Role of the Sucrose Synthase Encoding *PrSus1* Gene in the Development of the Parasitic Plant *Phelipanche ramosa* L. (Pomel)

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Phelipanche ramosa L. (Pomel) is a major root-parasitic weed attacking many important crops. Success in controlling this parasite is rare and a better understanding of its unique biology is needed to develop new specific control strategies. In the present study, quantitative polymerase chain reaction experiments showed that sucrose synthase encoding *PrSus1* transcripts accumulate at their highest level once the parasite is connected to the host (tomato) vascular system, mainly in the parasite tubercles, which bear numerous adventitious roots. In situ hybridization experiments revealed strong *PrSus1* expression in both shoot and root apices, especially in shoot apical meristems and in the vascular tissues of scale leaves and stems, and in the apical meristems and developing xylem in roots. In addition, immunolocalization experiments showed that a sucrose synthase protein co-localized with cell-wall thickening in xylem elements. These findings highlight the role of *PrSus1* in the utilization of host-derived sucrose in meristematic areas and in cellulose biosynthesis in differentiating vascular elements. We also demonstrate that *PrSus1* is downregulated in response to 2,3,5-triiodobenzoic acid-induced inhibition of polar auxin transport in the host stem, suggesting that *PrSus1* activity in xylem maturation is controlled by host-derived auxin.

Broomrapes (*Orobancha* spp. and *Phelipanche* spp.) are achlorophyllous root-parasitic plants. *Phelipanche ramosa* L. Pomel (syn. *Orobancha ramosa*) is a harmful weedy parasitic plant in various agrosystems (Parker 2009) (illustrations can be found on the Parasitic Plant Connection website). *P. ramosa* takes advantages of ideal growth conditions in the Mediterranean Sea Basin and in Asia Minor, where it parasitizes a large range of host crops, primarily tomato and tobacco. Parasite development is divided into preparasitic and parasitic stages. The preparasitic stage starts with seed preconditioning followed by germination, which is induced by molecules secreted in the rhizosphere by the roots of host plants. Most of the known germination stimulants are strigolactones (Yoneyama et

al. 2009). Germination leads to the emergence of a radicle which attaches to the host root surface. The parasitic phase starts with the penetration of the parasite into the host root through a differentiating haustorium which connects to the host vascular tissues. The haustorium contains both xylem and phloem (Hibberd and Jeschke 2001) and serves as both an attaching organ and a bridge for water and nutrient transfer from the host. The attached parasite acts as a root (Bar-Nun and al. 2008), then diverts hormones and photoassimilates from the host plant, mainly auxin and sucrose (Abbes et al. 2009; Aber et al. 1983; Bar-Nun et al. 2008). The parasite first develops a tubercle carrying numerous adventitious roots; the apical bud of this tubercle give rise to a subterranean shoot and then to a branched flowering spike after emergence from the soil. Success in controlling broomrape is rare (Perez de Luque et al. 2009; Rispaill et al. 2007) and there is an urgent need to better understand the physiological and metabolic features of this root-parasitic plant to develop new specific control strategies (Aly et al. 2009). The transfer of sucrose from the host and its rapid utilization by the achlorophyllous parasite corresponds to major functional processes of the host–parasite relationship. Sucrose is mainly used in *P. ramosa* for growth and accumulation of hexoses and, to a lesser extent, mannitol and starch (Delavault et al. 2002). In plants, both invertases (EC 3.2.1.26) and sucrose synthases (SUS) (EC 2.4.1.13) are involved in sucrose utilization (Sturm and Tang 1999). A recent study has shown that a dominant vacuolar invertase, PrSAIL, plays an active role in cell turgor and growth during the full life cycle of the parasite *P. ramosa* (Draie et al. 2011). Nevertheless, the involvement of SUS in parasite development needs to be clarified.

SUS are glycosyl transferases which convert sucrose into fructose and UDP-glucose in the presence of UDP (UDP-glucose: D-fructose 2-glucosyltransferase) and play multiple pivotal roles. SUS are commonly associated with glucan synthesis in starch deposition in plastids (Baroja-Fernández et al. 2009; Chourey and Taliercio 1994) and in the thickening of secondary cell walls in vascular tissues (Coleman et al. 2009; Salnikov et al. 2003). Their role in energy conservation is also assumed, notably under low oxygen conditions (Albrecht and Mustroph 2003; Ricard et al. 1998), maintaining cell growth in tissues naturally deprived in oxygen such as tubercles (Bologa et al. 2003), developing seed (Rolletschek et al. 2002), and phloem (van Dongen et al. 2003). Oxygen and related sugar deprivation can induce many SUS-encoding genes (Koch 1996; Koch

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Nucleotide sequences: *PrSus1* (JN048797), *PrSus1* promoter (JN204913), *PrEF1a1* (HM219554), *PrCCD7* (JN412814), and *PrIPT* (JN412815).

et al. 1992; Winter and Huber 2000). In phloem, SUS may be involved in energy delivery for sucrose loading and in callose formation in response to wounding and pathogen infection (Koch 2004). Various locations of SUS have been reported: they can be bound to plasmalemma associated with cellulose synthase and callose synthase (Delmer and Amor 1995; Salnikov et al. 2001; Subbaiah and Sachs 2001), to the cell wall (Salnikov et al. 2003; Ruan 2007; Persia et al. 2008), or to membranes of several types of organelles, including Golgi vesicles, plastids, and vacuoles (Buckeridge et al. 1999; Etxeberria and Gonzales 2003; Koch 2004), but also to actin proteins in the cytosol (Winter and Huber 2000; Winter et al. 1998). SUS has also been detected in mitochondria, where its function remains unclear (Subbaiah et al. 2006).

Many plant SUS-encoding genes (*Sus* genes) have been isolated and characterized (Sturm and Tang 1999). *Sus* genes belong to a small multigenic family in both monocot and dicot plants (Komatsu et al. 2002). Six *Sus* genes have been isolated from *Arabidopsis* and three from maize (Baud et al. 2004; Duncan et al. 2006). According to their intron/exon structure and size, *Sus* genes are organized into four phylogenetic groups, including a monocot SUS group, a dicot SUS1 group, a SUSA group, and a “new group” (Bieniawska et al. 2007; Komatsu et al. 2002; Harada et al. 2005). Unlike the genes of the SUSA group, many *Sus* genes belonging to the SUS1 group are induced by anoxia (Baud et al. 2004; Harada et al. 2005). In addition, sugar-responsive elements have been identified in *Sus* gene promoters (Grierson et al. 1994; Martin et al. 1993) and it is commonly observed that *Sus* genes from a given plant species are differently regulated by sugars (Koch et al. 1992). In contrast, few data are available on the implication of growth regulators in *Sus* gene regulation (Harada et al. 2005).

In the present study, we identified *PrSus1*, a gene belonging to the dicot SUS1 group (*PrSus1*) and isolated from the parasitic plant *P. ramosa*. Several experiments using different types of molecular and microscopy techniques revealed the spatial and temporal regulation of *PrSus1* during the infection process in addition to its upregulation by the host-derived auxin. Taken together, these results provide a useful foundation for understanding the role of *PrSus1* in cellulose synthesis in meristematic areas and vascular tissues of the parasitic plant *P. ramosa*.

RESULTS

Isolation of *PrSus1* cDNA from *P. ramosa*.

PrSUS1-encoding cDNA was obtained by reverse-transcriptase polymerase chain reaction (PCR) using sets of primers designed from conserved regions of plant SUS sequences. Using rapid amplification of cDNA ends (RACE) strategies, a full-length cDNA (2,786 bp) (accession number JN048797) was isolated. The *PrSus1* sequence encodes an 806-amino-acid protein, with a predicted molecular mass of 92.1 kDa, and shares the highest identity with two SUS proteins from *Solanum lycopersicum* (CAA09593, 88%) (Fuglevand et al. 1998) and *Coffea arabica* (CAJ32596, 87.8%) (Geromel et al. 2006). It presents a serine residue in the N-terminal region (Ser 11) which is conserved in most SUS proteins (Winter and Huber 2000). PrSUS1 features two SUS characteristics: an N-terminal extension and a glycosyl transferase group 1 domain. PrSUS1 also has a signal peptide for mitochondria. A phylogenetic analysis (data not shown) clustered PrSUS1 with the above-mentioned *S. lycopersicum* and *C. arabica* SUS in the dicot SUS1 group (Komatsu et al. 2002).

Development-related changes in *PrSus1* gene expression.

Patterns of gene expression were analyzed using real-time quantitative (q)PCR in a range of organs and stages of the

parasite development previously described by Draie and associates (2011). Accumulation of *PrSus1* transcripts was expressed relative to the transcript level of the constitutive elongation factor 1- α gene (*PrEF1 α 1*) (Fig. 1). *PrSus1* transcripts accumulated strongly in *P. ramosa* tissues once the parasite had attached to host roots. Accordingly, transcript levels were much higher in both tubercles and shoots than in germinated seed. Nevertheless, a gradual decline in *PrSus1* transcript accumulation was observed during the parasitic phase, from the highest level in young tubercles (Tub.III) and the lowest in emerged and flowering shoots (FS.V), particularly in developing fruit (F.V). Concerning *PrSus1* expression in seed, germinated seed exhibited higher *PrSus1* transcript levels than preconditioned seed.

Tissue-specific expression of *PrSus1* and SUS immunolocalization.

Because they displayed high accumulation in *PrSus1* transcripts (Fig. 1), young tubercles (Tub.III) (Fig. 2A and D) were chosen for in situ hybridization experiments from longitudinal sections of adventitious root and shoot apical meristems. Positive hybridization (purple stain) with the specific antisense probe indicated *PrSus1* transcript accumulation (Fig. 2C, F, and H), whereas no signal was observed after hybridization with the sense probe (Fig. 2B, E, and G). Transcripts accumulated strongly in both shoot and root apices (Fig. 2C and F). In contrast, no staining was detected in the starch-accumulating parenchyma of tubercles (Fig. 2C). In shoot apices, *PrSus1* mRNA accumulated in various tissues, including the medullary

Fig. 1. Development-related changes in the levels of *PrSus1* transcripts in *Phelipanche ramosa*. Transcript accumulation is expressed relative to *PrEF1 α 1* transcript levels. Data are means $\pm$ standard error ($n = 3$). Values with the same letter are not significantly different (analysis of variance, SNK test, $P < 0.05$). Preparasitic stages: preconditioned seed (PS) and germinated seed (GS); parasitic stages following attachment to tomato roots: growing tubercle (Tub.III); tubercle (Tub.IV) bearing the growing subterranean shoot (S.IV); tubercle (Tub.V) bearing the emerged flowering shoot; growing flowering shoot (apical part) (FS.V), and flowering shoot bearing developing fruits (F.V). Basal part (Bp.V) does not bear flowers and is larger and more fibrous than the flowering shoot (Draie and associates 2011).

meristem of the shoot apical meristem and vascular tissues of scale leaves and the stem (Fig. 2C). In root apices, in addition to the root apical meristem, differentiating xylem-conducting elements exhibited high accumulation of *PrSus1* mRNA (Fig. 2F and H). In contrast, mature xylem elements from basal parts of adventitious roots did not exhibit *PrSus1* mRNA accumulation (data not shown).

To complement in situ hybridization, SUS immunolocalization was performed on young tubercles (Tub.III) using an antibody raised against a denatured *Vicia faba* SUS (Ross and Davies 1992). Although labeling was absent in cross-sections probed with pre-immune serum (Fig. 3A), strong SUS labeling matched cell-wall thickening of xylem elements (Fig. 3B and C).

Fig. 2. In situ localization of *PrSus1* transcripts in adventitious roots and the apical bud of young *Phelipanche ramosa* tubercles. **A**, Longitudinal section of a young tubercle (Tub.III). **C**, **F**, and **H**, Sections (10 μ m thick) hybridized with antisense probe specific to *PrSus1* mRNA. **B**, **E**, and **G**, Sections hybridized with sense probe as a negative control. Positive hybridization signals are indicated by violet staining using a digoxigenin-labeled RNA immunodetection system. **D**, Young tubercle (Tub.III) attached to host root. Ar = adventitious root; dx = developing xylem; hr = host root; m = meristem; mx = mature xylem; p = scale-leaf primordium; sam = shoot apical meristem; sp = storage parenchyma; tu = tubercle; v = vascular tissues. Scale bars: A–F, 500 μ m; G and H: 36 μ m.

Isolation and sequence analysis of the *PrSus1* promoter.

A 2,175-bp 5'-flanking DNA fragment of the *PrSus1* gene was isolated by PCR amplification of a *P. ramosa* Genome-Walker library. The 5'-RACE analysis allowed the identification of a potential transcription start site (+1) of *PrSus1*. Nucleotide sequence comparison between the *PrSus1* cDNA and the partial *PrSus1* genomic sequence revealed that a 791-bp intron sequence was present in the 196-bp fragment of the 5' untranslated region. The structure of the *PrSus1* promoter (accession number JN204913) is shown in Figure 4. A TATA-box

sequence was located between nucleotides -34 and -28, and the closest CAAT-box sequence was located between -318 and -315. A promoter motif search using PLACE software (Higo et al. 1999) identified a number of putative auxin regulatory motifs, including an ACTTTA motif (NTBBF1), an auxin-regulated expression element that is required for tissue-specific expression in the meristem and vascular system (Baumann et al. 1999), and a TGACG motif corresponding to the auxin-regulated expression element ASF1 (Klinedinst et al. 2000) (Fig. 4).

Control of *PrSus1* expression by host-derived auxin.

To validate possible *PrSus1* control by auxin, the auxin transfer from host to parasite was manipulated by inhibiting polar auxin transport in host stem by applying 2,3,5-triiodobenzoic acid (TIBA), as previously described by Harb and associates (2004). Impact of the treatment on the expression of *PrSus1* and two auxin-signaling markers, *IPT* and *CCD7* genes, was analyzed. First, the auxin-responsive sequences *PrCCD7* and *PrIPT*, orthologous to pea (Johnson et al. 2006; Shimizu-Sato et al. 2009), were isolated from *P. ramosa* (accession numbers JN412814 and JN412815, respectively). As expected, *PrIPT* and *PrCCD7* genes were 3.6-fold induced and 5.9-fold repressed, respectively, in parasite tubercles growing on TIBA-treated host plants (Fig. 5). These findings indicate a decrease in auxin signaling in the parasite in response to TIBA. Interestingly, a threefold repression was also observed for *PrSus1* in tubercles growing on TIBA-treated host plants (Fig. 5), suggesting that *PrSus1*, like *PrCCD7*, is upregulated by host-derived auxin.

DISCUSSION

Once attached to host roots, achlorophyllous parasitic plants such as broomrapes must take up sucrose from the host plant (Abbes et al. 2009; Aber et al. 1983). As recently shown by Draie and associates (2011), sucrose unloading in *P. ramosa* is facilitated by rapid hydrolysis of the disaccharide mediated by the dominant PrSAII invertase. Nevertheless, this finding did not preclude a significant role of SUS in sucrose utilization in the parasite.

Using degenerate primers corresponding to highly conserved regions of plant *Sus* genes, we succeeded in isolating the *PrSus1* cDNA from *P. ramosa*, which shared some typical traits with plant SUS-encoding genes (Winter and Huber 2000). The deduced amino acid sequence includes a highly conserved serine residue in the N-terminal region (Ser 11) and two characteristics revealed by National Center for Biotechnology Information Conserved Domain Search: an N-terminal extension of SUS and a glycosyl transferase group 1 domain.

Fig. 3. Sucrose synthases (SUS) distribution in *Phelipanche ramosa* tubercles visualized using immunofluorescence microscopy. Cross-sections of a resin-embedded young tubercle (Tub.III): **A**, section probed with the pre-immune serum; **B** and **C**, sections probed with anti-*Vicia faba* SUS antibodies (Ross and Davies 1992). Sections were probed with Alexa-conjugated second antibodies (signal appears in white). Excitation light, 450- to 490-nm band-pass filter; emission light, >515 nm. Scale bars, 50 μ m.

Fig. 4. Auxin-responsive elements present in *PrSus1* promoter. A schematic diagram of the structure of the *PrSus1* gene with promoter, 5' untranslated region (5'UTR), first intron, partial coding sequence (CDS), TATA-box, CAAT-box, and transcription start site (+1). An analysis using PLACE software (Higo et al. 1999) identified auxin-responsive elements in the *PrSus1* promoter. **a**, Auxin-responsive elements corresponding to an NTBBF1 motif (ACTTTA). **b**, Auxin-responsive elements corresponding to an ASF1 motif (TGACG).

Its size of 92.1 kDa is typical of the monomeric form of SUS (approximately 90 kDa). According to the protein sequence, PrSUS1 belongs to the dicot SUS1 group (Komatsu et al. 2002). Both tubercles and shoots express *PrSus1* throughout parasite development (Fig. 1). Nevertheless, developing tubercles accumulate the highest level of transcripts (Tub.III), specifically in cellulose-synthesizing tissues of adventitious root apices and shoot apical buds, including the meristem and maturing vascular elements (Fig. 2). This distribution is consistent with the presence of an ACTTTA motif (NTBBF1) in the *PrSus1* promoter (Fig. 4); this motif is required for specific expression in meristems and vascular systems (Baumann et al. 1999). In root apices, *PrSus1* expression was associated with differentiating xylem elements, in accordance with immunofluorescence microscopy experiments showing the association of the protein with secondary cell-wall thickening (Figs. 2H and 3). Similarly, SUS has been localized to cellulose synthesis sites in tracheary elements of *Zinnia elegans*, where SUS accumulated near the plasma membrane and the microtubules under the thickenings of the secondary cell wall, and also in the cell wall (Salnikov et al. 2001). In other cellulose-synthesizing tissues, such as differentiating tobacco pollen tubes, SUS has been detected in Golgi vesicles, the plasma membrane underneath secondary thickening, and mostly in the cell wall, suggesting that SUS is transferred to the cell wall by a secretion mechanism involving Golgi vesicles (Persia et al. 2008). Moreover, SUS detection in cryogenically fixed and freeze-substituted cotton fibers (Ruan 2007; Salnikov et al. 2003) provides further support for a cell wall location of SUS in plants, acting during the synthesis of cell wall elements in association with callose synthase and cellulose synthase (Amor et al. 1995; Coleman et al. 2009; Gardiner et al. 2003). All of these findings suggest that PrSUS1 is involved in cellulose synthesis in the secondary thickenings of differentiating xylem elements in tubercles and adventitious roots of *P. ramosa*. As

observed for *Sus4* in tomato (Pien et al. 2001), *PrSus1* is also highly expressed in maturing vascular tissues of the shoot apical bud, in which xylem and phloem are sorely distinguished. Thus, PrSUS1 is probably crucial for cell wall integrity of both xylem and phloem tissues in the apical bud. In addition, because vascular tissues were not observed in radicles of germinated seed (Aber and Sallé 1983), the enhanced *PrSus1* expression observed in germinated seed in comparison with preconditioned seed may be related to cellulose enrichment in elongating radicles (Fig. 1).

Additional roles have been proposed for PrSUS1, including energy efficiency in meristems (Koch 2004) and starch deposition in plastids (Baroja-Fernández et al. 2009). Our findings do not preclude PrSUS1 from being the dominant enzyme for sucrose breakdown in meristematic cells. The presence of PrSUS1 is more likely related to the lower energy consumption in root and shoot apical meristems, because they strongly express *PrSus1* (Fig. 2C and F). However, PrSUS1 is probably not involved in starch deposition because no *PrSus1* transcript accumulation was observed in the starch-accumulating parenchyma of tubercles (Fig. 2C).

Although hormonal control of invertases is well documented (Ehness and Roitsch 1997; Sokolova et al. 2002), information on SUS is rare. Harada and associates (2005) show a positive correlation between stem elongation and the level of *PdSUS1* transcripts in turions of *Potamogeton distinctus* treated with synthetic auxin 2,4D and under anoxia. This correlation suggests that *PdSUS1* is upregulated by auxin. In the present study, two major findings suggest that *PrSus1* is also regulated by auxin. First, *PrSus1* is expressed strongly in auxin-rich tissues, including germinated seed (Fig. 1), meristems, and differentiating xylem elements (Fig. 2C, F, and H). Auxin production in germinating *Phelipanche ramosa* seed has been demonstrated by Slavov and associates (2004). In parallel, the role of auxin in vascular maturation and xylem differentiation is well documented in plants (Ohashi-Ito and Fukuda 2010; Yoshida et al. 2009). Developing *P. ramosa* tubercles exhibit root formation and disorganized vascular differentiation (Fig. 2A and D). These traits have also been described in the interaction between *P. ramosa* calli and tomato (Zhou et al. 2004), and are indicative of high auxin concentrations (Sachs 1991) in accordance with the fact that the tubercle acts as a strong sink for host-derived auxin (Bar-Nun et al. 2008). Second, inducing auxin deprivation of the parasite by growing them on TIBA-treated host plants, Harb and associates (2004) demonstrated that xylem differentiation in the parasite is controlled by host-derived auxin. We show that similar TIBA treatment of the host plant led to significant repression of *PrSus1* in tubercles (Fig. 5), suggesting that *PrSus1* is an auxin-responsive gene implicated in the maturation of xylem elements in *P. ramosa*. Changes in auxin response in tubercles has been demonstrated using two auxin-regulated genes as controls, the genes *PrIPT* and *PrCCD7* encoding an adenosine phosphate iso-pentenyl-transferase and a carotenoid cleavage dioxygenase, respectively (Brewer et al. 2009; Miyawaki et al. 2006; Tanaka et al. 2006). In pea, auxin derived from the shoot apex controls bud outgrowth by repressing *PsIPT2* and inhibiting local cytokinin biosynthesis in the nodal stem (Shimizu-Sato et al. 2009). Moreover, pea has two *IPT* genes, *PsIPT1* and *PsIPT2*, and both are markedly induced in the nodal stem following auxin depletion in response to decapitation (Tanaka et al. 2006). The gene *CCD7* is involved in the synthesis of strigolactones (Gomez-Roldan et al. 2008; Umehara et al. 2008), which interact with auxin in shoot branching control. The genes *rms5* and *MAX3*—orthologous to *CCD7* in pea and *Arabidopsis*, respectively—are highly repressed following decapitation or

Fig. 5. Effect of host plant (tomato) treatment with triiodobenzoic acid (TIBA) on the expression of two auxin-responsive genes (*PrIPT* and *PrCCD7*) and *PrSus1*. Young tubercles (Tub.III) were harvested 3 weeks after treatment with TIBA (10 mg g⁻¹ of lanolin). Transcript accumulation is expressed relative to *PrEF1a1* transcript levels. Data are means $\pm$ standard error ($n = 3$). For each gene, values with the same letter are not significantly different (analysis of variance, SNK test, $P < 0.05$).

inhibition of the auxin polar transport (Hayward et al. 2009; Johnson et al. 2006). Finally, the *in silico* analysis of *PrSus1* promoter revealed putative auxin-regulated expression elements NTBBF1 (Baumann et al. 1999) and ASF1 (Klinedinst et al. 2000) (Fig. 4). Their signification in auxin responsiveness needs additional investigations. Auxin controls xylem cell development in plants through direct and indirect pathways (Ohashi-Ito and Fukuda 2010; Yoshida et al. 2009). Although our study did not focus on xylogenesis control, it provides new insights into the auxin-mediated control of cellulose enrichment of secondary walls. Indeed, as attested by the presence of two auxin-regulated expression elements in the *PrSus1* promoter (Fig. 4) and the downregulation of *PrSus1* in parasites growing on TIBA-treated tomato plants (Fig. 5), the present study reveals, for the first time, an *SUS*-encoding gene that acts as an auxin-responsive gene in xylem differentiation in plants. Interestingly, there is some evidence that auxin can also induce cellulose synthase (*CeSA*)-encoding genes in *Arabidopsis* and *Z. elegans* through the presence of auxin-response elements in the *CeSA* promoter (Goda et al. 2004; Wu et al. 2009). The hypothesis that auxin may induce both cellulose synthases and the collaborating *SUS* to promote cellulose production in maturing vascular tissues in plants has not yet been tested.

Within the host–parasite interaction, we demonstrated that *PrSus1* is under the control of host-derived auxin. Given that polar auxin transport participates in the formation of a continuous vascular strand (Sachs 1991) and that the parasite acts as a strong sink for auxin (Bar-Nun et al. 2008), the formation of a continuous vascular strand between the host and the parasite and the differentiation of xylem elements in the parasite are under the control of the polar auxin transport in the host plant (Bar Nun et al. 2008; Harb et al. 2004). Consequently, the disruption of the auxin relationships between host and parasite through *PrSus1* inhibition may prevent parasite development. No genetic transformation tools have been developed for broomrapes; therefore, reverse genetics cannot be carried out to confirm the roles of *PrSus1* in the parasite. However, nucleic acids can be transferred between host and parasite through the phloem (Davis and Wurdack 2004; Westwood et al. 2009) and parasitic plant gene expression can be silenced by modifying host genes (Aly et al. 2009; Tomilov et al. 2008). Thus, *PrSus1*-silencing experiments are potential avenues for exploring the involvement of *PrSus1* in the establishment of the host–parasite interaction.

MATERIALS AND METHODS

Plant materials, cultivation, treatments, and sampling.

Seed were collected from flowering spikes of *P. ramosa* L. Pomel in France (Saint Jean d'Angely station, 2005) and stored in darkness at 25°C until use.

For molecular analyses on *P. ramosa* seed, seed were surface-sterilized as described by Labrousse and associates (2001). They were preconditioned on sterile, watered, glass fiber filters placed in 9-cm-diameter petri dishes for 7 days in darkness at 21°C. Preconditioning was carried out in water. Germination was induced by adding GR24 (1 µg liter⁻¹), a synthetic germination stimulant (Johnson et al. 1976).

Two-week-old tomato seedlings, previously cultivated in petri dishes, were transplanted into hydroponic co-culture systems to facilitate confrontation between tomato roots and *P. ramosa* GR24-germinated seed, as described previously (Lejeune et al. 2006). Inhibition of IAA polar transport by TIBA in host tomato plants was carried out following Harb and associates (2004). TIBA in lanolin (10 mg g⁻¹ [wt/wt]) was used by pasting a ring on the base of tomato seedlings 4 weeks after the

transplanting date into the hydroponic co-culture systems. Plants pasted with lanolin alone served as controls. Developing *P. ramosa* tubercles were harvested after 3 weeks of treatment.

All the plant materials were immediately frozen in liquid nitrogen and stored at –80°C prior to RNA extraction. Tissue samples for histological purposes were extemporaneously harvested.

Sample preparation and immunofluorescence labeling.

Young tubercles (Tub. III) were fixed overnight at 4°C in a mixture of 3% (wt/vol) paraformaldehyde and 0.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Fixed samples were rinsed using buffer and deionized water prior to dehydration through successive baths in 30, 50, 70, 85, 95, and 100% ethanol solutions. Samples were successively impregnated with 80/20, 60/40, 40/60, 20/80, and 0/100 (vol/vol) ethanol/London Resin White (SPI Supplies/Structure Probe, Inc., West Chester, PA, U.S.A.). The impregnated samples were then embedded in gelatin capsules and polymerized for 24 h at 55°C.

Semithin resin sections (1 µm thick) were collected on multiwell glass slides pretreated with Vectabond (British BioCell International, Cardiff, U.K.). Sections were successively incubated at room temperature in a blocking solution (3% [wt/vol] bovine serum albumin [BSA] and 0.1 M Na-phosphate buffer saline [PBS], pH 7.2) for 30 min and in solution containing primary antibodies raised against a denatured *V. faba* *SUS* (dilution 1:100 [vol/vol]) (Ross and Davies 1992) for 1 h. Sections were then incubated for 1 h in a solution of goat anti-rabbit immunoglobulin G Alexa 488–secondary antibody conjugates (dilution 1:100 [vol/vol]) (Life Technologies Corporation, Carlsbad, CA, U.S.A.). Antibodies were diluted in PBS containing 1% (wt/vol) BSA and 0.05% (wt/vol) Tween-20. Controls were performed using pre-immune serum instead of anti-*SUS* antibody at the same dilution.

Sections were examined using a Leica DMRD microscope equipped with epifluorescence (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). A 450- to 490-nm band-pass filter was used as excitation light, the fluorescence being detected above 515 nm.

Preparation of digoxigenin-labeled RNA probes for *in situ* hybridization.

Digoxigenin (DIG)-labeled RNA probes were prepared using an *in vitro* transcription kit (Riboprobe Combination Systems; Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The riboprobes were synthesized from the full-length *PrSus1* clones. Antisense and sense probes were transcribed from SP6 or T7 RNA polymerase promoters after linearization of the vector with *Apal* or *NdeI*, respectively. Full-length probes were treated by alkaline hydrolysis, as described previously by Winzer-Serhan and associates (1999), thus producing 250-bp fragments.

Sample preparation and *in situ* hybridization.

Young tubercles (Tub.III) were fixed on ice under vacuum in 4% (wt/vol) paraformaldehyde in 1× PBS (pH 7) for 3 h and then kept in fixative buffer overnight at 4°C. Once fixed, samples were washed, dehydrated slowly with ethanol as previously described, exchanged with Histochoice (Sigma, Saint-Quentin Fallavier, France), then embedded in paraffin. Sections (10 µm) were mounted on Polysine slides (VWR International, Strasbourg, France) and dried at 37°C overnight. Paraffin was removed through two successive 10-min baths in Histochoice. After an additional 15-min bath in methanol, slides were progressively hydrated in ethanol series (100, 95, 70, 50, and 30%) and then treated with successive baths: water (2 min), 1×

PBS (2 min), 0.2 M HCl (20 min), water (2 min), 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (20 min), and water (2 min). Slides were treated with proteinase K (Sigma) at 10 µg ml⁻¹ in 1× PBS for 30 min at 37°C, incubated in 1× PBS for 2 min at room temperature, fixed in 3.7% formaldehyde for 10 min, acetylated using 0.5% acetic anhydride in 0.1 M triethanolamine for 10 min, and then rinsed in 1× PBS. Sections were dehydrated in ethanol series before hybridization. Sections were hybridized with equal concentrations (1 ng µl⁻¹) of either sense or antisense probes in 1× Denhardt's (Sigma), 10% dextran sulfate, tRNA at 200 µg ml⁻¹, 50% formamide, RNasin (Roche Diagnostics, Meylan, France) at 10 U ml⁻¹, 1× salts (10 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 1 mM EDTA), and incubated at 55°C overnight in a wet chamber. Sections were washed twice in 50% formamide in 1× SSC for 2 h at 55°C, rinsed in NTE (10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 5 mM EDTA), incubated with RNase A (Fermentas GmbH, Villebon-sur-Yvette, France) at 10 µg ml⁻¹ in NTE for 30 min at 37°C, washed three times in NTE for 5 min at 37°C, placed in 50% formamide in 0.5× SSC for 1 h at 55°C, and then washed at room temperature in 1× PBS. Sections were successively incubated at room temperature in a blocking solution (0.5% [wt/vol] blocking reagent [Roche Diagnostics] 0.3% [vol/vol] Triton X100, 100 mM Tris-HCl [pH 7.5], and 150 mM NaCl) for 45 min, in buffer A (100 mM Tris-HCl [pH 7.5] containing 1% [wt/vol] BSA, 0.3% [vol/vol] Triton X100, and 150 mM NaCl) for 45 min, probed with anti-DIG serum (Roche Diagnostics) diluted 1:1000 in buffer A for 1 h in a wet chamber, washed three times in buffer A for 20 min each, and then three times in detection buffer (100 mM Tris-HCl, pH 9.5, containing 50 mM MgCl₂ and 100 mM NaCl) for 5 min each. Sections were incubated in a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution at 20°C. After suitable color development, reaction was stopped by rinsing in water and sections were mounted in aquatex (Merck, Strasbourg, France).

Extraction of total RNA and cDNA preparation.

Frozen tissues were ground in liquid nitrogen and total RNA was extracted using the RNeasy plant mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Extracts were treated with DNase I enzyme (0.02 U µl⁻¹)

(New England Biolabs, Ipswich, MA, U.S.A.). Integrity of the total RNA was checked by electrophoresis on 1% (wt/vol) agarose gel. RNA was quantified spectrophotometrically (A260/280, NadoDrop Spectrophotometer ND-1000; Labtech International Ltd, Ringmer, U.K.). Using oligo (dT20) as a primer, cDNA was prepared from samples (0.5 µg) of total RNA using the Superscript II Reverse Transcriptase kit (Life Technologies Corporation, Carlsbad, CA, U.S.A.).

Molecular cloning of *P. ramosa* cDNAs.

The cDNAs isolated from a flowering shoot (FS.V) were used for PCR amplifications. Degenerate primers corresponding to highly conserved regions were designed (Table 1). After denaturation at 94°C for 5 min, amplification consisted of 35 cycles of 45 s at 94°C, 45 s at 55°C, and 1.5 min 30 s or 45 s at 72°C for *PrSus1*. A final step of elongation was done at 72°C for 5 min. The amplified DNA fragment was purified and cloned into a pGEM-T Easy vector (Promega). Recombinant plasmid DNA was prepared and then sequenced by GATC Biotech (Konstanz, Germany). Based on the initial *PrSus1* sequence information, new primers were generated for RACE of each fragment using the Generacer kit (Life Technologies Corporation). RACE products corresponding to the SUS-encoding gene were cloned and sequenced. To amplify full-length cDNA, specific primer pairs were designed (Table 1). For *PrIPT* and *PrCCD7* cloning, a similar strategy was used (Table 1).

Real-time qPCR.

The determined sequences were used to design gene-specific primers for real-time qPCR. Six primer pairs were designed (maximum length, 150 bp; optimal melting temperature, 60°C; GC percentage between 30 and 80%) with Primer Express 3.0 software (Applied Biosystems, Foster City, CA, U.S.A.) (Table 1). The selected primers underwent an extensive search using the BLAST tool to avoid any significant homology with other known nucleotide sequences.

qPCR using SYBR Green technology was carried out on the Applied Biosystems 7300 real-time PCR system. Each reaction contained 300 nM each primer, 5 µl of cDNA sample (approximately 5 ng of input RNA), and 2× Power SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 µl. Thermal cycling was performed using the following cycling

Table 1. Primer pairs used in this study^a

Gene	Primer	Forward and reverse primers 5'→3'	Size (bp)	Application
<i>PrSus1</i>	SUS fwd deg	GGN GTN SAN TTY CTN AAY MGN CA	1,570	Partial cDNA cloning
	SUS rev deg	ACN GTN ARN CCR AAN GCY TCR TA	...	
<i>PrIPT</i>	IPT fwd deg	GGH GCH ACH GGN DCN GGV AAR TC	295	...
	IPT rev deg	GCN TSN AYG WAN GAR TTN GAN CCD CC	...	
<i>PrCCD7</i>	CCD7 fwd deg	AAR GTK ATG AAR AAY GTK GCN AAY AC	455	...
	CCD7 rev deg	TCN GTG AAN GCC CAR TCR TGD ATC AT	...	
<i>PrSus1</i>	FL fwd Pr-SUS1	ACC TTC AAA GGC AAA ACT CTT CAT TTG CT	2,786	Full-length cDNA cloning
	FL rev Pr-SUS1	AAC AAG AAA CTG AAT TAT TTT ATT TAT ATT AAT AC	...	
<i>PrIPT</i>	Pr-IPT fwd	ATT CAT CAA CTC TTT CAT TAT ATA CC	1,223	...
	Pr-IPT rev	TTC TTT ACA AAT AAG TCA TAA TAA ATT GAA	...	
<i>PrCCD7</i>	Pr-CCD7 fwd	CAT CAC TCC CTT CGG AAC ATG CCA TGC TAA	2,164	...
	Pr-CCD7 rev	TTT GTG TTC TTG TAC ACA ACA ACG GAT	...	
<i>PrEF1α1</i>	Q-Pr-EF1 UP	TTG CCG TGA AGG ATC TGA AAC	63	Quantitative PCR
	Q-Pr-EF1 DOWN	CCT TGG CAG GGT CGT CTT TA	...	
<i>PrSus1</i>	Q-Pr-SUS1 UP	CTC TAC GCC AAG AGT CCA AAG C	64	...
	Q-Pr-SUS1 DOWN	TTC TGT CGC CAC CAA CTA TGA C	...	
<i>PrIPT</i>	Q-Pr-IPT UP	CCA CCA CCT GCT CGG AAT TA	68	...
	Q-Pr-IPT DOWN	GAG CCA TGT CGC GGA AAT	...	
<i>PrCCD7</i>	Q-Pr-CCD7 UP	TGG ACG TGG CGG CTA AAA	63	...
	Q-Pr-CCD7 DOWN	TCT TTG GAG GCA TCT TGA ACA CT	...	
<i>PrSus1</i>	Pr-SUS1-GenWalk1	CTG TGT GCT CTT GAG AAC TTC TTG AAA AGC ATG ATC	na	Promoter cloning
	Pr-SUS1-GenWalk2	GAG GCT TCA AAA TTC CTT TGC CAT GGG CTT CA	...	

^a Abbreviations: PCR = polymerase chain reaction and na = not applicable.

conditions: 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. All qPCR runs contained negative controls with no cDNA template. Specificity of the PCR amplification was checked using a heat dissociation protocol (from 60 to 95°C) after the final cycle of the PCR. The similar efficiency of the primer sets was checked by performing qPCR on several dilutions of first strands. Results were standardized to the housekeeping *PrEF1a1* (accession number HM219554) gene expression levels which displayed a low cycle threshold (C_t) variation (standard deviation = 0.55 C_t) for all samples and experimental conditions.

Isolation of the *PrSus1* promoter region.

The 5' flanking sequence of *PrSus1* was isolated by a PCR-based genome-walking procedure using the Universal Genome-Walker kit (Clontech, Saint-Quentin-en-Yvelines, France.). Genomic DNA was extracted from a flowering shoot (FS.V) using the DNeasy plant mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Two non-overlapping gene-specific primers were designed based on the *PrSus1* cDNA sequence (Table 1). Primary PCR amplification was conducted using the PrSUS1-GenWalk1 and an adaptor primer 1. Cycling conditions consisted of an initial denaturation for 2 min at 94°C; followed by 7 cycles of 25 s at 94°C, 50 s at 68°C, and 3 min at 72°C; then followed by 32 cycles of 25 s at 94°C, 50 s at 62°C, and 3 min at 72°C; with a final 10-min extension at 72°C. The secondary PCR amplification was performed with the PrSUS1-GenWalk2 and a nested adapter primer 2. Cycling conditions consisted of an initial denaturation for 2 min at 94°C; followed by 5 cycles of 25 s at 94°C, 50 s at 65°C, and 3 min at 72°C; then followed by 20 cycles of 25 s at 94°C, 50 s at 58°C, and 3 min at 72°C; with a final 10-min extension at 72°C. PCR products obtained from the secondary PCR amplification were cloned and sequenced as described above.

Sequence analysis.

Bioinformatics analyses were performed using Vector NTI 9.1.0 software (Life Technologies Corporation). Sequence homologies were verified against GenBank databases using BLAST programs. Promoter motif searches were carried out using the PLACE software (Higo et al. 1999). Phylogenetic analyses were conducted in MEGA4 software (Tamura and Akutsu 2007). Amino acid sequences were aligned with Clustal W.

Statistical analysis.

An analysis of variance was performed on the results from qPCR analyses with the development stage or treatment as the tested factor using SigmaPlot version 10.0. Means of three independent RNA isolations were tested at $P < 0.05$ (SNK test).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Parasitic Plant Connection website:
www.parasiticplants.siu.edu/Orobanchaceae/Orobanch.Gallery.html
 NCBI BLAST programs: www.ncbi.nlm.nih.gov/blast
 NIAS DNA Bank PLACE software: www.dna.affrc.go.jp