Plant Mol Biol (2013) 81:273–286
DOI 10.1007/s11103-012-9997-8

Enhanced *Agrobacterium*-mediated transformation efficiencies in monocot cells is associated with attenuated defense responses

Wan-Jun Zhang · Ralph E. Dewey · Wendy Boss · Brian Q. Phillippy · Rongda Qu

Received: 4 June 2012 / Accepted: 6 December 2012 / Published online: 15 December 2012

Abstract Plant defense responses can lead to altered metabolism and even cell death at the sites of *Agrobacterium* infection, and thus lower transformation frequencies. In this report, we demonstrate that the utilization of culture conditions associated with an attenuation of defense responses in monocot plant cells led to highly improved *Agrobacterium*-mediated transformation efficiencies in perennial ryegrass (*Lolium perenne* L.). The removal of *myo*-inositol from the callus culture media in combination with a cold shock pretreatment and the addition of L-Gln prior to and during *Agrobacterium*-infection resulted in about 84 % of the treated calluses being stably transformed. The omission of *myo*-inositol from the callus culture media was associated with the failure of certain pathogenesis related genes to be induced after *Agrobacterium* infection. The addition of a cold shock and supplemental Gln appeared to have synergistic effects on infection and transformation efficiencies. Nearly 60 % of the stably transformed calluses regenerated into green plantlets. Calluses cultured on media lacking *myo*-inositol also displayed profound physiological and biochemical changes compared to ones cultured on standard growth media, such as reduced lignin within the cell walls, increased starch and inositol hexaphosphate accumulation, enhanced *Agrobacterium* binding to the cell surface, and less H_2O_2 production after *Agrobacterium* infection. Furthermore, the cold treatment greatly reduced callus browning after infection. The simple modifications described in this report may have broad application for improving genetic transformation of recalcitrant monocot species.

Keywords Cold shock · Glutamine · *myo*-inositol · Plant defense response · Transformation

Introduction

Agrobacterium tumefaciens is a gram-negative, soil-borne bacterium that naturally has a broad host range in plants, primarily dicot species. In nature, wild type *A. tumefaciens* causes a crown gall disease on host species, facilitated by the import and integration of a transfer DNA (T-DNA) from a tumor-inducing (Ti) plasmid from *A. tumefaciens* to the host cells (Gelvin 2003, 2005, 2010; Dafny-Yelin et al. 2008). This feature of *A. tumefaciens* has been widely exploited to introduce transgenes to plant, fungi, and even mammalian cells under laboratory conditions (Gelvin 2005; Dafny-Yelin et al. 2008). However, as a bacterium that is pathogenic to plants, *Agrobacterium* elicits a wide spectrum of defense responses within infected plant cells (Ditt et al. 2006; Torres et al. 2006; Zipfel et al. 2006). The recognition of pathogen-associated molecular patterns (PAMPs) (Torres et al. 2006; Zipfel et al. 2006; Dafny-Yelin et al. 2008) by the plant's innate immune system represents the first layer of defense against pathogenic bacterial attack. The recognition of PAMPs and the

Electronic supplementary material The online version of this article (doi:10.1007/s11103-012-9997-8) contains supplementary material, which is available to authorized users.

W.-J. Zhang (✉)
Department of Grassland Science, China Agricultural University, Beijing 100193, China
e-mail: wjzhang@cau.edu.cn

W.-J. Zhang · R. E. Dewey · R. Qu
Crop Science Department, North Carolina State University, Campus Box 7287, Raleigh, NC 27695, USA

W. Boss · B. Q. Phillippy
Plant Biology Department, North Carolina State University, Campus Box 7649, Raleigh, NC 27695-7649, USA

subsequent plant defense responses are also referred to as PAMP-triggered immunity (PTI) (Jones and Dangl 2006; Zipfel 2008). The cellular responses associated with PTI include rapid ion fluxes across the plasma membrane, the generation of reactive-oxygen-species, activation of mitogen-activated protein kinases and rapid changes in gene expression, such as the induced expression of pathogenesis related genes (*PRs*) (Asai et al. 2002; Djamei et al. 2007; Dafny-Yelin et al. 2008; Zipfel 2008; Gelvin 2010). Clearly, by using *Agrobacterium* as a vehicle for genetic transformation, an array of plant defense responses will be elicited during the course of infection.

In *Arabidopsis* it has been shown that susceptibility to *Agrobacterium* infection can be increased in plants possessing a mutant receptor kinase gene that is essential for perception of the bacterial PAMP factor EF-Tu (Zipfel et al. 2006). Dampening plant immunity by the expression of a *Pseudomonas syringae* type III effector that targets multiple pattern-recognition receptors also enhanced *Agrobacterium*-mediated transient expression of foreign genes in *Arabidopsis* (Tsuda et al. 2012). Alleviating defense responses of plants in vitro as a means of improving *Agrobacterium*-mediated transformation, however, remains a challenge.

Myo-inositol has numerous functions in eukaryotes, affecting a variety of developmental and physiological processes (Boss et al. 2006; York 2006; Michell 2008; Munnik and Nielsen 2011). It has been shown to be an important intermediate for plant cell wall polysaccharide synthesis in intact plants and detached plant tissues (Loewus 1965), as well as in cultured cells (Roberts and Loewus 1966; Loewus and Murthy 2000). Despite the fact that it can be synthesized *de novo* from glucose in plant cells (Thorpe et al. 2008), *myo*-inositol is a common constituent in standard plant culture media, as its addition is believed to improve plant regeneration (Murashige and Skoog 1962; Gamborg et al. 1968; Schenk and Hildebrandt 1972; Loewus 1990). Recently, a link between inositol metabolism and plant programmed cell death was revealed (Murphy et al. 2008; Meng et al. 2009; Chaouch and Noctor 2010), suggesting a direct role of *myo*-inositol and/or its derivatives in plant defense responses.

The metabolism of glutamine has also been implicated in the plant response to pathogen infection (Pageau et al. 2006; Liu et al. 2010). The *Arabidopsis* amino acid transporter mutant *lth1* showed enhanced disease resistance to a broad spectrum of pathogens, a response that was suppressed by L-glutamine (L-Gln) (Liu et al. 2010). L-Gln was also found to inhibit beta-aminobutyric acid (BABA)-induced plant resistance to a bacterial pathogen of *Arabidopsis* (Wu et al. 2010). Although the molecular mechanisms underlying how glutamine metabolism affects plant defense responses are poorly understood, these results suggested that L-Gln could play a role in mitigating plant defense responses and thus potentially could be modulated toward improving the efficiency of *Agrobacterium*-mediated plant transformation.

One of the long term goals of our research program is to utilize transformation technologies as a means of improving perennial ryegrass, a monocot plant species. *Agrobacterium*-mediated transformation of perennial ryegrass has been reported by several groups. Wu et al. (2005) reported a whole plant stable transformation efficiency of 14 % for perennial ryegrass after optimizing the timing of acetosyringone exposure and including an osmotic treatment. Cao et al. (2006) reported perennial ryegrass transformations efficiencies as high as 23 % after testing the effects of a variety of light, temperature and antioxidant conditions at different steps. Bajaj et al. (2006) established a transformation system with embryogenic cell lines, and the highest transformation efficiency obtained was 12 %. Other publications describing perennial ryegrass transformation reported efficiencies of 16 % or less (Altpeter 2006; Sato and Takamizo 2006; Wu et al. 2007). Because our initial efforts to obtain high efficiency transformation of perennial ryegrass by repeating conditions reported by other labs were not successful, there was a need to optimize the procedure. After comparing and testing an array of parameters, we established a protocol that was convenient for callus induction and proliferation, and enhanced *Agrobacterium*-mediated transformation. During this process we discovered that the removal of *myo*-inositol from the callus culture media, combined with the addition of L-Gln and a cold treatment during the transformation process dramatically improved the overall transformation efficiency. This enhancement in transformation efficiency was likely attributable to suppression of the plant defense response, as attenuation in the expression of certain *PRs*, increased *Agrobacterium* binding, and a reduction in H_2O_2 production was observed as a result of the modified treatment conditions. Similar results were observed when the same protocol was applied to transformation of rice suspension culture cells.

Results

Enhanced perennial ryegrass transformation using a combined treatment

Calluses of perennial ryegrass (cv. Monterey II) were transformed with *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pTJN33-gfp. Among the various factors investigated to improve perennial ryegrass transformation efficiency, the following were found to increase both transient and stable expression of the *gfp* reporter gene: (1) removal of *myo*-inositol from the callus

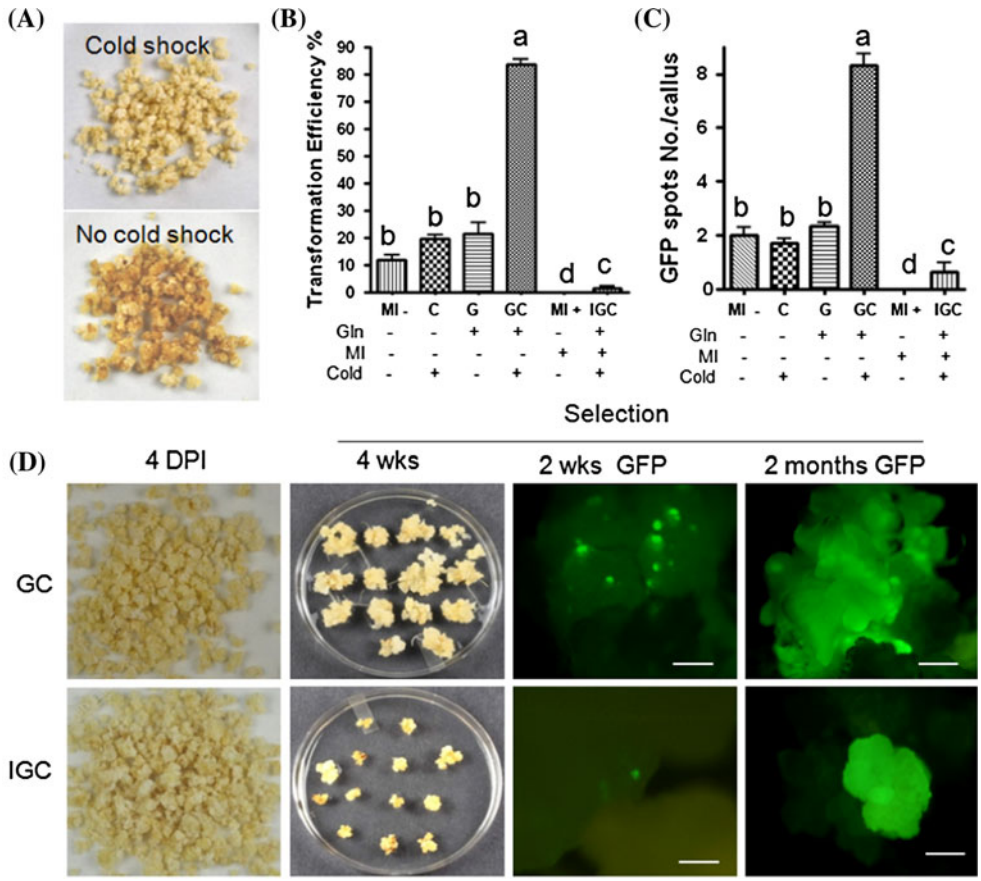

Fig. 1 Enhanced callus transformation of perennial ryegrass by combined treatments. **a** Cold-shock treatment prevents calluses from browning after *Agrobacterium* infection. The calluses were cultured on media containing *myo*-inositol (MI+). Photos were taken after 4 days of co-cultivation. **b** Factors that affect perennial ryegrass transformation. Four-month-old calluses cultured on media supplied with or without 100 mg l^{-1} *myo*-inositol (MI+ or MI−) were either incubated on ice for 20 min (Cold+) or at room temperature (Cold−). Addition of 100 μM L-Gln to the cold shock solution and bacterial suspension media (Gln+) was also tested. The *columns* and *bars* represent means and standard errors from three independent experiments using a total of 135 pieces of callus per treatment. *Different letters* indicate significant differences between treatments in a two-tailed student's *t* test ($p < 0.05$). **c** Means and standard errors are shown for the number of GFP spots per GFP positive callus from (b). Different letters indicate significant differences between treatments in a two-tailed student's *t* test ($p < 0.05$). **d** Removal of *myo*-inositol from the culture media enhances perennial ryegrass transformation under conditions of cold shock and L-Gln treatments. The calluses pre-cultured on media without *myo*-inositol showed faster recovery and more vigorous growth under selection of 5.0 mg l^{-1} PPT. Typical GFP expression in calluses 2 weeks and 2 months after selection on media either containing (IGC) or lacking (GC) supplemental *myo*-inositol is also shown. *Scale bar* = 1 mm

pre-culture media (MI−); (2) application of a cold shock prior to *Agrobacterium*-infection (C+); and (3) addition of 100 μM L-Gln (Gln+) to the solution prior to and during the infection (Fig. 1). The effects of these specific treatments on perennial ryegrass transformation, both alone and in combination, are presented below.

During perennial ryegrass transformation, we observed that the calluses were prone to turn brown after *Agrobacterium* infection, especially for those maintained on media supplied with 100 mg l^{-1} *myo*-inositol. Callus browning was significantly reduced, however, when a cold shock treatment was applied by immersing the calluses in 3 % maltose and placing the samples on ice for 20 min prior to infection (Fig. 1a). Moreover, it was observed that the addition of L-Gln to both the maltose solution and the *Agrobacterium* suspension media also had a positive effect on transformation frequencies. In preliminary experiments, several concentrations of L-Gln were tested and 100 μM L-Gln was found to be optimal for enhancing *Agrobacterium*-mediated transformation (data not shown); therefore this concentration was adopted in our standard protocol. As shown in Fig. 1b, although each of the three factors had a positive effect on transformation frequency, the combination of all three (labeled as GC) appeared to be synergistic, dramatically improving the transformation efficiency of perennial ryegrass.

It was clear from these studies that *myo*-inositol played a particularly key role among the three factors tested. The addition of *myo*-inositol to callus culture media seemed to have an inhibitory effect on transformation. In its presence,

minimal improvement in transformation efficiency was observed even when the L-Gln and cold treatments were applied (IGC in Fig. 1b). The efficiency of stably transformed calluses increased from 0 % in *myo*-inositol containing media (MI+), to 12 % for materials in media lacking *myo*-inositol (MI−), based on the number of calluses containing clusters of GFP-positive cells 1 month after selection was initiated. The efficiency was improved to about 20 % when the cold shock (C) or L-Gln (G) treatments were added individually. The combination of the three treatments (GC) resulted in a much higher transformation efficiency of 84 %. A similar trend was observed when the number of GFP spots per GFP positive callus was counted. The GC treatment yielded an average of about eight GFP spots per callus, in contrast to other treatments which averaged two or less GFP spots per callus (Fig. 1c). During the callus culture stage and immediately after infection, callus morphology looked similar whether the callus was cultured on media containing or lacking *myo*-inositol. However, when transferred to selection media containing 5.0 mg l^{-1} phosphinothricin (PPT), calluses from the GC treatment showed faster recovery and more vigorous growth. Significant differences in callus size between GC versus IGC treatments were very apparent by 4 weeks after selection (Fig. 1d). Even just 2 weeks after selection, multiple GFP spots were observed in the GC treated callus without *myo*-inositol, whereas the callus maintained on media with *myo*-inositol (IGC) showed sluggish growth, more cell death and fewer GFP spots per callus (Fig. 1d). Two months after selection, the GFP positive calluses from the GC treatment showed robust growth and obvious embryogenesis (Fig. 1d, 2 months GC), while those from the IGC treatment were much less vigorous and plant regeneration took about 2 months longer (data not shown).

Removal of *myo*-inositol from culture media does not negatively affect plant regeneration

In transformation experiments using plasmid pTJN33-gfp (Fig. 2a), we observed that the age of the starting callus also impacted transformation efficiency. When the three factors that define the GC treatment were applied to 2-, 3-, and 4-month-old calluses (corresponding to one, two and three subcultures), stable transformation efficiencies of 27, 72, and 84 % were observed, respectively. Longer culture periods were avoided as they had a tendency to reduce the capacity for subsequent plant regeneration, with or without supplemental *myo*-inositol (data not shown). Thus, calluses that had undergone three rounds of subculture (about 4-month-old) were selected for most of our transformation experiments.

To investigate whether removal of *myo*-inositol from callus culture media had a detrimental effect on the subsequent regeneration of whole plantlets, calluses showing GFP expression that were generated using the GC treatment that lacks *myo*-inositol were picked randomly and tested for regeneration competency. As shown in Figs. 2c, d and Table 1, the GFP positive calluses generated from transformation of 4-month-old calluses regenerated well. An average regeneration rate of 59 % was achieved in three independent transformation experiments, which suggests that removal of *myo*-inositol is not detrimental to plant regeneration per se. The transgenic plants rooted well on media containing 10 mg l^{-1} PPT (Fig. 2c), and grew normally in the greenhouse. Using a leaf painting assay, the transgenic plants showed resistance to a 15 mg l^{-1} PPT solution applied to the leaves (Fig. 2e). The presence of the transgene was verified by DNA Southern blot analysis using the *bar* coding sequence as a hybridization probe (Fig. 2f). When the same genomic DNAs were digested with *Hin*dIII, a restriction enzyme that cuts just once within the vector sequence (Fig. 2a), and hybridized to a *gfp* probe, any array of banding patterns was observed indicating that these transgenic plants were derived from independent transformation events (data not shown).

Myo-inositol supplementation has profound effects on cell wall lignin content, starch accumulation, *Agrobacterium* binding and inositol hexaphosphate ($InsP_6$) content

Although plant cells autonomously synthesize *myo*-inositol from glucose (Loewus and Murthy 2000), our experiments indicated that supplementation of *myo*-inositol to the culture media mediates multiple effects on callus metabolism. As shown in Fig. 3a, lignin was readily detected in cell walls of 4-month-old callus grown on media containing *myo*-inositol, whereas comparable callus growing on media without *myo*-inositol supplementation did not show appreciable lignin accumulation. This result suggests that the addition of *myo*-inositol to the culture media promotes the development of secondary cell walls. Moreover, starch metabolism within the callus was strongly affected. Calluses grown on media containing *myo*-inositol showed no obvious starch accumulation (as determined by iodine staining), while those maintained on media without *myo*-inositol showed intense staining for starch (Fig. 3a), indicating that supplementation of *myo*-inositol had a great effect on carbohydrate partitioning in the cultured callus cells. Additional studies also revealed that *myo*-inositol affects the binding of *Agrobacterium* to the perennial ryegrass cells. About four times the number of tightly bound *Agrobacterium* were observed on calluses when *myo*-inositol was removed from the culture media (Fig. 3b), suggesting that the metabolic changes caused by

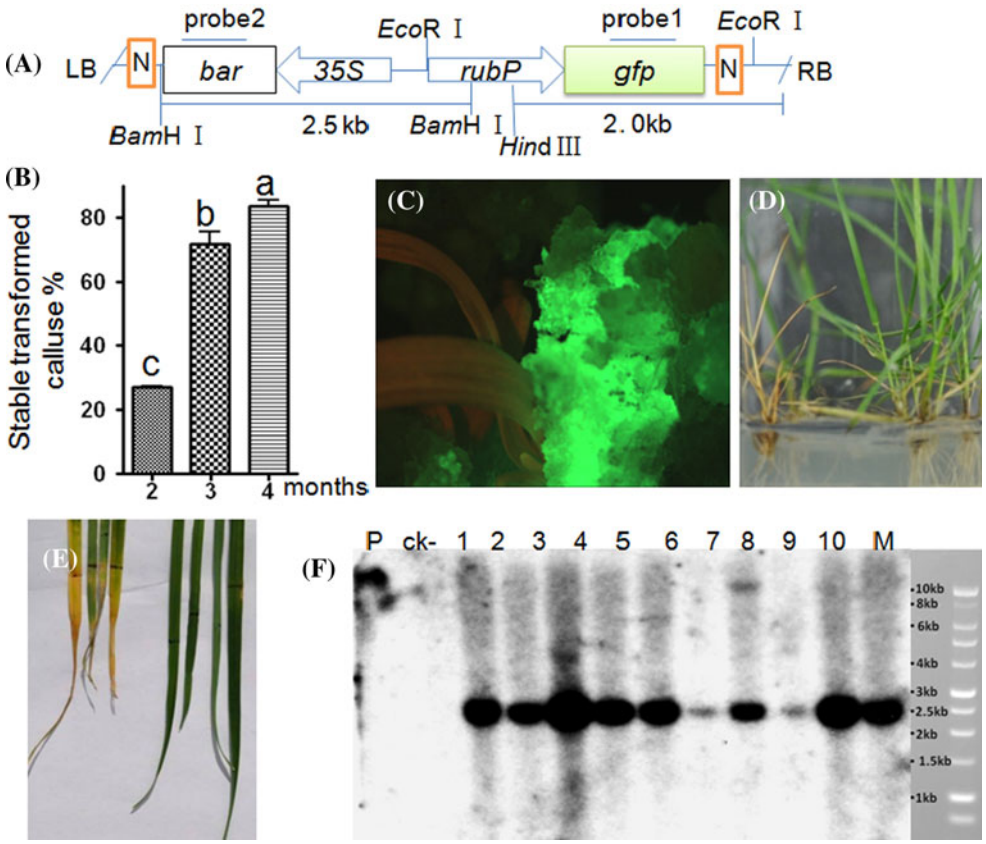

Fig. 2 Removal of *myo*-inositol from culture media does not prevent plant regeneration. **a** Schematic map of T-DNA region of plasmid pTJN33-gfp. *LB*, left border; *35S*, CaMV 35S promoter; *bar*, herbicide resistance gene; *rub*P, rice *rubi3* gene promoter; *N*, nopaline synthase terminator; *RB*, right border; The regions corresponding to the probes used in Southern blots are also shown. **b** Stable expression of a foreign gene (*gfp*) in perennial ryegrass callus as a function of callus age. Transformation was conducted using the GC treatment. Calluses were sub-cultured onto fresh media once a month. The *columns* and *bars* represent means and standard errors from three independent experiments using a total of approximately 180 pieces of callus. *Different letters* above the columns indicate significant differences under a two-tailed student's *t* test, $p < 0.05$. **c** Fluorescence microscopy of GFP-expressing calluses. **d** Transgenic plants rooting on MS medium containing 10 mg l^{-1} PPT. **e** Results of leaf painting with 15 mg l^{-1} PPT. Leaves of wild type plants (*left*) and transgenic plants (*right*) are shown 2 weeks after PPT application. **f** Southern blot analysis of transgenic perennial ryegrass plants. Ten randomly selected GFP positive plants were tested using a *bar* PCR product as a probe. P, 10 pg of non-digested pTJN33-gfp plasmid DNA (positive control); ck-: Digested genomic DNA of a non-transgenic plant (negative control); 1–10, Plant DNA was digested with *Bam*HI. A photograph of the molecular markers from the corresponding gel prior to transfer of the digested plant genomic DNAs is shown on the right of the panel. A 2.5 kb band is expected for transgenic plants containing an intact *bar* gene

Table 1 Plant regeneration efficiencies of randomly selected GFP positive calluses from three independent transformation experiments using 4-month-old calluses sub-cultured on *myo*-inositol free media

Exp.	No. of callus lines (A)	No. of callus lines that generated GFP plants (B)	Efficiency (B/A, %)
1	48	25	52.1
2	38	22	57.9
3	38	26	68.4
Total	124	73	58.9

the removal of *myo*-inositol may favor the processes involved in *Agrobacterium* binding to plant cells, and thus its potential infectivity.

Considering the key role of *myo*-inositol in the biosynthesis of various inositol phosphates, including $InsP_6$ (Loewus and Murthy 2000), and the various functions of inositol phosphates in signal transduction, chromatin remodeling, RNA export and DNA repair (Boss et al. 2006; York 2006; Gillaspy 2010), it is possible that the altered metabolism of inositol phosphates could affect the genetic transformation frequency. As an initial attempt to begin investigating possible mechanisms underlying the effects of *myo*-inositol on transformation efficiency, we measured $InsP_6$ concentrations within the callus after each subculture. As shown in Fig. 3c, we observed that the $InsP_6$ accumulation in the cultured callus increased with time in culture. Removal of *myo*-inositol from the culture media further increased $InsP_6$ and resulted in a more than

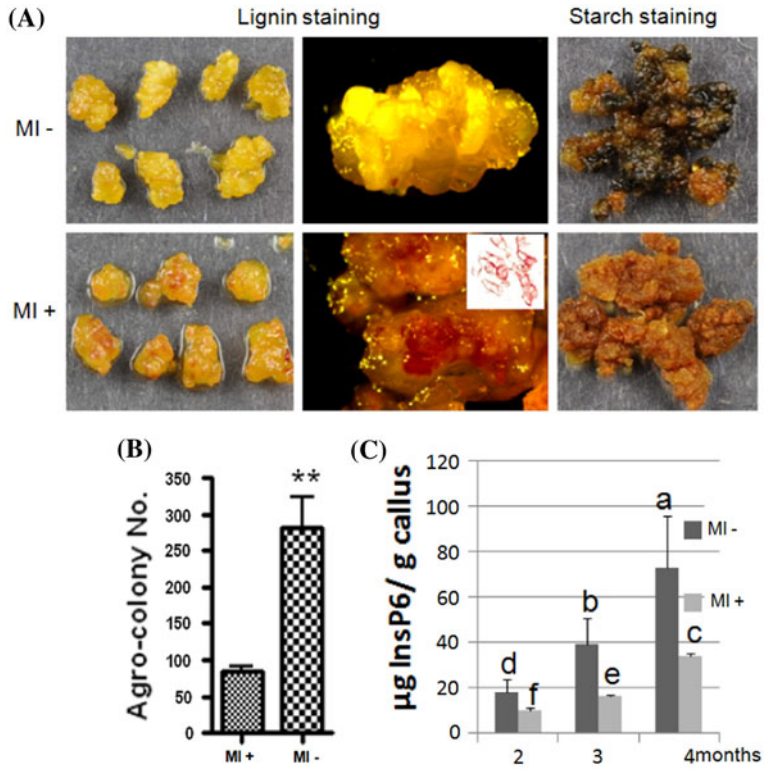

Fig. 3 Effects of *myo*-inositol on perennial ryegrass callus. **a** Lignin and starch staining of 4-month-old calluses cultured on media with (MI+) or without (MI−) 100 mg l^{-1} *myo*-inositol supplementation. Lignin deposits stain red, and starch accumulation shows a dark-blue coloration after staining with a saturated solution of phloroglucinol in 20 % HCl, and I_2-KI, respectively. The picture *inset* represents a magnification of crushed callus after staining, which shows the stained lignin located in cell walls. **b** Quantification of *Agrobacterium* tightly bound to calluses cultured on media with (MI+) or without (MI−) 100 mg l^{-1} *myo*-inositol. *Columns* and *bars* represent the means and standard errors of colony counts from three independent experiments. The *double asterisks* indicate significant differences using a one way ANOVA test ($p < 0.01$). **c** Comparison of inositol hexaphosphate ($InsP_6$) accumulation in callus (µg/g fresh weight) of various age maintained on media containing (MI+) or lacking *myo*-inositol (MI−). *Different letters* above the *columns* indicate significant differences under a two-tailed student's *t* test, $p < 0.05$. Data presented are mean ± SE (n = 3)

doubling of the $InsP_6$ levels in callus across all ages tested, indicating that both time in culture and supplementation with *myo*-inositol had major effects on inositol phosphate metabolism.

Removal of *myo*-inositol and the combined L-Gln and cold treatments reduce callus H_2O_2 generation and attenuate the plant defense response after *Agrobacterium* infection

Prior to our optimization of the perennial ryegrass transformation protocol, we frequently noticed browning and necrosis of the callus after *Agrobacterium* infection and observed that the browned calluses were slower to recover after co-cultivation. We speculated that the browning might be caused by H_2O_2 generation as part of the plant cell defense response. H_2O_2 generation in callus, 10 days after *Agrobacterium* infection, was tested using 3,3′-diaminobenzidine (DAB) staining (Thordal-Christensen et al. 1997). As shown in Fig. 4a, most of the callus tissue grown on *myo*-inositol-containing media without cold and L-Gln treatments (MI+) showed pronounced DAB staining, suggesting high levels of H_2O_2. Those calluses exposed to the cold and L-Gln treatments (IGC), and calluses grown on inositol-free media but without the cold and L-Gln treatments (MI−), stained less intensely with DAB. For the calluses exposed to all three treatments (GC), very little H_2O_2 was detected. Given that H_2O_2 production is a hallmark of plant defense responses (Wu et al. 1997), these results are supportive of the premise that the treatments used to enhance transformation efficiency mitigate the plant defense system triggered by *Agrobacterium*-infection.

As a more direct means of evaluating whether the observed improvement of *Agrobacterium*-mediated transformation involves altering the plant defense response, transcripts of some perennial ryegrass *PRs,* namely, *LP-PR1, 2, 3, 4, 5* and *10* (Tanaka et al. 2006; Zhang et al. 2011), were analyzed by semi-quantitative RT-PCR both before and 8 days after *Agrobacterium* infection (Fig. 4b). The results showed that the expression of *LP-PR1, LP-PR2, LP-PR3, LP-PR4* and *LP-PR5* were induced after *Agrobacterium*-infection, whereas *LP-PR10* expression was constant and

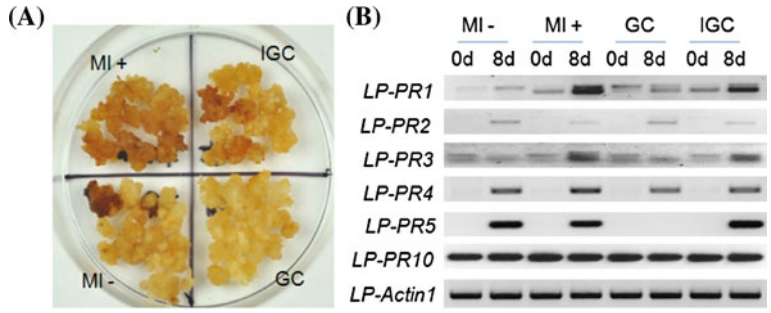

Fig. 4 Attenuated defense responses of perennial ryegrass callus using treatments associated with enhanced transformation efficiency. **a** H_2O_2 generation in calluses 10 days after *Agrobacterium* infection detected by staining with 1 mg l^{-1} DAB. Calluses with high levels of H_2O_2 stain brown. Similar results were obtained from three independent experiments. **b** Transcript analysis of perennial ryegrass *PRs* before and 8 days after *Agrobacterium* infection using semi-quantitative RT-PCR. Expression of the *LP-Actin1* gene was used as a template loading control

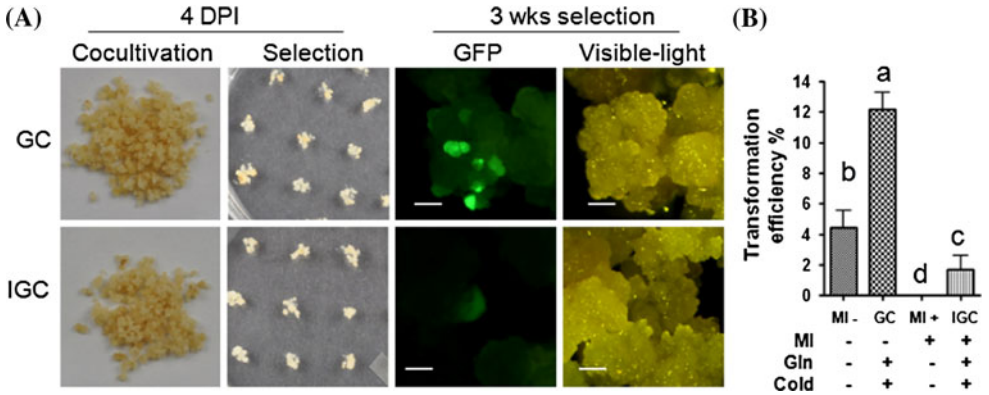

Fig. 5 Enhanced rice callus transformation. **a** Removal of *myo*-inositol from the culture media enhances rice (cv. Nipponbare) callus transformation. At 4 days after *Agrobacterium* infection (4 DPI) the calluses looked similar under both treatment conditions. After 3 weeks of selection on media containing 5.0 mg l^{-1} PPT, the calluses generated from the media without *myo*-inositol showed multiple GFP spots and robust growth, whereas calluses generated from media containing *myo*-inositol had fewer GFP spots and grew more slowly. *Scale bar* = 1 mm. **b** The combined treatment improves rice callus transformation efficiency. Data presented are the mean ± SE (n = 3) of three independent experiments using a total of approximately 180 pieces of callus. *Different letters* above the *columns* indicate significant differences under a two-tailed student's *t* test, $p < 0.05$

unaffected by the infection. Among the six *PRs* investigated, both *LP-PR1* and *LP-PR3* appeared to have two isoforms, and their expression was clearly influenced by the presence or absence of *myo*-inositol in the media. *Agrobacterium*-mediated induction of both *LP-PR1* and *LP-PR3* was much reduced in the treatments lacking *myo*-inositol (MI− and GC) when compared to the treatments containing *myo*-inositol in media (MI+ and IGC), and the relative expression patterns of the two isoforms were also altered. The expression pattern of *LP-PR5* was not affected by removal of *myo*-inositol alone (MI−) or by the cold and L-Gln treatments in the presence of inositol (IGC). However, *LP-PR5* induction was dramatically suppressed by the combined treatment without *myo*-inositol (GC), suggesting a synergistically negative effect of these treatments on *LP-PR5* induction. These results indicate that the treatments shown to enhance transformation efficiency do affect the plant defense response to *Agrobacterium* infection.

Enhanced rice callus transformation using the combined treatments

To test whether the combined treatments can also promote transformation in other monocot species, a rice (*Oryza sativa*) suspension cell line of cv. Nipponbare (Sivamani and Qu 2006) was cultured on the same media used for perennial ryegrass callus maintenance. No obvious morphological differences were observed among the calluses grown on media supplied with or without *myo*-inositol before or immediately after infection with *Agrobacterium*. However, 3 weeks after selection, calluses that were cultured on media lacking *myo*-inositol had multiple GFP spots and showed vigorous growth, whereas calluses generated from the media with *myo*-inositol had fewer GFP spots and grew more slowly (Fig. 5a). Stable callus transformation was determined 1 month after selection based on GFP expression. Statistical analysis of three independent

experiments showed that the GC treatment resulted in an average efficiency of 12 %, a rate that was significantly greater than that observed with other treatments (Fig. 5b). The treatment where *myo*-inositol was simply removed from callus culture media (MI−) had an efficiency of 4 % and the IGC treatment had an efficiency of approximately 2 %. No stably transformed calluses were observed when the rice cells were cultured using inositol-containing media and not provided with the L-Gln and cold treatments (MI+). These results suggest that the transformation response in rice is similar to that of perennial ryegrass in that removal of *myo*-inositol from the media improved transformation efficiency, and cold shock and L-Gln treatments further enhanced this process. As shown in Supplementary Fig. 1, 3-month-old rice calluses that were cultured on media supplied with or without *myo*-inositol for two subcultures showed similar patterns in lignin and starch accumulation as perennial ryegrass calluses.

Arabidopsis responds differently to supplementary *myo*-inositol in *Agrobacterium*-mediated root transformation

To investigate whether the treatments used to enhance transformation efficiencies in perennial ryegrass and rice would have a similar effect when applied to a dicot species, additional experiments were conducted in *Arabidopsis thaliana* using the wild-type *Agrobacterium* strain A208 (Fig. 6a). Root fragments of 1-month-old seedlings that had been germinated on media either with or without *myo*-inositol (MI+ or MI−) were infected in the presence or absence of the L-Gln and cold shock treatments and evaluated for crown gall formation. These results showed that the combination of the cold shock and 100 μM L-Gln treatments (GC and IGC, respectively) significantly promoted crown gall formation (Fig. 6a; Supplementary Fig. 2a). Compared to the MI− and MI+ treatments, the transformation efficiencies of the corresponding GC and IGC treatments increased by about 60 %. Although the L-Gln and cold treatments significantly enhanced transformation efficiency, supplementation with *myo*-inositol, whether alone or in addition to the L-Gln and cold treatments, had no effect on *Arabidopsis* root fragment transformation and crown gall formation, suggesting that the presence or absence of *myo*-inositol in the media had no obvious effect on these processes in *Arabidopsis*. Moreover, no differences in staining for lignin or starch accumulation were observed in the roots of *Arabidopsis* seedlings grown in the presence or absence of supplemental *myo*-inositol (Supplementary Fig. 2b).

To evaluate whether the plant defense response of *Arabidopsis* was altered due to the unique set of treatments in *Agrobacterium*-mediated transformation, the expression of *Arabidopsis PRs*, *AtPR1, 2, 3* and *4* (Doczi et al. 2007) was analyzed, before and 2 days after *Agrobacterium* infection, using semi-quantitative RT-PCR (Fig. 6b). The expression/induction patterns of these *PRs* in response to the applied treatments were quite different in *Arabidopsis* from that observed in perennial ryegrass. For example, in the absence of *myo*-inositol (MI− and GC), *AtPR1* expression was already high prior to infection and was not further induced by the infection. In the presence of *myo*-inositol (MI+ and IGC), however, its basal expression was low, but then strongly induced 2 days after infection. Expression of *AtPR2* and *AtPR4* was induced under all of the tested treatments, except that the induction of *AtPR2* using the GC treatment appeared to be weaker when compared to IGC. In contrast, the expression of *AtPR3* was constant and unaffected by either supplementary *myo*-inositol or *Agrobacterium* infection. The remarkable differences in the expression patterns of *PRs* with respect to the various treatments emphasizes the point that plant defense reactions to *Agrobacterium* infection and other cultural factors can differ substantially between monocots and dicots, and that these differences may contribute toward the variations in the transformation results observed in our studies.

Discussion

Agrobacterium-mediated perennial ryegrass transformation has been previously reported, but the transformation efficiencies were highly variable. The great complexity of genotypes that is characteristic of perennial ryegrass, together with differences in choice of explant selection are among the contributing factors leading to the development of numerous protocols by different laboratories. None of the reported protocols, however, appeared to be sufficiently robust to enable high throughput functional genomics research. To facilitate our long-term project goals, we designed numerous experiments to optimize the *Agrobacterium*-mediated transformation of perennial ryegrass. Of the many parameters tested, the removal of *myo*-inositol, the addition of glutamine, and a cold treatment appeared to be particularly beneficial in enhancing transformation efficiency. Using this optimized protocol, several functional genes have been delivered to the perennial ryegrass genome with high efficiency, and transgenic plants have been obtained in less than 3 months (unpublished results).

Myo-inositol, a common ingredient in plant culture media, is an important growth factor that plays a central role in plant growth and development (Loewus 1990; Loewus and Murthy 2000). In this report, we observed that removal of *myo*-inositol from culture media resulted in a series of physiological changes within the calluses, and

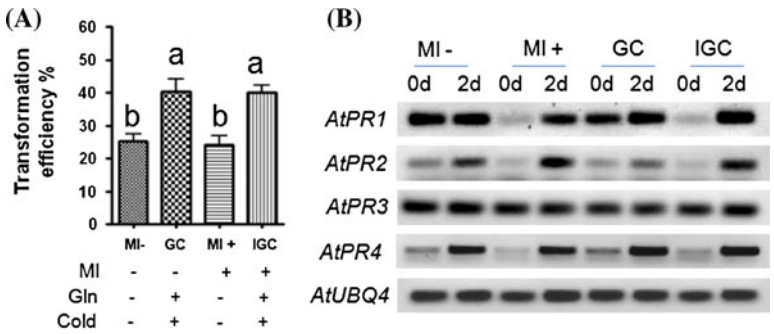

Fig. 6 *Arabidopsis* responds differently to *myo*-inositol in *Agrobacterium*-mediated root transformation. **a** L-Gln and cold shock treatments facilitated *Arabidopsis* root fragment transformation and crown gall formation, but *myo*-inositol had no obvious effect. Data presented are mean ± SE (n = 3) of gall formation from three independent experiments using a total of about 720 root fragments. *Different letters* indicate significant differences in a two-tailed student's *t* test ($p < 0.05$). **b** Transcript analysis of *Arabidopsis PRs* by semi-quantitative RT-PCR before and 2 days after *Agrobacterium* infection in response to the treatments shown in **a**. *AtUBQ4* was used as a template loading control

enhanced *Agrobacterium*-mediated genetic transformation of perennial ryegrass and rice, two monocot species. The concept of using *myo*-inositol as a constituent in culture media stemmed from early observations that certain natural complex supplements used in media formulations to promote growth, such as coconut milk, contained high levels of this compound (Loewus 1990). It has subsequently become one of the most common constituents found in media used today. Due to the near ubiquitous inclusion of *myo*-inositol in plant growth media, our observations of enhanced transformation efficiency through the omission of this compound in monocots is certainly the most surprising of the results presented in this study, especially considering the fact that a plethora of plant transformation protocols have been already established in which this compound is included as a component of the media. Our inability to recover stably transformed calluses from perennial ryegrass and rice in our experiments when *myo*-inositol was included in the culture medium was unexpected, particularly give that others have reported relatively high transformation efficiencies in perennial ryegrass with this constituent included in the media (Wu et al. 2005; Nishimura et al. 2006; Cao et al. 2006). It is clear that the plant transformation process is complex, and that there are multiple ways by which transformation efficiencies in monocot species may be enhanced, with or without modulating the inositol. Given the results of this study, however, it will be of interest to determine the extent with which existing monocot transformation protocols may be enhanced even further simply through the removal of this constituent.

In addition to its role in cell wall synthesis, extensive research has revealed that *myo*-inositol and/or its large family of derivatives are involved in signaling pathways in eukaryotes, being associated with ABA (Xiong et al. 2001), salt stress (Takahashi et al. 2001), gravity (Perera et al. 2006) and plant defense responses (Ortega and Perez 2001; Murphy et al. 2008; Meng et al. 2009). Our data strongly support the notion that *myo*-inositol can have profound effects on plant cellular metabolism, cell wall structure, and plant defense reactions to pathogen infection. In this report, we demonstrate that removal of *myo*-inositol from the culture media of perennial ryegrass callus directly affects the expression of *PRs*. The most straightforward interpretation is that the plant defense response has been attenuated under these conditions, leading to an enhancement in genetic transformation efficiency. To our knowledge, this is the first direct link between exogenous supply of *myo*-inositol, the expression of *PRs* and plant defense reactions. Our observations did not apply to the dicot plant *Arabidopsis*, however, indicating that there may be fundamental differences in the roles of exogenously added *myo*-inositol in regulating metabolism and gene expression between the two major plant classifications. These findings may lead to new approaches in mitigating plant defense pathways toward enhancing *Agrobacterium*-mediated plant transformation across an array of monocot plants, as well as providing new insights into the mechanisms of pathogen infection in plants.

Our results also demonstrated alterations in cell wall lignin content and *Agrobacterium* binding capacity resulting from the addition of *myo*-inositol to the culture media. Since monocot plants generally are not native hosts of *Agrobacterium*, they normally have fewer bacteria tightly bound to their cell walls after infection (DeCleene 1985). The cell walls of monocot grasses and dicot plants differ considerably in chemical composition. For example, the primary cell wall of grasses contains much higher levels of arabinoxylan, and much less pectin and xyloglucan than that of dicot plants (Fry 1988). Mutations that affected cell wall synthesis and structure, and resulted in altered transformation efficiency, have been isolated in *Arabidopsis*, suggesting the importance of cell wall structure and

Agrobacterium binding to the cell surface in the transformation of plants (Gelvin 2009).

In our study, the content of $InsP_6$, a product of inositol metabolism, steadily increased with the callus age during the 4 month culture period tested, and the content of $InsP_6$ was significantly higher when the media was free of *myo*-inositol, correlating positively with transformation efficiency. This trend is in contrast with some studies where a reduction, not increase, in $InsP_6$ content was reported to be associated with reduced plant basal immunity, and thus enhanced pathogen susceptibility (Murphy et al. 2008; Meng et al. 2009; Donahue et al. 2010). Each of these studies, however, was conducted using *Arabidopsis*, a species in which we observed the metabolism of exogenous inositol to be fundamentally different than perennial ryegrass or rice. In plants, $InsP_6$ represents an important phosphate reserve in seed tissue, which can subsequently be used to assist plant growth and development during germination. Recently, however, a wide range of important functions associated with cellular signaling have been characterized for $InsP_6$ in both plants and mammals (York 2006; Meng et al. 2009; Chaouch and Noctor 2010; Gillaspy 2010; Stevenson-Paulik and Phillippy 2010). For example, there are reports documenting that $InsP_6$ can regulate transcription (Shen et al. 2003), mRNA export from the nucleus (York et al. 1999), DNA repair (Hanakahi et al. 2000), calcium signaling (Lemtiri-Chlieh et al. 2003), programmed cell death (Meng et al. 2009), and basal immunity (Murphy et al. 2008). Any of these functions have the potential to affect the process of transgene integration and expression. Moreover, $InsP_6$ itself can act as a powerful antioxidant (Murphy et al. 2008), inhibiting reactive oxygen species generation and the browning of explants. Thus, the increase of $InsP_6$ within callus tissue that results from the removal of *myo*-inositol from the media could have multiple effects in promoting transformation. Studies on the role of inositol and its derivatives on plant defense are just emerging and further research will be needed to define the specific mechanism(s) the underlying the observations we report here.

Browning-rendered cell death or necrosis is a major obstacle in developing high efficiency *Agrobacterium*-mediated transformation protocols (Parrott et al. 2002). The obvious browning of callus, when maintained on media containing *myo*-inositol, was noticed during perennial ryegrass transformation. In comparison, the calluses grown on media lacking *myo*-inositol showed little or no obvious browning. In our experiments, cold shock treatment alone could substantially reduce callus browning after infection even in *myo*-inositol-containing media (Fig. 1a). The use of a cold pretreatment to reduce browning was previously reported in *Agrobacterium*-mediated sorghum transformation of immature seeds (Nguyen et al. 2007), and a cold treatment also enhanced somatic embryo formation in *Arabidopsis* (Márton and Browse 1991).

Assays for hydrogen peroxide (H_2O_2) generated by *Agrobacterium*-infected perennial ryegrass callus revealed that cold shock and L-Gln treatment of the callus cultured on media lacking *myo*-inositol generated much less H_2O_2, indicating an interaction between these treatments that correlated positively with enhanced genetic transformation. Cold shock has been reported to trigger calcium signaling (Knight et al. 1991), which, in turn, could directly affect plant defense responses to pathogen infection (Kudla et al. 2010). Moreover, callus treatment with L-Gln may also involve the attenuation of plant defense responses. Although L-Gln serves as a primary metabolite in plant growth, cellular Gln homeostasis was also recently linked with plant defense reactions. As the pathogen competes for host nitrogen reserves during infection, the depletion of Gln in infected tissues was shown to activate plant defense responses. In addition, the direct application of Gln onto leaves strongly inhibited H_2O_2 production and could thereby suppress cell death during pathogen infection (Liu et al. 2010). In another report, L-Gln was found to inhibit plant resistance to pathogens induced by a non-protein amino acid, beta-aminobutyric acid (BABA) (Wu et al. 2010). In our study, we demonstrated that L-Gln treatment of callus enhances *Agrobacterium* transformation in perennial ryegrass. Given the findings in the reports cited above, it is reasonable to speculate that this enhancement is being mediated through the mitigation of the plant defense response. Nevertheless, it should be noted that tissue culture and *Agrobacterium*-mediated transformation are very complicated systems and that numerous physical, chemical, and biological factors could have impacts. While our approaches showed a strong association between the attenuation of plant defense responses and improved transformation efficiency, alternative explanations may exist, and there are clearly ways to improve transformation efficiency that don't necessarily involve modification of the plant defense response per se.

Transformation efficiency of rice callus generated from suspension cells was also increased using the same procedure optimized for perennial ryegrass transformation, although the overall transformation efficiency of rice suspension cells in our experiments was not as high as that previously reported by others (Nishimura et al. 2006). One explanation for this discrepancy may lie in the fact that the rice cell line we used was more than 5 years old. The similar trend that we observed in both the perennial ryegrass and rice systems, however, suggests that mechanistically the interactions between *Agrobacterium* and monocot host cells may be similar in response to the exogenously supplied *myo*-inositol, L-Gln and cold treatments. With respect to the supplemental *myo*-inositol, however, the response between dicots and monocots

appears to be fundamentally different. The expression/induction patterns of the *PRs* investigated were quite different in *Arabidopsis* from the patterns observed for the corresponding genes in perennial ryegrass in response to *myo*-inositol. Furthermore, *myo*-inositol had no appreciable effect on *Arabidopsis* root cell lignin, starch metabolism or transformation efficiency, although we cannot exclude the possible effects of tissue type and/or age amongst the respective experiments.

In summary, we identified three novel, yet simple treatments for improvement of *Agrobacterium*-mediated transformation for monocot species, which in general are recalcitrant to this process. The specific combination of these treatments has synergistic effects and remarkably enhanced transformation frequencies for both perennial ryegrass and rice. The cold shock and L-Gln treatments also facilitated *Agrobacterium* infection of *Arabidopsis*, a dicot plant. Cumulatively, our results suggested that the observed enhancement in transformation efficiencies could be attributable to an attenuation of the plant defense response.

Experimental procedures

Explants and tissue culture

Callus of perennial ryegrass and rice, and root fragments of *Arabidopsis* were used for transformation. The callus of perennial ryegrass (*Lolium perenne* cv. MontereyII, seeds purchased from National Seed Co., Lisle, IL, USA) was initiated from wounded mature seeds on callus induction medium NPC [N6 salts (Chu et al. 1975) + 1.0 g l^{-1} proline + 1.0 g l^{-1} casein hydrolysate + 9.9 mg l^{-1} thiamine + 9.5 mg l^{-1} pyridoxine hydrochloride + 4.5 mg l^{-1} nicotinic acid + 30 g l^{-1}maltose + 3.0 g l^{-1} phytagel + 7.0 mg l^{-1} 2,4-D + 0.05 mg l^{-1} BAP, pH 5.8] with or without 100 mg l^{-1} *myo*-inositol. Briefly, the embryos of mature seeds were sliced longitudinally, and plated on callus induction media for callus initiation. The callus was sub-cultured in monthly intervals on NPC1 medium by reducing 2,4-D to 5.0 mg l^{-1} and increasing BAP to 0.1 mg l^{-1} in the NPC medium. The rice (*Oryza sativa* cv. Nipponbare) callus was propagated on NPC1 medium with or without 100 mg l^{-1} *myo*-inositol from suspension cells that were generated in our lab (Sivamani and Qu 2006). The rice calluses were sub-cultured two times (one subculture per month) before transformation. *Arabidopsis thaliana* (Col-0) seeds were germinated on B5 medium (Gamborg et al. 1968) with or without *myo*-inositol, and the root fragments (~0.5 cm) were collected from 1-month-old seedlings as described by Gelvin (2006). All the chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Agrobacterium and plasmid

Agrobacterium strain EHA105, harboring binary vector pTJN33-gfp, was used in perennial ryegrass and rice callus transformation. The T-DNA region of pTJN33-gfp (Fig. 2a) contains a *bar* gene driven by the CaMV *35S* promoter and a *Prubi3::gfp* construct from plasmid pJLU11 (Lu et al. 2008).

Overnight cultures of *Agrobacterium* (OD_{600} about 0.8) in YEP medium (citation) were pretreated with 200 μM acetosyringone for 2 h in an incubator shaker at 250 rpm, 28 °C. The bacteria were then collected by centrifugation at 3270*g* for 15 min, and resuspended in *Agrobacterium* suspension medium (NPC1 liquid medium lacking casein hydrolysate, with 200 μM acetosyringone, and with or without 100 μM L-Gln, pH 5.4) to an OD_{600} of about 0.8. The bacterial suspension was kept in an incubator shaker at 80 rpm, 28 °C, for about 30 min prior to use.

Plant transformation

For cold shock treatment, perennial ryegrass or rice calluses were immersed in a 3 % maltose solution on ice for 20 min prior to *Agrobacterium* infection. Calluses immersed in 3 % maltose and kept at room temperature were used as a control. For L-Gln treatment, 100 μM of L-Gln were added to the maltose solution and to *Agrobacterium* suspension cultures. The calluses were then incubated with *Agrobacterium* suspensions under vacuum (about −0.08 MPa, 10 min) to facilitate infection. After breaking the vacuum, the calluses were further incubated in the *Agrobacterium* suspension with slight agitation (80 rpm) for 20 min and then blotted dry with three layers of sterile filter paper for 30 min to remove excess *Agrobacterium*. Co-cultivation was performed by placing the calluses on two layers of filter paper soaked with 1 ml liquid NPC1 medium containing 200 μM acetosyringone in petri dishes sealed with micropore surgical tape, in a 25 °C growth chamber for 4 days. The efficiencies of stable callus transformation (the number of calluses with GFP/number of calluses infected × 100 %) and the number of GFP spots per callus were recorded a month after selection on media containing 5 mg l^{-1} phosphinothricin (PPT). Resistant callus of perennial ryegrass was induced for embryogenesis and shoot formation on MS medium (Murashige and Skoog 1962) containing 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 5 mg l^{-1} PPT. The shoots were rooted on MS basal medium containing 10 mg l^{-1} PPT.

For *Arabidopsis* transformation, seeds were germinated on B5 medium supplemented with or without *myo*-inositol. Root fragments of 1-month-old *Arabidopsis* seedlings were infected by wild type *Agrobacterium* strain A208 following the procedure of Gelvin (2006). Successful transformation

of root fragments was assessed according to crown gall formation on the hormone-free medium. The cold shock and L-Gln treatments were as described above. Transformation efficiency (the number of root fragments with crown galls/number of root fragments infected × 100 %) was calculated after culturing the infected root fragments on basal B5 medium with 150 mg l^{-1} timentin for 1 month.

Analysis of perennial ryegrass callus

To determine whether the removal of, or supplementation with, *myo*-inositol in culture media results in metabolic changes in lignin content, starch accumulation and *Agrobacterium* binding of perennial ryegrass callus, multiple assays were conducted. Lignin in callus cell walls was assayed by staining with a saturated solution of phloroglucinol in 20 % HCl solution (Stange et al. 2002). Starch accumulation was tested by staining with an I_2-KI solution, which contains 0.15 % [w/v] I_2 and 0.45 % [w/v] KI (Takahashi et al. 2003).

The binding of *Agrobacterium* was evaluated 2 days after infection as described previously (Matthysse 1987, Matthysse and Mcmahan 2001). Briefly, 20 mg of infected calluses were mixed with 1 ml *Agrobacterium* in its suspension media. After vortexing for 1 min, the non-bound and loosely-bound *Agrobacterium* were removed by pipetting. This operation was repeated 3 times with fresh media. Subsequently, calluses were homogenized by grinding with 20 µl glass beads (450–600 µm in diameter) in 1 ml of *Agrobacterium* suspension media and vortexed for 3 min to release the bacteria tightly bound to callus cell walls. Thereafter, 10 µl of the supernatant was diluted 10,000× to facilitate counting of the bacteria after plating. Ten µl of the final dilution was mixed with 50 µl sterilized water and spread on YEP solid media containing antibiotics. Two days later, the colonies were counted, with each colony assumed to be derived from a single bacterium tightly bound to the callus cell wall.

Analysis of $InsP_6$ in perennial ryegrass callus

Accumulation of inositol hexaphosphate ($InsP_6$) was analyzed with high-performance liquid chromatography (Phillippy et al. 2003). An $InsP_6$ standard was purchased from Sigma-Aldrich (P8801) as a reference. Briefly, 2 g of callus was ground to a fine powder in liquid nitrogen using a mortar and pestle, transferred to a 15 ml tube, followed by addition of 3 ml 0.75 M HCl. The mixture was boiled for 15 min and centrifuged at 3700*g* for 5 min. Two ml of supernatant were centrifuged again at 16,000*g* for 10 min, and the resulting supernatant was passed through a C18 Sep-pak column (Waters Co., Milford, MA, USA) that was pre-eluted with 2 ml methanol and 5 ml H_2O. The elution was collected, filtered through a 0.45 µm filter, and analyzed by isocratic ion chromatography.

Histochemical staining for H_2O_2

The generation of H_2O_2 was analyzed by DAB (3,3′-diaminobenzidine) staining as described by Thordal-Christensen et al. (1997). Four-month-old calluses from GC, IGC, MI+ and MI− treatments were sampled 10 days after *Agrobacterium* infection and placed in a solution of 1 mg ml^{-1} DAB for 15 h. To develop the reddish-brown coloration indicative of DAB polymers, the stained materials were immersed in 95 % ethanol and bathed in boiling water for 10 min.

RT-PCR and Southern blot analyses

The expression of pathogenesis-related genes (*PRs*) was analyzed using semi-quantitative RT-PCR. Total RNA of perennial ryegrass callus, before and 8 days after *Agrobacterium* infection, was isolated using TRIzol reagent as described by Invitrogen (Carlsbad, CA, USA). Total RNA of *Arabidopsis* root fragments, before and 2 days after *Agrobacterium*-infection was purified using the same protocol. RT-PCR was performed with the Superscript® One-Step RT-PCR system according to the manufacturer's instruction (Invitrogen). Gene specific primers were used in a 10 µl reaction, with annealing temperatures of 53, 55 or 60 °C for 20 s, based on their respective Tms (Suppl. Table 1). One hundred ng of total RNA, and 25 reaction cycles were used in each assay. The *LP-Actin1* and *AtUBQ4* genes were used as internal controls for equal RNA sample loading for perennial ryegrass and *Arabidopsis*, respectively. Each RT-PCR experiment consisted of three technical replications in addition to two biological replications. The amplified DNA products were separated on 1.2 % (w/v) agarose gels.

For Southern blotting assays, total genomic DNA was extracted from individual plants using the CTAB method (Gao et al. 1989). Twenty µg of plant genomic DNA was digested with *Bam*HI or *Hind*III, using *gfp* or *bar* PCR products as the hybridization probes, respectively (Fig. 2a). The enzyme digestion products were separated in a 0.9 % (w/v) agarose gel and blotted onto Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primers used for *gfp* amplification (probe 1) were 5′-TGACCCTGAAGTTCATCTGCACCA-3′ (forward) and 5′-TGTGGCGGATCTTGAAGTTCACCT-3′ (reverse). The primers used for *bar* (probe 2) amplification were 5′-TGCACCATCGTCAACCACTA-3′ (forward) and 5′-TGAAGTCCAGCTGCCAGAAA-3′ (reverse). The probes were labeled with [α-^{32}P]-dCTP using a random primer

labeling kit (Promega, Madison, WI, USA). The blotted membrane was hybridized using the MiracleHyb™ solution (Stratagene) according to the manufacturer's instructions. The hybridized membranes were exposed to CL-X Posure™ X-ray film (Thermo, Rockford, IL, USA) for autoradiography.

Acknowledgments We are grateful to Jin Tong for assistance in plant transformation, Dr. Minesh Patel for assistance with the Southern blots, Dr. Ann G. Matthysse of the University of North Carolina, Chapel Hill, for discussions on *Agrobacterium* binding, and Dr. Zhen-Ming Pei of Duke University for discussions on calcium signaling. We also thank Dr. Stan Gelvin for providing *Agrobacterium* strain A208. This work was supported by a grant from Bayer CropScience LP (Research Triangle Park, NC, USA).

References

Altpeter F (2006) Perennial ryegrass (*Lolium perenne* L.). In: Wang K (ed) Methods in molecular biology, vol 344: *Agrobacterium* protocols. Humana Press Inc, Totowa, NJ, pp 55–64

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez–Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415:977–983

Bajaj S, Ran Y, Phillips J, Kularajathevan G, Pal S, Cohen D, Elborough K, Puthigae S (2006) A high throughput *Agrobacterium tumefaciens*-mediated transformation method for functional genomics of perennial ryegrass (Lolium perenne L.). Plant Cell Rep 25:651–659

Boss WF, Davis AJ, Im YJ, Galvao RM, Perera IY (2006) Phosphoinositide metabolism: towards an understanding of subcellular signaling. Subcell Biochem 39:181–205

Cao MX, Huang JQ, He YL, Liu SJ, Wang CL, Jiang WZ, Wei ZM (2006) Transformation of recalcitrant turfgrass cultivars through improvement of tissue culture and selection regime. Plant Cell Tiss Organ Cult 85:307–316

Chaouch S, Noctor G (2010) *Myo*-inositol abolishes salicylic acid-dependent cell death and pathogen defence responses triggered by peroxisomal hydrogen peroxide. New Phytol 188:711–718

Chu CC, Wang CS, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sinica 18:659–668

Dafny-Yelin M, Levy A, Tzfira T (2008) The ongoing saga of *Agrobacterium*-host interactions. Trends Plant Sci 13:102–105

DeCleene M (1985) The susceptibility of monocotyledons to *Agrobacterium tumefaciens*. J Phytopath Z 113:81–89

Ditt RF, Kerr KF, de Figueiredo P, Delrow J, Comai L, Nester EW (2006) The *Arabidopsis thaliana* transcriptome in response to *Agrobacterium tumefaciens*. Mol Plant Microbe Interact 19:665–681

Djamei A, Pitzschke A, Nakagami H, Rajh I, Hirt H (2007) Trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. Science 318:453–456

Doczi R, Brader G, Pettko-Szandtner A, Rajh I, Djamei A, Pitzschke A, Teige M, Hirt H (2007) The *Arabidopsis* mitogen-activated protein kinase kinase *MKK3* is upstream of group C mitogen-activated protein kinases and participates in pathogen signaling. Plant Cell 19:3266–3279

Donahue JL, Alford SR, Torabinejad J, Kerwin RE, Nourbakhsh A, Ray WK, Hernick M, Huang X, Lyons BM, Hein PP, Gillaspy GE (2010) The *Arabidopsis thaliana* myo-inositol 1-phosphate synthase1 gene is required for *myo*-inositol synthesis and suppression of cell death. Plant Cell 22:888–903

Fry SC (1988) The growing plant cell wall: chemical and metabolic analysis. Longman Scientific & Technical co-published in United States With John Wiley & Sons Inc. New York, pp 196–205

Gamborg OL, Miller RA, Ojima K (1968) Plant cell culture I. Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gao YF, Zhu Z, Xiao GF, Zhu Y, Wu Q, Li XH (1989) Isolation of soybean kunitz trysin inhibitor gene and its application in plant insect-resistant genetic engineering. Acta Bot Sin 40:405–411

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool. Microbiol Mol Biol Rev 67:16–37

Gelvin SB (2005) Agricultural biotechnology: gene exchange by design. Nature 433:583–584

Gelvin SB (2006) Methods in Molecular Biology, vol 343: *Agrobacterium* Protocols, 2/e, volume 1. In: K Wang (ed) Humana Press Inc., Totowa, NJ

Gelvin SB (2009) *Agrobacterium* in the genomics age. Plant Physiol 150:1665–1667

Gelvin SB (2010) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. Annu Rev Phytopathol 48:45–68

Gillaspy GE (2010) Signaling and the polyphosphoinositide phosphatases from plants. In: Munnik T (ed) Lipid signaling in plants 16. Springer, Berlin, pp 117–130

Hanakahi LA, Bartlett-Jones M, Chappell C, Pappin D, West SC (2000) Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. Cell 102:721–729

Jones JD, Dangl JL (2006) The plant immune system. Nature 444:323–329

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature 352: 524–526

Kudla J, Batistič O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell 22:541–563

Lemtiri-Chlieh F, MacRobbie EAC, Webb AAR, Manison NF, Brownlee C, Skepper JN, Chen J, Prestwich GD, Brearley CA (2003) Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. Proc Natl Acad Sci USA 100:10091–10095

Liu G, Ji Y, Bhuiyan N, Pilot G, Selvaraj G, Zou J, Wei Y (2010) Amino acid homeostasis modulates salicylic acid–associated redox status and defense responses in *Arabidopsis*. Plant Cell 22:3845–3863

Loewus FA (1965) Inositol metabolism and cell wall formation in plants. Fed Proc 24:855–862

Loewus FA (1990) Inositol biosynthesis. In: Marré DJ, Boss WF, Loewus FA (eds) Inositol metabolism in plants. Wiley-Liss, New York, pp 1–9

Loewus FA, Murthy PPN (2000) Myo-inositol metabolism in plants. Plant Sci 150:1–19

Lu JL, Sivamani E, Li X, Qu R (2008) Activity of the 5′ regulatory regions of the rice polyubiquitin rubi3 gene in transgenic rice plants as analyzed by both GUS and GFP reporter genes. Plant Cell Rep 27:1587–1600

Márton L, Browse J (1991) Facile transformation of *Arabidopsis*. Plant Cell Rep 10:235–239

Matthysse AG (1987) Characterization of non-attaching mutants of *Agrobacterium* tumefaciens. J Bacteriol 169:313–323

Matthysse AG, Mcmahan S (2001) The effect of the *Agrobacterium tumefaciens attR* mutation on attachment and root colonization differs between legumes and other dicots. Appl Environ Microbiol 67:1070–1075

Meng PH, Raynaud C, Tcherkez G, Blanchet S, Massoud K, Domenichini S, Henry Y, Soubigou-Taconnat L, Lelarge-Trouverie C, Saindrenan P, Renou JP, Bergounioux C (2009) Crosstalks between *myo*-inositol metabolism, programmed cell death and basal immunity in *Arabidopsis*. PLoS ONE 4:e7364

Michell RH (2008) Inositol derivatives: evolution and functions. Nat Rev Mol Cell Biol 9:151–161

Munnik T, Nielsen E (2011) Green light for polyphosphoinositide signals in plants. Curr Opin Plant Biol 14:489–497

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Murphy AM, Otto B, Brearley CA, Carr JP, Hanke DE (2008) A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. Plant J 56:638–652

Nguyen TV, Thu TT, Claeys M, Angenon G (2007) *Agrobacterium*-mediated transformation of sorghum [*Sorghum Bicolor* (L.) Moench] using an improved in vitro regeneration system. Plant Cell Tiss Org Cult 91:155–164

Nishimura A, Aichi I, Matsuoka M (2006) A protocol for *Agrobacterium*-mediated transformation in rice. Nat Protoc 1:2796–2802

Ortega X, Perez LM (2001) Participation of the phosphoinositide metabolism in the hypersensitive response of Citrus limon against *Alternaria alternata*. Biol Res 34:43–50

Pageau K, Reisdorf-Cren M, Morot-Gaudry JF, Masclaux-Daubresse C (2006) The two senescence-related markers, GS1and GDH, involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. J Exp Bot 57:547–557

Parrott DL, Anderson AJ, Carman JG (2002) *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.). Physiol Mol Plant Pathol 60:59–69

Perera IY, Hung CY, Brady S, Muday GK, Boss WF (2006) A universal role for inositol 1,4,5-trisphosphate-mediated signaling in plant gravitropism. Plant Physiol 140:746–760

Phillippy BQ, Bland JM, Evens TJ (2003) Ion chromatography of phytate in roots and tubers. J Agric Food Chem 51:350–353

Roberts RM, Loewus F (1966) Inositol metabolism in plants. III Conversion of *Myo*-inositol-2-^{3}H to cell wall polysaccharides in sycamore (*Acer pseudoplatanus* L.) cell culture. Plant Physiol 41:1489–1498

Sato H, Takamizo T (2006) *Agrobacterium tumefaciens*-mediated transformation of forage-type perennial ryegrass (*Lolium perenne* L.). Grassl Sci 52:95–98

Schenk BV, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Shen XT, Xiao H, Ranallo R, Wu WH, Wu C (2003) Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. Science 299:112–114

Sivamani E, Qu R (2006) Expression enhancement of a rice polyubiquitin gene promoter. Plant Mol Biol 60:225–239

Stange RR Jr, Alesandro R, McCollum TG, Mayer RT (2002) Studies on the phloroglucinol-HCl reactive material produced by squash fruit elicited with pectinase: isolation using hydrolytic enzymes and release of p-coumaryl aldehyde by water reflux. Physiol Mol Plant Pathol 60:283–291

Stevenson-Paulik J, Phillippy BQ (2010) Inositol polyphosphates and kinases. In: Munnik T (ed) Lipid signaling in plants 16. Springer, Berlin, pp 161–174

Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4/5-trisphosphate independent of abscisic acid in *Arabidopsis* cell culture. Plant Cell Physiol 42:214–222

Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H (2003) Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of *Arabidopsis* and radish. Plant Physiol 132:805–810

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell 18:1052–1066

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11:1187–1194

Thorpe TA, Stasolla C, Yeung EC, de Klerk GJ, Roberts A, George EF (2008) The components of plant tissue culture media II: organic additions, osmotic and pH effects, and support systems. In: George EF, Hall MA, de Klerk GJ (eds) Plant propagation by tissue culture, 3rd edn. Springer, New York, pp 115–173

Torres MA, Jones JD, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. Plant Physiol 141:373–378

Tsuda K, Qi YP, Nguyen LV, Bethke G, Tsuda Y, Glazebrook J, Katagiri F (2012) An efficient *Agrobacterium*-mediated transient transformation of *Arabidopsis*. Plant J 69:713–719

Wu G, Shortt BJ, Lawrence EB, León J, Fitzsimmons KC, Levine EB, Raskin I, Shah DM (1997) Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. Plant Physiol 115:427–435

Wu YY, Chen QJ, Chen M, Chen J, Wang XC (2005) Salt-tolerant transgenic perennial ryegrass (*Lolium perenne* L.) obtained by *Agrobacterium tumefacie*-mediated transformation of the vacuolar Na^+/H^+ antiporter gene. Plant Sci 169:65–73

Wu YY, Chen QJ, Cui XH, Chen H, Chen J, Wang XC (2007) Efficient regeneration and *Agrobacterium*-mediated stable transformation of perennial ryegrass. Russ J Plant Physiol 54:524–529

Wu C, Singh P, Chen MC, Zimmerli L (2010) L-Glutamine inhibits beta-aminobutyric acid-induced stress resistance and priming in *Arabidopsis*. J Exp Bot 61(4):995–1002

Xiong L, Lee B, Ishitani M, Lee H, Zhang C, Zhu JK (2001) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. Genes Dev 15:1971–1984

York JD (2006) Regulation of nuclear processes by inositol polyphosphates. Biochim Biophys Acta 1761:552–559

York JD, Odom AR, Murphy R, Ives EB, Wente SR (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science 285:96–99

Zhang N, Zhang S, Borchert S, Richardson K, Schmid J (2011) High levels of a fungal superoxide dismutase and increased concentration of a PR-10 plant Protein in associations between the endophytic fungus neotyphodium lolii and ryegrass. Mol Plant Micro Interact 24(8):984–992

Zipfel C (2008) Pattern-recognization receptors in plant innate immunity. Curr Opin Immunol 20:10–16

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125:749–760