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Salicylic acid differentially affects suspension cell cultures of *Lotus japonicus* and one of its non-symbiotic mutants

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Abstract Salicylic acid (SA) is known to play an important role in the interaction between plant and micro-organisms, both symbiotic and pathogen. In particular, high levels of SA block nodule formation and mycorrhizal colonization in plants. A mutant of *Lotus japonicus*, named *Ljsym4-2*, was characterized as unable to establish positive interactions with *Rhizobium* and fungi (NOD^-, MYC^-); in particular, it does not recognize signal molecules released by symbiotic micro-organisms so that eventually, epidermal cells undergo PCD at the contact area. We performed a detailed characterization of wild-type and *Ljsym4-2* cultured cells by taking into account several parameters characterizing cell responses to SA, a molecule strongly involved in defense signaling pathways. In the presence of 0.5 mM SA, *Ljsym4-2* suspension-cultured cells reduce their growth and eventually die, whereas in order to induce the same effects in wt suspension cells, SA concentration must be raised to 1.5 mM. An early and short production of nitric oxide (NO) and reactive oxygen species (ROS) was detected in wt-treated cells. In contrast, a continuous production of NO and a double-peak ROS response, similar to that reported after a pathogenic attack, was observed in the mutant *Ljsym4-2* cells. At the molecular level, a constitutive higher level of a SA-inducible pathogenesis related gene was observed. The analysis in planta revealed a strong induction of the *LjPR1* gene in the *Ljsym4-2* mutant inoculated with *Mesorhizobium loti*.

Keywords *Lotus japonicus* cell cultures · Salicylic acid · Cell death · H_2O_2 · NO

Introduction

Plant-interacting microbes differ with respect to the nature of the response that they elicit in their respective hosts. In an incompatible plant-pathogen interaction, the host plant induces a defense response—be it the hypersensitive response (HR), systemic acquired resistance or both—that limits pathogen invasion and spreading. However, in the case of symbiotic bacteria, such as those of the genus *Rhizobium*, an obvious defense response is usually not elicited. Instead, a beneficial relationship is established that results in nodule formation and atmospheric nitrogen fixation.

An emerging picture from recent studies indicates that legumes utilize similar mechanisms to recognize pathogens and symbiotic microbes (Miya et al. 2007; Wan et al. 2008). Both *rhizobia* and successful pathogens suppress plant defenses when establishing an infection. Plant defense-like phenotypes are induced by legumes or by bacterial mutants unable to carry out an efficient nodulation program (Carlson et al. 1987; Campbell et al. 2002; Veershlingam et al. 2004). In the same way, normally, after initial nodule formation, the host inhibits the progress of

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This report is dedicated to the memory of Prof. M. Terzi.

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additional rhizobial infections in order to limit nodule number, and this response leads to an HR-like response at the sites of aborted infection threads in *Medicago sativa* roots (Vasse et al. 1993). NO production in *Lotus* is induced early and transiently after *Mesorhizobium loti* inoculation (Shimoda et al. 2005; Nagata et al. 2008). At the molecular level, these defense-like phenotypes are associated to a transcript profiling showing an early transient up-regulation of several defense and stress-response genes after *Rhizobium* inoculation (Kouchi et al. 2004; Lohar et al. 2006).

Salicylic acid (SA) is a phenolic compound, which plays important roles in plant physiology. SA has been shown to be a key molecule in plant disease resistance, involved in the induction of both HR and systemic acquired resistance (Durner et al. 1997; Feys and Parker 2000). SA, when exogenously added to plants, can enhance defense gene induction (i.e., *PR1)*, the production of hydrogen peroxide (H_2O_2), and cell death (Draper 1997); moreover, its levels can also affect interactions between plants and symbiotic micro-organisms. Exogenous SA addition inhibited indeterminate nodulation of *Vicia sativa* (van Spronsen et al. 2003), and in plants expressing the bacterial *NahG* gene (encoding salycilate hydroxylase), where SA was not accumulated, both infection and nodulation were significantly increased (Gaffney et al. 1993; Stacey et al. 2006). Moreover, Medina et al. (2003) found that tobacco plants expressing *NahG* showed enhanced mycorrhizal fungal infection, whereas plants constitutive for SA expression exhibited reduced infection.

Nodulation in legumes is activated in response to rhizobial signaling molecules called nodulation (nod) factors that are perceived by a few cells in the emerging root hair zone behind the root apical meristem, gaining a transient competence for entering the nodule developmental program. In the last 10 years, several mutants blocked at the early stages of the nod factor-dependent transduction pathway, have been characterized (Schauser et al. 1999; Stracke et al. 2002; Endre et al. 2002; Madsen et al. 2003; Radutoiu et al. 2003; Anè et al. 2004; Levy et al. 2004; Kalò et al. 2005; Imaizumi-Anraku et al. 2005; Smit et al. 2005; Kanamori et al. 2006; Gonzales-Rizzo et al. 2006; Tirichine et al. 2007). Some of these caused inhibition both for rhizobial and mycorrhizal symbiosis, leading to the identification of common signaling steps (Stracke et al. 2002; Endre et al. 2002; Anè et al. 2004; Levy et al. 2004; Imaizumi-Anraku et al. 2005; Kanamori et al. 2006). Several genes such as *ENOD11* that are induced during nodulation are also activated during mycorrhizal infection (Albrecht et al. 1999; Gualtieri and Bisseling 2000; Journet et al. 2001), and there are parallels with some aspects of both types of infection (Kistner and Parniske 2002). In most of the mutants blocked in both symbiotic interactions, the calcium spiking response, activated early during the nodulation program, is repressed. This has been taken to suggest that Ca^{2+} spiking is a signaling step during mycorrhizal symbiosis. Recently, rapid and transient elevations in cytosolic free Ca^{2+} were recorded in soybean cell cultures in response to treatment with the culture medium of *Gigaspora margarita* spores (Navazio et al. 2007).

The *Ljsym4-2* mutant was isolated in *Lotus japonicus* by EMS mutagenesis and reported as unable to form nodules with *M. loti* and to form arbuscules upon inoculation with either of the two arbuscular mycorrhizal fungi, *Glomus intraradices* or *Gigaspora margarita* (Schauser et al. 1998; Bonfante et al. 2000).

Microscope analysis suggested that *LjSym4-2* is involved in a process occurring in epidermal cells (Bonfante et al. 2000; Novero et al. 2002), where infection threads are not formed upon contact with *M. loti*, and perifungal membranes do not develop around *G. margarita*. The *Ljsym4-2* mutant allele was subsequently isolated by positional cloning leading to the identification of the CASTOR gene, and fluorescence studies indicated that the nod-factor-induced calcium spiking signal was lacking in this genetic background (Imaizumi-Anraku et al. 2005).

As a consequence of the abortion of fungal infection, all of the epidermal colonized cells of *Ljsym4-2* appeared morphologically dead (Bonfante et al. 2000), a commonly observed response of resistant plants to pathogen attacks (Heath 1997).

Suspension cell cultures, as already reported for different species, provide an excellent experimental system where several parameters of cell growth and death can be precisely defined (Carimi et al. 2003, 2004; Zottini et al. 2006). For this reason, we produced a suspension cell culture from the *L. japonicus* mutant and wild type plants to study the cell death events induced in *Ljsym4-2* in detail. In this work, the characterization of programmed cell death (PCD) induced by SA is reported, and differences between the two cell lines are described. Low levels of SA are able to induce PCD only in suspension cell cultures of *Ljsym4-2* where an higher level of a SA-inducible *LjPR1* gene is observed. Moreover, two key molecules of the SA signaling pathway, nitric oxide (NO) and H_2O_2, show different behavior in mutant cells with respect to wt ones. Finally, the pattern of the *LjPR1* expression in *L. japonicus* seedlings at 48 h after inoculation with *M. loti* shows an induction in wild type seedlings and a clear burst of expression in the *Ljsym4.2* mutant background. However, the analysis of transgenic hairy roots transformed with the *nahG* gene suggests that the triggering of the defense-like response in the mutant roots does not seem to be the cause of the deficient symbiotic phenotype.

Materials and methods

Cell cultures, plant materials and treatments

Wild type *Lotus japonicus* (ecotype Gifu B-129) cell lines, and mutant cell lines *Ljsym4-2* were generated from roots of young seedlings and routinely subcultured in Gamborg's B5 medium with 9.2 μM 2,4-dichlorophenoxy-acetic acid (2,4-D, Duchefa), 2% sucrose. For subculture cycles, 1.2 ml of packed cell volume was placed in 100-ml Erlenmeyer flasks containing 20 ml liquid medium. Cells were subcultured in fresh medium at 10-day intervals and maintained in a climate chamber on a horizontal rotary shaker (80 rpm) at 25 ± 1°C with a 16-h photoperiod.

The pH of the media was adjusted to 5.5 ± 0.1 with NaOH before autoclaving at 121°C for 15 min. The growth regulator (2,4 D), when needed, was filter-sterilized and added directly to the medium. To determine the effect of salicylic acid (SA), 4-d-old wild type and mutant cells were incubated with 0.5 to 3 mM SA. Catalase (75 U/ml^{-1}, Sigma–Aldrich, Milan Italy) was added into the medium together with SA.

The NOS L-arginine-based inhibitor N^G-monomethyl-L-arginine (L-NMMA) as well as the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were from Alexis Biochemicals, Vinci, Italy. L-NMMA (1 mM) or cPTIO (0.4 mM), when required, were added to the cells 1 h before SA treatments.

Cell death was determined by spectrophotometric measurements of the uptake of Evan's blue stain, as described by Shigaki and Bhattacharya (1999).

To determine dry weight, integer cells were separated from the culture medium and cell debris through a vacuum filtration unit (Sartorius, Florence, Italy). The collected cells were dried overnight at 60°C. For cell suspension-cultured experiments, a randomized complete block design was used with three replicates (individual Erlenmeyer flasks). Each experiment was repeated three times.

The experiments on plant seedlings were performed on *Lotus japonicus* wild type and *Ljsym 4-2* mutant. Seeds were surface-sterilized by immersion in 4% (v/v) sodium hypochlorite, 2% (v/v) Triton X-100 for 20 min on a rotating plate agitator, and rinsed five times (10 min each) with sterile distilled water. Sterilized seeds were incubated overnight at 4°C, then transferred to Petri dishes containing 1% solidified agar medium and incubated for 1 day at 4°C and 1 day in the dark at 25°C. The dishes were then transferred to the light at 25°C for 4 days. Successively, wt and mutant roots were inoculated with *M. loti* (OD 580–600 nm) for 48 h; then the roots were cut and collected for RNA extractions.

Agrobacterium rhizogenes transformation and plant inoculation

The procedure was previously described by Martirani et al. (1999). Six day-old seedlings were cut in the root hair emergence zone at about 0.5–1 cm from the growing root tip. The freshly cut surface was inoculated with *Agrobacterium rhizogenes* grown overnight in liquid medium. After 10 days, when micro calli with emerging roots appeared clearly at the wound sites, composite plants were transferred to slanted fresh nitrogen-free nodulation medium agar. Every root tip was inoculated with 10 μl (5×10^6 *Rhizobium* cells) of an overnight-grown *R. loti* suspension culture and roots were kept in the dark. The plants were grown at 23°C with a 16-h photoperiod and nodulation was tested at 4 weeks post inoculation.

DNA analysis

Cells grown in liquid media were harvested, frozen in liquid N_2 and stored at −80°C. For DNA extraction, cells were ground using a mortar and pestle with liquid N_2. Genomic DNA was isolated as described by Doyle and Doyle (1987) and quantified by measurement of the OD-260 nm as described by Sambrook et al. (1989). For DNA fragmentation analysis, 10 μg of each sample was electrophoresed on 1% (w/v) agarose gels containing 1× TAE (40 mM Tris–acetate, 1 mM EDTA) and stained with ethidium bromide.

Nuclear morphology

Lotus japonicus wt and mutant *Ljsym4-2* cells were prepared for microscope analysis according to a previously described procedure (Traas et al. 1992) with minor modifications. Briefly, cells were fixed by adding 0.5 ml of a solution containing 4% (v/v) paraformaldehyde in Hepes, EGTA, $MgSO_4$ (HEM) buffer (100 mM Hepes pH 6.9, 10 mM EGTA, 10 mM $MgSO_4$) to 0.5 ml culture. After 15 min, cells were washed three times in HEM buffer and finally resuspended in HEM containing 0.2% (w/v) Triton X-100 (Sigma–Aldrich, Milan Italy) and 1 μg ml^{-1} of the DNA specific dye 4′,6-diamidino-2-phenylidone (DAPI) (Alexix Chemical, Vinci, Italy). The cells were overlaid on poly-L-lysine-coated (Sigma–Aldrich) microscope slides, and nuclei were visualized using a Leica DMR epifluorescence microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) with an excitation filter of 330–380 nm and a barrier filter of 400 nm. For nuclear morphology experiments, a randomized complete block design was used with three replicates (individual Erlenmeyer flasks). Each experiment was repeated at least three times. For each

point and treatment, 100 representative nuclei were counted. Images acquired by fluorescence microscopy were processed using Corel Photo-Paint (Corel Corporation, Dallas, TX, USA).

Extracellular NO quantification by fluorimetric analysis with DAF-2

NO was determined by binding to 4,5-diaminofluorescein (DAF-2; Alexis Biochemicals) in a fluorimetric assay (Nakatsubo et al. 1998). Fluorescence measurements were performed with a Perkin Elmer LS-55 Luminescence Spectrometer with an excitation wavelength of 495 nm, an emission wavelength of 515 nm, and with a slit width of 3 nm. We followed the procedure of Carimi et al. (2005) with slight modifications: 25.5 mg of cells were taken from the flask and transferred into 2 ml buffered medium consisting of 50 µl of 0.5 M sodium phosphate buffer (pH 7.2), 1 ml of H_2O, 2.5 µM DAF-2 and B52 medium to the final volume. After 15 min incubation at 25°C on a rotating plate agitator, the DAF-2-loaded cell suspension was transferred to a 10-mm quartz cuvette, and the fluorescence emission was determined at 24°C with slow stirring. When the assay was performed in presence of NOS inhibitor L-NMMA or NO scavenger cPTIO, the cells were pre-incubated 1 h with the respective inhibitor/scavenger before SA addition. Then, at each considered time (1 and 5 h after SA addition), the cells were taken from the flask and incubated with the DAF-2 containing buffer for 15 min, as mentioned above, before NO measurement. All reactions were carried out at least in duplicate and their reproducibility was checked. Each experiment was repeated three times. Relative fluorescence is expressed as the ratio to the control values obtained with untreated cells.

Imaging of intracellular NO productions

The cell-permeable diacetate derivative diaminofluorescin-FM (DAF-FM DA, Alexis Biochemicals) was used as a fluorescent probe for the detection of NO in cells (Kojima et al. 1998). In the presence of NO and O_2, DAF probes are converted to the fluorescent triazole derivative thereby increasing the quantum yield of fluorescence more than 180-fold (Nakatsubo et al. 1998). A 4-day-old suspension cell culture, corresponding to 50 µl of packed cells, was added to a solution containing 0.5 µM DAF-FM DA in a final volume of 1 ml of B52 medium. After 15 min incubation at 25°C on a rotating plate agitator, cells were washed with 1.5 ml B52 medium for 20 min. For microscopic analysis cells were layered onto a coverslip.

DAF-FM DA fluorescence was estimated using confocal laser scanning microscopy (excitation 488 nm, emission 515–530 nm; Nikon PCM2000). The images acquired from the confocal microscope were processed using the software Corel Photo-Paint (Corel Corporation, Dallas, TX, USA) and relative pixel intensities determined using Scion Image software (Scion Corporation, Frederick, MD, USA).

Extracellular H_2O_2 quantifications

H_2O_2 was measured according to Jiang et al. (1990) in the extracellular phase. Briefly, 1 ml of suspension cell cultures was harvested by centrifugation (10,000 g, 3 min, 25°C), and the H_2O_2 concentration was measured in the supernatant. An aliquot of supernatant (500 µl) was added to 500 µl of assay reagent (500 µM ferrous ammonium sulphate, 50 mM H_2SO_4, 200 µM xylenol orange, and 200 mM sorbitol). After 45 min of incubation, the peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} was determined by measuring the absorbance at 560 nm of the Fe^{3+}-xylenol orange complex.

RNA analysis

Cells and seedlings were harvested, frozen in liquid N_2 and stored at –80°C. RNA was isolated using Trizol (Invitrogen, San Giuliano Milanese, Italy) following the manufacturer's instructions and treated with DNase I (Ambion Inc., Austin, TX, USA). Total RNA from each sample (2.5 µg) was reverse-transcribed using PowerScript reverse transcriptase (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer's instructions. For the semi-quantitative RT-PCR, the 18S rRNA was used as an internal standard using the primers from the Quantum RNA Universal 18S Internal Standards Kit (Ambion). The primers used for RT-PCR analysis of *LjPR1* were 5′-TGGGATGACACCGTAGCTGCTTTT-3′ (forward) and 5′-CCGCACTGTTTTCCACTAGCACAA-3′ (reverse). The following cycling conditions were used: 95°C for 60 s followed by 26 and 28 cycles for *LjPR1* and 11 cycles for 18S at 94°C for 30 s, 61°C for 30 s and 72°C for 30 s using a Hybaid PCR express thermal cycler (VWR, Milan, Italy). PCR products were visualized on 1% (w/v) agarose gels containing ethidium bromide (Sambrook et al. 1989). Products of RT-PCR were purified using the QIAquick PCR purification Kit (Qiagen, Milan, Italy), and sequenced. Real-time PCR was performed with a DNA Engine Opticon 2 System, MJ Research (MA, USA) using SYBR to monitor dsDNA synthesis. The ubiquitin (*UBI*) gene (AW719589) was used as an internal standard.

The sequences of the gene-specific primers used for real-time RT PCR were the following: 5′-GCCAATC AACGTAAGGGAGA-3′ (forward) and 5′-CGCACTG TTTTCCACTAGCA-3′ (reverse). The concentration of primers was optimized for each PCR reaction and each amplification was carried out in triplicate. The PCR program used was as follows: 95°C for 13 min and 39 cycles of 94°C

for 15 s, 63°C for 15 s. and 72°C for 15 s. Data were analyzed using Opticon Monitor Analysis Software Version 2.01 (MJ Research). The relative level of expression was calculated with the following formula: relative expression ratio of the gene of interest is $2^{-\Delta CT}$ with $\Delta CT = CT_{gene}$ minus CT_{UBI}. Analysis of the melting curve of PCR products at the end of the PCR run revealed a single narrow peak for each amplification product, and fragments amplified from total cDNA were gel-purified and sequenced to assure accuracy and specificity.

Statistical analysis

Statistical analyses were performed by the Student's *t*-test.

Results

Cell growth and spontaneous senescence in suspension cell cultures of *L. japonicus* wild type and Ljsym4-2

To study cell-death events induced in *Ljsym4-2* in detail, we produced suspension cell cultures from the *L. japonicus* mutant and wild type plants. Both cell cultures were characterized for their physiological parameters (Fig. 1). Wild type and *Ljsym4-2* cells were sub-cultured with a cycle of ~10 days: this period comprised the exponential growth phase up to the stationary phase. If the medium was not changed at the proper time (10–14 days), a decrease in dry weight was observed (decline phase) (Fig. 1a, a′). This decrease correlated well with a rapid and similar increase in cell death in both cell lines, as suggested by the analysis of their survival curves (Fig. 1b, b′). Agarose gel analysis of DNA, isolated from wild type and mutant suspension cultures at different times after culture initiation, showed that DNA laddering (a characteristic feature of PCD) occurred in a time-dependent fashion (Fig. 1c, c′) in both cell lines. In fact, if the medium was not changed after 10 days, DNA laddering was observed 21 days after culture initiation (when cell death reaches 40%) and was more pronounced afterwards.

Comparing the physiological parameters of the two cell lines (Fig. 1), we observed some slight differences, i.e., the growth peak was reached at 14 days in mutant cells instead of 11 days of wt cells, and a slower increase in the percent

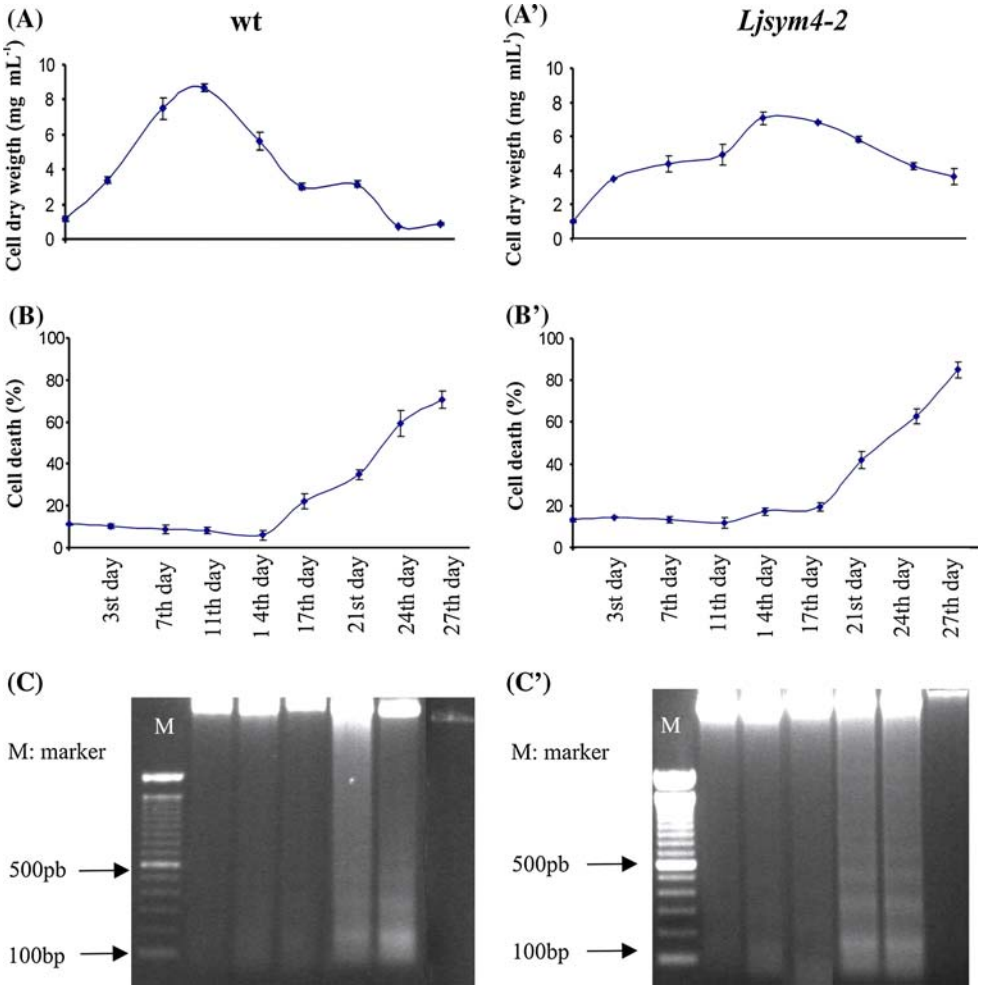

Fig. 1 Growth and senescence of *Lotus japonicus* wt and *Ljsym4-2* mutant cell cultures. Dry weight of wt (**a**) and *Ljsym4-2* (**a′**). Cell death estimated by Evan's blue staining of wt (**b**) and *Ljsym4-2* (**b′**). Agarose gel analysis of DNA extracted from wt (**c**) and *Ljsym4-2* (**c′**) collected at different days (11, 14, 17, 21, 24, 27) after culture initiation

of cell death was detected in the mutant cell population. However, these differences are commonplace among cell lines generated independently.

Effects of different levels of SA on wild type and mutant Lotus cell cultures

In plants, SA can affect interactions with both symbiotic and pathogenic micro-organisms. For this reason, the effects of different levels of SA were analyzed in our experimental system. In particular, two different SA concentrations (0.5 and 1 mM) were tested on 4-day-old proliferating cell-suspension cultures of wild type and *Ljsym4-2* for 5 days. The dry weight of *Lotus* cells was differentially and significantly dependent on SA concentration (Fig. 2a). The addition of 0.5 mM SA to the culture medium of wt cells had a moderate effect on cell growth and on cell viability, 10.5% cell death versus 6.5% present in control cells (Fig. 2b), but in mutant cells, it reduced cell growth to 50% and increased the percent of cell death three-fold (Fig. 2b). A 1 mM concentration of SA affected cell growth of both cell lines, but a more severe effect (70% reduction) was reported for mutant cells. In the presence of 1 mM SA, a four-fold increase in cell death was reported for the mutant cell culture, whereas only a doubling in cell death was detected in wt cells (Fig. 2b). No intra-nucleosomal DNA cleavage was observed in wt cells treated with 0.5 and 1 mM SA, whereas pronounced nuclear DNA degradation was evident in mutant cells treated with either SA concentration (Fig. 2c).

Nuclear morphology, observed at the two SA concentrations, was investigated using DAPI staining coupled with fluorescence microscopy. A strong increase in the percentage of condensed plus stretched nuclei was already detected after 48 h of treatment in mutant but not in wt cells (Fig. 2d).

This different sensitivity of wt and mutant cells to SA was confirmed in three cell lines, independently produced (data not shown).

Components of the signaling pathway induced by SA

To investigate components involved in the SA signaling pathway, H_2O_2 and NO production were analyzed in wt and mutant cells.

The accumulation of H_2O_2 was measured by xylenol orange assay in wt and *Ljsym4-2* cells incubated in the presence of 0.5 and 1 mM SA. As shown in Fig. 3a, an increase (24%) in H_2O_2 production can be detected 20 min after treatment in wt cells incubated with 1 mM SA. No effect was apparent when wt cells were treated with 0.5 mM SA. When *Ljsym4-2* cells were similarly treated, a

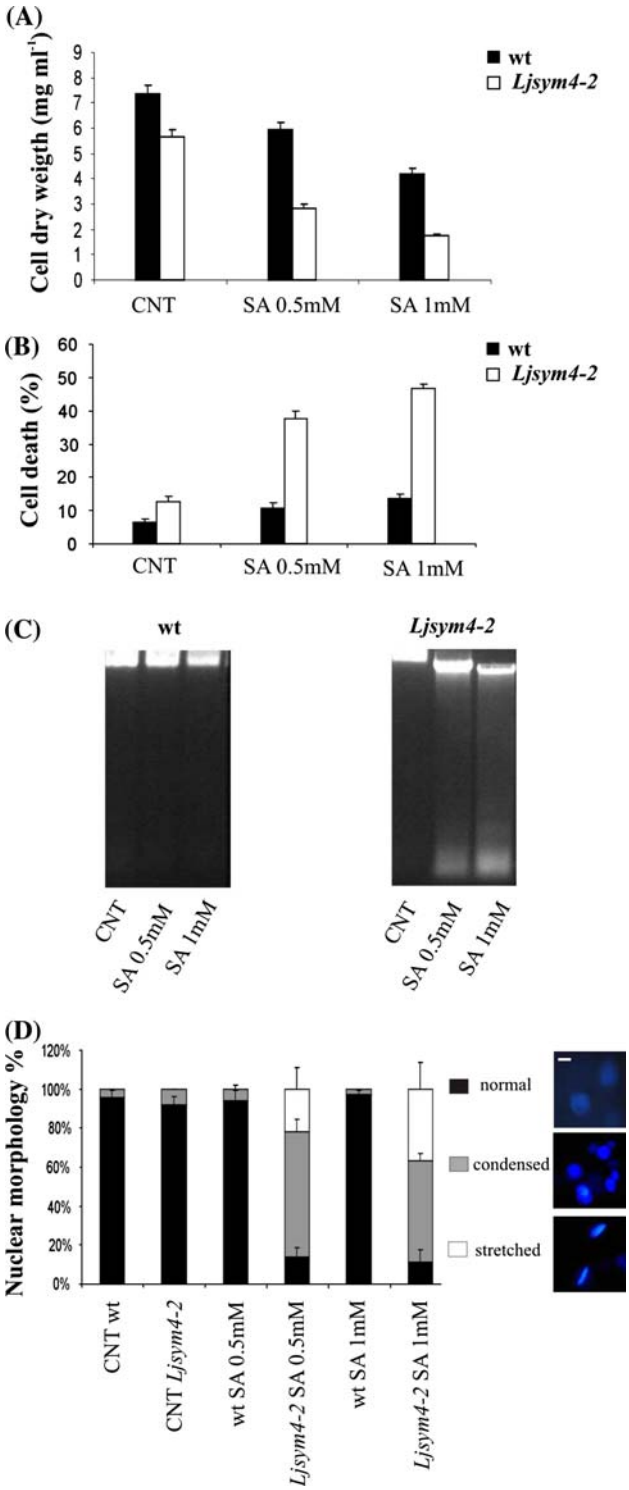

Fig. 2 Effects of different levels of SA on *Lotus japonicus* wt and *Ljsym4-2* mutant cells. Four-day-old cells treated with two SA concentrations (0.5 and 1 mM) for 5 days (**a**, **b**, **c**). **a** Cell dry weight of wt and *Ljsym4-2*. **b** Cell death estimated by Evan's blue staining of wt and *Ljsym4-2* cells. **c** Agarose gel analysis of DNA extracted from wt and mutant cells. **d** Nuclear morphology of wt and *Ljsym4-2* cells treated for 48 h (*Bar* 20 μm). Values represent mean ± SE of six independent experiments

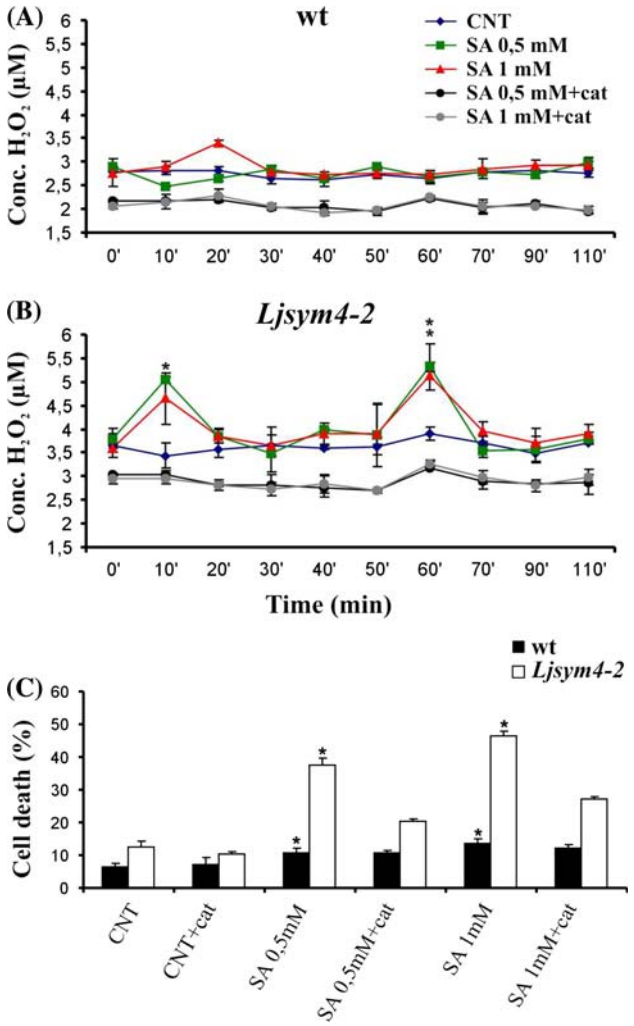

Fig. 3 Measure of H_2O_2 production in *Lotus japonicus* wt and *Ljsym4-2* mutant cells. Four-day-old cells were treated with two SA concentrations (0.5 and 1 mM) ± catalase (75 U/ml), and H_2O_2 was measured with xylenol orange assay in wt (**a**) and in mutant cells (**b**). **c** Cell death (%) of wt and mutant cells treated with SA (0.5 and 1 mM) ± catalase (75 U/ml) for five days. Values represent mean ± SE of five independent experiments. *Asterisks* indicate values that are significantly different from those of untreated cells by Student's *t* test (* *P*, 0.01, ** *P*, 0.05)

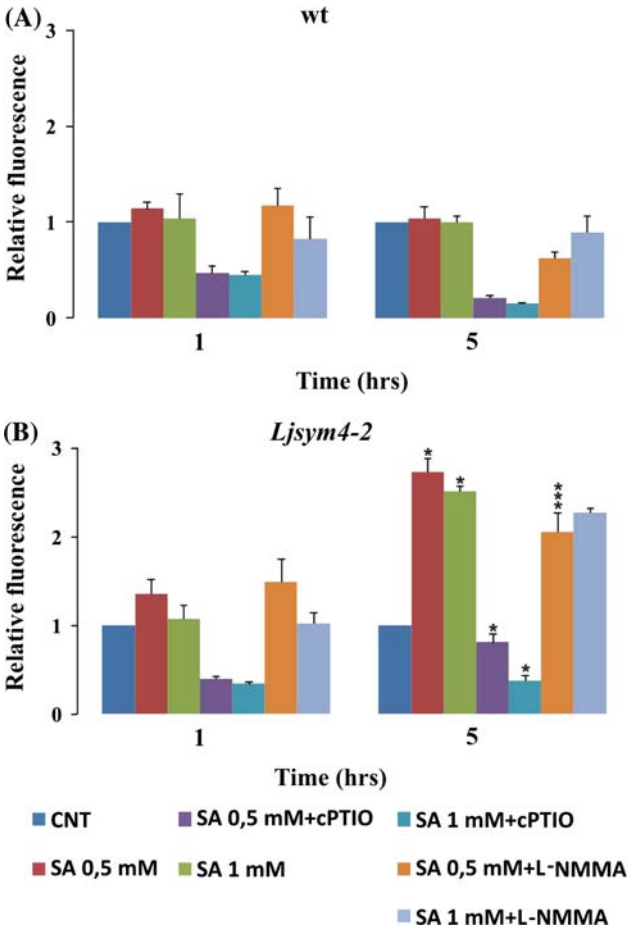

Fig. 4 NO released from wt and *Ljsym4-2* mutant cells treated with SA. Four-day-old cells were treated with two SA concentrations (0.5 and 1 mM) ± c-PTIO (0.4 mM) and L-NMMA (1 mM) and the amount of NO released from wt (**a**) and mutant (**b**) cells was measured at 1 and 5 h using the DAF-2 probe. *Asterisks* indicate values that are significantly different from those of untreated cells by Student's *t* test (* *P*, 0.01, ** *P*, 0.05). Three *asterisks* indicate value that is not significantly different from cells treated with SA only without L-NMMA

biphasic increase of H_2O_2 was reported in the presence of either SA concentration. A first peak occurred at 10 min, slightly higher than that observed in wt cells at 20 min. A second similar peak appeared 1 h later. Biphasic increases of reactive oxygen intermediates (ROI) in response to pathogenic attack in several plant cell systems have been reported (Levine et al. 1994; Chandra et al. 1996).

In order to decipher the role of H_2O_2 in SA-induced PCD, we measured the effect of catalase on cell death induced by SA. Pre-treatment with catalase (75 U ml^{-1}) eliminated the increased peaks, reduced the basal accumulation of H_2O_2 (Fig. 3a, b), and almost halved cell death in mutant cell cultures (Fig. 3c).

It has been recently demonstrated that SA induces NO production through an NOS-like route in Arabidopsis roots and cell cultures (Zottini et al. 2007). In order to understand if NO is a signaling molecule also in our experimental conditions, NO production upon SA treatment was evaluated. NO production was determined by using the fluorescent probe DAF-2, which allows the measurement of NO released from the cells (see "Materials and methods"). In Fig. 4a, the release of NO in wt cells at two different times (1 and 5 h) in the presence of SA 0.5 and 1 mM, is shown. No differences in fluorescence levels were detected in control and wild type SA treated cells, while in mutant cells, the presence of both SA concentrations induced an increase of the florescence level, at 5 h treatment (Fig. 4b). Pre-treatment with the NO scavenger cPTIO cancelled the accumulation of NO, while pre-

treatment with the NOS inhibitor L-NMMA does not seem to induce a statistically significant effect. (Fig. 4b). The effect of SA on NO accumulation was also determined at the intracellular level by using the cell-permeable fluorescent probe DAF-FM DA combined with confocal microscopy analyses. NO levels increased more and constantly in mutant cells compared to wt cells, in agreement with the results obtained by measuring the NO released by the cells (data not shown).

In order to prove the role of NO in PCD induced by SA, we measured the effect of the NO scavenger cPTIO (0.4 mM) and the NOS inhibitor L-NMMA (1 mM) on cell growth and cell death induced by 0.5 and 1 mM SA treatments (Fig. 5). In this biological assay, pre-treatment either with cPTIO or L-NMMA increased cell growth and reduced cell death to a larger extent in mutant cell cultures where PCD was induced (Fig. 5b′: mutant cells in the presence of 0.5 and 1 mM SA show, respectively, 38 and 46% cell death). These results, in particular the ones obtained with cells pre-treated with cPTIO, allow us to suggest that NO is a signaling component of this form of PCD.

Our results on signaling components involved in SA-induced pathways showed that the time lapse in which the double peak of H_2O_2 is produced in *Ljsym4-2* mutant cells, 10 and 60 min (Fig. 3b), preceded the time of NO production (Fig. 4b). Hence, we performed an experiment where mutant cells pre-treated with catalase were analyzed for NO production at several time points. The results, presented in Fig. 6, showed the absence of NO production

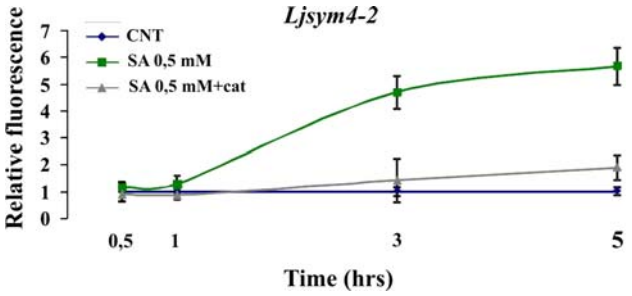

Fig. 6 NO released from *Ljsym4-2* mutant cells treated with SA in the presence of catalase. Mutant cells were treated with 0.5 mM SA ± catalase (75 U/ml). The amount of NO released from cells up to 5 h was measured using the DAF-2 probe. Values represent mean ± SE of three independent experiments

under these experimental conditions, suggesting a key role of H_2O_2 on the induction of NO production. Pre-treatment with catalase (75 U/ml) reduced the accumulation of H_2O_2 and almost halved cell death (Fig. 3).

Different sensitivity of wt and mutant cells to SA in PCD induction

Results described so far revealed a different sensitivity of wt and mutant cells toward to SA. In order to identify the SA concentration able to induce an increase of cell death in wt cells comparable to that detected in the mutant cells, we incubated wt cells with increased levels of SA (1.5 and 3 mM). These two additional SA concentrations showed a severe effect on cell growth and induced a significant

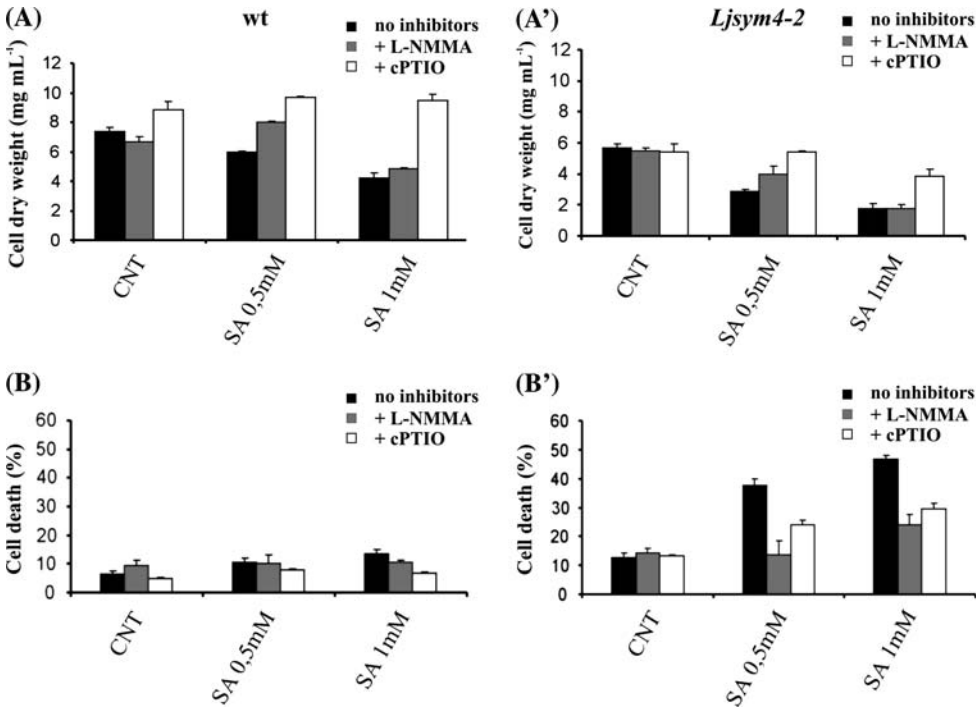

Fig. 5 Effects of a NO scavenger and an NOS-inhibitor on cell growth and cell death of wt and *Ljsym4-2* cultured cells. Four-day-old cells were treated with two SA concentrations (0.5 and 1 mM), c-PTIO (0.4 mM), and L-NMMA (1 mM) for five days. Cell dry weight of wt (**a**) and *Ljsym4-2* (**a′**). Cell death estimated by Evan's blue staining of wt (**b**) and *Ljsym4-2* (**b′**) cells. Values represent mean ± SE of three independent experiments

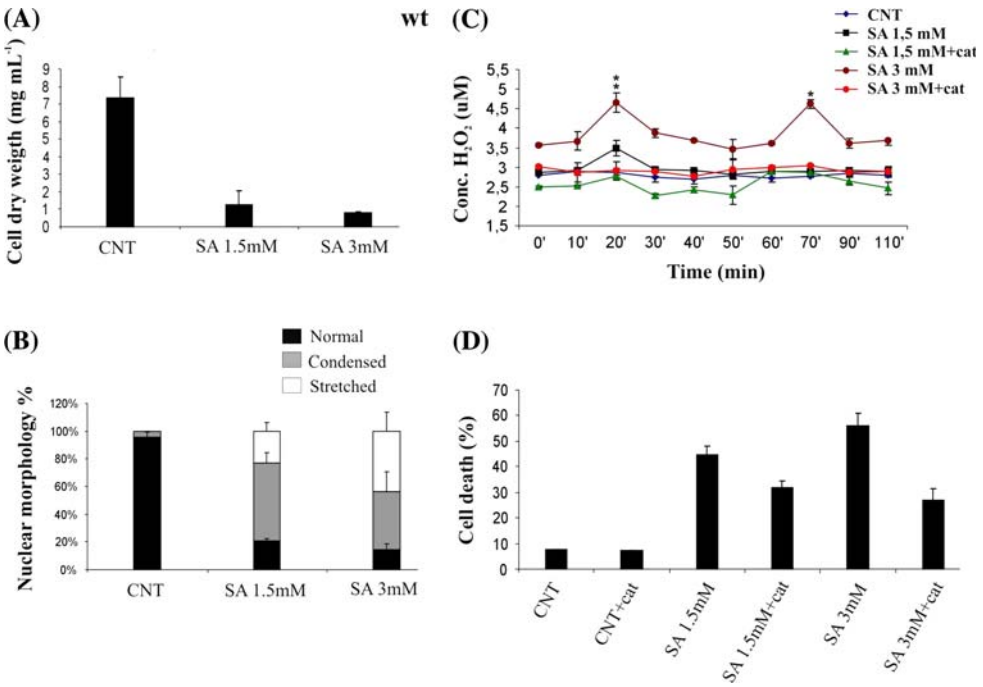

Fig. 7 Effect of 1.5 and 3 mM SA concentrations on *Lotus japonicus* wt cells. Four-day-old wt cells were incubated for 5 days in presence of the two SA concentrations: **a** Dry weight, **d** Cell death estimated by Evan's blue staining with the two SA concentrations ± catalase (75 U/ml). **b** Percentage of normal and apoptotic (condensed and stretched) nuclei in cells treated with SA 1.5 and 3 mM for 48 h. **c** H_2O_2 production measured by xylenol orange assay in four-day-old cells treated with the two SA concentrations ± catalase (75 U/ml). *Asterisks* indicate values that are significantly different from those of untreated cells by Student's *t* test (* *P*, 0.01, ** *P*, 0.05)

increase in the percentage of cell death in wt suspension cultures (Fig. 7a, d). The addition of 1.5 mM SA to the medium of wt cells induced a level of cell death (40%) similar to that detected in *Ljsym4-2* cells incubated with 0.5 mM SA. Nuclear morphology observed at the two concentrations showed an increase in the percentage of condensed plus stretched nuclei, confirming the induction of PCD in these treated cells (Fig. 7b).

As shown in Fig. 7c, an increase in H_2O_2 production can be detected 20 min after treatment of wt cells with 1.5 mM SA and a more relevant increase with 3 mM SA. The presence of 3 mM SA induced a second peak at 70 min, correlating a biphasic increase of reactive oxygen intermediates with the induction of cell death. Also in this case, as shown in Fig. 3, pre-treatment with catalase (75 U/ml) reduced the accumulation of H_2O_2 and the percent of cell death in the culture (Fig. 7d).

The NO released from cells incubated with 1.5 and 3 mM SA could not be directly measured because high levels of SA (more than 1 mM) interfered with the DAF-2 assay. Thus, the NO accumulation in wt cells treated with 1.5 and 3 mM SA was determined at the intracellular level by using the cell-permeable fluorescent probe DAF-FM DA. The results (Fig. 8a) showed a clear increase of NO in a dose- dependent manner.

In order to confirm the role of NO in the PCD pathway induced by high levels of SA in wt cells, we measured the effect of the NO scavenger cPTIO (0.4 mM) and the NOS inhibitor L-NMMA (1 mM) on wt cell growth and cell death induced by 1.5 and 3 mM SA treatment. Also in this case, the results clearly showed that pre-treatment with cPTIO and L-NMMA increased cell growth and reduced cell death (Fig. 8b, c), confirming the role of NO in the signaling pathway of this form of PCD.

To complete the picture, a last experiment was performed. Wild type cell cultures were pre-treated with L-NMMA (1 mM) and analyzed for H_2O_2 production. In this case, cPTIO could not be used because of its interference with xylenol orange present in the H_2O_2 assay under our experimental conditions. The results in Fig. 8d show a reduction of H_2O_2 suggesting a role for L-NMMA in this process.

Modulation of LjPR1 gene expression in wt and mutant cell cultures

Pathogenesis related (PR) proteins are normally used as markers of a SA-dependent plant defense response. In order to test SA-dependent pathway in wild type and *Ljsym4-2* cells we used, as molecular marker, the *L. japonicus* predicted gene LjSGA_043037.1 (http://www.kazusa.or.jp/lotus/blast.html) (renamed here as *LjPR1*), orthologue of the *A. thaliana PR1* gene (At2G14610; E value $3e^{-50}$).

The analysis of the amount of *LjPR1* transcript, measured by semi-quantitative RT-PCR (Fig. 9a), showed very low basal level of expression in control wild type cells, whereas it was clearly induced 24 h after treatment with 0.1 and to a lesser extent, with 0.5 mM SA. In the *Ljsym4-2* cells, the level of expression of *LjPR1* was very high both in untreated and SA-treated cells, confirming a constitutive alteration of the SA sensing in this mutated background.

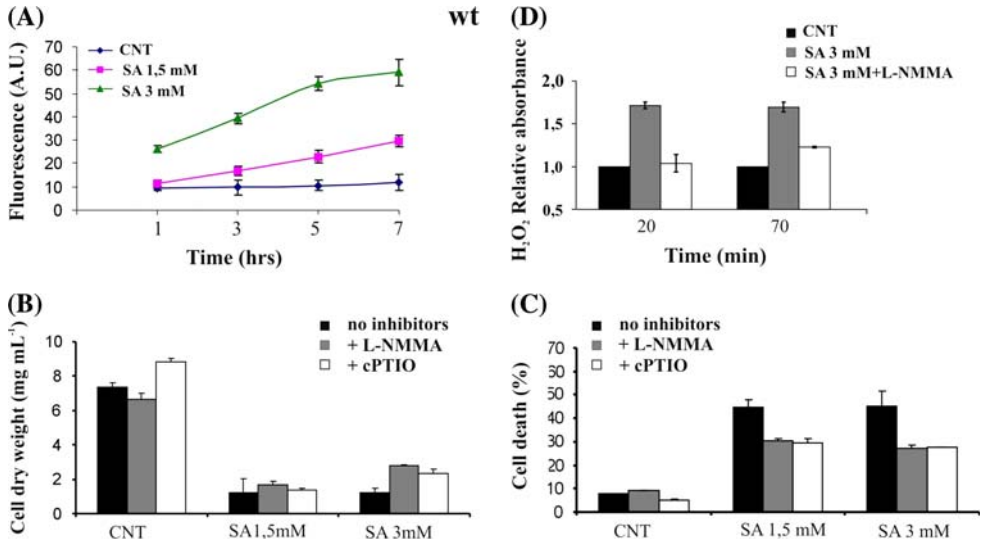

Fig. 8 SA-treated wt cells: intracellular levels of NO, cell growth, cell death in the presence of a scavenger and an inhibitor of NO and H_2O_2 in presence of a NOS inhibitor. Four-day-old cells were treated with 1.5 and 3 mM SA for 7 h and **a** intracellular levels of NO were visualized using a cell-permeable NO-sensitive probe (DAF-FM DA). Cell dry weight (**b**) was measured and % cell death **c** estimated by Evan's blue staining in wt cells treated with SA and with cPTIO (0.4 mM) or L-NMMA (1 mM). **d** Measure of H_2O_2 production in *Lotus japonicus* wt cells treated with 3 mM of SA ± L-NMMA (1 mM). *Values* represent mean ± SE of three independent experiments

Characterization of plant responses to *M. loti* in wild type and mutant *Ljsym4-2* seedlings

In order to test in planta the physiological relevance of the altered level of the SA-dependent pathway characterized in cells, we compared the basal level of expression of the *LjPR1* gene in wild type and *Ljsym4-2* mutant *L. japonicus* seedlings and followed the profile of its expression after *M. loti* inoculation. Roots of wild type and mutant *Ljsym4-2* seedlings, grown in N starvation conditions, were inoculated with *M. loti*, and the level of expression of the *LjPR1* gene was measured, by qRT-PCR analysis at the time of inoculation 0 and 48 h later. As shown in Fig. 9b the amount of *LjPR1* transcript did not change in the *Ljsym4-2* un-inoculated roots when compared to wild type. Furthermore, wild type roots showed a clear increase (about 2 fold) in the *LjPR1* transcript level at 48 h p.i. consistently with the reported early induction of defense genes during the establishment of the symbiotic interaction (Kouchi et al. 2004; Lohar et al. 2006). The level of expression of *LjPR1* at 48 h p-i. was of one order of magnitude higher in the *Ljsym4-2* seedlings (Fig. 9b).

In order to see whether the altered SA dependent pathway could represent the cause of the nod-phenotype reported for the *Ljsym4-2* mutant (Bonfante et al. 2000) we constructed composite *L. japonicus* transgenic plants using transformation with *Agrobacterium rhizogenes* carrying the pROK2-NAHG, or pHKN 29 (Kumagai and Kouchi 2003; 35S-GFP construct), or pIG121-HM (Hiei et al. 1994; 35S-gusA construct) and the appearing roots were tested for nodulation by inoculating with *M. loti*. Composite transgenic plants obtained after *A. rhizogenes* infection of wild type seedlings showed a full nodulation capacity (7–10 nodules per plant), whereas *Ljsym4-2* plants transformed with each of the constructs were unable to form nodules although the presence of the GFP and GUS markers allowed an estimation of 30–60% of transformed roots in three independent experiments (data not shown). This result suggests that the alteration of the SA pathway in the *Ljsym4-2* background is not the main cause of the symbiotic phenotype.

Discussion

An intriguing behavior of *Ljsym4-2* mutant plant is that, as a consequence of the abortion of a symbiotic fungal infection, all epidermal colonized cells appear morphologically dead (Bonfante et al. 2000). This is a common response of resistant plants to attack by pathogens, but not an ordinary response of legume plants to a symbiotic fungal infection. Hence, a mutation in the *LjSym* gene prevents mycorrhizal formation and activates a cell death pathway in the presence of symbiotic fungi.

We thought it worthwhile to study cell-death events induced in *Ljsym4-2* in detail, and for this reason, we produced suspension cell cultures from the *L. japonicus* mutant and wild type plants and characterized them. The physiological parameters of cell growth and senescence measured in both cell cultures showed similar patterns with

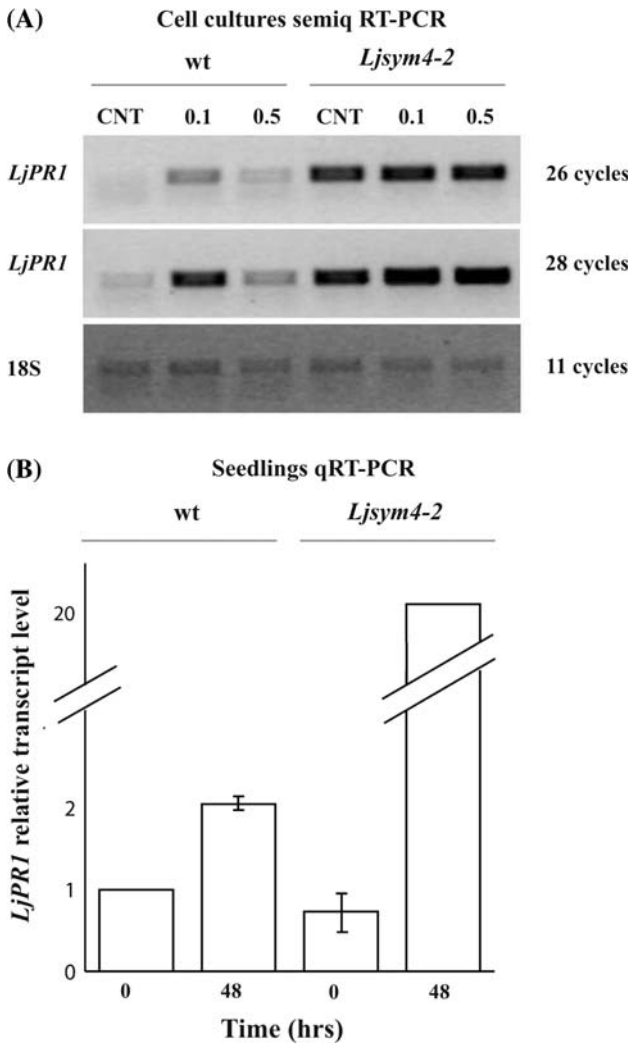

Fig. 9 Analysis of *LjPR1* expression in wt and *Ljsym4-2* mutant cultured cells and seedlings. **a** *LjPR1* expression in wt and *Ljsym4-2* cultured cells after incubation with different concentrations of SA (0.1 and 0.5 mM) for 24 h. The expression of *LjPR1* was analyzed by semiq RT-PCR at two different PCR amplification cycles (26 and 28) whereas as internal standard the 18S transcript was analysed after 11 cycles of PCR amplification. The results are representative of two independent biological repetition. **b** Relative *LjPR1* transcript levels in roots of wild type and *Ljsym4-2* roots at the moment of *M. loti* inoculation 0 and 48 h later. Transcript levels were determined by qRT-PCR, normalized to that of the internal control ubiquitin (see Materials and methods) and plotted relative to *LjPR1* transcript levels at time 0 wild type seedlings. *Bars* represent the mean and SD of data obtained with RNA extracted from two biological replicates and three technical replicates each

slight differences justified by the independent origin of the two cell lines (Fig. 1). This result indicates that, in mutant cells, the cell death events induced during spontaneous senescence of the cell population occurred normally (Carimi et al. 2003, 2004).

Because SA acts in plants as a signaling molecule involved in several physio-pathological processes, we decided to investigate its effects on wt and mutant suspension cultures. Both cell lines were incubated in the presence of two (0.5 and 1 mM) SA concentrations and these treatments induced cell death in mutant but not in wt cells (Fig. 2). This meant that we were imposing on wt cells modulations in SA concentrations still perceived as physiological, whereas on mutant cells, the same signal induced cell death.

Then, we investigated the components of the signaling pathway induced by SA, specifically, H_2O_2 and NO, which have been reported to be important molecules in plant cell death induction (Delledonne et al. 2001). Results involving H_2O_2 production allowed us to distinguish between the two cell lines: mutant cells respond to SA (0.5 and 1 mM) by inducing the appearance of a double peak of H_2O_2 absent in wt cells (Fig. 3b). Biphasic increases of reactive oxygen intermediates (ROI) have been reported in several plant cell systems in response to pathogenic attack (Levine et al. 1994; Chandra et al. 1996). Thus, this response of mutant cells to SA suggests these SA concentrations, physiological for wt cells, were perceived by mutant cells as a death signal.

The total amount of NO (cell-released and intracellular) differed in mutant and wt cells (Figs. 4, 5). In mutant cells, the levels of NO is clearly increased in the presence of both 0.5 and 1 mM SA, whereas in wt cells, the levels remained low. An accurate analysis of the two components, H_2O_2 and NO, involved in the signaling pathway induced by SA allowed us to establish that H_2O_2 production preceded NO formation, behaving potentially as a signaling molecule for NO production (Fig. 6) (Lum et al. 2002; Bright et al. 2005). In addition, we demonstrated that both these components, as previously shown in PCD induced during the hypersensitive response (Delledonne et al. 2001; de Pinto et al. 2006), were necessary for inducing cell death.

The data on NO detection were obtained by using the DAF fluorometric method that we know to be an indirect way to detect NO (Planchet and Kaiser 2006). However, in the present paper experiments were always performed in parallel with experiments including the use of cPTIO as NO scavenger, a strategy usually adopted to confirm NO results (Besson-Bard et al. 2008).

The effects of 0.5–1 mM SA on mutant cells were comparable with those observed on 3 mM SA-treated wt cells when we measured cell survival, appearance of a double peak of H_2O_2, (Fig. 7) and production of NO (which remained constant for a few hours) (Fig. 8a), thus confirming a different sensitivity of the two lines with respect to SA.

In order to try to define a possible sequence of events in H_2O_2, and NO production, as last experiment, we pretreated wt cells with L-NMMA, H_2O_2, production was analyzed and a drastic reduction of it, was observed

(Fig. 8d). This result is an intriguing one. In fact, even if L-NMMA was not able to reduce in short times NO production in SA treated cells (Fig. 4b), this NOS inhibitor reduced drastically the cell death rate in a 4-day vitality assay (Fig. 5b′). Unfortunately, this experiment was not also performed in presence of cPTIO because of its interference with components present in the H_2O_2 assay.SA is a key molecule in inducing the expression of defense genes. In particular, in *Arabidopsis*, *PR1* expression after a pathogenic attack correlates with plant response capability (Shah 2003). Then, in order to verify at the molecular level the different sensitivity of wt and mutant cells to SA, a *L. japonicus* gene orthologue to the *A. thaliana PR1* gene was identified by a bioinformatic analysis. The induction of *LjPR1* by SA was clearly detected in wt *Lotus* cells (Fig. 9a). An interesting result was obtained in *Ljsym4-2* mutant cells where the *LjPR1* gene resulted always well expressed either in absence or in presence of SA, confirming a constitutive alteration of this mutant in this signaling pathway.

The analysis of *LjPR1* expression in plant seedlings did not show the different basal level of expression between wild type and mutant (Fig. 9b). This could be explained by the less homogeneity of the seedlings, as experimental system, compared to cell cultures. The induction of defense and stress-responsive pathways during symbiotic interactions has been reported previously (Colebatch et al. 2004; Kouchi et al. 2004; Lohar et al. 2006) and an initial induction followed by a rapid suppression of these genes may be part of the physiological cascade of events leading to a successful symbiotic interaction (Kouchi et al. 2004; Lohar et al. 2006). In agreement with these reports we observed a significant induction of the *LjPR1* gene at 48 h p.i. in wild type seedlings. Interestingly, the observed burst of the level of expression observed in the *Ljsym4-2* seedlings at 48 h p.i. suggests the triggering of a defense-related pathway in the mutant background (Fig. 9b). However, such a conclusion, must be further investigated in planta through a more complete analysis of the molecular pathway leading to the plant defense response. Recently, a strong induction of genes encoding phenylalanine ammonia lyase (*PAL*), in *L. japonicus* roots inoculated with *M. loti* mutants unable to form infection threads structures (*cgs* mutants; D'Antuono et al. 2008) was reported. A question that remains to be elucidated is to what extent plant defense responses are physiologically triggered during nodulation. The differences in the level of *LjPR1* between wt and *Ljsym4-2* mutant at 48 h p.i. represent a striking picture of the physiological and pathological level of plant defense response triggered by a successful and unsuccessful symbiotic interaction, respectively.

The interference between SA-dependent defense response and symbiotic pathways has been previously reported. In fact, addition of exogenous SA inhibits both indeterminate and determinate nodulation in legume roots (Martinez-Abarca et al. 1998; Lian et al. 2000; van Spronsen et al. 2003). Recently, it was shown that reduction of the endogenous SA level by transgenic expression of the bacterial *nahG* gene also correlated with a significant increase in the number of infections and mean nodule number when compared with wild type controls (Stacey et al. 2006). The same approach in *Nicotiana tabacum* led to an enhancement of mycorrhizal fungal infection (Medina et al. 2003). However, the triggering of a defense-related response is not necessarily directly involved in the specific interaction with rhizobia mediated by Nod factors, but could represent a parallel pathway triggered during the infection. In the case of the *Ljym4-2* mutant, we did not observe the rescue of the nod- phenotype in transgenic hairy roots transformed with a constitutively expressed copy of the *nahG*. This result must be considered with caution since we did not provide direct evidences for a biologically significant increase of the SA level in the *Ljym4-2* roots. However, a possible interpretation would be that the putative induction of the defense response, deducible by the strong induction of the SA inducible *LjPR1* gene (Fig. 9), is not the main cause of the defective symbiotic phenotype. In other words, the induction of the defense response pathway could take place in the *Ljsym4-2* genetic background after *M. loti* inoculation, probably as a consequence of the block of the infection progress.

The plant mutation *Ljsym4-2* identifies the CASTOR gene and determines a block in the Nod factor-dependent pathway by inhibiting the calcium spiking response but not calcium influx in root hairs (Imaizumi-Anraku et al. 2005; Miwa et al. 2006). CASTOR encodes for a nuclear membrane-localized cation channel (Riely et al. 2007; Charpentier et al. 2008). It has been suggested that there are at least two possible mechanisms by which CASTOR and its orthologue POLLUX could contribute to Ca^{2+} spiking. In fact, potassium flowing from the root hair cytoplasm into the nuclear envelope could either alter the membrane potential, opening voltage-gated calcium channels responsible for the spiking signal, or may act as a counter-ion, which compensates for the rapid release of positive charge from the calcium store during each spike (Oldroyd and Downie 2008; Charpentier et al. 2008). Lack of calcium spiking is associated to the block of cortical cell divisions and nodule primordium formation because the Ca^{2+} oscillation would not be perceived by the calcium–calmodulin-dependent protein kinase CCaMK (Levy et al. 2004; Mitra et al. 2004), which controls the activity of putative transcriptional activators, such as NSP1, NSP2 and

ERN (Kalò et al. 2005; Smit et al. 2005; Murakami et al. 2006; Middleton et al. 2007).

Our results performed in suspension cell cultures show that a mutation in the allele *Ljsym* changes the cell sensitivity to SA by inducing cell death in mutant cells when not in wild type.

The different behavior, detectable at the cellular level, invokes a general cell function for the *Ljsym* gene not limited to root tissues. In this context, it is worth mentioning that the two orthologues CASTOR and POLLUX are ubiquitously present and highly conserved in both legumes and non-legumes (Chen et al. 2009). In tobacco cell suspension culture, it has been reported that SA induces calcium influx (Lin et al. 2005). We can hypothesize that an increase in calcium concentration inside mutant cells can be "misinterpreted" in cells lacking calcium spiking and, as a consequence, a double peak of H_2O_2 and an increase in NO level are induced leading to a program of cell death. At the moment, we are not able to suggest a detailed molecular mechanism that links the function of the *Ljsym* gene and perception and signal transduction induced by SA, but investigations are underway to clarify these links.

Conclusions

In suspension cell cultures, we have compared the SA sensitivity of wild type and *Ljsym4-2* mutant showing clear differences. Different parameters of the SA-dependent signaling pathway were significantly altered in the *Ljsym4-2* cells compared to wt cells. The most relevant result is the different sensitivity of wild type and mutant cells to SA in cell death induction. These results gave us the opportunity to begin some ad hoc experiments on plant seedlings that suggest the induction of a plant defense-like response. The double approach—cell culture and plant seedlings—should help in the future to better understand the beneficial and pathogenic role of SA in plants.

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