

Developmental and Wound-, Cold-, Desiccation-, Ultraviolet-B-Stress-Induced Modulations in the Expression of the Petunia Zinc Finger Transcription Factor Gene *ZPT2-2*¹

Alexander R. van der Krol*, Remco M.P. van Poecke³, Oscar F.J. Vorst², Charlotte Voogt, Wessel van Leeuwen, Tanja W.M. Borst-Vrensen, Hiroshi Takatsuji, and Linus H.W. van der Plas

Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4 6703 BD Wageningen, The Netherlands (A.R.v.d.K., R.M.P.v.P., O.F.J.V., C.V., W.v.L., T.W.M.B.-V., L.H.W.v.d.P.); and Laboratory of Developmental Biology, National Institute of Agrobiological Resources, 2–1–2 Kannondai, Tsukuba, Ibaraki 305, Japan (H.T.)

The *ZPT2-2* gene belongs to the *EPF* gene family in petunia (*Petunia hybrida*), which encodes proteins with TFIIIA-type zinc-finger DNA-binding motifs. To elucidate a possible function for *ZPT2-2*, we analyzed its pattern of expression in relation to different developmental and physiological stress signals. The activity of the *ZPT2-2* promoter was analyzed using a firefly luciferase (*LUC*) reporter gene, allowing for continuous measurements of transgene activity in planta. We show that *ZPT2-2::LUC* is active in all plant tissues, but is strongly modulated in cotyledons upon germination, in leaves in response to desiccation, cold treatment, wounding, or ultraviolet-B light, and in petal tissue in response to pollination of the stigma. Analysis of mRNA levels indicated that the modulations in *ZPT2-2::LUC* expression reflect modulations in endogenous *ZPT2-2* gene expression. The change in *ZPT2-2::LUC* activity by cold treatment, wounding, desiccation, and ultraviolet-B light suggest that the phytohormones ethylene and jasmonic acid are involved in regulating the expression of *ZPT2-2*. Although up-regulation of expression of *ZPT2-2* can be blocked by inhibitors of ethylene perception, expression in plants is not induced by exogenously applied ethylene. The application of jasmonic acid does result in an up-regulation of gene activity and, thus, *ZPT2-2* may play a role in the realization of the jasmonic acid hormonal responses in petunia.

The petunia (*Petunia hybrida*) gene *ZPT2-2* belongs to the *EPF* gene-family, which encodes transcription factors containing a canonical TFIIIA-type zinc finger motif (Kubo et al., 1998). More than 200 different cDNAs from different eukaryotic systems have been found to encode this type of zinc finger motif, and many of their products play an important role in development (Rosenberg et al., 1986; Tautz et al., 1987). The *EPF* family contains at least 21

members in petunia, and each member of this family is preferentially expressed in different floral organs (Takatsuji et al., 1994; Kubo et al., 1998). *ZPT2-2* (renamed from *EPF2-5*) was first identified by its homology to *ZPT2-1* (renamed from *EPF1*), which was originally isolated as a petal-specific DNA-binding protein for the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene (Takatsuji et al., 1992). *ZPT2-2* and *ZPT2-1* bind to the same DNA sequence in the promoter of the *EPSPS* gene (Takatsuji et al., 1996).

The *EPSPS* enzyme is part of the shikimate biosynthesis pathway, leading to synthesis of chorismate, which is a precursor for aromatic amino acids, lignin, flavonoids, and alkaloids (Hermann, 1995). The *EPSPS* gene is expressed at low levels in vegetative tissue and at high levels in floral petal tissues (Benfey et al., 1990). It is known that stress induces an increase in carbon flow through the shikimate pathway to provide precursor molecules for stress-related compounds such as lignin, flavonoids, and alkaloids (Hermann, 1995); however, it is not known whether the petunia *EPSPS* gene is transcriptionally activated by stress.

The expression patterns of *ZPT2-1* and *ZPT2-2* are very similar to that of *EPSPS* (Takatsuji et al., 1992, 1994). This, along with the DNA-binding activity in vitro, suggest that *ZPT2-2* and *ZPT2-1* might be involved in the transcriptional regulation of *EPSPS*. However, assigning a function to the *ZPT2-2* has failed so far by lack of mutant plants for the *ZPT2-2* gene. Ectopic expression or suppression of *ZPT2-2* gene activity by co-suppression or antisense genes has yielded no viable plants (H. Takatsuji, unpublished data). Indirectly, this suggests that *ZPT2-2* fulfills a crucial role in plant growth and development. An alternative way to elucidate the function of this gene could be a detailed analysis of its expression pattern. Both petal and stamen tissue contain high levels of *ZPT2-2* mRNA, while *ZPT2-2* mRNA level was undetectable in other plant tissues under normal growth conditions by northern-blot analysis (Takatsuji et al., 1994).

The apparent specific expression of *ZPT2-2* in the second and third floral whorl suggested that this gene may be under control of B-type floral homeotic *MADS* box genes (Coen and Meyerowitz, 1991; van der Krol and Chua, 1993;

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² Present address: CPRO-DLO, Droevendaalsesteeg 1, Postbus 16 6700 AA Wageningen, The Netherlands.

³ Present address: Laboratory of Entomology, Wageningen University, Binnenhaven 7 6709 PD Wageningen, The Netherlands.

* Corresponding author; e-mail Sander.vanderkrol@algem.pf.wau.nl; fax 31-317-484740.

Takatsuji et al., 1994). In petunia, second- and third-whorl organ identity has been shown to be under control of the B-type MADS box genes *pMADS1* (van der Krol et al., 1993) and *fbp1* (Angenent et al., 1992). Indeed, two putative MADS box DNA-binding sites have been identified around position -930 relative to the transcription start site within the *ZPT2-2* promoter (Takatsuji et al., 1994). Thus, one possible function for the *ZPT2-2* gene could be a role in the realization of petal and stamen identity.

A limited expression analysis of transgenic plants expressing a *ZPT2-2::GUS* reporter gene has previously been reported by Takatsuji et al. (1992, 1994). Here we continue the expression analysis of the 2.1-kb *ZPT2-2* promoter activity, using the firefly luciferase (LUC) gene as a reporter. The high sensitivity by which photons can be detected allowed for expression analysis in tissues in which *ZPT2-2* gene expression previously went unnoticed. Furthermore, the non-destructive monitoring of LUC activity allows for (semi)continuous measurements of gene activity at the level of whole plant or plant tissue. We could thus "film" transient modulations in LUC activity during germination and in petals in response to pollination, giving both a spatial and temporal resolution of the *ZPT2-2* promoter activity. The LUC reporter system has thus led to the identification of new sites of *ZPT2-2* gene expression and has revealed the dynamic behavior in the *ZPT2-2* promoter activity under different physiological conditions.

MATERIALS AND METHODS

Construction of *ZPT2-2::LUC* Reporter Plants

The *ZPT2-2::LUC* reporter gene was constructed by fusing a 2.1-kb promoter fragment of the *ZPT2-2* gene (Takatsuji et al., 1994) at the ATG initiation codon to the initiation codon of the LUC coding sequence. For the fusion an *NdeI* site was introduced at the site of the initiation codon in both the *ZPT2-2* and the *LUC*⁺ gene (Promega, Madison, WI). The *ZPT2-2::LUC* chimeric reporter gene (pGM86) was introduced into petunia (*Petunia hybrida*) V26 plants by *Agrobacterium tumefaciens*-mediated transformation. Regeneration on selective medium resulted in the isolation of nine independently transformed petunia lines. Primary transformants were backcrossed to wild-type V26 and seeds were collected for the measurement of LUC activity in progeny plants.

Plant Growth, Seed Germination, and Pollination of Flowers

Plants were grown on soil in phytotrons at 25°C with 16 h of light, and at 20°C with 8 h of dark. For germination experiments, transgenic seeds were placed in a 8.5-cm Petri dish on two layers of filter paper with 3 mL of a 0.1 mM luciferin solution. The Petri dish was sealed and placed in a dark box under a 2D luminometer (Hamamatsu Photonics, Hamamatsu, Japan) for the measurement of LUC activity. For pollination experiments a freshly opened flower from a transgenic plant carrying the *ZPT2-2::LUC* reporter gene was emasculated, cut at the base of the pedicel, and

placed in a flask containing a 0.1 mM luciferin solution either with or without 100 μ M silver thiosulfate (STS). Pollination was performed 12 h later by applying fresh pollen of petunia line W115 on the stigma of the flower.

Measurements of in Planta LUC Activity with the 2D Luminometer

The LUC reporter plants were repeatedly sprayed with 1 mM luciferin (Promega) starting 1 d before imaging with the two-dimensional luminometer. Luciferin readily penetrates cells of most petunia tissues. It is a stable compound in plants and may persist for weeks. Repeated spraying with luciferin results in an equilibrium in luciferin influx and luciferin consumption by the LUC activity. Changes in photon production can therefore either be ascribed to a change in LUC reporter gene activity or to changes in the physiology of the cells that alter substrate (oxygen, luciferin, or ATP) influx. Changes in the physiology of the cell are not promoter specific and can be verified by comparison with other LUC reporter gene activity under similar conditions. Photon production by LUC was imaged in planta with an intensified CCD camera (Hamamatsu) in slice mode (gain 9.8). For expression studies in germinating seeds and pollinated flowers, each image was obtained by 30-min photon accumulation. Images were thus collected every 30 min over a period of 3 d, and analyzed with Argus 50 software (Hamamatsu). LUC activity in images is represented in false colors using the color scale shown in Figure 1. The range of relative LUC activities that the color scale spans is given in the legend of each figure.

Wounding, Cold, and UV-B Light Treatment of Leaves

The LUC activity measurements in leaves of *ZPT2-2::LUC* plants in response to wounding were performed by making 3-mm discs (or by cutting leaves) of presprayed plants and placing them on two layers of filter paper with 0.5 mM luciferin. Photon production was registered in the dark with the 2D luminometer in 10- or 15-min intervals over a period of 1 to 2 d. For the cold-induction experiments, LUC activity in whole plants (presprayed with luciferin for 5 d) was imaged in a 20-min time interval, after which one set of plants was kept at 4°C and the other set at 24°C for 12 h in the dark. After 12 h, LUC activity was again imaged during a 20-min time interval. Note that no new luciferin was applied during the temperature treatments.

For the UV-B light treatment, isolated leaves from a *ZPT2-2::LUC* plant (presprayed with luciferin) were irradiated for 6 h with 15 μ mol m⁻² s⁻¹ UV-B light (TL12 tubes, Philips, Eindhoven, The Netherlands) wrapped in a single layer of UV-C blocking cellulose acetate. The given flux density represents the emission in the spectral range between 295 and 345 nm. Control leaves were shielded from UV light by a glass sheet. Following the light treatment, LUC activity in the leaves was imaged during a 20-min interval.

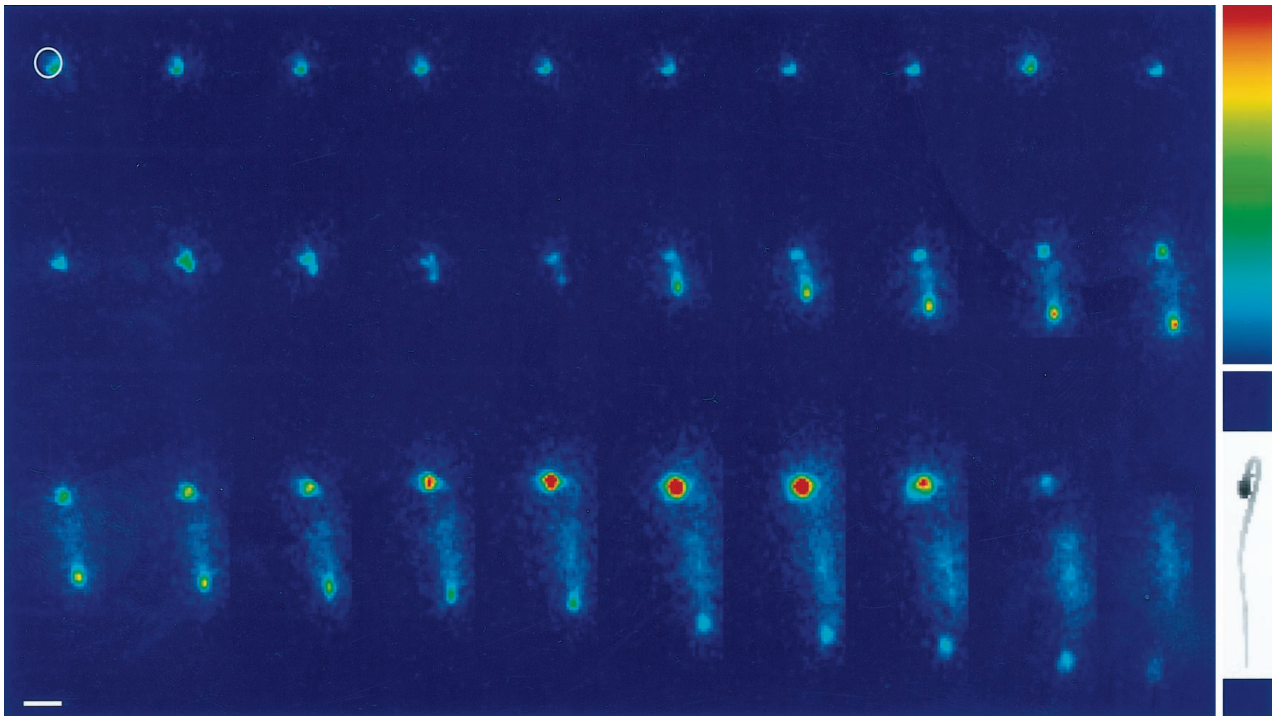


Figure 1. *ZPT2-2::LUC* activity in a germinating petunia seed. A seed from a *ZPT2-2::LUC* transformant was imbibed in 0.5 mM luciferin. Individual images of in vivo LUC activity were obtained by integration of photon production during 30 min. Consecutive images were obtained over a 3-d period. Selected images are shown (time interval between images is 2 h). The white circle in the first image indicates the size of the petunia seed. Note the elevated LUC activity in the root tip and the transient increase in LUC activity in the cotyledons of the seedling. Inset top right, Color scale used for the representation of LUC activity. The color scale ranges from 0 (blue) to 256 (red) relative units of LUC activity. Inset bottom right, Light image of the seedling. Space bar = 1 mm.

Desiccation and Jasmonic Acid Treatment of *ZPT2-2::LUC* Plants

For desiccation experiments, two branches from a *ZPT2-2::LUC* plant (presprayed with luciferin for 5 d) were placed in a vase with or without water. After 24 h the LUC activity in both branches was monitored during a 30-min interval. For the jasmonic acid (JA) treatment two branches from a *ZPT2-2::LUC* plant were placed in a vase with water or water plus JA (50 μM). After 15 h, the LUC activity in the branches was imaged during a 30-min interval.

Analysis of *ZPT2-2* mRNA Levels in Different Tissues

Isolation of plant RNA was as described previously (van der Krol et al., 1993). Total RNA (20 μg) was size-fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with a ^{32}P -labeled *ZPT2-2* cDNA probe as described previously (van der Krol et al., 1993). Alternatively, mRNA was converted to cDNA and a semi-quantitative amplification by PCR was performed using *ZPT2-2* specific primers and 1 μg of cDNA as template. As a control the same amount of cDNA was used in a PCR reaction using *gapdh* or *ubiquitin* specific primers (Souer et al., 1996). Samples were analyzed after 25, 30, or 35 cycles after fractionation on agarose gel or by probing Southern blots with a ^{32}P -labeled *ZPT2-2* cDNA probe.

RESULTS

ZPT2-2::LUC Activity during Germination

A *ZPT2-2::LUC* reporter gene was constructed by ligating a 2.1-kb promoter fragment from the *ZPT2-2* gene to the *ff-LUC* coding sequence. The chimeric gene was cloned into a binary vector, and leaf discs from petunia line V26 were used for *A. tumefaciens*-mediated transformation. Nine independent transformed lines were obtained, and for each line seeds were obtained by backcrossing each line to wild-type V26 plants. Seeds were collected, and expression analysis of the *ZPT2-2::LUC* gene activity was performed on transgenic progeny plants. For expression analysis during germination the seeds were imbibed in the dark on wet filter paper with 0.5 mM luciferin. Each image of the LUC activity was obtained by integration of the photons emitted during a 30-min interval (gain 9.8), and sequential imaging was over a 3-d period. Selected images of this analysis are shown in the top of Figure 1.

LUC activity in the seeds could be detected as early as 3 h after imbibition, and seemed to be localized at the site of the root apex. Upon germination (approximately 29 h after imbibition), the LUC activity briefly declined but was subsequently re-established in the root tip, in which it stayed active but declined at the end of a 60-h period (Fig. 1, top). The LUC activity in cotyledons (still partly covered by the seed coat) transiently increased after germination,

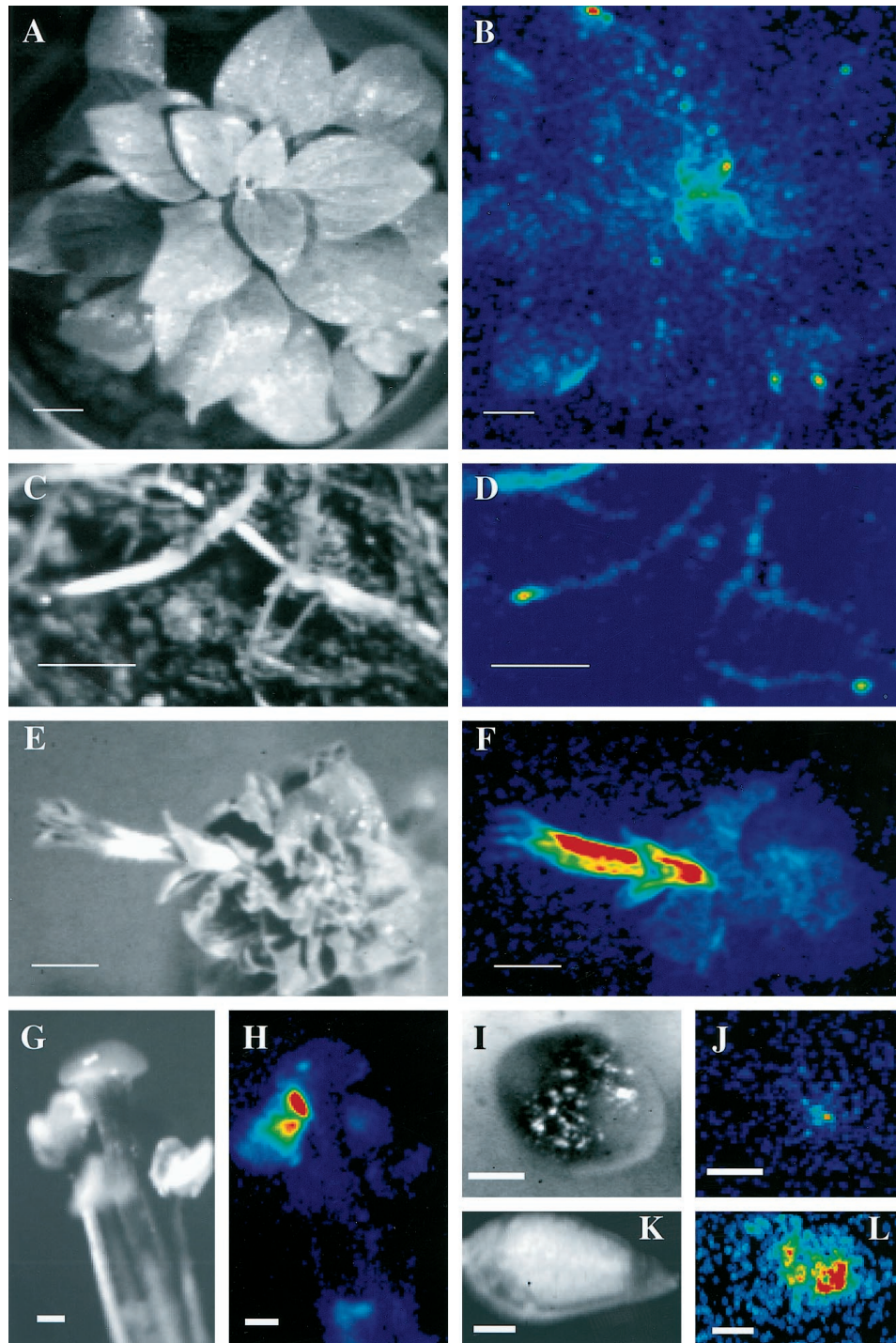


Figure 2. In vivo *ZPT2-2::LUC* activity in vegetative and flowering plants. A plant expressing the *ZPT2-2::LUC* gene was repeatedly sprayed with luciferin, after which the in planta LUC activity was imaged during a 20-min interval. Light images, A, C, E, G, I, and K. *ZPT2-2::LUC* activity images, B, D, F, H, J, and L. The LUC activity is represented in a false color scale ranging from 0 to 256 (see legend to Fig. 1). A and B, Vegetative shoot (plant 4 weeks old); C and D, roots (plant 4 weeks old); E and F, inflorescence (plant 6 weeks old); G and H, stamen and style; I and J, transgenic pollen on non-transgenic stigma; K and L, developing seeds 5 d post-pollination. Thick space bars = 1 mm; thin space bars = 1 cm. Note that the resolution of the images is limited by the resolution of the intensifier (350 × 350 dpi).

reaching a peak in expression 40 h after germination. The elevated LUC activity in the root tip was seen in seedlings of all nine transgenic lines carrying the *ZPT2-2::LUC* gene.

The duration of the transient increase of expression in cotyledons varied among individual plants, varying from hours to days. This variation did not seem to depend on the

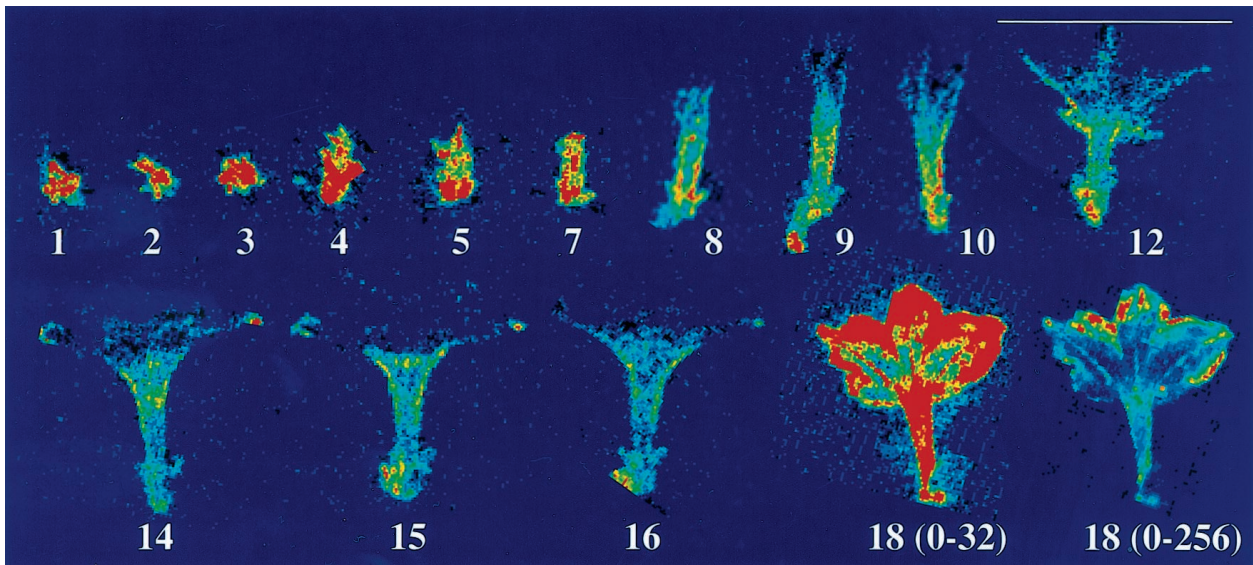


Figure 3. *ZPT2-2::LUC* activity in the same flower during 18 d of development on the plant. A *ZPT2-2::LUC* plant was sprayed daily with luciferin and LUC activity was imaged in the same floral bud during a 30-min interval over an 18-d period. Numbers correspond to the day at which the image was taken. The LUC activity is represented in false colors (scale 0–16; see legend to Fig. 1). The last image (d 18) is shown twice, once in scale 0 to 16 and once in scale 0 to 256.

site of transgene insertion, since the variation also occurred among genetically identical seeds (transgenic seeds from a backcross of a transgenic line carrying a single insert).

***ZPT2-2::LUC* Is Active in All Parts of Mature Transgenic Plants**

One of the great advantages of the LUC reporter system is that the imaging of LUC activity with the 2D luminometer can give a global view of the reporter gene activity in whole, mature plants instead of in small, isolated plant tissues (Fig. 2). Image analysis of photon production in the *ZPT2-2::LUC* plants showed that the activity was detected in all parts of the plants during vegetative growth. The images of in planta LUC activity show a higher photon production/density in young leaves compared with mature, expanded leaves (Fig. 2, A and B). In both young and mature leaves, the expression of *ZPT2-2::LUC* was not evenly distributed, but showed localized patches of up to 15-fold higher photon production compared with the average activity in leaves. In mature, soil-grown roots, the *ZPT2-2::LUC* gene is active throughout the root at a low level, with elevated levels in the root tip (Fig. 2, C and D).

In flowering plants LUC activity was detected throughout the inflorescence (stem, bracts, floral bud; Fig. 2, E and F), and was clearly elevated in petal tissue. Detection of the LUC activity in intact mature stamen was not possible due to the lack of penetration of the luciferin substrate into locules (data not shown). When luciferin was applied on locules after anthesis, high LUC activity could be observed in the pollen (Fig. 2, G and H). Figure 2, I and J, shows the activity of *ZPT2-2::LUC* in pollen applied to a non-transgenic petunia stigma that was pre-treated with luciferin.

At 5 d after pollination of a non-transgenic petunia plant with *ZPT2-2::LUC* pollen the LUC activity could be imaged in developing seeds. For imaging of LUC activity the carpel leaves were removed (Fig. 2, K and L). Analysis of in vivo *ZPT2-2::LUC* gene expression during later stages of seed development was not possible due to the lack of penetration of the luciferin substrate into the seed (data not shown).

***ZPT2-2::LUC* Activity during 20 d of Flower Development**

The change in *ZPT2-2::LUC* activity during floral development was monitored on the plant by imaging LUC activity in the same floral bud every day over an 18-d period (Fig. 3). The images show how the *ZPT2-2::LUC* promoter activity increases in the tube and limb tissues until opening of the petals. Prior to petal senescence at d 18 (non-pollinated flower), the activity of *ZPT2-2::LUC* shows a dramatic increase in the petals. As a control we also imaged the activity of a cauliflower mosaic virus 35S::*LUC* and a lipid transferase (*LTP*)::*LUC* reporter gene in transgenic petunia flowers. For both these reporter genes the LUC activity in petals declined steadily after opening of the petals, and the decline in LUC activity was accelerated at the onset of wilting (data not shown). It is therefore concluded that the increase in photon production in *ZPT2-2::LUC* petals prior to wilting is specific to the response of the *ZPT2-2* promoter.

Pollination Induces Up-Regulation of *ZPT2-2::LUC* Activity in Petals

It has been shown previously that in petunia the developmental control of petal wilting is accelerated by pollina-

tion of the stigma (Gilissen and Hoekstra, 1984; Whitehead et al., 1984; Hoekstra and Weges, 1986). Without pollination, wilting of petals on isolated flowers commences approximately 8 d after anthesis. However, when the petunia stigma is pollinated at anthesis, the petals wilt within 2 to 3 d. We imaged the change in *ZPT2-2::LUC* gene activity in petals in response to pollination in 30-min intervals over a 3-d period. Figure 4A shows selected images of LUC activity in petals of a flower until 72 h post-pollination (time interval between images is 3.5 h). The figure shows that the *ZPT2-2* promoter activity increases in response to pollination, reaching a peak in activity at 26 h post pollination. Quantification of the photon production in the petal tube shows that the up-regulation of *ZPT2-2::LUC* activity in petals can already be detected at 2 h post pollination (not shown).

It has been shown before that the action of the phytohormone ethylene plays a role in the process of petunia petal wilting (Whitehead et al., 1984; Woltering et al., 1997). We tested whether ethylene signaling plays a role in the modulation of expression of the *ZPT2-2* gene by testing the effect of a blocker of ethylene perception (STS) and the expression of *ZPT2-2::LUC* in petals. Figure 4B shows selected images of LUC activity in petals of a flower pre-treated with STS at different times after pollination (3.5-h interval between images). This figure shows that pollination did not induce any change in petal LUC activity;

however, the basal level of expression of this reporter gene in the petals was not affected by the STS treatment. Similar results were obtained using the inhibitor MCP (data not shown).

Wounding-Induced Regulation of *ZPT2-2::LUC* Activity in Leaves

During our analysis of *ZPT2-2::LUC* plants, we noticed an elevated LUC activity at sites of injury, indicating that *ZPT2-2* may be up-regulated by wounding. To test this, we quantified the *ZPT2-2::LUC* activity in leaf discs over a 17-h period. Figure 5A shows that, upon wounding, the LUC activity in the discs shows a dramatic increase, reaching a peak at 2 to 3 h after the wounding. At 17 h post wounding, the LUC activity was still elevated compared with the initial LUC activity in the discs. The maximum level and response dynamics in discs taken from the same leaf were always comparable, but the level of induction in older leaves was lower than in young leaves (Fig. 5A, compare response of the discs from leaf 3 and 9).

Figure 5, B and C, shows an image of the induction of LUC activity in a sliced leaf. Besides the increase in LUC activity near the wound surface, an increased activity in the main veins, away from the wound surface, can also be seen. This indicates a transport of an inducing signal through the leaf. Figure 5, D and E, shows a closeup of the

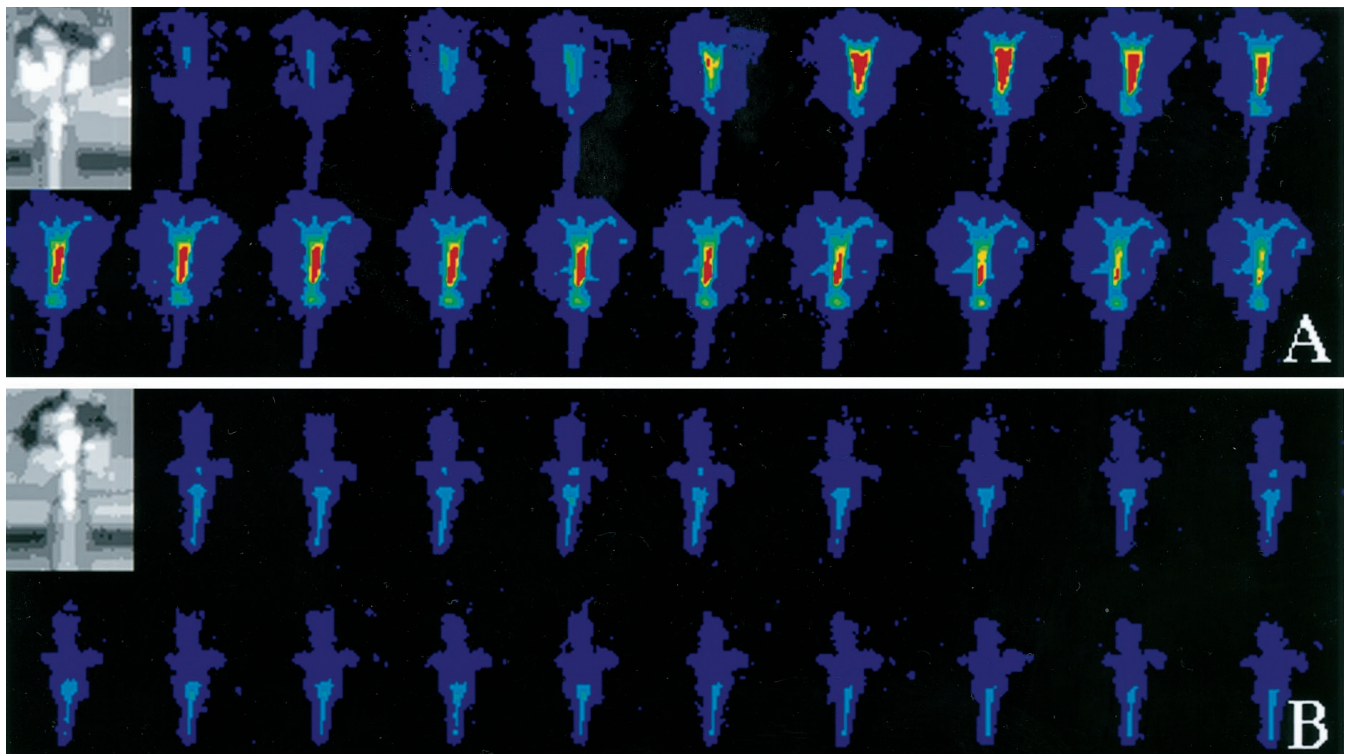


Figure 4. *ZPT2-2::LUC* activity in petals in response to pollination. A flower from a *ZPT2-2::LUC* plant was harvested prior to anthesis and put in a small vase with 0.1 mM luciferin (A) or 0.1 mM luciferin plus 0.1 mM STS (B). After 12 h of equilibration, the flowers were pollinated and in vivo LUC activity was imaged in 30-min intervals during a 3-d period. The time interval between the images was 3.5 h. The LUC activity is represented in false colors (color scale range from 0–256; see legend to Fig. 1). Note that the transient increase in LUC activity in the tube and corolla tissue (A) is blocked by the STS treatment (B). The insets show the light images of the flowers in the vase.

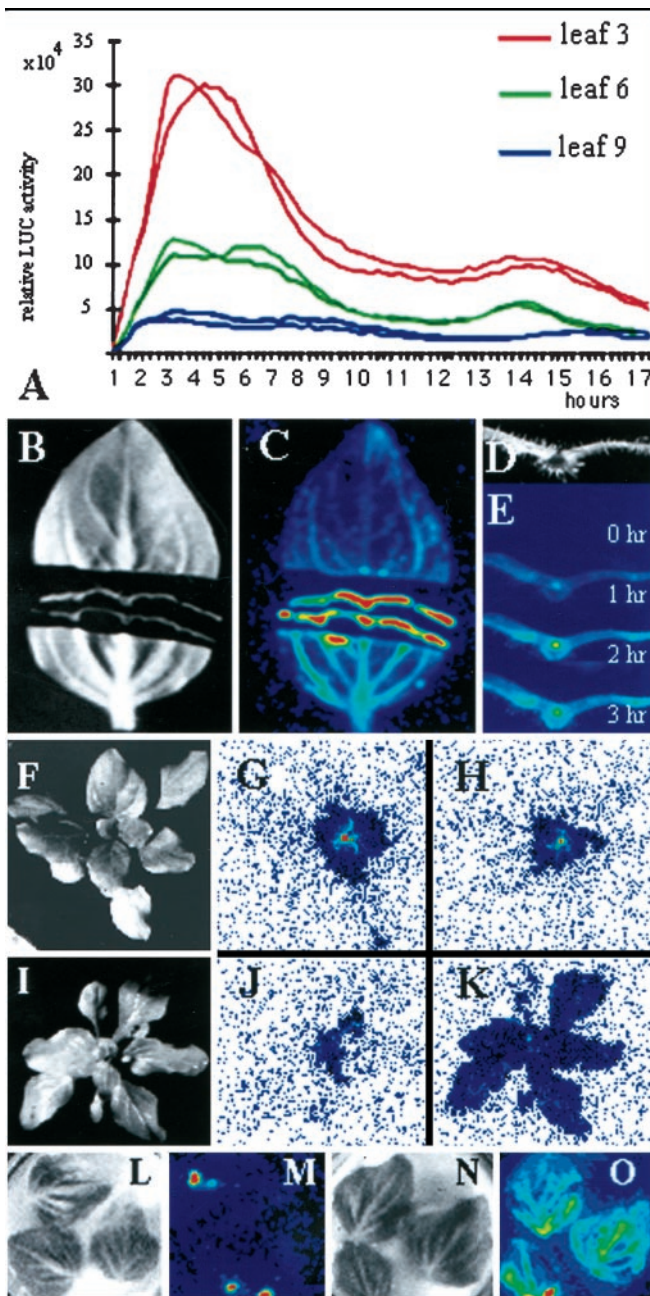


Figure 5. *ZPT2-2::LUC* activity in response to different stress signals. A, *ZPT2-2::LUC* activity during a 24-h period in (2×) two small leaf discs taken from leaf 3, 6, or 9 from the top of the plant. B, Light image of sectioned leaf; C, *ZPT2-2::LUC* activity image acquired in 20-min interval 1 h after sectioning of the leaf. D, Light image of leaf section; E, *ZPT2-2::LUC* activity image acquired in 20-min interval in leaf section at 0, 1, 2, and 3 h after sectioning. F, Light image of plant treated at 24°C; G, *ZPT2-2::LUC* activity image acquired in 20-min interval in plant before treatment at 24°C. Note that here the background color has been changed from black to white. H, *ZPT2-2::LUC* activity image acquired in 20-min interval in plant after a 12-h treatment at 24°C. Note that here the background color has been changed from black to white. I, Light image of plant treated at 4°C. J, *ZPT2-2::LUC* activity image acquired in 20-min interval in plant before treatment at 4°C. Note that here the background color has been changed from black to white. K, *ZPT2-2::LUC* activity image acquired in 20-min interval in plant after a 12-h treatment at

leaf slice and the change in the LUC activity over a time period of 3 h. The *ZPT2-2::LUC* gene is activated in all cells of the leaf (epidermis, mesophyll cells, cortex, and in vascular tissue).

Cold Stress Induces Up-Regulation of *ZPT2-2::LUC* Activity in Leaves

We also tested whether the *ZPT2-2* gene expression could be induced by cold stress. Two transgenic plants expressing *ZPT2-2::LUC* were pre-sprayed with luciferin and LUC activity was imaged at room temperature over a 20-min period (Fig. 5, F and I, light image; G and J, LUC activity image). The plants were then placed in the dark at either 24°C or 4°C. After 12 h, the activity of the LUC reporter was imaged during a 20-min period at room temperature (Fig. 5, H and K). After 12 h at 24°C, the *ZPT2-2::LUC* shows a slight decrease in activity (Fig. 5H). In contrast, in the plant treated for 12 h at 4°C, the LUC activity had increased throughout the plant (Fig. 5K). This increased activity rapidly declined within 2 h at room temperature (not shown). Note that no new luciferin was applied during or after the 12-h temperature treatments.

UV-B Light Induces Up-Regulation of *ZPT2-2::LUC* Activity in Leaves

It has been well documented that UV-B light can impose stress on plant tissue (for review, see Jansen et al., 1998). We therefore tested the effect of UV-B irradiation on *ZPT2-2::LUC* expression in leaves. Isolated leaves were illuminated with white light either supplemented or not supplemented with UV-B light for 6 h. The expression of *ZPT2-2::LUC* was subsequently imaged during a 15-min period. The leaves illuminated with only white light show a low level of LUC activity throughout the leaf and a high level of LUC activity localized at the wound surface (Fig. 5, L and M). The leaves illuminated with white light supplemented with UV-B light showed elevated LUC activities throughout the leaf, mainly associated with the veins (Fig. 5, N and O). Quantification of the average LUC activity in the leaves shows that in UV-B-illuminated leaves LUC activity is about 5-fold higher than those in the control leaves.

Desiccation Induces Up-Regulation of *ZPT2-2::LUC* Activity in Shoots

Since the pollination-induced up-regulation of *ZPT2-2::LUC* activity in petals precedes the wilting of the petals, we tested whether this response could also be observed upon desiccation of leaf tissues. Two branches of a

4°C. Note that here the background color has been changed from black to white. L, Light image of leaves illuminated for 6 h with white light. M, *ZPT2-2::LUC* activity image acquired in 20-min interval in leaves illuminated for 6 h with white light. N, Light image of leaves illuminated for 6 h with white plus UV-B light. O, *ZPT2-2::LUC* activity image acquired during a 20-min interval in leaves illuminated for 6 h with white plus UV-B light.

ZPT2-2::LUC plant (sprayed with luciferin) were placed in a vase either with or without water. The LUC activity was imaged in 30-min intervals over a 35-h period. Photon production in the shoots was quantified and expressed as a percentage of the initial value (Table I). In the control shoot (on water) the LUC activity remained more or less constant over the 35-h period (indicating that the pre-spray with luciferin was sufficient to maintain LUC activity for prolonged time). In the desiccating shoot the activity increased to up to 7-fold of the initial LUC activity. After 3 d these isolated leaves still look healthy and had retained full turgor. The leaves on this shoot had lost their turgidity and look completely wilted, but apparently the cells in these leaves had enough activity to show the increase in *ZPT2-2* promoter activity. Shoots of a plant carrying a cauliflower mosaic virus *35S::LUC* reporter gene only showed a decrease in LUC activity upon desiccation (data not shown), indicating that the up-regulation of LUC activity is specific for the *ZPT2-2* promoter.

Modulations of *ZPT2-2::LUC* Activity Are Comparable to Modulations in Endogenous *ZPT2-2* Gene Expression

We tested whether the sites of *ZPT2-2::LUC* expression are a reflection of endogenous *ZPT2-2* gene expression. Similarly, we tested whether the modulations in *ZPT2-2::LUC* expression are a reflection of changes in endogenous *ZPT2-2* gene activity. To determine the expression of the endogenous *ZPT2-2* gene in different tissues, we isolated RNA from different plant organs at different stages of development or under different abiotic stresses. *ZPT2-2* mRNA levels were semi-quantified by reverse transcriptase (RT)-PCR using *ZPT2-2* specific primers. RNA was isolated from seedling, leaf (wounded or desiccated), petal (before and after pollination), and stamen tissues. The mRNA converted to cDNA, which was used in the RT-PCR reactions.

The amount of *ZPT2-2* specific PCR product was semi-quantified in relation to the amount of the *gadh*-specific PCR product from the same cDNA sample. From the PCR amplification experiment it can be concluded that *ZPT2-2*

Table I. Relative LUC activity in desiccating and non-desiccating shoots taken from the same plant

Shoots from a *ZPT2-2::LUC* plant that was pre-sprayed with luciferin were placed in a vase with water (water) or without water (dry). LUC activity was quantified at $t = 0$ (set at 100%) and every subsequent 5 h for a period of 35 h. Note that no extra luciferin was added during the treatment.

Time <i>h</i>	Relative LUC Activity	
	Water	Dry
	%	
0	100	100
5	117	163
10	114	260
15	112	415
20	109	519
25	117	602
30	115	671
35	109	763

Table II. Relative steady-state level of endogenous *ZPT2-2* mRNA in different tissues

The hybridization signal to *ZPT2-2* specific RT-PCR products was quantified and related to the hybridization signal to *gadh* specific RT-PCR product, amplified from the same cDNA pool. The relative signal of *ZPT2-2* PCR product in unstressed leaves was arbitrarily set at 100%. p.fl., Pollinated flower; np.fl., non-pollinated flower.

Sample	<i>ZPT2-2</i> mRNA
	%
Control leaf	100
Desiccated leaf	709
Wounded leaf	2.758
Petal (np.fl.)	6.260
Petal (p.fl.)	42.431
Anther young	2.912
Anther mature	3.460
Seedlings	707

transcripts are indeed present in all tissues sampled. The *ZPT2-2* transcript level in non-stressed leaves was arbitrarily set at 100% (Table II). Quantification of the relative *ZPT2-2* mRNA levels in leaves after wounding (2 h) or desiccation (24 h) showed a 7- and 27-fold increase in *ZPT2-2* mRNA level. This increase is similar to what has been observed for the *ZPT2-2::LUC* reporter gene expression after wound or desiccation stress. Table II also shows that the induction of *ZPT2-2::LUC* in petals was up-regulated in response to pollination. These analyses indicate that the modulation in expression of the *ZPT2-2::LUC* reporter gene is a reflection of the changes in endogenous *ZPT2-2* gene expression.

Hormonal Regulation of *ZPT2-2* Gene Expression

We tested whether *ZPT2-2::LUC* expression could be induced by application of endogenous ethylene or JA. When two shoots from the same transgenic plant were incubated with or without ethylene (10 nL L^{-1}) for 12 h, no difference in LUC activity was observed (data not shown), indicating that ethylene alone is not sufficient for the modulation in *ZPT2-2* gene expression. Figure 6 shows the LUC reporter gene activity in two shoots taken from the same reporter plant, with one shoot on water and one shoot on $50 \mu\text{M}$ JA (after 15 h). The figure shows that, in contrast to

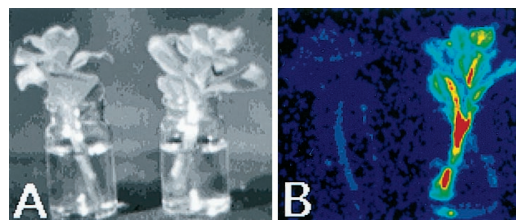


Figure 6. Induction of *ZPT2-2::LUC* activity in shoots by JA. Two shoots taken from the same *ZPT2-2::LUC* plant were placed in a vase with water (left) or water plus $50 \mu\text{M}$ JA (right). A, Light image; B, LUC activity image after 15 h, in a false color scale ranging from 0 to 256 (see legend to Fig. 1).

ethylene, JA is sufficient for the up-regulation of *ZPT2-2::LUC* gene expression.

DISCUSSION

LUC Reporter Reveals Low Levels and Dynamic Behavior of *ZPT2-2* Promoter Activity

The lack of *ZPT2-2* mutant plants makes it difficult to assign a specific function to this gene. However, an extensive analysis of the expression pattern of *ZPT2-2* during plant development and a comparison with the expression of other known genes may give clues about the developmental processes or physiological responses in which *ZPT2-2* plays a role. Previous expression analysis identified the *ZPT2-2* gene as floral specific. Expression on northern blots was detected only in the second and third floral whorls (A.R. van der Krol, unpublished data; Takatsuji et al., 1994), indicating that *ZPT2-2* may be under control of the B-type floral homeotic MADS box genes *fbp1* (Angenent et al., 1992) and *pMADS1* (van der Krol et al., 1993).

We used the LUC reporter system to analyze the activity from the *ZPT2-2* promoter in transgenic plants. The sensitivity by which photons can be detected allowed for the identification of sites with extremely low promoter activity that previously went unnoticed (e.g. the expression in leaves, root, pollen, and developing seeds). The non-destructive method of measuring gene activity through the LUC reporter enabled us to monitor gene expression in the same tissue over prolonged periods of time. We could thus follow the expression of *ZPT2-2::LUC* on the plant in the same flower over a period of 20 d (Fig. 3). In this manner we identified a strong up-regulation of *ZPT2-2* gene activity at the onset of petal wilting. Our in planta studies also revealed transient modulation in *ZPT2-2* activity during germination in cotyledons (Fig. 1) and petals in response to (pollination-induced) wilting (Figs. 3 and 4). At this time it is not known whether the transient nature of these modulations in *ZPT2-2* activity are due to a transient occurrence of a signal, a desensitization to a continuous signal, or a combination of these two.

The ease by which LUC activity can be measured in plants using the two-dimensional luminometer makes it easy to test the effect of different stress conditions on the expression of *ZPT2-2::LUC*. We thus were able to identify up-regulation of *ZPT2-2::LUC* expression in leaves upon wounding, UV-B light stress, cold stress (see Fig. 5), or desiccation stress (Table I). Both the level and dynamics of the wound response were reproducible between discs taken from the same leaf (Fig. 5A). However, between leaves both the strength and dynamics of the response varied, with the youngest leaf showing the highest and fastest response (compare response discs from leaf 3, 6, and 9, Fig. 5A). Imaging of the wound response in leaf sections shows that the increase in *ZPT2-2::LUC* activity is observed in all cell layers of the leaf upon wounding (Fig. 5, D and E). The images that were obtained during the (semi) continuous measurements of LUC activity were compiled in stacks, which can be viewed as videos at <http://www.spg.wau.nl/pf/movies.html>.

Modulations in *ZPT2-2::LUC* Reporter Gene Activity Coincide with Modulations in Endogenous *ZPT2-2* Gene Activity

The low *ZPT2-2::LUC* activity in transgenic plants suggested a low level of *ZPT2-2* gene activity in vegetative tissues that previously went unnoticed. Expression of the endogenous *ZPT2-2* gene in these tissues was confirmed using RT-PCR and RNA samples from leaf (Table II) and root (not shown). The steady-state levels of *ZPT2-2* mRNA in the vegetative plant tissue were on average less than 3% of that observed in petals or stamen. We also demonstrated that the increase of *ZPT2-2::LUC* activity in leaves by desiccation and wound stress are a true reflection of modulations in endogenous *ZPT2-2* gene activity. The (semi) quantification of *ZPT2-2* mRNA steady-state levels in desiccated and wounded leaves showed a 7- and 27-fold increase, respectively, compared with the *ZPT2-2* mRNA levels in control leaves (Table II). These induction levels coincide well with those observed for in vivo *ZPT2-2::LUC* reporter gene activity upon desiccation and wounding (9- and 30-fold, respectively, see Fig. 5A; Table II).

Complex Hormonal Control of *ZPT2-2* Gene Expression

In flowering petunia plants, the petals wilt about 8 d after anthesis. This process of petal wilting can be accelerated by pollination of the style (Hoekstra and Weges, 1986; Hoekstra and van Roekel 1988; Tang et al., 1994; Tang and Woodson, 1996). It has been shown that removal of the style at 4 h after pollination can no longer inhibit this pollination-induced wilting of petals, indicating that 4 h after pollination a signal has reached the petals from the stigma. Once perceived by the petal, this signal is no longer required to complete the petal wilting process (Gilissen and Hoekstra, 1984). With our (semi) continuous measurement of *ZPT2-2::LUC* activity in the tube, we observed that at 2 h after pollination of the stigma, the LUC activity had already increased in the petal tube. This indicates that a pollination-induced signal is perceived by the petals within 2 h after pollination of the stigma. No modulation in petal LUC activity was seen in control, unpollinated flowers over a period of 3 d (data not shown).

Our analysis of the expression of *ZPT2-2::LUC* indicates that in petals there are two modes of *ZPT2-2* gene expression: a low basal level and an inducible expression that may be dependent on endogenous phytohormone levels. Inhibition of ethylene perception blocks the up-regulation of *ZPT2-2* gene expression in petals after pollination, suggesting that ethylene acts as an inducing signal in flowers in this regulatory system. However, incubation of whole plants or flowers in ethylene did not induce expression of the *ZPT2-2::LUC* gene within 24 h (data not shown), indicating that ethylene is required but not sufficient for induction.

We also tested whether the transient increase of *ZPT2-2::LUC* activity in cotyledons (Fig. 1) requires a functional ethylene signal transduction pathway. Seeds were germinated on wet filter papers in sealed Petri dishes either with or without 1 $\mu\text{L L}^{-1}$ MCP. The effect of blocking

ethylene receptors was evident from the lack of root hair development on MCP-treated seedlings. Imaging of the LUC activity in these seedlings showed that the transient up-regulation of *ZPT2-2::LUC* in cotyledons was not blocked and thus does not seem to require active ethylene sensing (data not shown).

It is known that the phytohormone JA mediates the response of plants to cold, wound, and UV-B light stresses (Sembdner and Parthier, 1993; Creelman and Mullet, 1995). Furthermore, the sites of the plant that show elevated levels of *ZPT2-2* activity (i.e. root tip, young leaves, and petals) have been shown to be the sites of JA accumulation in plants (Meyer et al., 1984). These observations suggest that JA may be involved in the induction of *ZPT2-2* gene activity and, indeed, JA can induce *ZPT2-2* gene expression in shoots (Fig. 6). A further characterization of how the phytohormone signaling of ethylene and JA are integrated to modulate the activity of *ZPT2-2* gene expression will be published elsewhere.

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