Identification of novel microRNAs involved in plant stress response

Introduction

MicroRNAs (miRNA) are important regulators of gene expression in a wide range of organisms, including vertebrates, invertebrates, fungi, plants and viruses.

In plants, one of the best characterized microRNAs is the phosphate-responsive miR399 (Fujii *et al.*, 2005; Bari *et al.*, 2006). It is an essential part of the PHR1 signaling pathway, and is positively regulated by the transcription factor AtPHR1. Mir399 promotes the degradation of PHO2 (ubiquitin E2 conjugase) which in turn is thought to function as a negative regulator of the high-affinity phosphate transporters of the PHT1-family (Aung *et al.*, 2006; Bari *et al.*, 2006).

In this study, we used the miRCURY LNA™ microRNA Array, v11.0 -Other Species, to identify additional microRNAs involved in the *Arabidopsis* phosphate stress response. Many genes affected by phosphate starvation are also sugar responsive (Müller *et al.*, 2007). For this reason, we also performed miRNA expression profiling on plants fed with sucrose.

Material and method

RNA isolation

Total RNA was extracted, using Trizol, from leaf material of *A. thaliana* thaliana wild-type (WT) plants grown on Rockwool supplied with a limited and sufficient amount of phosphate, respectively. In parallel, RNA was extracted from leaf material of WT plants fed with sucrose. The quality of the RNA was evaluated using an Agilent 2100 Bioanalyzer.

RNA labeling

The RNA was labeled using the miRCURY LNA™ microRNA Power Labeling Kit (Exiqon, Denmark) according to the manufacturer's instructions. One µg total RNA from sample and common reference pool was labeled with Hy3™ and Hy5™ fluorescent labels, respectively.

Microarray analysis

The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array, v.11.0 – Other Species (Exiqon, Denmark). This array can be used to detect 4168 mature microRNAs from 85 species and covers 98.1% of all currently annotated *A. thaliana* microRNAs (miRBase v. 14.0). The hybridization was carried out according to the manufacturer's instructions on a Tecan HS4800™ hybridization station (Tecan, Austria). Following hybridization, the microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and stored in an ozone-free (< 2.0 ppb) environment to prevent

Figure 1

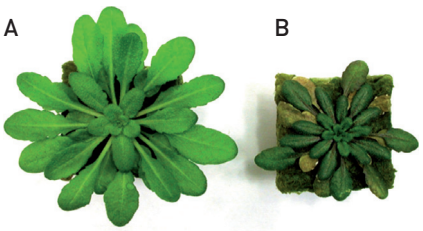

Figure 1. Effects of phosphate starvation on *Arabidopsis*. Plants were grown with limiting amounts of phosphate (40µM Pi) for 3 weeks. During the last week half of the plants were supplied with a nutrient solution containing 4mM Pi (A), whereas the other half was retained at 40µM Pi (B).

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Figure 2

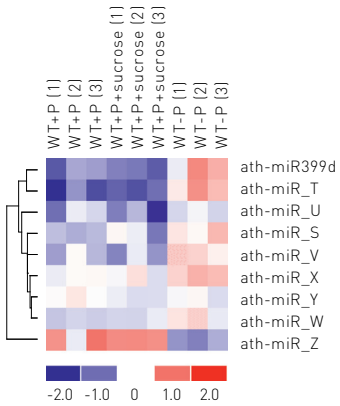

Figure 2. MicroRNA expression levels are affected by phosphate starvation. Several miRNAs were found to be induced during starvation. Interestingly, one miRNA was found to be down-regulated in plants grown with a limited amount of phosphate. Each experiment was carried out in triplicate.

bleaching of the fluorescent dyes. Image analysis was carried out using the ImaGene™ 8.0 software (BioDiscovery, Inc., USA). Quantified signals were background corrected (Normexp with offset value 10 – Ritchie *et al.*, 2007) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. A student's T-test was performed on the data. The statistical cut-off was set to $p < 0.05$.

Results

In order to identify novel miRNAs involved in the phosphate stress response, we performed microarray miRNA expression profiling on RNA from *Arabidopsis* plants fed on varying amounts of phosphate and sucrose.

As expected, the expression of miR399d was strongly induced in wild-type plants starved for phosphate. The impact of the limited and sufficient amounts of phosphate can clearly be observed in Figures 1 and 2.

Furthermore, we were able to identify several novel phosphate responsive miRNAs demonstrating that the miRNA-based regulation of the phosphate starvation response goes beyond the PHR1-miRNA399 loop (Figure 2). In addition, a single novel microRNA (ath_miR_Y) was found to be differently expressed in plants fed with additional sucrose compared to plants fed with sufficient phosphate only. This miRNA is likely a part of a regulatory mechanism controlling plant carbohydrate levels.

The results of this study clearly show the benefits of using the miRCURY LNA™ microRNA Array platform for global miRNA expression profiling in plants.

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