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Original Paper

Characterization of the promoter of the *Wiv-1* (*Lin6*) gene encoding a wound-inducible cell wall-bound acid invertase in tomato

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Abstract Fragments of different length (4 kb and 1 kb) of the 5′ upstream region of the *Wiv-1* (*Lin6*) gene encoding a wound-inducible cell wall-bound acid invertase in tomato were fused to the beta-glucuronidase (GUS) gene and expressed in tobacco and tomato plants. The GUS activity detected was similar in all transformed lines, suggesting that the 1 kb region contains most of the regulatory motifs responsible for the characteristics of the *Wiv-1* promoter. GUS activity was observed in vascular tissues of transgenic tomato and tobacco. In stem sections of transgenic tobaccos, the strongest activity was observed in the internal phloem of the nodes adjacent to the bases of petioles. The GUS activity in leaves of transgenic tobacco was elevated by addition of soluble sugars, especially sucrose. Previous studies suggested that the Wiv-1 enzyme participated in the regulation of carbohydrate contents in tomato leaves (Ohyama and Hirai 1999). The results obtained in the present study suggest that the Wiv-1 enzyme controls the loading of sucrose at the phloem of nodes and *Wiv-1* regulation depends on the concentration of sugars in source leaves.

Key words: Acid invertase, beta-glucuronidase, *Lycopersicon esculentum*, Wounding.

Acid invertase (EC 3.2.1.26) is a well-characterized enzyme that hydrolyzes sucrose to hexoses in various sink organs of plants. Multiple isozymes are known which differ in subcellular localization and biochemical characters such as their isoelectric points (pIs) (Sturm and Chrispeels 1990). Soluble acid invertases have acidic pH optima and acidic pIs and mostly are localized in vacuoles (Leigh et al. 1979). They are believed to control levels of hexoses in sink (Yelle et al. 1991; Klann et al. 1993, 1996; Ohyama et al. 1995; Zrenner et al. 1996) and source (Scholes et al. 1996) tissues. Extracellular or apoplastic acid invertases are ionically bound to cell walls (Hisajima and Arai 1978); they have acidic pH optima and basic pIs. Cell wall-bound acid invertases proposedly participate in phloem unloading (Miron and Schaffer 1991; Dali et al. 1992; Ruan and Patrick 1995), regulation of sink strength (Weber et al. 1995; Cheng et al. 1996), and gravitropism (Wu et al. 1993). Both soluble and cell wall-bound invertases are involved in responses to wounding and pathogens (Sturm and Chrispeels 1990; Ohyama et al. 1995, 1998; Matsushita and Uritani 1974; Zhang et al. 1996).

In tomato (*Lycopersicon esculentum*), genes for acid invertase isozymes have been isolated and characerized thoroughly (Elliott et al. 1993; Klann et al. 1993, 1996; Sato et al. 1993; Ohyama et al. 1995; Godt and Roitsch 1997; Fridman et al. 2000; Sinha et al. 2002; Fridman and Zamir 2003). Four genes (*Lin5*, *Lin6*, *Lin7*, and *Lin8*) for extracellular isozymes have been identified (Godt and Roitsch 1997). The wound-inducible gene *Lin6* (*Wiv-1*, Ohyama et al. 1998) is proposed to control sink strength in pollen and the transport of sugars from mature leaves (Ohyama and Hirai 1999). Although its induction by sugars has been observed in photo-autotrophic suspension culture cells (Sinha et al. 2002), the localization of the protein and the response of the gene to soluble sugars in tomato plants are unknown.

Here we report the characterization of the promoter of the cell wall-bound acid invertase gene *Wiv-1* by transgenic approaches using beta-glucuronidase (GUS) as a reporter. Responses of the promoter to wounding and soluble sugars as well as its tissue specificity were

Abbreviation: GUS, beta-glucuronidase: RCF, relative centrifugal force.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB247265.
This article can be found at http://www.jspcmb.jp/

analyzed, and the putative physiological roles of the enzyme encoded by *Wiv-1* in the living plant are discussed.

Materials and methods

Isolation of the Wiv-1 (Lin6) gene

Genomic DNA was isolated from leaves of tomato (*Lycopersicon esculentum* Mill. cv. House-Odoriko) by the standard method (Murray and Thompson 1980). The DNA was partially digested by *Sau3*AI and then fractionated on a discontinuous sucrose density gradient (10–17.5–25–32.5–40% sucrose (w/v) in 20 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 8.0). After centrifugation at 72,000×*g* (RCFmax) for 17 hr at 20°C, fractions containing fragments of 15–20 kb were collected and used for the construction of a lambda EMBL3 library (Sambrook and Russell 2001). The library was screened by plaque hybridization with *Wiv-1* cDNA (Ohyama et al. 1998) as a probe; one positive clone, *LEWAIG1-1*, was isolated. A part of an insert of *LEWAIG1-1* was excised by *Bam*HI digestion and then subcloned into the pUC18 vector (designated *pBEWIGP112*, Figure 1). The 5′ upstream region of the *Wiv-1* insert in *pBEWIGP112* was sequenced by an automatic sequencer (model 373A; Applied Biosystems, Foster City, CA, USA).

Construction of vectors

The subclone *pBEWIGP112* was used to construct binary vectors (Figure 1). Two portions of the 5′ upstream regions (either 4 kb or 1 kb upstream of the start codon ATG) of the insert in *pBEWIGP112* were amplified by PCR using either of two primer pairs. For amplification of the complete 5′ upstream region (about 4 kb) of the clone, the pair 5′-ACCGTCACATA-GTCAACTTGT-3′ (W5 prime) and 5′-CTTTAATTCTTTCTT-TTTGTGTT-3′ (W28-6: corresponds to the sequence 1–23b upstream of the start codon) was used. Alternatively, the pair 5′-CATACTACACTTCGATCTATGG-3′ (Wiv-1-1053 s) and W28-6 was used for the amplification of the 1 kb 5′ upstream region. Thermal cycles consisting of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C were repeated 25 times. The amplified 4 kb 5′ region was cloned into the pCR2.1 vector (Invitrogen Corp., San Diego, CA, USA), while the 1 kb 5′ region was cloned into the pT7Blue vector (Novagen, Inc., Madison, WI, USA). These subclones were designated pCRWP-5 and pWP1K-16, respectively. pCRWP-5 was excised by *Hin*dIII and *Xho*I and cloned into the pBI101 (Clontech Laboratories, Inc., Palo Alto, CA, USA) vector digested by *Hin*dIII and *Sal*I. Similarly, pWP1K-16 was excised by *Hin*dIII and *Bam*HI and then inserted into *Hin*dIII and *Bam*HI sites of pBI101. The resultant plasmids containing the 4 kb or the 1 kb 5′ region were termed pW4F and pW1K, respectively (Figure 1), and were used for the transformation of *Rhizobium* (*Agrobacterium*) by electroporation (Sambrook and Russell 2001).

Rhizobium-mediated transformation of tomato and tobacco

The transformation of tobacco (*Nicotiana tabacum* SR1 cv. petit habana) and tomato (*Lycopersicon esculentum* cv. Micro-Tom, Meissner et al. 1997) was performed as described previously (Ohyama et al. 1995; Yamada and Bohnert 2000). *Rhizobium radiobactor* (formerly *Agrobacterium tumefaciens*) LBA4404 carrying either pW4F or pW1K was used as the donor. Regenerated transgenic tobacco and tomato plants were maintained on MS media or were cultivated in a greenhouse (Ohyama et al. 1995; Yamada and Bohnert 2000).

Wounding of leaves

Discs (8 mm diameter) were cut from leaves of transgenic tobacco grown in a greenhouse. Wounding of these discs was performed as described previously (Ohyama et al. 1995).

Induction of GUS activity by sugars

Sugar induction experiments using tobacco plants were performed according to Wenzler et al. (1989). T_1 tobacco plants transformed with pBI121 (35S promoter:: *GUS*) were used as controls.

Histochemical and enzymatic analyses of GUS expression

Histochemical analysis of GUS activity in transformed plants was performed as described previously (Ohta et al. 1990; Fukuoka et al. 1998). GUS-stained tissue sections were post-stained with 0.01% aniline blue as detailed before (Hedley et al. 2000). Enzymatic analysis was performed according to the method of Ohta et al. (1990).

Results

Isolation of the 5′ upstream region of Wiv-1 and introduction of GUS constructs

A genomic clone, *LEWAIG1-1*, containing the gene for the wound-inducible cell wall-bound acid invertase, *Wiv-1*, was isolated from tomato (Figure 1). Southern analysis revealed that the clone contained the complete open reading frame and about 4 kb of the 5′ upstream region (data not shown). The 5′ upstream sequence (accession no AB247265) of *LEWAIG1-1* was almost identical with that of the *Lin6* gene previously described (Fridman and Zamir 2003). The region between −190 and −700 contained Box II (CCA/TGG) and GATA ($A(N)_3GATA$) motifs (data not shown) that are known to be involved in phloem-specific expression (Yin et al. 1997; Hedley et al. 2000). To analyze the characteristics of the promoter in the 5′ upstream region of *LEWAIG1-1* and to specify the regulatory region, the complete (4 kb) or partial (1 kb) 5′ region was fused to the *GUS* gene (Figure 1, Jefferson et al. 1987) and was introduced into tobacco and tomato plants. GUS activity of all of the obtained transgenic plants was examined histochemically and we confirmed that spatial GUS expression pattern induced by each construct was similar in all samples tested (data not shown). From those transgenic plants,

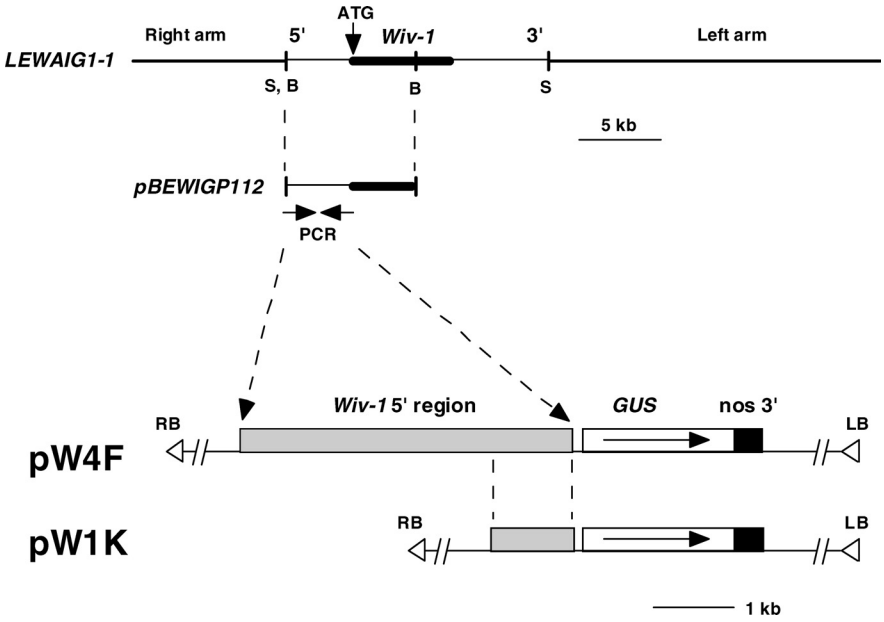

Figure 1. Construction of the binary vectors pW4F and pW1K. The *Bam*HI-excised fragment of the genomic clone *LEWAIG1-1* containing the 5′ upstream region of the gene *Wiv-1* was subcloned into pUC18 (designated *pBEWIGP112*). Two fragments of the 5′ region of about 4 kb or 1 kb upstream from the start codon (ATG) were amplified by PCR and then inserted upstream of the GUS gene of pBI101. pW4F contained the 4 kb fragment, while pW1K contained the 1 kb fragment. LB and RB indicate the left and right borders of the T-DNA, respectively. *Bam*HI and *Sal*I sites are indicated by B and S, respectively.

one T_0 tobacco line (4F46) from 40 lines transformed with the plasmid pW4F (4 kb 5′ region:: *GUS*, Figure 1) as well as two tobacco T_0 lines (K21 and K25) from 40 lines and one tomato T_0 line (28-12) from 2 lines transformed with the plasmid pW1K (1 kb 5′ region:: *GUS*, Figure 1) were selected as high GUS expressors carrying the functional transgenes for further experiment. These lines were maintained in a greenhouse for subsequent histochemical and enzymatic analyses.

Histochemical analysis of GUS expression controlled by the Wiv-1 promoter

Relative levels of GUS activity in various tissues of selected transgenic tobacco and tomato lines are compared in Table 1. Expression patterns in the 4F46 tobacco line transformed with the 4 kb 5′ region and in the K21 and K25 tobacco and the 28-12 tomato lines transformed with the 1 kb 5′ region were similar. Strong signals were found in vascular tissues of nodes, particularly adjacent to the bases of petioles (Figure 2A, C, E), and in pollen grains (Table 1). Stem sections of transgenic tobaccos showed that the strongly stained regions corresponded to internal phloem (Figure 2B, D). Strong GUS activity also occurred in vascular tissues of tomato fruits (Figure 2F).

Wounding and sugar induction experiments in transgenic tobacco

In leaves of all the transgenic tobacco lines, GUS activity continuously increased during a period of 96 h after wounding (Figure 3), similarly as reported for the tomato Wiv-1 enzyme (Ohyama et al. 1998).

To characterize sugar-dependent gene induction, detached leaves of the tobacco T_0 lines 4F46 (transformed with pW4F) and K21 (transformed with pW1K) as well as the control T_1 line 135-1 carrying the 35S promoter:: *GUS* construct (Jefferson et al. 1987) were cultured on MS media supplemented with various concentrations of soluble sugars for 3 weeks at 25°C in darkness. The GUS activity in 4F46 and K21 leaf tissue was elevated by sucrose (Figure 4) in a concentration-dependent manner. High levels of mannitol (234 mM) applied in addition to 30 mM sucrose could not substitute for high sucrose concentrations, so that purely osmotic effects could be ruled out (Figure 4). Glucose also induced the GUS activity although glucose tended to be less effective than sucrose, at least when GUS activity was expressed on a per fresh weight basis (Figure 4). No significant effects were observed in leaves of the control line 135-1 (Figure 4).

Discussion

The tissue specificity of GUS expression was similar in all transgenic lines of tobacco and tomato (Table 1). Moreover, in leaves of all the transgenic tobacco lines, GUS activity increased for 96 h after wounding (Figure 3), resembling the wounding profile of the endogenous Wiv-1 mRNA in tomato (Ohyama et al. 1998). Furthermore, sugar-dependent induction of GUS-activity

Table 1. Relative strength of GUS histochemical staining.

Tissue	Tobacco lines			Tomato line
	F46	K21	K25	28-12
Leaf	−	−	−	−
Stem (node)~basal petiole	++++	+++	++	++
Pollen	++	+++	++	++
Stigma	−	−	−	+
Ovary	++	+	−	++
Anther	−	−	−	+
Petal	−	−	−	−
Calyx	−	−	−	±
Root	±	±	+	NE
Fruit at 20–30DAA				−
Fruit at 40–45DAA				++
Outer pericarp				+
Vascular tissues				++
Ripe fruit (at 55-DAA)				++
Outer pericarp				−
Vascular tissues				++
Seed surface	NE	NE	NE	++

NE: not examined; ++++: strong activity detected; −: no activity detected; DAA: days after anthesis. Fruits were examined in tomato only.

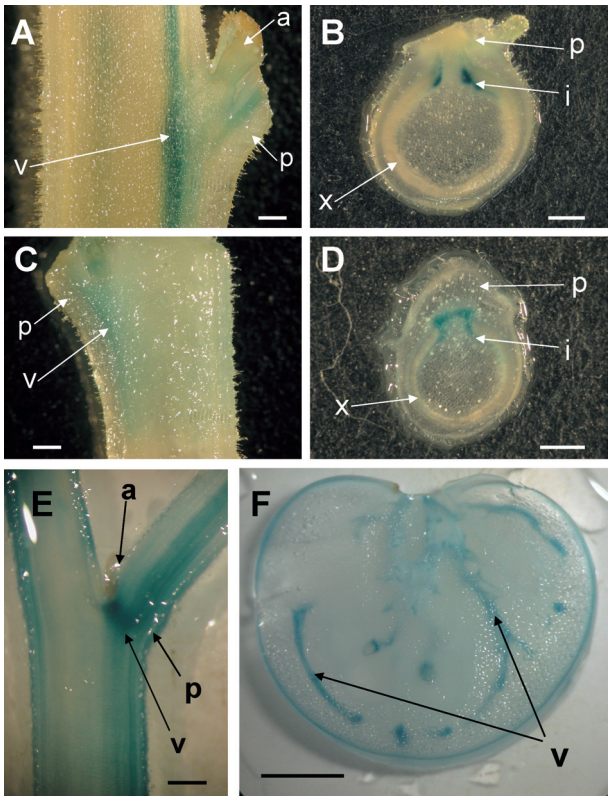

Figure 2. Histochemical analysis of GUS activity in transformed plants carrying 4 kb (tobacco line 4F46, transformed with pW4F) or 1 kb (tobacco line K21 and tomato line 28-12, transformed with pW1K) of the 5′ upstream region of *Wiv-1* fused to the *GUS* reporter gene. Longitudinal (A) and transverse (B) sections of stems (bar=1 mm), from greenhouse tobacco line 4F46. Longitudinal (C) and transverse (D) sections of stems (bar=1 mm), from greenhouse tobacco line K21. (E) Longitudinal stem section (bar=1 mm) from tomato line 28-12 grown on MS media. (F) Fruit at late green stage (35–45 days after anthesis; bar=5 mm) from greenhouse tomato line 28-12. P, petiole base; a, axillary bud; i, internal phloem; x, xylem; v, vascular tissue.

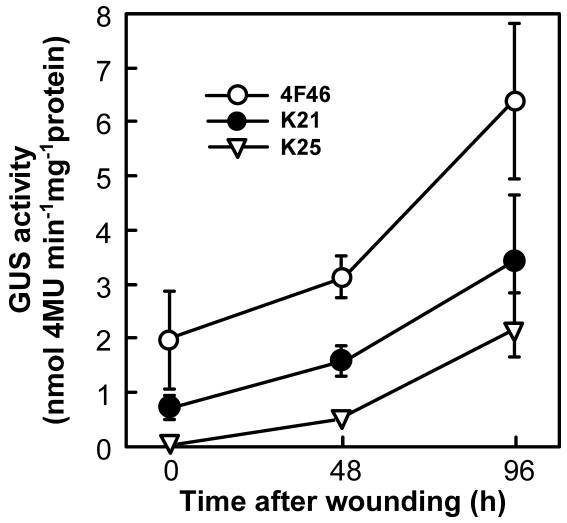

Figure 3. Wound-induction of GUS activity in transgenic tobacco. Leaf segments from plants transformed with pW4F (line 4F46) or pW1K (lines K21 and K25) were incubated at 25°C in the dark for 96 h, and the GUS activity in leaf extracts was determined. Columns represent means of three experiments with standard errors.

in 4F46 tobacco resembled that in K21 tobacco (Figure 4). These data suggest that the decisive regulatory motifs controlling the characteristics of the *Wiv-1* promoter are present in the 1 kb 5′ upstream region, and that the promoter functions similarly in tobacco and tomato. While, in wounding and sugar-induction experiments, basal level of GUS activity in 4F46 tobacco was higher than that in K21 tobacco, and the responses to wounding and sugars in K21 seemed to be more prominent than those in 4F46 (Figure 3, 4). Further experiments using more than one transgenic plant are necessary to clarify the possibility that the region upstream of the 1 kb

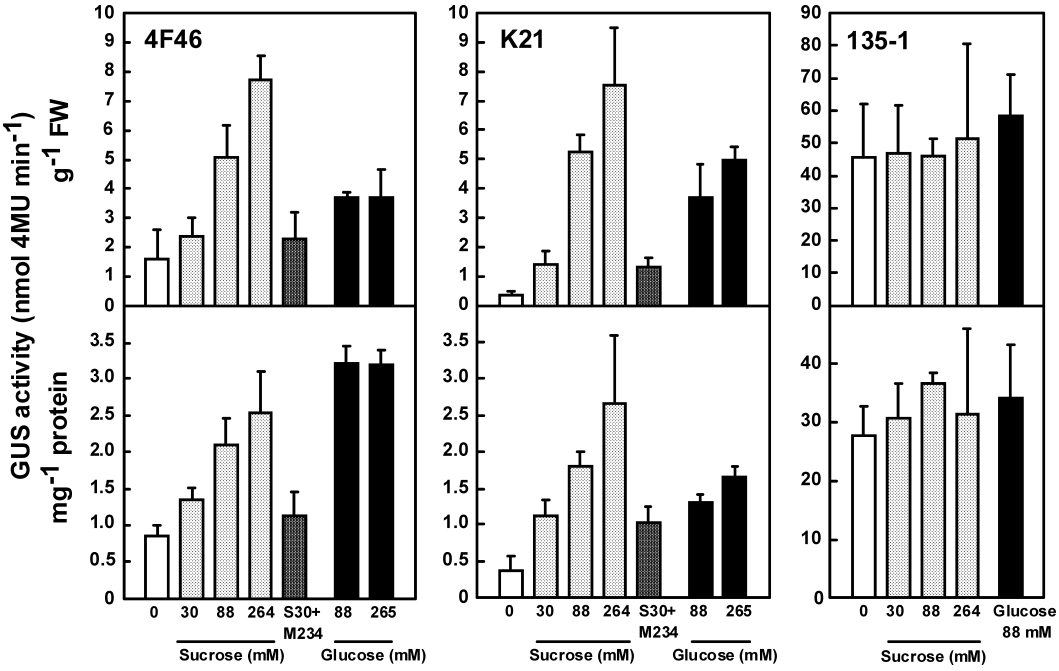

Figure 4. Induction of GUS activity in transgenic tobacco by application of sugars. Detached leaves from tobacco plants of the lines 4F46 (transformed with pW4F), K21 (transformed with pW1K), and 135-1 (transformed with 35S:: *GUS*) were cultured on sugar-free MS media supplemented with sucrose (0, 30, 88, or 264 mM), glucose (88 or 264 mM), or sucrose (30 mM) plus mannitol (234 mM; S30+M234) at 25°C in darkness. After 3 weeks, the GUS activity in leaf extracts was determined. Columns represent means of three experiments with standard errors.

promoter contains cis-regulatory elements that might affect the downstream basal promoter.

GUS appeared restricted to the internal phloem in stems (nodes) of tobacco (Figure 2A, B, C, D), and to vascular tissues of fruit and nodes (plus basal petioles) of tomato (Figure 2E, F), suggesting that the *Wiv-1* (*Lin6*) promoter is a vascular tissue (phloem)-specific promoter. Pronounced GUS activity also was observed in pollen of all transgenic plants (Table 1), supporting the hypothesis that the activity of cell wall-bound acid invertase mediates the sink strength of pollen, probably by facilitating the uptake of hexoses (Ylstra et al. 1998; Ohyama and Hirai 1999; Goetz et al. 2001).

The metabolizable sugars glucose and sucrose induced the expression of *GUS* gene driven by the *Wiv-1* promoter (Figure 4). These data confirmed the suggestion that metabolizable sugars in general seemed to induce sink-specific enzymes including invertases, whereas photosynthetic genes were repressed (Sinha et al. 2002). While, the GUS activity was induced not only by sucrose (substrate of invertase) but also by glucose (product) (Figure 4). These data imply that the Wiv-1 enzyme is an effective factor contributing to the signal transduction pathway of hexoses for wounding or pathogen responses (Sinha et al. 2002).

We have previously reported that the antisense inhibition of *Wiv-1* expression caused a reduction of the carbohydrate (especially sucrose) contents in mature leaves (Ohyama and Hirai 1999). On the other hand, over-expression of apoplast invertase leads to an increase in carbohydrates in mature leaves (Sonnewald et al. 1991). We here demonstrated that GUS expression driven by the *Wiv-1* promoter was dose-dependently induced by sucrose (Figure 4). Moreover, GUS activity was induced in vascular tissues of detached leaves (leaf blades and petioles) treated with sucrose or glucose (data not shown). These responses were not due to an osmotic stimulus since equimolar mannitol was not effective (Figure 4). Taken together, our data suggest that the Wiv-1 enzyme that accumulates in the phloem of nodes adjacent to the petioles is involved in the regulation of sucrose loading, and that the level of the enzyme is probably controlled by the sugar concentration in source leaves.

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