± 0.17	1.0
Naringenin chalcone	0.48 ± 0.05	0.83 ± 0.21	1.7
Total	1.85 ± 0.38	5.31 ± 1.14	2.9

Upon visual inspection, fruits of the three *CRY2-OX* lines show increased pigmentation, both at the immature green and at the red ripe stage (Fig. 2, E and F). We spectrophotometrically measured the levels of lycopene, the major red pigment and lipophilic antioxidant in tomato fruits, at the red ripe stage. The data show an approximately 1.5- to 2-fold increase in this pigment (Fig. 3E).

The pigment (carotenoid and flavonoid) content of the pericarp of red ripe fruits of line 52.3 was also analyzed by HPLC (Table II). Again, lycopene shows a significant (1.7-fold) increase, with other carotenoids (β-carotene and lutein) showing more modest and variable (for β-carotene) increases. Water-soluble antioxidants (phenylpropanoids, flavonoids) also show a significant increase (2.9-fold), with rutin, the major flavonoid, showing the highest increase (4.3-fold).

It has been shown previously that accumulation of lycopene in ripening tomato fruits is associated with the increase in transcript levels of lycopene biosynthetic genes, like *phytoene synthase 1* (*Psy1*) and *phytoene desaturase* (*Pds*; Giuliano et al., 1993), and the repression of lycopene-catabolizing genes like *lycopene β-cyclase* (*β-Lcy*; Pecker et al., 1996). The mRNA levels of these genes, as well as of other genes for carotenoid biosynthesis and of cryptochrome genes, were measured at the red ripe stage through RT-QPCR. Transcripts involved in lycopene synthesis (*Psy1*, *Zds*), as well as *CRY1a* and *CRY1b* transcripts, do not show significant variations in *CRY2-OX* fruits with respect to their wild-type counterparts (Table III). Actually, the *Pds* transcript shows a slight repression instead of an induction, consistent with the model that we had previously proposed, i.e. that the activity of the *Pds* promoter is feedback regulated by carotenoid levels (Corona et al., 1996). Instead, transcripts of the two genes involved in lycopene cyclization (*β-Lcy*, *B*) show

Table III. *Real time RT-PCR quantitation of mRNA levels in ripe fruit pericarps*

Gene	Relative Expression Levels (*CRY2-OX 52.3*/Wild Type)[a]
CRY2	5.57 ± 0.96
CRY1a	1.00 ± 0.08
CRY1b	0.97 ± 0.16
Psy1	0.86 ± 0.04
Psy2	0.90 ± 0.12
Pds	0.75 ± 0.09
Zds	0.89 ± 0.08
β-Lcy	0.59 ± 0.06
B	0.54 ± 0.08

[a]Normalized for actin expression.

an approximately 2-fold repression in *CRY2-OX* fruits. We and others have shown previously that fruit-specific silencing of either of the two transcripts (*β-Lcy* or *B*) results in increased lycopene levels (Ronen et al., 2000; Rosati et al., 2000). Thus, the data are consistent with the idea that *CRY2* overexpression increases fruit lycopene content through the repression of lycopene cyclase genes rather than through the induction of earlier genes in the carotenoid pathway.

Virus-Induced Gene Silencing

VIGS has been advocated as a tool for large-scale functional genomics in plants (Baulcombe, 1999). It has been used for the functional characterization of several plant genes, including those involved in responses to pathogens or in primary or secondary metabolism, but not genes for plant photosensory receptors. Potato virus X (PVX) has been used extensively for inducing VIGS in *Nicotiana benthamiana* (Angell and Baulcombe, 1999). We constructed a PVX-based silencing vector (*PVX*::*CRY2*), containing the 3′ nontranslated region of the *CRY2* transcript cloned in the pPVX201 plasmid (Baulcombe et al., 1995). This region was chosen because it shows negligible homology to the *CRY1* transcripts, thus minimizing the possibility of gene silencing. As no reports are available on the use of PVX for inducing VIGS in tomato, a DNA-based infection method was developed (see ''Materials and Methods''). Wild-type and *CRY2-OX* plants grown under long-day conditions were infected with *PVX*::*CRY2* and, as a control, with PVX alone. At 3 weeks postinfection (5 weeks postsowing), the silencing of the *CRY2* transcript in *PVX*::*CRY2*-infected plants was approximately 3-fold in wild-type and 5-fold in *CRY2-OX* plants (Table IV). The higher levels of silencing observed in transgenic plants are in agreement with what was observed previously (Baulcombe, 1999). The levels of the *CRY1a* and *CRY1b* transcripts were unaltered in *PVX*::*CRY2*-infected plants, as were those of all cryptochrome genes after infection with PVX alone (Table IV).

The silencing of the *CRY2* transgene in *CRY2-OX* plants was accompanied by a reversion of several of the *CRY2-OX* phenotypes: three phenotypes that were clearly suppressed by silencing but not by infection with PVX alone were internode shortening (Figs. 2G and 5A), anthocyanin pigmentation of leaf veins (Fig. 2H), and late flowering (Fig. 5B). Infection with PVX alone caused a significant degree of internode shortening both in wild-type and *CRY2-OX* plants (Fig. 5A). Therefore, the minor internode elongation observed in *CRY2*-silenced, wild-type plants (Fig. 5A) is probably the result of two contrasting effects: internode shortening induced by PVX infection and internode elongation induced by silencing of *CRY2*. No clear effects of *CRY2* silencing on flowering time of wild-type plants were observed.

The lateral branching phenotype was also suppressed in silenced plants. However, the interpretation of the data was complicated by the fact that infection with PVX alone has a clear inhibitory effect on the growth of axillary branches (data not shown).

DISCUSSION

In Arabidopsis, cry2 plays multiple roles in regulating photomorphogenesis at low irradiances and flowering time (Lin and Shalitin, 2003; Yanovsky and Kay, 2003). *CRY2* overexpression in tomato results in several photomorphogenic and flowering phenotypes, some of which are expected on the basis of the experience gained with Arabidopsis while others are novel (Fig. 6). Similar to their Arabidopsis counterparts, tomato *CRY2* overexpressors show a light quality-dependent short-hypocotyl phenotype under low irradiances (Lin et al., 1998). However, the enhancement of the short-hypocotyl phenotype at high irradiances, the anthocyanin accumulation, and the dwarfism of adult plants observed in *CRY2-OX* tomato are associated with *CRY1*, rather than *CRY2*, overexpression in Arabidopsis (Lin et al., 1996, 1998). We have previously shown that impairment of tomato cry1a function reduces anthocyanin levels and increases internode and hypocotyl length (Ninu et al., 1999; Weller et al., 2001). The presence of cry1-like phenotypes in cry2-overexpressing lines suggests that cry2

Table IV. *Real-time RT-PCR quantitation of mRNA levels (normalized for actin expression) in uninfected, PVX-infected, and silenced leaves measured 3 weeks after infection*

For further details, see legend to Figure 5.

Gene	Line	Uninfected	PVX Infected	Silenced
CRY2	Wild type	1.0 ± 0.3	1.12 ± 0.07	0.3 ± 0.11
	CRY2-OX	5.4 ± 1.7	4.6 ± 1.0	0.9 ± 0.2
CRY1a	Wild type	1.0 ± 0.02	1.15 ± 0.2	0.99 ± 0.2
	CRY2-OX	0.89 ± 0.17	1.11 ± 0.2	0.83 ± 0.9
CRY1b	Wild type	1.0 ± 0.15	1.21 ± 0.24	0.83 ± 0.14
	CRY2-OX	1.16 ± 0.2	1.19 ± 0.28	0.8 ± 0.12

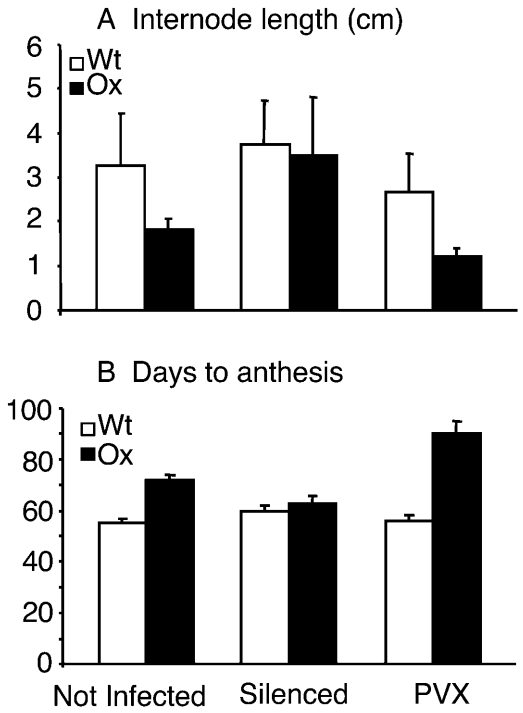

Figure 5. Consequences of VIGS. Plants grown in long day under high-irradiance white light were infected 2 weeks after seed sowing. A, Average length of the first 5 internodes (3 weeks postinfection). B, Flowering time.

plays roles partially redundant with those of cry1 in the control of tomato photomorphogenesis.

The pigment content of *CRY2-OX* leaves and fruits suggests that cry2 controls some aspect of pigment biosynthesis/accumulation. It is possible that this control is at least partially exerted at the transcriptional level. We had previously shown that the Arabidopsis nuclear *Psy* gene, encoding the first dedicated step in the plastid-localized carotenoid biosynthesis pathway, is positively regulated by blue light through an unidentified photoreceptor (von Lintig et al., 1997). *CRY2* overexpression in tomato increases flavonoid and lycopene levels in ripe fruits. This phenotype is reminiscent of the tomato *high pigment* mutants *hp1* and *hp2*, which display shortened hypocotyls and internodes, anthocyanin accumulation, and highly pigmented fruits. *Hp1* and *Hp2* encode the tomato orthologs of negative regulators of light signal transduction in Arabidopsis: Det1 and Ddb1 (Mustilli et al., 1999; Liu et al., 2004). Our data suggest that, consistent with the observed accumulation of lycopene, *Cry2* overexpression represses two genes in the carotenoid biosynthetic pathway (lycopene β-cyclases) in fruits. Previous findings had indicated that transgenic silencing of these genes results in increased fruit lycopene levels (Ronen et al., 2000; Rosati et al., 2000). Microarray profiling of *CRY2-OX* plants is under way to determine which genes, in pigment biosynthesis as well as in different biochemical pathways, are regulated by *CRY2* overexpression.

Several laboratories, including ours, have achieved the targeted increase in tomato fruits of individual antioxidant compounds, such as the carotenoids lycopene, β-carotene, and zeaxanthin and the flavonoids rutin and chlorogenic acid, through metabolic engineering of individual biosynthetic steps (Romer et al., 2000; Rosati et al., 2000; Muir et al., 2001; Dharmapuri et al., 2002; Fraser et al., 2002; Niggeweg et al., 2004) or of regulatory transcription factors (Bovy et al., 2002). Manipulation of light-sensing pathways (Liu et al., 2004; this study) is, to our knowledge, a novel strategy to achieve the simultaneous increase of multiple antioxidant compounds impacting on fruit nutritional value. However, as a word of caution, we wish to add that metabolomic profiling of *CRY2-OX* fruits shows a high degree of metabolic perturbation (L. Giliberto, A. Fernie, N. Schauer, and G. Giuliano, unpublished data) whose nutritional impact has still to be carefully evaluated.

Flowering time is an important trait, influencing both the time it takes for a plant to reproduce in the wild and the time it takes to produce harvestable fruits and seeds for human consumption. In Arabidopsis,

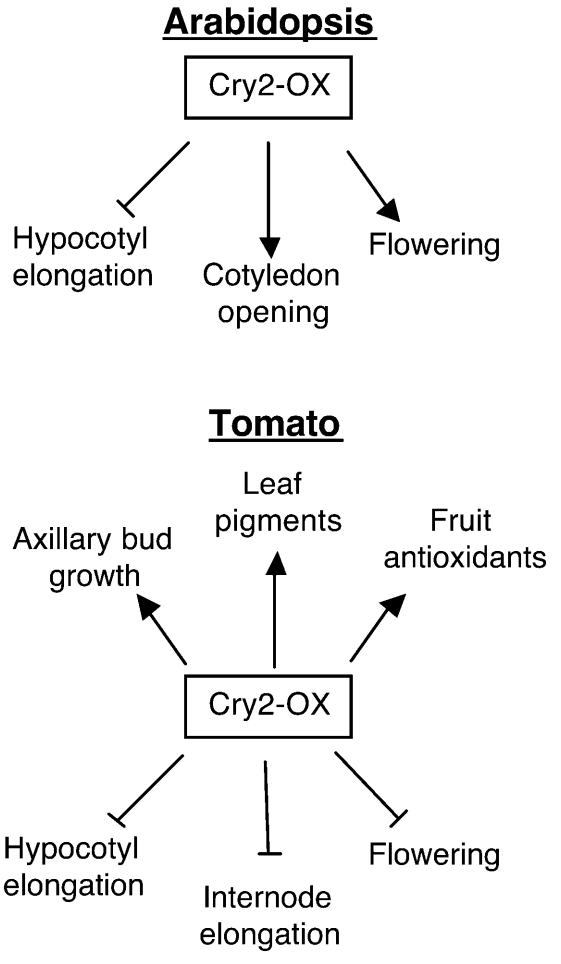

Figure 6. Responses modified by cry2 overexpression in tomato (this study) and Arabidopsis (Lin et al., 1998; Guo et al., 1998).

a long-day plant, the photoreceptors cry2 and phytochrome A act positively and phytochrome B acts negatively to regulate flowering in response to light (Koornneef et al., 1998; Mockler et al., 2003; Valverde et al., 2004). Signals from these photoreceptors and those from the endogenous circadian clock are integrated at the level of CONSTANS (CO), a transcriptional regulator positively influencing the mRNA levels of FLOWERING LOCUS T (FT), which in turn, when reaching certain thresholds, activate the transition from vegetative growth to flowering (Yanovsky and Kay, 2002, 2003; Valverde et al., 2004). In rice (*Oryza sativa*), a short-day plant, phytochromes but not cryptochromes have been implicated in the photoperiodic control of flowering (Izawa et al., 2002; Hayama et al., 2003). The short-day phenotype of rice has been suggested to derive from a different regulatory relationship between CO and FT, which is inductive in Arabidopsis and inhibitory in rice (Hayama et al., 2003). Cultivated tomato behaves differently from both Arabidopsis and rice: It is generally considered a day neutral plant, although it flowers earlier when daylength is increased without proportionally decreasing light intensity (Table I). An interesting question is how, in a day-neutral plant, cry2 overexpression can delay flowering in a photoperiod-independent fashion, without influencing the number of leaves made before the first inflorescence appears. Further experiments measuring the activity of *CO*, *FT*, and meristem identity genes in plants with altered cry2 activity are needed to clarify this point.

Another important agricultural trait is axillary branching. In tomato, the excessive outgrowth of axillary branches negatively affects fruit production so that they have to be manually removed, increasing labor costs. Mutants in some tomato genes, such as *lateral suppressor*, prevent the initiation of axillary meristems (Schumacher et al., 1999). At variance with the role of such genes, *CRY2* overexpression releases the inhibition of axillary meristems—a phenomenon known as apical dominance—rather than altering their number or position. Apical dominance is a complex phenomenon involving the hormones auxin and cytokinin (Chatfield et al., 2000) as well as other, yet unidentified signaling molecules (Booker et al., 2004). We have not found gross alterations of overall hormone levels in *CRY2-OX* plants. However, cytokinin has been shown to act over short distances in paracrine signaling (Faiss et al., 1997). Thus, it is possible that more localized alterations in hormone levels have escaped our attention. An alternative possibility is that cry2 regulates a signal at or downstream of the hormone-sensing step. Both possibilities are presently being investigated.

This is the first time, to our knowledge, that VIGS has been used to functionally study a plant photosensory receptor. In some cases (flowering time, internode length, anthocyanin accumulation), VIGS clearly reverted the *CRY2-OX* phenotypes, whereas PVX infection did not. In other cases (chlorophyll and carotenoid accumulation, lateral branching), PVX infection itself interfered with the phenotypes studied, complicating the interpretation of the results. Two other drawbacks of the PVX system are its inherent instability in tomato, the low levels of silencing obtained in wild-type plants, and the variable silencing observed in fruits. Other silencing vectors have now been developed, which may overcome some of these problems (Liu et al., 2002).

In conclusion, cry2 is a central player in tomato development. Its manipulation through transgenic overexpression and VIGS results in the alteration of a large set of developmental and biochemical responses both in vegetative and reproductive tissues (Fig. 6). In Arabidopsis, cry2 controls photomorphogenic responses through the interaction with the photomorphogenic repressor Cop1, resulting in a modulation of Hy5 abundance (Wang et al., 2001) and flowering time through the modulation of CO mRNA and protein levels (Yanovsky and Kay, 2002; Valverde et al., 2004). Efforts are under way to determine the signal transduction pathway in tomato.

MATERIALS AND METHODS

Standard molecular biology protocols were followed as described (Sambrook et al., 1989). The *CRY2* full-length cDNA of tomato (*Solanum lycopersicum*; Perrotta et al., 2000) was cloned in the *Sma*I and *Sac*I sites of the Agrobacterium-based vector pBI121 (Jefferson et al., 1987), under the control of the cauliflower mosaic virus *35S* promoter. Agrobacterium-mediated transformation was performed according to a published protocol (van Roekel et al., 1993). To establish truly independent transformation events, only one regenerant was taken from every cocultivated tomato explant. For fruit and seed production, plants were grown in the greenhouse under controlled conditions (16 h light, 25°C:8 h dark, 20°C).

All germination and growth experiments were conducted in growth chambers at a constant temperature of 25°C. Light sources were as follows: white was provided by Osram (Munich) 11-860 lamps and Osram Fluora 77 lamps (2:1 ratio). Blue was provided by Osram 67 lamps, additionally filtered through a Lee (Hampshire, UK) Dark Blue plastic filter (ref. 119). Red was provided by Osram 60 lamps, additionally filtered through a Lee Primary Red plastic filter (ref. 106). Irradiances were adjusted by using Lee neutral density filters to 40 and 5 μmol m^{-2} s^{-1} (high and low irradiance) for white, and 15 and 2 μmol m^{-2} s^{-1} (high and low irradiance) for blue and red.

Seeds were harvested from ripe fruits (10–15 d after breaker stage), treated for 20 min in 100 mM HCl, washed extensively in tap water, dried overnight on filter paper, and stored at room temperature in capped tubes over silica gel. For measuring hypocotyl lengths, seeds were imbibed on two layers of filter paper, moistened with tap water, in Magenta boxes (Sigma, Milan). Germination was induced with 16 h of high-fluence white light, ensuring a homogeneous germination rate. The seeds were transferred under the various light conditions for 7 d.

Data (pigment content, HPLC profiles, internode length, days to anthesis, transcript levels) were collected from at least three independent experimental replicates. For hypocotyl length, at least 12 seedlings per experiment were measured in duplicate experiments.

Total RNA was isolated using a published protocol (Lopez-Gomez and Gomez-Lim, 1992), retrotranscribed using oligo$(dT)_{16}$, and then quantified through real-time quantitative PCR using an Applied Biosystems 7000 and the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The oligonucleotides used (designed using the Primer Express program) were the following: *CRY2*, GGGATCGTTTAATGCAAGCTATAATT and CGAGTTATCAAACACAACTTCAACAG; *CRY1a*, CGTGAGCTTGATCGGATTGA and CGGACATATTCCCCATGAGG; *CRY1b*, CCTACCTGATGGTTGTGAGTTCC and CATTTGGATCAAACTTGTATCCCTC; *Psy1*, TGGCCCAAACGCATCATATA and CACCATCGAGCATGTCAAATG; *Psy2*, GTTGATGGCCCTAATGCATCA and TCAAGCATATCAAATGGCCG; *Pds*, GTGCATTTTGATCGCATTGAAC and GCAAAGTCTCTCAGGAGGATTACC; *Zds*, TTGGAG-

CGTTCGAGGCAAT and AGAAATCTGCATCTGGCGTATAGA; *β-Lcy*, TCGTTGGAATCGGTGGTACAG and AGCTAGTGTCCTTGCCACCAT; *B*, TGTTATTGAGGAAGAGAAATGTGTGAT and TCCCACCAATAGCCATAACATTTT; and *Actin*, AGGTATTGTGTTGGACTCTGGTGAT and ACGGAGAATGGCATGTGGAA.

The protocol of Porra (Porra et al., 1989) was used for spectrophotometric determination of leaf chlorophyll and carotenoids. For anthocyanin measurement, leaf tissue (100 mg) or 3 seedlings were extracted overnight in 0.5 mL of methanol containing 50 mM HCl. After addition of 0.375 mL of water and 0.5 mL of chloroform, the mixture was vortexed, centrifuged, and the OD_{535} of the upper phase determined. For spectrophotometric lycopene determination, approximately 0.5 g of pericarp tissue was weighed, brought to 1 g with distilled water, and homogenized with an Ultraturrax homogenizer (IKA, Staufen, Germany) at maximum speed. After addition of 1 mL of chloroform, the mixture was homogenized again, centrifuged, and 20 μL of the lower phase diluted in 1 mL of acetone, and the OD_{474} was determined.

For HPLC determinations, flavonoids, phenylpropanoids, and carotenoids were extracted from freeze-dried ripe tomato fruit (skin and pericarp tissue). A homogenous powder was created by homogenization in a freezer-mill (6750) apparatus (Glen Creston, Middlesex, UK). Carotenoids were extracted from 100 mg of freeze-dried powder as described by Fraser et al. (2000). In brief, chloroform (3 mL) was added to the powder, followed by methanol (1 mL). The suspension was mixed and incubated on ice for 20 min. To the mixture, 50 mM Tris-HCl, pH 7.5 (1 mL), was added and then centrifuged at 3,500 rpm in order to form a partition. The resulting hypophase was removed and the aqueous phase reextracted with chloroform. The chloroform extracts were pooled and reduced to dryness under a stream of nitrogen. These extracts were stored at −20°C under an atmosphere of nitrogen prior to analysis by HPLC. Samples were prepared for HPLC by redissolving the carotenoid residue in ethyl acetate (50 μL) and centrifugation at 12,000 rpm to remove particulate material. Component carotenoids were separated and identified using a Waters (Milford, MA) Alliance 2695 HPLC system with online photodiode array detection (scanning from 200–600 throughout chromatography). The stationary phase used was a reverse-phase C30 5-μm media (YMC, Wilmington, NC). Resolving column dimensions were 250 × 4.6 mm coupled to a 20 × 4.6-mm guard column. Carotenoids were bound to the stationary phase in methanol: water (99:1) containing 0.01% (w/v) ammonium acetate (12 min) and then eluted with and *tert*-butyl methyl ether using a 0% to 65% linear gradient over 30 min. The column was maintained at 25°C throughout chromatography. Carotenoids were identified by comparison with authentic standards on the basis of retention times and characteristic spectral properties. Quantitation was achieved from dose-response curves created at 470 nm for lycopene and 450 nm for both β-carotene and lutein.

Flavonoids (including glycone derivatives) and phenylpropanoids were extracted from freeze-dried powder (50 mg) with methanol (1 mL) containing salicylic acid (20 μg) as the internal standard. The mixtures were incubated at 90°C for 60 min. After cooling on ice, the suspensions were centrifuged at 3,500 rpm for 5 min. The resulting supernatants were removed and passed through a 0.4-μm filter. Separation and identification of flavonoids and phenylpropanoids was performed using a Dionex HPLC system with online photodiode array detection (Dionex, Camberley, Berks, UK). A C18 reverse-phase column (250 × 4.6 mm with 25 × 4.6-mm guard column from Hichrom, Berks, UK) maintained at 25°C was used to separate component flavonoids and phenylpropanoids. The mobile phases consisted of 2% aqueous methanol containing 0.015% HCl (A) and acetonitrile (B). Initial chromatographic conditions consisted of 95% A and 5% B for 10 min, followed by a linear gradient to 50% B over 30 min. Identification was achieved by cochromatography and comparison of spectral characteristics with authentic standards. Quantitation was achieved from dose-response curves created with authentic standards.

For *PVX*::*CRY2* construction, the 3′ nontranslated region of the tomato *CRY2* cDNA was amplified using the oligonucleotides AGATCGATCAGCTGTGAATACTTCACAT and ACGCGTCGACAGGATATCGTCATTCCACT (introducing *Cla*I and *Sal*I restriction sites at the ends of the molecule) and cloned in the *Cla*I and *Sal*I sites of the pPVX201 plasmid (Baulcombe et al., 1995).

For PVX infection, 2-week-old plants grown under long-day conditions were transferred to the dark for 6 to 7 h before inoculation. Two leaves per plant were inoculated, each with 20 μL of a solution containing 10 mg/mL plasmid DNA, 50 mM KH_2PO_4, pH 7.0, and 0.5% (w/v) Celite type 45, 10 to 40 μm (Serva, Heidelberg). After gentle rubbing with a glass spatula and brief (10 min) drying, the leaves were gently washed with tap water and the plants transferred to the dark overnight, before starting the normal light cycle.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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