Proline Accumulation and *AtP5CS2* Gene Activation Are Induced by Plant-Pathogen Incompatible Interactions in *Arabidopsis*

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Submitted 22 September 2003. Accepted 17 November 2003.

Accumulation of free L-proline (Pro) is a typical stress response incited by osmotic injuries in plants and microorganisms. Although the protective role of Pro in osmotic stress is not well understood, it is thought to function as compatible osmolyte or as a scavenger of reactive oxygen species (ROS). Here we show that, in *Arabidopsis thaliana*, Pro biosynthesis can be activated by incompatible plant-pathogen interactions triggering a hypersensitive response (HR). Pro accumulates in leaf tissues treated with *Pseudomonas syringae* pv. *tomato* avirulent strains (*avrRpt2* and *avrRpm1*) but remains unchanged in leaves infected with isogenic virulent bacteria. Incompatible interactions lead to transcriptional activation of *AtP5CS2*, but not *AtP5CS1*, encoding the rate limiting enzyme in Pro biosynthesis pyrroline-5-carboxylate synthase (P5CS). *AtP5CS2::GUS* and *AtP5CS2::LUC* transgenes were induced inside and around the HR lesions produced by avirulent *Pseudomonas* spp. in transgenic plants. Pro accumulation was faster and stronger when stimulated by *avrRpm1* than by *avrRpt2*, and was compromised in the low-salicylic acid plants *NahG* and *eds5* when signaled through the RPS2-dependent pathway. In addition, Pro content and *AtP5CS2* expression were enhanced by ROS in wild-type plants, suggesting that ROS may function as an intermediate signal in *AtP5CS2*-mediated Pro accumulation.

Additional keyword: ProDH.

Higher plants can accumulate free L-proline (Pro) in response to different environmental stresses, such as drought and salinity (Delauney and Verma 1993; Rhodes et al. 1986), cold (Savoure et al. 1997; Xin and Browse 1998), or UV radiation (Saradhi et al. 1995). Pro accumulation is thought to function as a compatible osmolyte that stabilizes membranes and subcellular components (Delauney and Verma 1993; Rhodes et al. 1986), including the mitochondrial electron transport complex II (Hamilton and Heckathorn 2001). In addition, Pro is proposed to scavenge free radicals (Saradhi et al. 1995; Siripornadulsil et al. 2002; Smirnov and Cumbes 1989) and to ameliorate shifts in redox potential by replenishment of the NADP⁺ supply (Delauney and Verma 1993; Hare and Cress 1997).

A positive correlation between Pro accumulation and osmotic stress tolerance has been reported in several experimental systems (Gilmour et al. 2000; Nanjo et al. 1999). However, a stringent causal relationship between Pro accumulation and stress tolerance is not yet firmly established. Whereas some Pro-accumulating *Arabidopsis* mutants, such as *eskimo1*, display enhanced stress tolerance (Xin and Browse 1998), others, such as *sos1*, are hypersensitive to stress (Liu and Zhu 1997). Moreover, Pro accumulation may result in toxicity under specific metabolic conditions (Hellman et al. 2000; Mani et al. 2002).

In higher plants, Pro is synthesized from glutamate or ornithine and the first pathway is considered to be dominant under stress conditions (Delauney and Verma 1993; Roosens et al. 1999). In the glutamate pathway, Pro biosynthesis is initiated through phosphorylation and reduction of glutamate to glutamyl-5-semialdehyde (G5SA) by the bifunctional of Δ^1 -pyrroline-5-carboxylate synthase (P5CS). G5SA is converted to Pro through pyrroline-5-carboxylate (P5C) by the Δ^1 -pyrroline-carboxylate reductase (P5CR) (Delauney and Verma 1993; Hu et al. 1992). The γ -glutamyl kinase activity of P5CS represents the rate-limiting step in this pathway and is sensitive to feedback inhibition by Pro (Roosens et al. 1999; Zhang et al. 1995). Pro catabolism involves its oxidization into glutamate by a two-step reaction including two mitochondrial enzymes, proline dehydrogenase (ProDH) and P5C-dehydrogenase (P5CDH) (Deuschle et al. 2001; Kiyosue et al. 1996; Verbruggen et al. 1996).

In *Arabidopsis thaliana*, drought and salt stress differently activate the expression of two related P5CS genes: *AtP5CS1* (*At2g39800*) and *AtP5CS2* (*At3g55610*). *AtP5CS1* is responsible mainly for Pro accumulation during salt and drought stress. Expression of *AtP5CS1* is activated by an ABA-dependent signal transduction pathway and modulated by light and brassinosteroid (Ábrahám et al. 2003; Savoure et al. 1997; Strizhov et al. 1997; Yoshida et al. 1995). *AtP5CS2* transcription is slightly elevated by salt stress (Strizhov et al. 1997) and induced by cold through the CBF3 transcription factor (Gilmour et al. 2000). Pro catabolism is controlled by ProDH (*At3g30775*), whose transcription is activated by enhanced Pro levels and is repressed under osmotic stress conditions (Kiyosue et al. 1996; Verbruggen et al. 1996).

Infections with virulent races of *Pseudomonas syringae* lead to the development of disease in *Arabidopsis* plants (Whalen et al. 1991). In contrast, the perception of avirulent races of the bacterium stimulates a complex set of plant defenses leading to disease resistance. Thus, *A. thaliana* Col-0 plants expressing the *RPS2* and *RPM1* resistance (*R*) genes recognize, directly

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or indirectly, the bacterial avirulent products encoded by the *avrRpt2* or *avrRpm1* genes, respectively, activating gene-for-gene mediated disease resistance and hypersensitive response (HR) (Bent et al. 1994; Grant et al. 1995; Mindrinos et al. 1994).

Cross-talks between abiotic and biotic defense programs have been suggested (Genoud and Métraux 1999; Singh et al. 2002). Early common protective responses include alterations at plasma membrane ion channels, up-regulation of the oxidative metabolism producing reactive oxygen species (ROS), and calcium influx (Lamb and Dixon 1997; Mittler 2002). The importance of salicylic acid (SA) in pathogen-induced disease

resistance and hypersensitive cell death is well documented (Alvarez 2000; Delaney et al. 1994; Gaffney et al. 1993). Through the potentiation of oxidative burst, SA can control both biotic and abiotic defense programs (Borsani et al. 2001; Shirasu et al. 1997).

Using genetically defined pathosystems involving *A. thaliana* and *P. syringae* to either activate gene-for-gene disease resistance (avirulent pathogen; incompatible interaction) or produce disease (virulent pathogen; compatible interaction), we evaluated whether Pro metabolism is altered under biotic stress conditions. Here we demonstrate that Pro is accumulated specifically in *Arabidopsis* leaf tissues developing incompatible interactions involving *avrRpt2:RPS2* or *avrRpm1:RPM1* recognition and leading to HR. This response involves the transcriptional activation of *AtP5CS2* but not *AtP5CS1*, and it can be induced by ROS. Moreover, Pro accumulation signaled through the RPS2 pathway depends on SA as well.

RESULTS

Pro accumulates in *Arabidopsis* tissues developing incompatible interactions.

Expanded leaves of *A. thaliana* Col-0 plants were infiltrated at two discrete sites with approximately 10 μ l of bacterial suspensions (5×10^6 CFU/ml) of isogenic virulent (*vir*) and avirulent (*avrRpt2* and *avrRpm1*) races of *P. syringae* pv. *tomato* DC3000 or dilution solution (mock inoculation). Bacteria were diluted in 0.4 M sucrose instead of the 10 mM MgCl₂ classically used for such purpose (Alvarez et al. 1998; Nawrath and Métraux 1999). Infiltration of 10 mM MgCl₂ alone increased the osmotic potential of leaf tissues from -3.71 ± 0.75 Mpa to -1.89 ± 0.11 Mpa and produced a slight increase in Pro content in those tissues (1.5-fold; 1 to 3 h post-infection [hpi]) (data not shown). In contrast, infiltration of 0.4 M sucrose altered neither the osmotic potential (-3.15 ± 0.58 Mpa) nor the Pro levels in inoculated tissues, when tested at 24, 48, and 72 h after infiltration. Pathogens infiltrated in this solution produced the typical HR (*avrRpt2* and *avrRpm1*) or disease (*vir*) symptoms in *A. thaliana* Col-0 plants (Alvarez et al. 1998; Bent et al. 1994; Grant et al. 1995; Mindrinos et al. 1994) (Fig. 1B). Moreover, sucrose infiltration did not modify the basal expression pattern of Pro-metabolic genes at these time points (Fig. 2).

The average of Pro levels of individual untreated leaves from 5- to 6-week-old plants was 46.5 ± 15.6 μ g per g of fresh weight (Fig. 1A). Pro levels started to increase at 12 hpi with *avrRpm1* and at 24 hpi with *avrRpt2*. Such Pro enhancement preceded the macroscopic detection of net HR lesions in the challenged tissues, which became visible at 24 hpi with *avrRpm1* (data not shown) or 48 hpi with *avrRpt2* (Fig. 1B). The *avr*-treated tissues accumulated Pro until 72 hpi, reaching 7- and 12-fold increase over the basal values when inoculated with *avrRpt2* or *avrRpm1*, respectively. By 5 days postinfection (dpi), both responses declined and leaves displayed net signs of cellular collapse (data not shown). In contrast, Pro levels remained essentially unchanged until 5 dpi in plants treated with either *vir* or sucrose (Fig. 1A). These results indicated that Pro accumulation is elicited specifically upon R or avirulence (avr) recognition and that kinetic and quantitative differences are displayed when the response is achieved by stimulation of the RPM1- or RPS2-dependent pathways.

Local differences in Pro content were analyzed at and near the infiltration sites for *avrRpt2* treatments. Bacterial suspensions or 0.4 M sucrose were infiltrated as spots of 3 to 4 mm in diameter. Infiltrated and noninfiltrated tissues from the same leaves were excised separately in 9- to 12-mm-diameter

Fig. 1. Free L-proline (Pro) accumulates in *Arabidopsis* leaves treated with avirulent races of *Pseudomonas*. **A**, Pro levels in entire leaves, infiltrated at two spot sites with 0.4 M sucrose (suc), or bacterial suspensions (5×10^6 CFU/ml) of virulent (*vir*) or avirulent (*avrRpm1* and *avrRpt2*) races of *Pseudomonas syringae* pv. *tomato*. Values represent the average $\pm$ standard deviation of three independent experiments including at least five leaves per treatment. **B**, Typical leaf symptoms developed by wild-type and *NahG* plants after infiltration of the *avrRpt2* bacterial suspensions used for Pro determination; hpi = hours postinfiltration. **C**, Local Pro accumulation in *avrRpt2*-treated *Arabidopsis* leaves. Bacterial suspensions were infiltrated at spots of 3 to 4 mm in diameter. At 72 hpi, 9- to 12-mm-diameter discs containing infiltrated (central) or noninfiltrated (periphery) areas were excised from the same leaves for Pro determination as indicated in the diagram.

leaf discs and used for Pro quantification. At 72 hpi, only leaf samples taken from the *avrRpt2*-infiltrated areas displayed a net increase in Pro content, whereas the noninfiltrated tissues had essentially unaltered Pro concentration (Fig. 1C). The *vir*- and sucrose-inoculated samples showed no differences in Pro content at 72 hpi (data not shown).

Expression of Pro metabolism genes in pathogen-inoculated tissues.

Pro levels are tightly controlled by biosynthetic and catabolic activities. To monitor the Pro biosynthetic and catabolic pathways during pathogen infection, we analyzed the transcription levels of the *A. thaliana* *AtP5CS1* and *AtP5CS2* genes controlling the glutamate biosynthetic pathway, and the *ProDH* and *P5CDH* genes participating in Pro degradation. Northern blot experiments revealed that *AtP5CS2*, but not *AtP5CS1*, was activated by *avrRpt2* inoculation (Fig. 2) and reached up to eightfold increased levels at 72 hpi. Such *AtP5CS2* activation paralleled Pro accumulation in *avrRpt2*-treated tissues (Fig. 1A). In contrast, *AtP5CS2* expression was almost unaffected by *vir* or mock inoculations (Fig. 2). On the other hand, *AtP5CS1* expression was not altered by any of these treatments (Fig. 2). Upon *avrRpt2* inoculation, *ProDH* transcript levels increased 3 to 4 times from 0 to 72 hpi, while no alterations were observed in *vir*- or mock-inoculated leaves (Fig. 2). In addition, *P5CDH* expression was not modified in *vir*-, *avrRpt2*-, or sucrose-infiltrated *Arabidopsis* leaves (Fig. 2). These results suggest that Pro accumulation in HR developing tissues principally derives from the induction of *AtP5CS2*.

In order to localize *AtP5CS* activation in response to *P. syringae* pv. *tomato* inoculation, transgenic *Arabidopsis* plants expressing the β -glucuronidase (*GUS*) or firefly luciferase (*LUC*) reporter genes under the control of *AtP5CS1* and *AtP5CS2* promoters (Ábrahám et al. 2003) were infiltrated by *vir* and *avrRpt2*. The spatiotemporal pattern of *GUS* and *LUC* activities were analyzed along with development of disease or HR. Very low basal *GUS* activity was detected in naive *AtP5CS2:GUS* plants (Fig. 3A), which is consistent with previous observations about the low abundance of *AtP5CS2* transcripts in leaf tissues (Strizhov et al. 1997). *AtP5CS2:GUS* expression was activated in leaves infiltrated with the avirulent pathogen (Fig. 3A). This response initially was detected by 24

Fig. 3. Spatiotemporal pattern of *AtP5CS1* and *AtP5CS2* expression in hypersensitive response. Transgenic *Arabidopsis* plants containing the *AtP5CS1* and *AtP5CS2* gene promoters fused to the β -glucuronidase (*GUS*) or firefly luciferase (*LUC*) reporter genes were inoculated at two spot sites with 0.4 M sucrose (suc), or bacterial suspensions (5×10^6 CFU/ml) of virulent (*vir*) or avirulent (*avrRpm1* and *avrRpt2*) races of *Pseudomonas syringae* pv. *tomato*. **A**, *GUS* activity was detected by X-gluc histochemical staining at 48 h postinfiltration. **B**, *LUC* expression was monitored by luminescence imaging at the indicated times after infiltration. Yellow lines indicate the borders of lesions.

Fig. 2. *AtP5CS2* and *ProDH* are activated by infiltration of avirulent bacteria. Leaves were infiltrated at two spot sites with 0.4 M sucrose (suc), or bacterial suspensions (5×10^6 CFU/ml) of virulent (*vir*) or avirulent (*avrRpm1* and *avrRpt2*) races of *Pseudomonas syringae* pv. *tomato*. **A**, Northern blot analysis of genes *AtP5CS1*, *AtP5CS2*, *ProDH*, and *P5CDH* on total RNA samples isolated from *avrRpt2*-infiltrated leaves. Hybridization with 5S rRNA was used as loading control. **B**, Relative values of the *AtP5CS1*, *AtP5CS2*, *ProDH*, and *P5CDH* transcript levels detected by Northern hybridization. Values were normalized to 5S rRNA content on each well.

hpi at the inoculation sites and enhanced by 48 and 72 hpi, when it often was visible in neighboring tissues surrounding the infiltration sites. The *vir* infiltration occasionally led to *AtP5CS2::GUS* activation, whereas mock inoculations did not activate *AtP5CS2::GUS* over this period. Identical treatments were performed with transgenic plants carrying the *AtP5CS1::GUS* construct, whose expression was not induced by any *P. syringae* pv. *tomato* inoculation (Fig. 3A).

AtP5CS1 and *AtP5CS2* promoter activities also were monitored in living tissues of transgenic plants expressing the firefly luciferase gene fusions, where images of luminescence could be compared directly with the development of symptoms (Fig. 3B). LUC activity was first detected in *avrRpt2*-treated leaves of *AtP5CS2::LUC* plants at 24 hpi at the center of the infiltration site. One or two days later, LUC activity usually was localized in infiltrated tissues or sometimes shifted to

regions surrounding the HR lesions (Fig. 3B). LUC activity was not detected far from the infiltrated regions of leaves treated with *avrRpt2*.

AtP5CS1::LUC transgenic plants had no increased luminescence upon bacterial inoculation. These plants occasionally showed LUC activity in necrotic, dry, or mechanically damaged tissues that had no correlation with the type of infiltration. These results confirmed that *AtP5CS2* is the major gene activated at the transcriptional level by plant–pathogen incompatible interactions around the infection site.

SA and ROS modulate Pro accumulation.

SA is known to be required for the proper activation of local and systemic pathogen-induced defense networks (Alvarez 2000). Transgenic *NahG* plants are depleted in SA by overexpression of salicylate hydroxylase, which oxidizes SA into catechol. These plants are more susceptible to pathogen attack and are unable to develop confined HR lesions or activate systemic acquired resistance (Alvarez 2000; Delaney et al. 1994; Gaffney et al. 1993) (Fig. 1B). The *eds5* mutant fails to accumulate SA after pathogen attack and EDS5, homologue to multidrug and toxin extrusion transporters, is suspected to be involved in SA biosynthesis (Nawrath and Métraux 1999). In order to test whether *avr*-induced Pro enhancement depends on SA accumulation, Pro levels were quantified in *avrRpt2*- or *avrRpm1*-infiltrated *NahG* and *eds5* plants, which developed cell death in response to these treatments (Fig. 1C; data not shown). Similar basal Pro levels were detected in leaf tissues of *NahG*, *eds5*, and wild-type plants (Fig. 4A). *NahG* and *eds5* plants were compromised in Pro accumulation elicited by *avrRpt2*, reaching only 28% (*NahG*) or 44% (*eds5*) of the Pro levels found in wild-type plants at 72 hpi (Fig. 4A). *AtP5CS2* expression was not induced in *NahG* plants upon perception of *avrRpt2*, as happened in wild-type plants (Fig. 2), and neither was this gene activated in response to the *vir* pathogen (Fig. 4B). On the other hand, *AtP5CS1* expression was not modified by *avrRpt2*, *vir*, or mock inoculations in *NahG* plants (data not shown). Interestingly, *NahG* and *eds5* plants only weakly suppressed the Pro accumulation activated by *avrRpm1* recognition, containing 88% (*NahG*) and 72% (*eds5*) of the levels detected in wild-type plants at 72 hpi (Fig. 4A). These results indicated that the stimulation of a unique R gene-dependent pathway activates similar responses in both low-SA plants and that each pathway is differently affected by SA deficiency to signal Pro accumulation.

The requirement of SA for producing a sustained enhancement of Pro levels and *AtP5CS2* activation in RPS2-mediated incompatible interactions prompted us to analyze whether SA itself induced such responses. Wild-type plant leaves were infiltrated with 10, 50, and 150 μ M SA and Pro content was determined at 0, 3, 6, and 9 hpi. The SA concentrations utilized in these treatments were in the same range found in *A. thaliana* leaf tissues developing incompatible interactions (Bi et al. 1995). A transient Pro increase (fourfold) was detected at 6 hpi with 50 and 150 μ M SA, but this response was not sustained by 9 hpi (data not shown). Thus, exogenous SA treatments were not able to reproduce the Pro accumulation effect generated by *avrRpt2* infiltration. When *AtP5CS2::GUS* or *AtP5CS2::LUC* plants were infiltrated with 150 μ M SA, only faint or background activity of the reporter genes was detected in treated leaves at 7 hpi (Fig. 5C and D).

SA potentiates the oxidative burst and hypersensitive cell death in genetically defined disease resistance (Lamb and Dixon 1997; Shirasu et al. 1997). Therefore, we evaluated whether externally generated ROS can modulate Pro accumulation. *Arabidopsis* leaves were infiltrated locally with the enzymatic mix of xanthine and xanthine oxidase (X-XO) that

Fig. 4. Requirement of salicylic acid (SA) for L-proline (Pro) accumulation induced through the RPM1- or RPS2-dependent pathways. **A**, Pro accumulation in entire leaves of wild-type, *NahG*, and *eds5* *Arabidopsis* plants infiltrated with *avrRpm1* (upper panel) or *avrRpt2* (lower panel). Leaves were infiltrated at two spot sites with 0.4 M sucrose, or bacterial suspensions (5×10^6 CFU/ml) of virulent (*vir*) or avirulent (*avr*) races of *P. syringae* pv. *tomato*. Samples were collected at the indicated times. hpi = hours postinfiltration. Values represent the average of three independent experiments. **B**, Northern blot assays developed with *AtP5CS2* probes in *NahG* leaves infected with *vir* or *avrRpt2* (*avr*) bacteria.

produces superoxide anion in situ (Jabs et al. 1996). Upon X-XO treatment, a progressive and sustained increase in Pro content occurred during the initial 9 hpi (Fig. 5A). Moreover, positive correlation was found between Pro levels and concentrations of xanthine oxidase enzyme employed in infiltration (Fig. 5B). These results indicated that leaf tissues can sensitively increase their Pro content upon ROS accumulation. Histochemical GUS assay detected *AtP5CS2:GUS* activity in leaf tissues infiltrated with X-XO (Fig. 5C). Similar results were obtained with transgenic *AtP5CS2:LUC* plants, where luminescence could be detected at X-XO infiltration sites (Fig. 5D). These results indicated that ROS can mediate the activation of *AtP5CS2* promoter in situ and eventually function during biotic stress.

DISCUSSION

Pro content increases in *Arabidopsis* leaf tissues developing incompatible interactions with *P. syringae* pv. *tomato*.

In this work, we describe that Pro accumulation, a typical plant osmotic stress response, also occurs in response to biotic stress. Our results demonstrate that free Pro content increases in *Arabidopsis* leaf tissues that activate an HR upon recognition of avirulent races of *P. syringae* pv. *tomato* (*avrRpt2* and *avrRpm1*). Pro accumulation is detected as early as 12 to 24 h after treatment with avirulent bacteria, and the magnitude of increase (7- to 12-fold at the third day) is comparable with that reported for abiotic stresses (Delauney and Verma 1993; Kishor et al. 1995; Roosens et al. 1999). These values are not substantially exceeded by transgenic plants designed to over-produce Pro by manipulation of Pro metabolic genes (Nanjo et al. 1999) or by some of the *Arabidopsis* mutants that constitutively accumulate this imino acid (Xin and Browse 1998). On the other hand, Pro content was not enhanced in *Arabidopsis* leaves developing necrosis in response to virulent *Pseudomonas* infection.

Biosynthesis and catabolism of Pro are tightly regulated processes involving two key enzymatic activities, P5CS and ProDH (Delauney and Verma 1993, Hare and Cress 1997). In *A. thaliana*, P5CS contributes to Pro accumulation under abiotic stress conditions mainly through ABA-dependent transcriptional activation of the *AtP5CS1* gene (Savouré et al. 1997; Strizhov et al. 1997; Yoshiba et al. 1995). *ProDH*, which controls Pro catabolism, becomes transcriptionally activated by enhanced Pro levels, but can be repressed during osmotic stress, permitting Pro accumulation under such conditions (Kiyosue et al. 1996; Verbruggen et al. 1996). Our data showed that transcription of *AtP5CS2* and, to a lesser extent, *ProDH*, was induced in response to the avirulent but not to the virulent pathogens, whereas *AtP5CS1* and *P5CDH* transcription did not change under these conditions (Fig. 2).

The *avrRpt2*-induced Pro accumulation correlates with the increase in *AtP5CS2* transcript levels during 72 hpi, suggesting enhancement of the biosynthetic pathway in the infected leaves through *AtP5CS2* gene activation. *ProDH* expression also is induced over this period (Fig. 2); however, the continuous rise in Pro content in *avr*-treated tissues (Fig. 1) would suggest that Pro biosynthesis is more effective than Pro catabolism at this stage. In *avrRpt2*-infiltrated leaves, *ProDH* transcription probably was activated by the accumulating Pro, as happens in nonstressed plants in the absence of osmotic repression (Kiyosue et al. 1996; Verbruggen et al. 1996). Therefore, our findings suggest that, under biotic stress conditions, Pro levels are controlled by transcriptional regulation of *AtP5CS2* and *ProDH* genes. Further studies will be required to reveal possible modifications in the activity of P5CS and ProDH enzymes in response to pathogen attack.

ABA-responsive *AtP5CS1* is the major gene induced under osmotic stress (Strizhov et al. 1997; Yoshiba et al. 1995) but does not seem to be responsible for the increased biosynthesis of Pro in *avrRpt2*-treated plants. *AtP5CS2* is moderately activated by cold or osmotic stress and previously has been associated with cell division events and housekeeping functions (Ábrahám et al. 2003; Gilmour et al. 2000; Strizhov et al. 1997). Therefore, specific activation of *AtP5CS2* during plant-pathogen incompatible interaction describes a novel function of this gene in plant defense.

Pro is considered a compatible osmolyte in osmotic stress conditions (Delauney and Verma 1993), and has been suggested to function as a scavenger of hydroxyl radicals, controlling redox homeostasis as well (Hare and Cress 1997; Siripornadulsil et al. 2002; Smirnov and Cumbes 1989). Our results show that

Fig. 5. Reactive oxygen species accumulation mediates L-proline (Pro) enhancement and *AtP5CS2* activation. **A**, Pro accumulation in entire *Arabidopsis* leaves locally infiltrated either with 1 mM xanthine (X), or 1 mM xanthine and xanthine oxidase (X-XO) at 0.5 U/ml. **B**, Lineal dependence of Pro response on a concentration range of xanthine oxidase able to signal hypersensitive cell death on *Arabidopsis* leaves (Jabs et al. 1996). Pro levels were measured at 9 h postinfiltration. **C**, Histochemical detection of β -glucuronidase (GUS) activity in *AtP5CS2:GUS* *Arabidopsis* leaves locally infiltrated with 1 mM xanthine (X), 1 mM xanthine and xanthine oxidase (X-XO) at 0.5 U/ml, or 150 μ M salicylic acid (SA). GUS activity was analyzed at the indicated times. hpi = hours postinfiltration. **D**, Luciferase imaging of transgenic *AtP5CS2:LUC* *Arabidopsis* leaves treated with X-XO or SA as described above and analyzed at 7 hpi.

Pro accumulation occurs in and around the HR lesion (Fig. 1C). Scavenger properties of Pro may have importance in protecting pathogen-infected plant tissues once increased amount of ROS are generated. In HR-developing tissues, only *AtP5CS2* and *ProDH* transcript levels were enhanced, suggesting that Pro biosynthetic and catabolic pathways are induced in the absence of *P5CDH* activation. Enhanced Pro turnover can lead to P5C accumulation when ProDH and P5CDH activities are uncoupled (Deuschle et al. 2001; Hellmann et al. 2000). Interconversion of P5C and Pro can influence NADP⁺ or NADPH levels (Hare and Cress 1997) and, therefore, might influence the extension of oxidative damage in HR-developing tissues. Moreover, P5C could activate apoptotic cell death under certain circumstances (Hellmann et al. 2000; Maxwell and Davis 2000). Further experiments are required to investigate putative functions of Pro and P5C in biotic stresses leading to HR development.

Plant signals involved in Pro accumulation and *AtP5CS2* activation.

A substantial accumulation of Pro was detected from 12 to 24 hpi with *P. syringae* pv. *tomato* avirulent pathogens that activate either the RPM1- or the RPS2-dependent defense pathways (Fig. 1A). Pro increase occurs in strict dependence on R and avr recognition for at least 5 days after pathogen inoculation (Fig. 1A). This response is earlier and more strongly achieved when it is induced by the RPM1 pathway. Likewise, gene expression profile changes and HR development are faster and more strongly induced by the RMP1 compared with the RPS2 pathway (Ritter and Dangel 1996; Tao et al. 2003). Pro accumulation may result from the activation of still unknown signaling components common to both defense cascades, which are proposed to share major signal transduction pathways (Tao et al. 2003).

Thus, up-regulation of Pro biosynthesis may require specific signaling factors of the RPM1- or RPS2-dependent pathways leading to disease resistance and HR. It is known that RPM1-induced defenses are less affected by SA deficiency than those signaled through RPS2. *NahG* plants depleted in SA reduce more severely the RPS2- than RPM1-mediated resistance and development of hypersensitive cell death (Rate and Greenberg 2001; Tao et al. 2003). Similarly, R-gene mediated resistance, hypersensitive cell death, and global gene expression changes are reduced in a stronger manner for the RPS2 pathway in the *ndr1* mutant which is impaired in pathogen-induced SA accumulation (Tao et al. 2003). Therefore, we characterized features of RPM1- and RPS2-mediated Pro accumulation in two types of SA-deficient plant, the transgenic *NahG* line and the *eds5* mutant. In these plants, Pro accumulation clearly was reduced when generated via RPS2 but almost unaffected for its RPM1-dependent activation. Thus, Pro accumulation displays similar differences in SA sensitivity to the classical defense responses described above, suggesting that it may involve common signaling components.

Pro accumulation was similarly reduced in *NahG* and *eds5* plants during RPS2-dependent activation (Fig. 4). Thus, irrespective of whether lack of SA is caused by impaired SA biosynthesis in *eds5* (Nawrath and Métraux 1999) or by SA degradation in *NahG* (Delaney et al. 1994), reduction of RPS2-dependent Pro accumulation in both plants must result from SA deficiency. Some *NahG* phenotypes were, in contrast, proposed to be caused by the accumulation of catechol instead of SA deficit (van Wees and Glazebrook 2003).

Our results showed that exogenous SA triggers only a limited accumulation of Pro and no or only weak activation of *AtP5CS2* in wild-type plants (Fig. 5C and D). These findings suggest that, during induction of the RPS2-mediated re-

sponses, SA is important but not sufficient to activate *AtP5CS2* transcription and Pro enhancement. The signal flow for RPS2-mediated defenses could be weaker and probably more sensitive to SA than that involving RPM1 (Tao et al. 2003). SA might function then by activating early steps in RPS2-dependent responses but requiring additional signals to maintain enhanced Pro levels. Transcriptional activation of *AtP5CS2* by *avrRpt2* mainly occurs in and around the HR lesion (Fig. 3), where SA may reach its higher levels in the challenged leaves (Enyedi et al. 1992). Further studies will be required to elucidate whether SA locally contributes to activate *AtP5CS2*-mediated Pro enhancement in HR.

The requirement of SA for proper activation of local defenses and HR has been extensively characterized (Alvarez 2000; Delaney et al. 1994). SA can potentiate, among other HR components, the early development of the oxidative burst preceding hypersensitive cell death (Shirasu et al. 1997). Looking for SA-dependent HR components that may contribute to signal Pro accumulation in response to *avrRpt2*, we evaluated the effect of ROS. We found that both Pro levels and *AtP5CS2* promoter activity were enhanced by an external treatment generating superoxide anion in *Arabidopsis* leaves. Thus, the localized oxidative burst triggered at *avr*-treated tissues may positively modulate *AtP5CS2* activation and Pro accumulation. Generation of ROS is a common plant response to pathogen infection and to different adverse environmental conditions such as high light, UV-B irradiation, osmotic stress, soil salinity, cold, or high temperatures (Lamb and Dixon 1997; Shinozaki and Yamaguchi-Shinozaki 2000). ROS levels in photosynthetic tissues are controlled by SA during salt and osmotic stresses (Borsani et al. 2001). Therefore, common elements of pathogen defense and abiotic stress responses can be modulated by ROS.

Differences in transcriptional regulation of *AtP5CS1* and *AtP5CS2* suggest that alternative mechanisms regulate Pro biosynthesis in biotic and abiotic stress conditions. Although osmotic stress leads to *AtP5CS1* induction and *ProDH* repression through an ABA-mediated signal transduction pathway, incompatible pathogen interactions result in SA-dependent or independent *AtP5CS2* activation, probably involving ROS as intermediate signal.

MATERIALS AND METHODS

Plant material.

A. thaliana wild-type, *eds5* (Nawrath and Métraux 1999), and *NahG* (Delaney et al. 1994) plants of the Columbia ecotype (Col-0) (genotype *RPS2/RPS2* and *RPM1/RPM1*) were used in this study. Promoter analysis was performed with *A. thaliana* Col-0 transgenic plants carrying the 2.5- or 1.8-kb promoter fragments of *AtP5CS1* or *AtP5CS2* genes, respectively, fused to the *uidA* (*GUS*) (Ábrahám et al. 2003) or firefly luciferase (*LUC*) reporter genes. Plants were maintained in growth chambers under a photoperiod with 12 h of light and 12 h of dark at 22°C and watered every 2 days.

Plant infiltration.

Plants were used for infiltration at the age of 5 to 6 weeks. The virulent strain *P. syringae* pv. *tomato* DC3000 (Whalen et al. 1991) and the isogenic avirulent races *avrRpt2* (Innes et al. 1993) and *avrRpm1* (Ritter and Dangel 1996) were used for bacterial infiltration. Bacteria were grown overnight in King's B medium supplemented with antibiotics and further used to infiltrate fully expanded leaves at a concentration of 5×10^6 CFU/ml as described (Alvarez et al. 1998). Plants developing net HR lesions (*avr*) or disease symptoms (*vir*) were selected for further studies. To generate superoxide anion accumulation

into intercellular spaces of leaf tissues, a mix of xanthine (1 mM) and xanthine oxidase (0.25 to 0.75 U/ml) was utilized as described (Jabs et al. 1996). Experiments were repeated three times.

Determination of Pro content.

Free proline content in leaf tissues was measured by colorimetric methods (Bates 1973). Values were referred to L-proline (Sigma, St. Louis) calibration curves. Six entire leaves were used for each measurement. Values represent the average of at least three independent experiments.

Measurement of osmotic potential.

The osmotic potential of leaf tissues infiltrated with 10 mM MgCl₂ or with 0.4 M sucrose was determined by psychrometry. Infiltrated or uninfiltrated leaf tissues were pressed onto filter paper discs which immediately were sealed in C-52 Wescor sample chambers connected to an HR-33 T dew point Wescor microvoltmeter. Readings were taken at regular intervals until a stable value was obtained, indicating equilibrium. Voltage readings were referred to a standard curve made from a series of NaCl solutions.

RNA hybridizations.

Total RNA was prepared according to Alvarez and associates (1998) and 10 µg of each sample was used to monitor transcript accumulation by RNA blot hybridization. *AtP5CS1* and *AtP5CS2* probes were obtained by polymerase chain reaction (PCR) using specific primers pairs (Ábrahám et al. 2003). DNA probes for the *Arabidopsis ProDH* and *P5CDH* genes were PCR amplified with gene-specific primers 5'-ACAGCGATAAACCGAAACATC-3' and 5'-TAGCCACAGGAAATGTTCTTACC-3' (*ProDH*), and 5'ATGTTGGAGCATATGG-3' and 5'-GTGACGAGTTCGTAGG-3' (*P5CDH*). Northern blots were hybridized with radiolabeled cDNA of 5S rRNA as loading control. Signals were detected by Phosphor-Imager and quantified by NIH Image computer software. Experiments were repeated three times.

GUS and LUC activity.

Plants were grown and infiltrated as described above. GUS expression was monitored in *AtP5CS1::GUS* or *AtP5CS2::GUS* plants at 24, 48, 72, and 96 hpi by X-Gluc histochemical staining (Ábrahám et al. 2003). In order to detect bioluminescence signals, transgenic plants carrying the *AtP5CS1::LUC* or *AtP5CS2::LUC* constructs were sprayed with 1 mM luciferin solution and light emission was detected by a CCD camera (Visilux Imager, Visitron Systems GmbH, Mannheim, Germany) using 20 min of integration time. LUC expression was monitored at the same time points as those of GUS. Luminescence images were processed by Metaview 4.5r6 software.

ACKNOWLEDGMENTS

This work was supported by collaboration grant number ARG-7/99 of OMFB-Hungary/SETCyP-Argentina to M. E. Alvarez and L. Szabados; grants from Fundación Antorchas, SECyT-UNC, and Agencia Nacional de Promoción Científica y Tecnológica (BID 1201/OC-AR PICT 01-10123) to M. E. Alvarez; EU 5th Framework Project OPTI-2, QLRT-2000-00103 to L. Szabados; and OTKA grant no. T029430 and T-032428 to L. Szabados. We thank L. Ortega and E. Taleisnik for help with measurement of osmotic potential, A. Király and C. Mas for technical assistance, J. P. Métraux for *eds5* seeds, J. Dangel for *P. syringae* pv. *tomato* isolates, and E. Taleisnik and C. Koncz for critical reading of the manuscript.

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