Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet

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SUMMARY

Inducible plant defence responses in sugar beet (*Beta vulgaris* L.) leaves are repressed during the early phase of infection by the fungus *Cercospora beticola*. In this report, we show that the concentration of the plant hormone abscisic acid (ABA) increases in sugar beet leaves during *C. beticola* infection. After an initial burst of ABA induced by inoculation of the fungus, elevated ABA concentrations were detected during the fungal penetration and colonization phases 3–9 days after inoculation. Fifteen days after inoculation, with visible onset of the necrotic phase of infection, the strongly elevated ABA concentrations in infected leaves were at levels similar to drought-stressed plants. A synthetic promoter composed of four copies of the ABA-responsive element (ABRE) A2 and the coupling element CE3 of the ABA-inducible barley gene *HVA1* was strongly induced by ABA and *C. beticola* infection in transgenic sugar beet leaves. Analysis of the spatial pattern of promoter activity revealed that the ABA-inducible promoter was locally activated at the fungal infection sites. Furthermore, expression of the basic leucine zipper transcription factor AREB1 was induced by drought stress and fungal infection in the sugar beet. Application of ABA reduced the promoter activity of the phenylalanine ammonia lyase (*BvPAL*) gene, and this effect was observed with the –34 to +248 *BvPAL* promoter region. This region is equivalent to the core promoter, which is necessary for the suppression of *BvPAL* expression by *C. beticola*, as recently shown. These data indicate that ABA accumulation and activation of the ABA-dependent signalling cascade are the primary cause of suppression of *BvPAL* expression during infection of sugar beet leaves.

INTRODUCTION

Cercospora beticola is the economically most important fungal pathogen in relation to sugar beet. This pathogen causes typical leaf spots consisting of necrotic cells that reduce the photosynthetically active area of the plant and ultimately the sugar yield of the roots. The fungus penetrates the leaf through the stomata and grows during the hemibiotrophic infection cycle within the intracellular space of the leaf. After intense colonization of the leaf tissue, the parenchyma and epidermal cells collapse in the vicinity of the fungal hyphae and the final necrotic zone appears, leading to the typical sporulating leaf spots (Feindt *et al.*, 1981a).

The molecular analysis of the interaction between *C. beticola* and sugar beet is still in its early phases and many questions remain to be answered. The genetics and biochemistry of *C. beticola* virulence have not been examined as extensively as that of the related fungi *Cercospora kikuchii*, *Cercospora nicotianae* and *C. zeae-maydis* (Weiland and Koch, 2004). Resistance to *C. beticola* is polygenic inherited and quantitative. Quantitative trait loci (QTL) of *C. beticola* resistance have been mapped to four (Nilsson *et al.*, 1999; Setiawan *et al.*, 2000) and to six of the nine sugar beet chromosomes (Schäfer-Pregl *et al.*, 1999). Resistance genes analogues (RGAs), as candidates of resistance genes, have been linked to QTL (Hunger *et al.*, 2003). Several pathogenesis-related sugar beet proteins with chitinase (Nielsen *et al.*, 1993) and glucanase (Gottschalk *et al.*, 1998) activity and other antifungal proteins (Nielsen *et al.*, 1997) have been suggested as candidate genes.

Recently it was shown that inducible plant defences of sugar beet are repressed during the development of *Cercospora* leaf spot disease. In the early phase of infection, the expression of the phenylalanine ammonia lyase (*BvPAL*) and cinnamic acid 4-hydroxylase (*BvC4H*) genes was repressed at both the transcript and the enzyme activity levels. A specific region within the *BvPAL* promoter, –34 to +45, with respect to the start site of transcription, is required for the reduction of transcription, indicating that the *PAL* core promoter is involved in the detection of the unknown repression signal (Schmidt *et al.*, 2004).

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Many microbial plant pathogens successfully induce disease due, in part, to their ability to suppress the inducible defence responses in their hosts. Compared with our understanding of bacterial pathogens, our knowledge about the mechanism of defence repression caused by fungal pathogens and the chemical nature of the responsible suppressors is limited. The saponin degradation product β 2-tomatine mediates suppression of plant defences in tomato and *Nicotiana benthamiana* by interfering with fundamental signal transduction processes (Bouarab *et al.*, 2002; Martin-Hernandez *et al.*, 2000). Supprescine A and B, which are secreted by the pea pathogen *Mycosphaerella pinoides*, cause a delay in the elicitor response or PAL transcription and a delay in the accumulation of pisatin, the main phytoalexin of peas (Yamada *et al.*, 1989). The delay of the defence responses coincides with an inhibition of ATPase activity in pea plasma membranes (Shiraishi *et al.*, 1994). Additional suppressors isolated from germination fluid or culture fluid of phytopathogenic fungi have been reviewed by Shiraishi *et al.* (1994).

The phytohormone abscisic acid (ABA) plays important roles in the adaptation of vegetative tissues to abiotic environmental stresses, such as drought and high salinity, as well in seed maturation and dormancy (Marion-Poll and Leung, 2006; Uno *et al.*, 2000). Microarray analysis of 7000 *Arabidopsis thaliana* cDNA clones revealed that 3–4% of the genes were responsive to ABA (Takahashi *et al.*, 2004). ABA-responsive genes are likely to contain the ABA-responsive element ABRE consensus sequence (PyACGTGGC) in their promoters. Although other *cis*-acting regulatory elements also function in ABA-dependent gene expression, ABRE is the most important *cis*-acting element that regulates ABA-dependent osmotic responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). A single copy of ABRE is insufficient to direct ABA-dependent expression, and either an additional copy of ABRE or a coupling element, such as CE1 or CE3, is required for full ABA-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). The ABRE is a binding site for basic leucine zipper (bZIP) transcription factors, referred to as ABRE-binding proteins (AREBs) or ABRE-binding factors (ABFs), as shown by Choi *et al.* (2000) and Uno *et al.* (2000). Expression of the bZIP transcription factor AREB1 is upregulated in vegetative tissues by ABA, as well as drought and high salinity stresses. In *A. thaliana*, AREB1 is a key positive regulator of ABA signalling in vegetative tissues under drought stress (Fujita *et al.*, 2005).

Compared with the plant hormones ethylene, jasmonic acid and salicylic acid, which play important roles in disease resistance, the role of ABA in plant disease resistance is not well defined (Mauch-Mani and Mauch, 2005). Application of exogenous ABA or inhibition of ABA biosynthesis revealed that increased ABA levels correlated with increased susceptibility of plants to fungal (Henfling *et al.*, 1980; Mohr and Cahill, 2003; Ward *et al.*, 1989) and bacterial pathogens (Asselbergh *et al.*, 2008). Mutants impaired in ABA signalling or ABA biosynthesis show an enhanced

resistance to pathogens (Asselbergh *et al.*, 2008; Audenaert *et al.*, 2002; Mohr and Cahill, 2003).

In this study, we analysed the accumulation of ABA during the infection of sugar beets by *C. beticola*, and studied the stimulation of the ABA signal transduction cascade by the fungal pathogen.

RESULTS

ABA accumulates in *C. beticola* infected sugar beet leaves and in the fungal culture fluid

To analyse the role of ABA in the interaction between the sugar beet and *Cercospora beticola*, we first assessed ABA concentrations in mixed leaf samples of 12-week-old sugar beet plants of the susceptible cultivar 3D0018. The plants had been infected with *C. beticola* (40 000 mycelium fragments and spores/mL) or mock-inoculated in the greenhouse and showed a reduced expression of the phenylalanine ammonia lyase (*BvPAL*) gene at the early phase of fungal infection (Schmidt *et al.*, 2004).

Immediately after inoculation, the ABA concentration in the *C. beticola* inoculated leaves was an average of 8.9 pmol ABA/mg compared with 6.0 pmol ABA/mg in mock-inoculated leaves as determined by ELISA. The ABA concentration in the inoculated leaves declined to the level of the uninfected leaves over the following 2 days (1 and 2 dpi). At 3–9 dpi, increased levels of ABA were detected in the *C. beticola* inoculated leaves, with a statistically significant increase of up to 110% on day 5, 45% on day 7, 50% on day 8, and 60% on day 9 (Fig. 1). Microscopic analysis (data not shown) of the infected leaves revealed that the elevated ABA levels starting at day 5 coincided with initiation of the penetration and colonization phase of *C. beticola*. During the biotrophic phase of *C. beticola* infection, no visible disease symptoms were apparent.

After the appearance of typical 2–4-mm leaf spots, indicating the onset of the necrotic phase of the fungal life cycle, sugar beet leaves were again analysed for ABA. The ABA concentration of leaves without symptoms and those with slight, moderate and strong leaf spot symptoms were determined 15 days after inoculation. The ABA concentrations of slightly, moderately and strongly infected leaves were clearly increased compared with the uninfected leaves (Fig. 2A). The increase of ABA concentration correlates with the severity of the disease symptoms.

The infection of sugar beet leaves by *C. beticola* resulted in an increase of ABA concentrations to different levels at the different time points during the infection process. After an initial increase of ABA caused by the inoculation procedure itself, a moderate increase of ABA was observed during the penetration and colonization phases at days 5–9. Finally at day 15, a 3.5- to 5.3-fold increase of ABA was detected in partially necrotic leaves at the end of the fungal infection cycle.

Fig. 1 Accumulation of ABA during the biotrophic period of *Cercospora beticola* infection in sugar beet leaves. Determination of ABA in *C. beticola* infected and mock-inoculated sugar beet leaves by competitive ELISA. The solid black squares represent the infected samples and the white circles the uninfected samples. Sugar beet plants were infected with 40 000 mycelium fragments and spores/mL of *C. beticola* in the greenhouse and then analysed immediately (0 dpi = days post inoculation) and at specific time points up until 9 dpi, during the biotrophic period of infection. The fungal development on sugar beet leaves is shown. The data shown are the means of six replicates from two mixed samples of leaves, and each mixed sample consisted of six middle-sized leaves taken from six plants. The bars indicate the standard deviation. The asterisk indicates significant difference compared with the non-infected control at the same time point ($P < 0.05$). The statistical analysis was performed with a two-sided *t*-test.

Table 1 Detection of abscisic acid in the culture fluid of *Cercospora beticola* after 2 weeks of growth in Steinberg medium.

	Culture fluid of <i>C. beticola</i>	Steinberg medium without <i>C. beticola</i>
ABA (pmol/mL)	3.5 ± 1.0	0

Mean ± SD is shown.

The increased ABA concentrations during the infection process as well as the accumulation of ABA in necrotic leaves correlated with an increase in fungal biomass. These data and the statistically significant ABA increase of 50% in leaves immediately after *C. beticola* inoculation allowed us to speculate that the increased ABA is at least partially of fungal origin. As members of the genus *Cercospora* are known to produce ABA (Assante *et al.*, 1977) we analysed the ability of *C. beticola* to produce the plant hormone. *C. beticola* was grown in liquid culture and the ABA concentration was measured. A concentration of 3.5 pmol ABA/mL was detected in the fungal culture filtrate, while no ABA was detectable in the control medium (Table 1). Furthermore, we determined the ABA concentration of the inoculation suspension. A concentration of 3.6 nmol ABA/mL was detected in the inoculant of 40 000 mycelium fragments and spores/mL. These results confirmed the ability of *C. beticola* to synthesize the stress hormone ABA.

ABA concentrations of *C. beticola* infected leaves are similar to those detected in drought-stressed leaves

ABA is involved in the regulation of the abiotic stress response of plants, and elevated ABA levels are found in drought-stressed plants (reviewed by Marion-Poll and Leung, 2006). To compare the concentration of ABA during biotic and abiotic stresses, the ABA concentration in sugar beet leaves during drought stress was determined. Fully developed sugar beet plants grown for 10 weeks in 1-L pots in the greenhouse were exposed to drought stress during the summer by discontinuing the water supply to the soil. As soon as 24 h later, an initial reduction of leaf turgor of several leaves was visible. After 30 h, water stress became obvious as older leaves showed wilting. The wilting process continued until 78 and 96 h, when wilting was observed in all leaves of the plants.

The ABA concentration in well-watered control plants was in the range 5.8–7.0 pmol ABA/mg at each time point. In comparison, the ABA concentration of the water-stressed plants increased continuously from 5.8 pmol ABA/mg at the beginning of the experiment to 7.2, 18.4, 36.7 and 96.6 pmol ABA/mg at 24, 30, 78 and 96 h, respectively. At the final time point (102 h), which was characterized by a strong wilting of the leaves, the endogenous ABA concentration was 85.2 pmol ABA/mg leaf tissue (Fig. 2B). These results showed that the ABA concentrations of *C. beticola* infected leaves during the necrotic phase of infection are in the physiological range of levels of ABA concentrations in wilting sugar beet leaves during the first hours and days after water stress.

The *BvAREB1* transcription factor is induced by drought stress and *C. beticola* infection

The abscisic acid responsive element binding protein 1 (AREB1) is a basic domain leucine zipper transcription factor that binds the ABRE motif in the promoter region of ABA-inducible genes and functions as transacting activator. As the ABA and *C. beticola* induced 4x(CE3-A2) promoter contains an ABRE motif, we analysed the expression of the sugar beet *AREB1* gene under drought stress and fungal infection. The cDNA clone of the *AtAREB1* sugar beet orthologue was identified as a partial cDNA clone in a unique sugar beet cDNA collection (Herwig *et al.*, 2002) and converted to a full-length cDNA clone by RT-PCR. The 1856-bp *BvAREB1* cDNA clone encodes a basic domain leucine zipper transcription factor of 489 amino acids, which is 52% identical to *AtAREB1* and shows highest similarity to *AtAREB1* with an *e*-value of 3×10^{-81} compared with all *A. thaliana* genes.

Expression of *BvAREB1* in leaves of drought-stressed and control plants, which had been analysed for levels of ABA, was determined by RNA blot analysis. Expression of *BvAREB1* was weakly detectable at the start; after the older leaves began to

Fig. 2 Accumulation of ABA and *BvAREB1* transcripts during the necrotic period of *Cercospora beticola* infection and in leaves of drought-stressed sugar beet. (A) Accumulation of the plant hormone ABA during the necrotic period of *C. beticola* infection. ABA concentrations in leaves without symptoms and leaves with slight, moderate and strong leaf spot symptoms were determined by ELISA at 15 dpi. The data shown are the means of six replicates. The standard deviation is shown by bars. The asterisk indicates significant difference compared with the uninfected control at the same time point ($P < 0.05$). The statistical analysis was performed with a two-sided *t*-test. (B) Accumulation of the plant hormone ABA during wilting of sugar beet leaves caused by water stress in the greenhouse. ABA levels were measured in leaves of water-stressed plants and well-watered control plants by ELISA 0, 24, 30, 72, 96 and 102 h after stopping the water supply. The data shown are the means of six replicates. The bars represent the standard deviation. The asterisk indicates significant difference from the control at the same point in time ($P < 0.05$). The statistical analysis was performed with a two-sided *t*-test. (C) Accumulation of *BvAREB1* transcripts in sugar beet leaves with moderate and strong disease symptoms. *BvAREB1* expression in the samples, previously analysed for ABA, was determined by semi-quantitative RT-PCR. Expression was normalized against levels of GAPDH expression to confirm equal cDNA amounts, and *BvPR1* was used as a marker for defence gene activation. (D) Expression of the transcription factor *BvAREB1* is induced in drought-stressed plants 72, 96 and 102 h after stopping the water supply as shown by RNA blot analysis. No difference between control plants (–) and stressed plants (+) was detectable at the beginning of the experiment and after 30 h. *BvAREB1* transcripts are marked by arrows. Equal loading of total RNA was confirmed by ethidium bromide staining (data not shown).

wilt, 30 h after stopping the water supply, no difference in *BvAREB1* expression between control and stressed plants was observed. However, transcripts of the transcription factor were induced in the leaves of stressed plants compared with the watered control plants after 78 and 96 h (Fig. 2D). ABA concentrations in these leaves were approximately six- and 14-fold higher than levels in the control plants at these time points, respectively, as shown in Fig. 2B.

After a drought stress-mediated accumulation of *BvAREB1* mRNA was observed, in accordance with data from the *A. thaliana* orthologue (Choi *et al.*, 2000; Fujita *et al.*, 2005; Uno *et al.*, 2000), the responsiveness of *BvAREB1* expression to pathogens was analysed. In order to determine *BvAREB1* expression at the necrotic phase of *C. beticola* infection (15 dpi), leaves with varying severity of disease symptoms that had been analysed for

their ABA concentration were analysed by RT-PCR. Induction of *BvAREB1* was detectable in leaves with moderate and strong leaf spot symptoms compared with symptomless and slightly damaged leaves (Fig. 2C). Fungal infection of leaves also induced the expression of the *BvPR1* defence gene, in contrast to absence of expression in uninfected leaves (Fig. 2C).

Multiple copies of the *cis*-acting element CE3-A2 confer ABA responsiveness to a synthetic promoter in sugar beet

To date, a large number of ABA-regulated genes have been identified in *A. thaliana* and rice but not in sugar beet. We predicted that the pathogen-induced increase of ABA would result in transcriptional activation of ABA-responsive genes; thus,

an ABA-specific promoter in combination with a reporter gene was developed to analyse the change of ABA signalling during fungal infection. In order to ensure high ABA specificity, a synthetic promoter composed of *cis*-acting sequences known to be required for ABA inducibility was generated. The construction and use of a synthetic promoter with specificity for a plant hormone has already been successful in auxin studies (Sabatini *et al.*, 1999; Ulmasov *et al.*, 1997).

The abscisic acid response complexes 1 (ABRC1) and 3 (ABRC3) are required and sufficient for ABA induction of the genes *HVA22* and *HVA1* in aleurone and leaf tissue of barley (Shen *et al.*, 1996). ABRC1 is composed of the ACGT-box A3 (GCCACGTACA), which is a reverse complement to the ABRE *cis*-acting element and the coupling element CE1 (TGCCACCGG). ABRC3 consists of the ABRE *cis*-acting element A2 (CCTACGTGGC) and the coupling element CE3 (ACGCGTGTCTC). We constructed two synthetic promoters, 4x(CE3-A2) and 4x(A3-CE1), consisting of four copies of the *cis*-elements CE3-A2 and A3-CE1, respectively. The sequences were then cloned upstream of the 35S minimal promoter and the *Photinus pyralis* luciferase gene (Fig. 3A). The induction of the 4x(CE3-A2) and 4x(A3-CE1) promoters by ABA was tested in a transient assay by particle bombardment of sugar beet leaves that were sprayed with 100 μM *+/- cis-trans* ABA immediately prior to transformation. A construct containing the duplicated 35S promoter (d35S) and the luciferase gene from *Renilla reniformis* was used as an internal control as previously described (Schmidt *et al.*, 2004). The promoterless vector MS23-*LUC* and the 4x(A3-CE1) promoter construct failed to show ABA responsiveness. However, the 4x(CE3-A2) promoter was reproducibly induced by ABA treatment (Fig. 3B). Together, this shows that the synthetic promoter containing the ABRC3 of the barley gene *HVA1* confers ABA-responsiveness to sugar beet leaves in a transient assay.

Strong induction of the 4x(CE3-A2) promoter by ABA in leaves of transgenic sugar beet

The ABA-inducible 4x(CE3-A2) luciferase construct was transformed into the sugar beet cultivar 3DC4156 and transgenic plants were generated. To analyse the strength and time course of ABA-induction of the promoter, *in vitro* plants were immersed for 15 s into a 100 μM solution of *+/- cis-trans* ABA, and the reporter gene activity was determined at specific time points, up until 96 h after inoculation. The 4x(CE3-A2) promoter was rapidly and transiently induced by ABA application (Fig. 3C), and as soon as 1 h after ABA treatment, a nine- to 20-fold induction was observed for the transgenic lines PR101/6 and PR101/35, respectively. Maximal reporter gene activity was observed 24 h after hormone application and the activity declined until 72 and 96 h (Fig. 3C). The induction rate after 24 h was 168- and 91-fold for the lines PR101/6 and PR101/35, respectively. Although an

even higher induction rate could be detected 48 h after ABA treatment based on a lower reporter gene activity of the control plants for PR101/35, the maximal promoter activity was detected 24 h after ABA stimulation.

To analyse the specificity of the promoter, 1-aminocyclopropane-1-carboxylic acid (ACC) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) responsiveness was determined in comparison with an ABA treatment. Ethylene is generated in plants by oxidation of ACC. BTH induces systemic acquired resistance in plants via the same signal transduction pathway as salicylic acid (SA) but does not cause an accumulation of SA (Friedrich *et al.*, 1996; Lawton *et al.*, 1996). *In vitro* plants of the line PR101/11 were immersed for 15 s into a 100 μM solution of *+/- cis-trans* ABA, a 1.4 mM solution of BTH or placed on MS agar plates supplemented with 100 μM ACC. The induction rate after 48 h was 266-fold for ABA and six-fold for BTH. ACC treatment did not activate the promoter. Therefore, the 4x(CE3-A2) promoter is strongly induced by ABA, very slightly activated by BTH and not activated by ethylene.

To gain more insight into the spatial expression of the 4x(CE3-A2) promoter, histochemical luciferase assays were performed and visualized using a CCD camera. Figure 4A and 4B show that the 4x(CE3-A2) promoter is uniformly active in the blade of leaves of *in vitro* plants after ABA treatment. However, promoter activity seems to be strongly reduced or absent in the petioles of the plants. Promoter activity of transgenic plants in the absence of ABA application was hardly detectable and within the range of the background activity of non-transgenic sugar beets.

Induction of the 4x(CE3-A2) promoter by *C. beticola* infection

Transgenic lines that had shown strong ABA responsiveness were analysed for their response to fungal infection. *In vitro* plants were inoculated with *C. beticola* and the 4x(CE3-A2) promoter activity was measured immediately after inoculation at specific time points up until 7 days post-inoculation (dpi). The progress of fungal infection was followed by microscopic analysis.

Initiation of fungal growth on the epidermis and occasional penetration of stomata by *C. beticola* was detected at 1 dpi in the *in vitro* assay. Intensive growth of hyphae on the epidermis and frequent penetration of the stomata by hyphae was typically observed at 2 dpi. The colonization of parenchyma tissue by hyphae growing in the intercellular spaces and the first appearance of small necrotic spots are characteristic of 3 dpi. At 4 dpi, the micronecrotic spots frequently appeared, indicating the onset of the necrotic phase of infection. The last investigated time point (7 dpi) was characterized by the development of large necrotic areas. Compared with disease development in the greenhouse, the onset of disease in the fungal *in vitro* assay is faster; each step of the infection process is accelerated in the *in vitro* assay as

Fig. 3 The synthetic promoter 4x(CE3-A2) is responsive to exogenous ABA and *Cercospora beticola* infection in sugar beet leaves. (A) Schematic representation of the promoter reporter gene fusions of the 4x(CE3-A2)-LUC and 4x(A3-CE1)-LUC constructs. Four copies of the barley CE3-A2 or A3-CE1 *cis*-elements are upstream of the 35S minimal promoter and the *Photinus pyralis* luciferase gene. The nucleotide sequences of the CE3-A2 and A3-CE1 elements are shown below each construct. (B) ABA induction of the synthetic promoter 4x(CE3-A2) as shown by a transient assay. The reporter constructs 4x(CE3-A2)-LUC, 4x(A3-CE1)-LUC or the promoterless plasmid MS23-LUC-m3 (vector) were co-transformed with the p70SRUC normalization vector into leaf discs by particle bombardment. Leaf discs were sprayed either with 100 μM *cis/trans* ABA or 5% DMSO as a control. (C) Transient ABA induction of the 4x(CE3-A2) promoter in leaves of transgenic sugar beets. Two reporter gene lines (PR101/6 and PR101/35), which harbour the synthetic 4x(CE3-A2) promoter upstream of the luciferase gene, and the non-transgenic cultivar 3DC4156 (control) were sprayed with 100 μM *cis/trans* ABA or 5% DMSO. Specific luciferase activity ($K \times \text{RLU/mg}$ tissue) was determined 1, 24, 48, 72 and 96 h after hormone application. The average value of four replicates per treatment and the ABA inducibility of the promoter at each time point are given. The experiment was repeated twice with similar results. White columns (5% DMSO), black columns (100 μM ABA in 5% DMSO). (D) Induction of the 4x(CE3-A2) promoter by *Cercospora beticola* infection in sugar beet leaves. The reporter gene lines, PR101/6 and PR101/35, and the non-transgenic cultivar 3DC4156 were infected with *C. beticola*. Specific luciferase activity ($K \times \text{RLU/mg}$ tissue) was determined daily after infection. The average value of four replicates per treatment and the fungal induction of the promoter per time point are given. The experiment was repeated twice with similar results. White columns (non-infected), black columns (infected).

shown in Table 2, independent of the concentration of inoculant (data not shown).

All transgenic lines that had demonstrated a clear response to ABA were strongly activated by *C. beticola* infection, as shown in Fig. 3D. Line PR101/6 already showed a six-fold induction of promoter activity at 1 dpi. On average, a significant four-fold increase of promoter activity was observed in the analysed lines during the fungal penetration phase (2 dpi). A strong increase of promoter activity (28- to 34-fold induction) was observed during the parenchyma colonization phase at 3 dpi. The maximum

promoter activation with a 42- to 83-fold induction of reporter gene activity was observed at the onset of the necrotic phase, at 4 dpi. At the final time point analysed, 7 dpi, the promoter activity had reached a plateau, and in some cases had already declined, as observed for PR101/6.

Cellular specificity of 4x(CE3-A2) promoter activity was visualized by the histochemical luciferase assay in several independent transgenic lines using the *in vitro* assay. The reporter gene activity was sharply restricted to a small zone of leaf tissue surrounding the necrotic areas (Fig. 4C,D). Compared with the uniform

Table 2 Stages of infection by *Cercospora beticola* under *in vitro* and greenhouse conditions.

	<i>C. beticola</i> infection of <i>in vitro</i> plants	<i>C. beticola</i> infection of greenhouse plants
Fungal growth on the epidermis	1 dpi	1–5 dpi
First penetration of stomata	1 dpi	4 dpi
Intensive growth on the epidermis and frequent penetration of stomata	2 dpi	5 dpi
Colonization of the parenchyma tissue	3 dpi	5–9 dpi
Appearance of necrotic spots	4 dpi	10–12 dpi
Large necrotic spots and initiation of sporulation	7 dpi	15 dpi

dpi, Days post inoculation.

Fig. 4 Histochemical localization of luciferase activity in ABA-treated and *Cercospora beticola* infected sugar beet leaves. The left column (A, C, E) shows specimens under dark field conditions detected by a CCD camera after application of luciferin; the right column (B, D, F) contains the same specimens observed with bright field optics. (A, B) Reporter gene line PR101/11, expressing the synthetic promoter 4x(CE3-A2) upstream of the luciferase gene, and the non-transgenic control 48 h after application of 100 μ M *cis/trans* ABA. A PR101/11 plant in the absence of ABA treatment is shown as a control. (C, D) Local induction of the 4x(CE3-A2) promoter activity in PR101/11 around the lesions caused by *C. beticola* infection in the *in-vitro* assay at 8 dpi. Luciferase activity was absent in the leaf disc of the uninfected PR101/11 line (top plant). (E, F) Local induction of 4x(CE3-A2) promoter activity around a *C. beticola* lesion observed in two leaf discs (1, 2) from PR101/11. Reporter gene activity was not detectable around the lesion of a non-transgenic plant (3) or in the leaf disc of a uninfected transgenic PR101/11 plant (4). The plants were infected in the greenhouse and leaf discs were stained for luciferase activity.

reporter gene activity induced by exogenous application of ABA (Fig. 4A,B), the 4x(CE3-A2) promoter is activated very locally during the plant–pathogen interaction at the developing necrotic spots. In order to analyse the promoter activity in plants in the

greenhouse, the transgenic line PR101/35 was transferred to the greenhouse and infected with *C. beticola*. The histochemical luciferase assay detected confined local reporter gene activity around the leaf spots caused by *C. beticola*. Reporter gene activity

Fig. 5 ABA reduces the activity of the *BvPAL* minimal promoter. (A) Schematic representation of the -34-*BvPAL*-*LUC* reporter gene construct with the *BvPAL* minimal promoter and the 5'-UTR from position +1 to +147. Stippled box = UTR, hatched box = coding region of *BvPAL*. (B) Reduction of the activity of the *BvPAL* minimal promoter by ABA in a transient assay. Normalized reporter gene activity of -34-*BvPAL*-*LUC* in ABA-treated and untreated leaf discs of sugar beet. The *p70SRUC* vector was co-transformed with the *BvPAL* reporter gene vector to control for differences in transformation efficiency.

was not detectable in the uninfected leaves of PR101/35 or in *C. beticola*-infected leaves of non-transgenic plants (Fig. 4E,F). Therefore, the 4x(CE3-A2) promoter is activated at the fungal infection site under greenhouse conditions similarly as in the *in vitro* assay.

ABA reduces the activity of a *BvPAL* minimal promoter

To determine the ability of ABA to repress *BvPAL* promoter activity, a transient expression assay was performed. Sugar beet leaves were bombarded with both the reporter gene construct -34-*BvPAL*-*LUC* (Fig. 5A), containing a minimal *BvPAL*-promoter from position -34 to +248, relative to the transcription starting site, upstream of the *P. pyralis* luciferase gene, along with an internal control for transformation efficiency. The internal standard contained the *Renilla reniformis* luciferase gene under the control of two copies of the 35S promoter. A recent study showed that the activity of the *BvPAL* minimal promoter retains approximately 30% of the activity of the full-length *BvPAL* promoter (Schmidt *et al.*, 2004). Expression levels of ABA-treated and untreated leaf discs were analysed. In the ABA-treated leaf discs, the normalized reporter gene activity was strongly reduced to 35% of the activity of the untreated control group (Fig. 5B). As the measured luciferase activity of the *P. pyralis* reporter gene was normalized against the luciferase activity of the *R. reniformis* reporter, the possibility that the observed ABA effect is a result of a general reduction of gene expression can be ruled out. Thus, ABA acts as a negative regulator of *BvPAL* expression, and can exert its effect on the *BvPAL* minimal promoter.

DISCUSSION

In this study, we have shown that the plant hormone ABA accumulates in sugar beet leaves during the plant's interaction with the fungal pathogen *Cercospora beticola*. After an initial burst of ABA caused by the pathogen immediately after inoculation, the level of ABA gradually decreased during day 1 and day 2 to the level of uninoculated plants. The early decline of ABA in inoculated plants is probably caused by enhanced ABA degradation by the host. On days 3–9 after inoculation, ABA increased in infected leaves, and this elevation was significantly different from levels in uninfected plants during days 5–9. The elevated levels of hormone coincide with the early phase of infection, which is characterized by intensive fungal growth on the epidermis, penetration of stomata and colonization of parenchymous tissue. In particular, the invasion and multiplication of the pathogen in the leaf tissue during days 5–9 is accompanied by a significant increase of ABA. The appearance of necrotic spots in the late or necrotic phase of infection is accompanied by a strong 3.5- to 5.3-fold ABA increase. The measured ABA concentrations correspond to enhanced hormone levels in sugar beets in the early and middle phase of drought stress. The necrotrophic phase of infection, evident by the *Cercospora* leaf spots, is characterized by an intensive fungal growth inside the necrotic spots, resulting in the fungal stomata. Stomata are the starting point for sporulation of *C. beticola* from the necrotic spots. These data indicate that increased ABA during infection correlates with increased fungal biomass, and that the elevated ABA concentrations are at least partially of fungal origin. This conclusion is supported by two findings. First, a significant increased ABA concentration in leaves was observed immediately after spraying fungus on the leaves. Second, the plant hormone was detected in the *C. beticola* culture fluid and inoculant, confirming the ability of *C. beticola* to produce ABA.

Although *C. beticola* was observed to be capable of producing the plant hormone ABA in culture fluid, it cannot be ruled out that the elevated ABA concentrations are caused partially by fungal stimulation of the plant ABA biosynthetic pathway. The infection by the hemibiotrophic pathogen could mimic a physiological situation similar to drought stress. Pioneering work by Kettner and Dörffling (1995) demonstrated the accumulation of ABA in tomato leaves after infection with *Botrytis cinerea*. Using a tomato genotype and a fungal isolate, both impaired in ABA production, the authors concluded that ABA metabolism in infected tomato plants is influenced by four factors: stimulation of fungal ABA biosynthesis by the tomato, release of ABA or its precursor by the fungus, fungal stimulation of the ABA biosynthetic pathway of the plant, and inhibition of ABA degradation in the host. The ability of plant pathogens to produce plant hormones is not restricted to ABA production. Gibberellin is produced by the fungal pathogen *Gibberella fujikuroi*, and cytokinin produced

by many pathogens can promote pathogen success through retardation of senescence in infected leaves (reviewed in Jones and Dangl, 2006).

The application of the promoter–reporter gene technique generated additional insight into the temporal and spatial changes in ABA concentration during fungal infection. Although reporter gene analysis is a routine method for model organisms, e.g. *Arabidopsis thaliana*, the technique is not a standard for every crop as appropriate transformation protocols or reporter genes are not always available. We demonstrated successful application of the synthetic promoter 4x(CE3-A2) luciferase reporter constructed by our group in sugar beet plants. The luciferase reporter gene had previously been successfully used in analysing various organ- and cell-specific promoters in transgenic beets (Oltmanns *et al.*, 2006; Stahl *et al.*, 2004). Promoter analysis demonstrated that the promoter containing the AREB *cis*-acting element A2 is rapidly and transiently activated by ABA application with a maximum induction 24 h after ABA application and a uniform expression pattern in the blade of the leaves. However, after *C. beticola* infection, the ABA-responsive promoter was locally activated at the infection sites in transgenic sugar beets in both the *in vitro* assay and under greenhouse conditions. These results demonstrated two points: first, the ABA-responsive ABRC3 complex of barley retains its hormone sensitivity in the sugar beet; and second, ABA accumulates locally and not systemically during the host–parasite interaction in the sugar beet. Although maximum promoter stimulation was observed in the *in vitro* assay at the onset of the necrotic phase (4 dpi), the promoter was already strongly activated in the reporter gene lines at 3 dpi in the absence of necrotic spots. Time-course analysis of promoter induction after ABA treatment showed a delay of 24 h between exogenous ABA application and maximum reporter gene activity. Taking into account the time course of promoter stimulation, the strong promoter activation at 3 and 4 dpi revealed that ABA already strongly accumulates at the infection site at 2 and 3 dpi during the penetration and colonization phase of *C. beticola*. The promoter results were in accordance with the ABA measurements during the early and late infection phase and confirmed the involvement of the hormone by an independent experimental approach.

The third line of evidence for changes in ABA concentration during *C. beticola* infection is the induced expression of the bZIP transcription factor BvAREB1 and therefore the activation of the ABA signal transduction cascade. We identified BvAREB1 as the nearest orthologue of AtAREB1 in the sugar beet and showed that *BvAREB1* transcripts accumulate after drought stress and *C. beticola* infection. Expression of *AREB1* is upregulated by drought, high salinity and ABA in vegetative tissue of *A. thaliana* (Fujita *et al.*, 2005). ABA activation of a 42-kDa kinase resulted in multisite phosphorylation of AtAREB1, leading to induction of ABA responsive genes with at least two ABRE motifs in their promoters (Furihata *et al.*, 2006). Consistent with the role of ABA

in the activation of AtAREB1, we observed a correlation between increased ABA concentrations and enhanced *BvAREB1* expression in fungal infected sugar beets.

Comparing the *C. beticola* infection of greenhouse and *in vitro* plants, the response of the *in vitro* plants to infection was faster progression through each stage of infection. The reason for the shortened infection period is unclear but could be due to the smaller size of the *in vitro* leaves and the thinner epidermis, which favours the release of plant-derived substrates supporting the growth of the fungus. Feindt *et al.* (1981b) described that the susceptibility of sugar beet leaves in a single plant is dependent on age, and that the resistance of leaves gradually increases with the age and size of the leaves. The higher susceptibility of younger leaves correlates with enhanced spore germination, hyphal growth and penetration frequency of stomata by *C. beticola*, identical to our observations with the *in vitro* plants.

The increased ABA concentration at the infection site in the sugar beet and the activation of the ABA signal transduction cascade will negatively interfere with the activation of defence genes, a process referred to as suppression of PAMP-triggered immunity (PTI) or effector triggered susceptibility (ETS), according to Jones and Dangl (2006). The ability of ABA to suppress the expression of the *PAL* gene, a commonly used marker of defence gene activation, has already been demonstrated in soybean (Ward *et al.*, 1989) and tomato (Audenaert *et al.*, 2002). Consistent with these findings, a strong reduction of sugar beet *BvPAL* promoter activity was observed after ABA application. The identical minimal *BvPAL* promoter (from position –34 to +248) was negatively regulated by *C. beticola* infection (Schmidt *et al.*, 2004) and by ABA application. The promoter region, which is sufficient to explain the repression of *BvPAL* expression during *C. beticola* infection, was narrowed to the region between –34 and +45 (Schmidt *et al.*, 2004). We postulate that suppression of *BvPAL* gene expression as observed in the early phase of *C. beticola* infection (Schmidt *et al.*, 2004) is induced by the increased accumulation of ABA at the infection sites. ABA, either produced directly by *C. beticola* during infection or produced by the sugar beet after fungal stimulation, acts as a suppressor of plant defence and contributes to the suppression of one or more components of PTI.

Phenylalanine ammonia lyase catalyses the first step in the general phenylpropanoid pathway in plants, the conversion of L-phenylalanine to *trans*-cinnamic acid (CA). CA is the precursor of several compounds associated with the secondary metabolic pathway, e.g. lignins, flavonoids and phytoalexins (Lois *et al.*, 1989). The influence of *PAL* expression on the disease resistance of plants has been analysed in detail using transgenic approaches. *PAL*-co-suppressed tobacco plants are not able to establish systemic acquired resistance (SAR) after infection with tobacco mosaic virus (TMV) (Pallas *et al.*, 1996) and are more susceptible to the fungal pathogen *Cercospora nicotianae* (Maher *et al.*,

1994). Down-regulation of PAL enzyme activity of approximately 50% accelerates the onset of *Cercospora* disease symptoms and the severity of disease (Maher *et al.*, 1994). Over-expression of PAL in tobacco results in increased production of the phenylpropanoid compound chlorogenic acid and reduced susceptibility to infection with *C. nicotianae* (Shadle *et al.*, 2003; Way *et al.*, 2002).

To explain the negative effect of ABA in disease resistance, an indirect interference of biotic stress signalling by ABA has been suggested (Mauch-Mani and Mauch, 2005). Audenaert *et al.* (2002) described an antagonistic effect of ABA on SA-mediated defence signalling in tomato. A reduction of lignin and SA concentrations in *A. thaliana* leaves after ABA treatment was reported by Mohr and Cahill (2007). Anderson *et al.* (2004) showed that in *A. thaliana*, ABA strongly reduced the transcript levels of jasmonate- and ethylene-responsive defence genes, such as *PDF1.2*, a basic chitinase, a PR4 protein and a lectin-like protein. Furthermore, they showed a dominant antagonistic effect of ABA on jasmonate–ethylene signalling. Suppression of many defence-related genes, including those important for phenylpropanoid and lignin biosynthesis, pathogen perception and antimicrobial processes, have been identified by a genome-wide expression analysis of ABA-treated *A. thaliana* leaves (Mohr and Cahill, 2007). It remains to be seen if either SA or JA–ethylene signalling in the sugar beet is similarly influenced by ABA, or if SA biosynthesis in the sugar beet is impaired by *BvPAL* gene repression.

EXPERIMENTAL PROCEDURES

Plant and fungal material and inoculation procedure

Sugar beets (*Beta vulgaris* L.) of the genotype 3D0018 and 3DC4156 (KWS SAAT AG, Einbeck, Germany) were grown in 1-L pots under greenhouse conditions from spring to autumn. *C. beticola* infection of the sugar beet genotype 3D0018 was performed with 40 000 mycelium fragments and spores/mL of the isolate Ahlburg in the greenhouse as previously described (Schmidt *et al.*, 2004).

Reporter gene sugar beet lines, which were inoculated with ABA or infected with *C. beticola*, were cultivated as *in vitro* plants in a growth chamber (RUMED Licht-Thermostat Typ 1301, Laatzen, Germany) at 25 °C with a 16/8-h photoperiod. Propagation and cultivation of *in vitro* lines was performed in plastic containers on MS agar. To infect plants with *C. beticola*, the *in vitro* plants were immersed for 15 s in a suspension of 400 000 mycelium fragments and spores/mL of the fungal isolate Ahlburg or in a diluted suspension of V8 juice as a control.

Absciscic acid determination

Quantitative determination of the plant hormone abscisic acid (ABA) in sugar beet leaves was performed with a competitive

ELISA (Phytotetek ABA Test Kit, Agdia, IN, USA) according to the manufacturer's instructions. For each sample, three leaf discs (1 cm diameter) were cut from plants growing in the greenhouse with a cork-borer, the fresh weight (60–70 mg) was determined, and samples were subsequently frozen in liquid nitrogen. Powdered tissue was extracted with 600 µL of 80% acetone at 4 °C overnight. After centrifugation, the ABA concentration of the supernatant was determined in duplicate via ELISA; standards, with and without ABA were included (Sigma, A-1049). The presence of infected and control leaves was determined in six samples from multiple plants, and the means and standard deviations were calculated. The ABA determinations were repeated three times.

ABA production by the isolate Ahlburg of *C. beticola* *in vitro* was measured. The fungal isolate was grown for 2 weeks in liquid Steinberg medium (VanEtten and Stein, 1978) at 25 °C in the dark on a rotary shaker. After filtration of the culture, the ABA concentration of the filtrate was determined with a competitive ELISA as described above.

Reporter gene constructs

Reporter gene constructs suitable for analysis in transient assays or in transgenic sugar beet analysis were constructed using the luciferase gene from *Photinus pyralis* as the reporter. The *cis*-element CE3-A2 was constructed by annealing the phosphorylated upper and lower strand oligonucleotides 5'-CTAGTACGCGT-GTCCTCCCTACGTGGCT-3' and 5'-CTAGAGCCACGTAGGGAG-GACACGCGTA-3'; the *cis*-element A3-CE1 was constructed by annealing the phosphorylated oligonucleotides 5'-CTAGTGCCA-CGTACACGCCAAGCACCCGGTGCCATTGCCACCGGT-3' and 5'-CTAGACCGGTGGCAATGGCACCGGGTGCTTGGCGTGACGTGGCA-3'. The underlined nucleotides are not part of the described CE3-A2 and A3-CE1 sequences (Shen *et al.*, 1996) and introduce an *SpeI* site at the 5'-end and an *XbaI* site at the 3'-end after annealing. The oligonucleotides were annealed in 1× STE (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA) by heating to 95 °C for 5 min and cooling to 4 °C at 0.2 °C/s.

The CE3-A2 and A3-CE1 *cis*-elements were cloned into the *SpeI* and *XbaI* sites of the MS23-*LUC*-m3 vector, upstream of the 35S minimal promoter. The resultant vectors were named CE3-A2-*LUC* and A3-CE1-*LUC*. Promoters containing multiple copies of elements were created by a cloning strategy described by Rushton *et al.* (2002). Briefly, CE3-A2-*LUC* and A3-CE1-*LUC* were digested with either *SpeI* or *XbaI* along with *SacI*, which cuts the plasmids on the outside of the synthetic promoters. Ligation of two such fragments creates the plasmids 2× (CE3-A2)-*LUC* and 2× (A3-CE1)-*LUC* with double the number of elements. This was repeated to generate the constructs 4x(CE3-A2)-*LUC* and 4x(A3-CE1)-*LUC*. The cloning of construct -34-*BvPAL*-*LUC* has been previously described (Schmidt *et al.*, 2004).

To transform the sugar beet, the synthetic promoter along with the luciferase gene from 4x(CE3-A2) was excised as a *Hind*III-*Sac*I fragment and ligated into the binary vector pGPTV-KAN (Becker *et al.*, 1992). This binary T-DNA construct, pGPTV-Kan-4x(CE3-A2)-*LUC*, was then directly transformed into the *Agrobacterium tumefaciens* strain GV3101, which harbours the resident plasmid pMP90 (Koncz and Schell 1986).

RNA blot hybridizations and RT-PCR

Total RNA was isolated according to a previously published method (Logemann *et al.*, 1987). Radioactive probes were generated by random hexamer priming using the Amersham Multiprime DNA Labelling System (Freiburg, Germany). Radioactive probes were generated by labelling 20 ng of DNA with 50 μ Ci P^{32} -dATP (6000 Ci/mmol; Amersham Biosciences Europe GmbH). An 800-bp DNA fragment of the coding region of the *BvAREB1* cDNA was used as a probe. Electrophoretic separation of RNA, transfer to Hybond nylon membranes (Amersham Biosciences Europe GmbH), hybridization with radioactive probes and exposure of the membrane to X-ray films were performed according to standard protocols (Ausubel *et al.*, 1988).

RT-PCR was performed with total RNA from sugar beet leaves. One microgram of total RNA was used to construct first-strand cDNA using the SuperScriptTM First-Strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. RT-PCR for *BvAREB1* expression using the primers AGGAGCAATGGGGACTCATCCTCTGTATC and TTCAGCCTGTTCTTCCGTAAGTCTTG generated a 243-bp fragment. Transcripts of the *BvPR1* gene (accession number AM932128) were confirmed by 327-bp PCR products with the primers CACCCCTAGAAAAATGAATTTAC and ACCACATCTGTACTGCAGTTGTG. The following primers were used for detection of *BvGAPDH* expression: GCGTCGACCGATTACATG and GTCGGTGAAGACACAGTGA.

Cloning of *BvAREB1*

The *AtAREB1* orthologue cDNA clone of the sugar beet was identified as the partial cDNA clone plt3_005_c10 by a tblastx analysis in a unique sugar beet cDNA collection (Herwig *et al.*, 2002). The partial cDNA clone plt3_005_c10 (575 bp long) contains 353 bp of the 3'-end of the coding region of the transcription factor. The 1856-bp full-length *BvAREB1* cDNA clone (accession number AM932127) was constructed by 5'-RACE using the BD SMARTTM PCR cDNA Synthesis Kit (BD Biosciences) and total RNA from *C. beticola* infected leaves. Using the gene-specific primer CATGGCCCGTTTGTCTCTCTAAGCTTTT and the Smart II primer AAGCAGTGGTATCAACGCAGATACGCGGG as the universal primer, the full-length *BvAREB1* cDNA clone was amplified using the AdvantageTM 2 PCR Kit (BD Biosciences). After cloning the PCR product into the pGEM-T Easy vector (Promega, Mannheim,

Germany), the DNA sequence was determined by the DNA sequencing service of MWG Biotech (München, Germany).

Plant transformation

Agrobacterium tumefaciens-mediated transformation of the sugar beet (*Beta vulgaris*, var. 3DC4156) was performed with bacteria containing the binary T-DNA plasmid pGPTV-Kan-4x(CE3-A2)-*LUC* as previously reported (Lindsey and Gallois, 1990; Stahl *et al.*, 2004).

Reporter gene analysis

Transient biolistic experiments with leaves from 12- to 16-week-old sugar beets were performed using the PDS-1000/He system (Biorad) as previously described (Schmidt *et al.*, 2004; Stahl *et al.*, 2004). In brief, the MS23-*LUC*-m3, 4x(CE3-A2)-*LUC*, 4x(A3-CE1)-*LUC* or -34-*BvPAL*-*LUC* constructs were mixed with the internal standard p70SRuc, which contains two copies of the 35S promoter upstream of the *Renilla reniformis* luciferase, at a ratio of 1:1 (w/w). DNA was precipitated onto gold microcarriers.

Leaf discs of sugar beets were placed on MS + 0.4 M mannitol agar plates and bombarded with the DNA coated microcarriers, at a pressure of 10.7 MPa (1550 psi), with the distance between the stopping screen and the leaf sample set at 12 cm, and in a vacuum of 0.0965 MPa (28.5 inches Hg). After bombardment, leaf samples were incubated for 4 h at 25 °C in the light of a growth chamber. The activity of both luciferase genes was quantified using the dual luciferase assay (Promega). Relative reporter gene activity was calculated as previously described (Schmidt *et al.*, 2004).

For analysis of ABA induction of the MS23-*LUC*-m3, 4x(CE3-A2)-*LUC* and 4x(A3-CE1)-*LUC* constructs, leaf discs were placed onto MS + 0.4 M mannitol agar plates and sprayed with a solution of 5% (v/v) dimethyl sulfoxide (DMSO) and 100 μ M $\pm$ ABA or with 5% (v/v) DMSO as control. Immediately after bombardment, the discs were sprayed again with ABA or the control solution. The leaves were then incubated for 20 h and the reporter gene activity was determined. Experiments were repeated four times. To determine the ABA responsiveness of the -34-*BvPAL*-promoter, 100 μ M $\pm$ ABA was applied to MS + 0.4 M mannitol agar plates, and leaf discs were pre-incubated for 4 h before bombardment, and incubated for 24 h after bombardment.

For quantitative luciferase reporter gene assays of transgenic sugar beets, specific amounts of leaf tissue from *in vitro* plants were homogenized in 4 volumes of cell culture lysis reagent (CCLR, Promega, Mannheim, Germany). Complete leaves of the plants were analysed. For leaf tissue, 0.1 g tissue was homogenized in 400 μ L CCLR. Ten microlitres of the supernatant, corresponding to 2.5 mg of tissue, was mixed with 100 μ L luciferase assay reagent (Promega). Relative luciferase units (RLUs) were measured

with a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany) as described in the manufacturer's protocol (Promega). For each transgenic line, treatment and time point, four plants were analysed and the mean of the specific luciferase activity (RLU/s $\times$ mg tissue) and the standard deviation were calculated. Experiments were repeated twice with similar results.

For the histochemical luciferase assays, *in vitro* plants were analysed after ABA treatment or fungal infection. Whole plants were sprayed 24 h prior to camera detection with a solution of 5% (v/v) dimethyl sulfoxide (DMSO) and 100 μ M luciferin. The plants were photographed under white light, and luciferase activity was then detected in the dark using a MicroMax digital CCD camera (Visitron Systems, Puchheim, Germany). For some photos, single leaves were removed from the plants and placed on MS agar plates.

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