4.3. Effect of transgenes conferring enhanced pathogen resistance on the interaction with symbiotic fungi in rice

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Scientific background

Microbial diseases cause substantial losses to crops. In order to reduce yield loss the development of novel breeding strategies is urgently needed. Transgenesis has been recognized as a powerful approach to enhance disease resistance. However, the designs of transgenic plants often target resistance to foliar pathogens without considering effects on root interactions. Frequently, pathogens are capable of infecting multiple plant organs and tissues. For the rice blast fungus *Magnaporthe oryzae* for instance, it was shown that the fungus efficiently invades not only leaves but also roots, causing disease in either case[1]. The fungal infection structures differ considerably between leaf and root invasion and involve different independent genetic components in the fungus.
Root associations also include beneficial microbial interactions such as the ancestral arbuscular mycorrhizal (AM) symbiosis. This symbiosis is an integral component of most terrestrial ecosystems and more than 80% of the vascular plants enter into this association including most crop species. Understanding the effects of transgenes conferring enhanced resistance to pathogens on the AM symbiosis is of utmost ecological importance.

Because the AM symbiosis represents one of the most ancient symbioses[2], that arose more than 400 MY ago, it can be regarded as an ancestral form of plant-microbe interactions. It has recently been discovered that arbuscular mycorrhiza and evolutionarily younger plant-microbe interactions require certain genetic components of the plant in common. One of these components, the *Lotus japonicus* receptor-like kinase SYMRK, is required for early recognition of rhizobia, AM fungi and nematodes[3,4]. Another example is given by the plant-released strigolactones that are recognized by AM fungi as well as by parasitic plants[5].

It can therefore not be excluded that boosting plant resistance against pathogens might simultaneously affect susceptibility to the symbiotic AM fungi. This aspect needs therefore to be carefully examined.

Fungal invaders can be gradually classified into obligate biotrophs (arbuscular mycorrhizal fungi), to hemi-biotrophs and necrotrophs. Biotrophs are microbes that feed on living cells while necrotrophs kill host cells and feed on their content. Intense research on foliar *Arabidopsis thaliana* diseases over the past years has revealed that distinct defense signaling pathways become activated dependent on the mode of infection of the invaders[6]. Salicylic acid (SA) is implicated in the induction of the systemic acquired resistance (SAR) and of programmed cell death usually associated with infection by biotrophs, while jasmonic acid (JA) and ethylene are involved in the defense against necrotrophic pathogens[6]. In *Arabidopsis thaliana* the *AtNPR1* gene is known as a central regulator of the SA-mediated SAR[7]. In *Arabidopsis*, *NPR1* over-

expression confers resistance to biotrophic pathogens, such as the fungus *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae*[7]. In parallel, NPR1 is also involved in suppression of the JA-associated gene activation[8]. This illustrates the central role of this regulator in the coordination of plant defense signaling.
Rice lines over-expressing the *OsNPR1* ortholog have an enhanced foliar resistance to the bacteria *Xanthomonas oryzae*[11] and to the fungus *Magnaporthe oryzae* (Chern M., personal communication).
Other key regulators of the plant defense mechanisms relevant for this study include the WRKY transcription factor that is involved in regulation of NPR1and the small GTPase OsRac known to induce a series of defense reactions. Overexpression of each of these factors in rice has been shown to confer resistance to pathogens [12-17].

Approach

1. In a first approach, existing transgenic lines with enhanced resistance to rice diseases were analyzed to determine the effect of the respective transgene on root interactions. For this purpose the hemibiotrophic fungal pathogen *Magnaporthe oryzae* and the biotrophic arbuscular mycorrhizal fungus *Glomus intraradices* were used. This pathogen was chosen because: 1) As a hemi-biotroph[1, 18] it exhibits an infection style that is related to Glomus for a direct comparison of biotrophic interactions with opposite outcome. 2) the fungus infects both leaves and roots allowing to compare the effect of the transgenes on leaf and root susceptibility. To be able to follow fungal development within rice tissue by fluorescent microscopy, we used the GFP transformed *M. oryzae* strain Guy11[1].
The rice lines used all affect the expression levels of central regulatory components of the defense response presented above:

- *OsNPR1*[9], a line over-expressing the regulator of the SA-mediated response,
- *OsWRKY71*[15], a line over-expressing the transcription factor, and
- *OsRac1*[17], a line exhibiting constitutive-active expression of the small GTPase.

This report will focus on the results obtained with line *OsNPR1*.

2. The second approach targets complementary aspects of plant defense such as e.g. phytoalexin production or cellular dynamics related to biotrophy and involves the generation of novel transgenic material. Global expression profiling data available in our laboratory[19] served as a basis for this study. We identified rice genes that transcriptionally responded to colonization of rice roots by the biotroph *Glomus intraradices,* the hemibiotroph *Magnaporthe oryzae* and the necrotroph *Fusarium moniliforme*. For further studies we focused on genes differentially expressed in all three interactions and in the biotroph and hemi-biotroph but not in the necrotroph interaction. RNAi lines have been generated targeting a total of five genes and combinations of different genes. These lines are currently investigated and the results will be presented at a later time.

Results

In the rice line *OsNPR1* expression of *OsNPR1* in the roots was confirmed (Fig. 1)

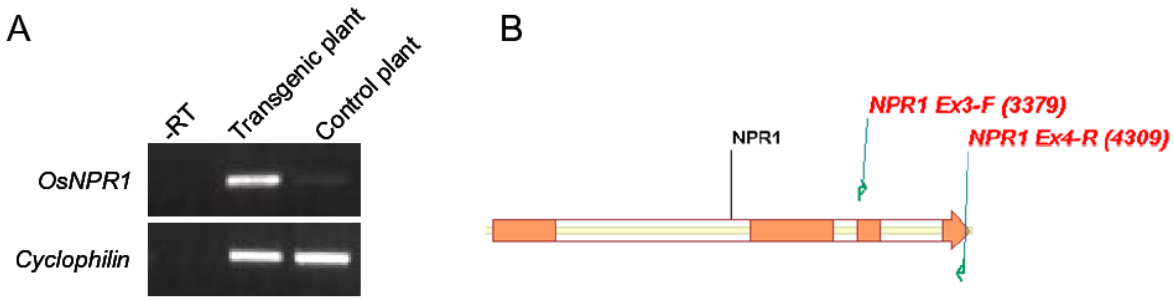

Figure1: (A) Analysis of expression in *OsNPR1* over-expression and in empty vector control plants using RT-PCR. The –RT control confirmed absence of genomic DNA contamination (RNA pooled from both plant genotypes). Rice cyclophilin served as a constitutive standard. The positions of the *OsNPR1* primers relative to the exon-intron structure of the gene are indicated (**B**); orange boxes: exons, empty boxes: introns, green arrows: primers.

In order to then evaluate the impact of *OsNPR1* over-expression on the susceptibility of rice roots to *M. oryzae* and to *G. intraradices*, rice lines carrying the empty vector or the over-expression construct were inoculated with conidia of *M. oryzae* or with spores of *G. intraradices*. *M. oryzae* had invaded the outer cell layers of the root in empty vector control and in wild-type plants at 4 days post inoculation (dpi). Thick and bulbous hyphae passed from one cell to another (Figure 2A, arrow heads) as previously described[1]. Fungal structures had entered into the vascular tissue at 12 dpi (Figure 2B). Surprisingly, comparable morphology and kinetics of intraradical hyphal growth was observed in *OsNRP1* over-expressing plants (Figure 2C and D). Plant cell death was not observed indicating biotrophic fungal growth. No disease symptoms could be observed until 2 to 3 weeks post inoculation in both plant genotypes. These observations suggest that although over-expression of *OsNPR1* resulted in enhanced foliar resistance to *M. oryzae* roots exhibited wild-type susceptibility.

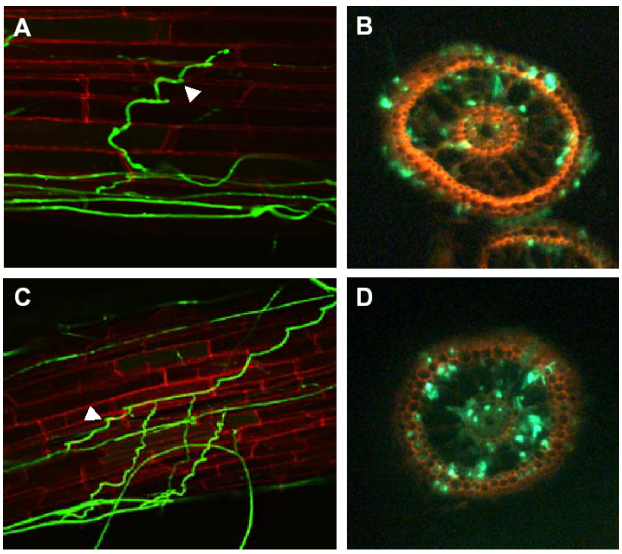

Figure 2: Micrograph of propidium iodide stained root tissue from control (**A**, **B**) and *OsNPR1* overexpressing plants (**C**, **D**) infected with GFP-transformed *M. oryzae* strain Guy11. Confocal micrographs show similar fungal morphology in epidermal cells of control (**A**) and over-expressing (**C**) plants 6 days post inoculation. Cross sections at 12 days post inoculation indicate that the fungus had reached the vascular tissue of both plant genotypes. The arrow heads indicate fungal hyphae when passing from cells to cell.

Roots inoculated with *G. intraradices* were harvested at 6-10 weeks post inoculation. Root colonization was quantitatively and qualitatively evaluated. Infected wild-type and control plants showed longitudinal growth of intraradical hyphae and the development of highly branched arbuscules (Figure 3 A and B). In *OsNPR1* over-expressing plants, the degree of colonization was comparable to the corresponding control plant. Fungal structures, including intraradical hyphae and arbuscules (Figure 3C and D) developed in a similar manner as in the control.

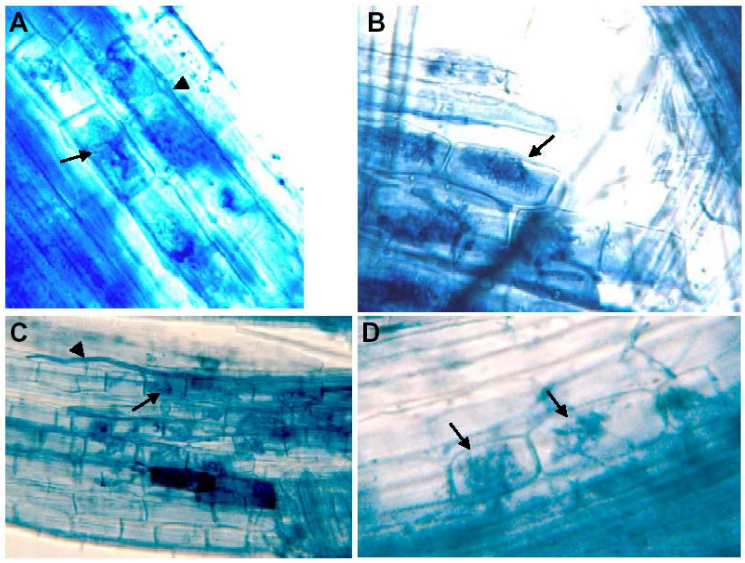

Figure 3: Micrograph of trypan blue stained root tissue from control (**A**, **B**) and *OsNPR1* overexpressing plants (**C**, **D**) colonized by *G. intraradices*. Fungal structures are revealed in dark blue. Intraradical hyphae (arrow heads) and arbuscules (arrows) show similar development in wild type and transgenic plants.

Concluding remarks

In this study, a rice line earlier reported to display enhanced resistance to the foliar pathogens *X. oryzae* and *M. oryzae* was used for root inoculation with *M. oryzae* and *G. intraradices*. We observed that the pathogenic blast fungus as well as the beneficial AM fungus infected roots of the *OsNPR1* over-expression line in a comparable fashion relative to roots of control or wild-type plants. Since *M. oryzae* is known to use a different genetic program for root infection than for leaf infection. Molecular mechanisms underlying resistance of leaf tissue might be irrelevant or bypassed during root infection. We thus showed for the first time that enhanced resistance to foliar infection of rice might not be equivalent to enhanced resistance of the entire plant against the same pathogen. It is thus of crucial importance to include examination of root resistance properties into the characterization of transgenic plant lines. Furthermore, wild-type levels of colonization and absence of morphological alterations in fungal structures of *G. intraradices* indicated that over-expression of *OsNPR1* did not affect root colonization by an AM fungus. Hence, development of the AM symbiosis occurs independent of elevated levels of OsNRP1.

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Collaborators

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