Introduction

Manganese (Mn) is an essential micronutrient with many important functions in plant metabolism. The most important role of Mn is its involvement in the oxygen evolving complex in photosystem II. This complex is responsible for photo-induced water oxidation which releases molecular oxygen and hydrogen ions while initiating the photosynthetic electron flow. In the shikimate pathway, Mn activates the enzyme, phenylalanine ammonia-lyase, leading to biosynthesis of various key metabolites, *e.g.* the cell wall component lignin. In addition, Mn is an essential inorganic cofactor of Mn superoxide dismutase, responsible for the scavenging of reactive oxygen species in peroxisomes and mitochondria (Marschner, 1995; Pedas et al., 2005).

Due to its essential functional properties, Mn deficiency causes a significant reduction of crop yields. Mn deficiency problems are observed in many areas of the world, but are especially widespread in Australia, USA, Asia and Northern Europe (Campbell and Nable, 1988). It has recently been estimated that approximately 30% of soils in China are suffering from Mn deficiency, causing severe yield and quality reductions (Yang et al., 2007). In major parts of Scandinavia, Mn deficiency has now become the foremost plant nutritional disorder (Hebbern et al., 2005). Soils on which Mn deficiency do most often occur are calcareous sandy soils with a high content of organic matter and with neutral- to alkaline pH. Such conditions favor microbial or chemical oxidation and thereby immobilization of Mn^{2+} in the plant-unavailable form, MnO_2. Furthermore, the mobility and uptake of Mn^{2+} is severely limited by diffusion, as Mn^{2+} has an effective diffusion coefficient of 10^{-10} cm^2 s^{-1}, and thus diffuses less than one millimeter per year. The first visual symptoms of Mn deficiency are slack and soft leaves with interveinal chlorosis, which is a result of a reduced carbohydrate content and chlorophyll degradation, respectively (Campbell and Nable, 1988).

The ability to grow in soils containing low levels of plant-available Mn^{2+} varies greatly among different plant species and among genotypes within the same species, a phenomenon commonly referred to as differential Mn efficiency (Ascher-Ellis et al., 2001). Genotypes of winter barley (*Hordeum vulgare*) are known to respond differently to low Mn^{2+} availability (Hebbern et al., 2005). The observed genotypic differences are so striking that it appears to be of major practical, as well as scientific relevance to identify and characterize the underlying processes. Such knowledge will enable engineering of new genotypes able to grow efficiently on soils suffering from Mn deficiency.

Despite the many important physiological functions of Mn in the plant cell, the amount that is required for optimal growth is relatively low, and it is remarkable that the Mn^{2+} uptake capacity greatly exceeds this requirement (Pedas et al., 2005). For this reason, Mn toxicity is a widespread phenomenon on acid and waterlogged soils as Mn^{2+} becomes more available at low pH and at a low redox potential (Marschner, 1995). Acid soils cover approximately 30% of the Earth's surface (von Uexküll and Mutert, 1995), and Mn toxicity, together with Al toxicity, is a major limiting factor for plant production. To avoid toxic effects caused by high Mn^{2+} soil solution concentrations, plants must be able to regulate their Mn^{2+} homeostasis. This can be achieved by controlling the uptake of Mn^{2+} from the soil, root Mn^{2+} sequestration and compartmentalization, xylem Mn^{2+} loading and transport, and Mn^{2+} sequestration and storage in stem and leaf cells. The molecular basis for transport of Mn^{2+} across membranes in plant cells is poorly understood but several transport protein families have been shown to play a role in Mn^{2+} transport (Pittman, 2005). The following will briefly describe the successful identification and characterization of three genes encoding Mn^{2+} transport proteins involved in Mn^{2+} acquisition and homeostasis in barley.

Mn^{2+} acquisition

Differential Mn efficiency has been examined in several studies over the last decades but the underlying physiological mechanisms are still incompletely understood (Ascher-Ellis et al., 2001). Several factors have been suggested to be involved: Efficient translocation of Mn^{2+} between roots and shoot; exudation of Mn^{2+}-chelating and/or Mn^{4+}-reducing compounds from roots; Mn content in the germinating seed; and Mn requirement of Mn-dependent enzymes. In a previous study we identified a high-affinity uptake system for Mn^{2+} in barley, operating at concentrations below 130 nM and showed that the Mn-efficient genotype Vanessa assimilated Mn^{2+} with four times the rate of the Mn-inefficient genotype Antonia (Pedas et al., 2005). The physiological relevance of the observed difference in Mn^{2+} uptake rates was confirmed by demonstrating a several fold higher net uptake of Mn^{2+} in the efficient compared to the inefficient genotype. The Mn^{2+} concentration in the bulk soil solution is normally in the low micromolar range but due to diffusion limitations, the concentration may further be reduced to nanomolar levels in the rhizosphere. Under such conditions, a high ability of roots to capture Mn^{2+} in competition with Mn-oxidizing rhizosphere microorganisms becomes very favorable for the plant.

The observed genotypic differences in Mn^{2+} uptake kinetics led to the identification of the first Mn^{2+} transport protein from a graminaceous species (Pedas et al., 2008). This protein, isolated from barley roots, was shown to be localized to plasma membrane when transiently expressed in onion epidermal cells (Fig. 1A).

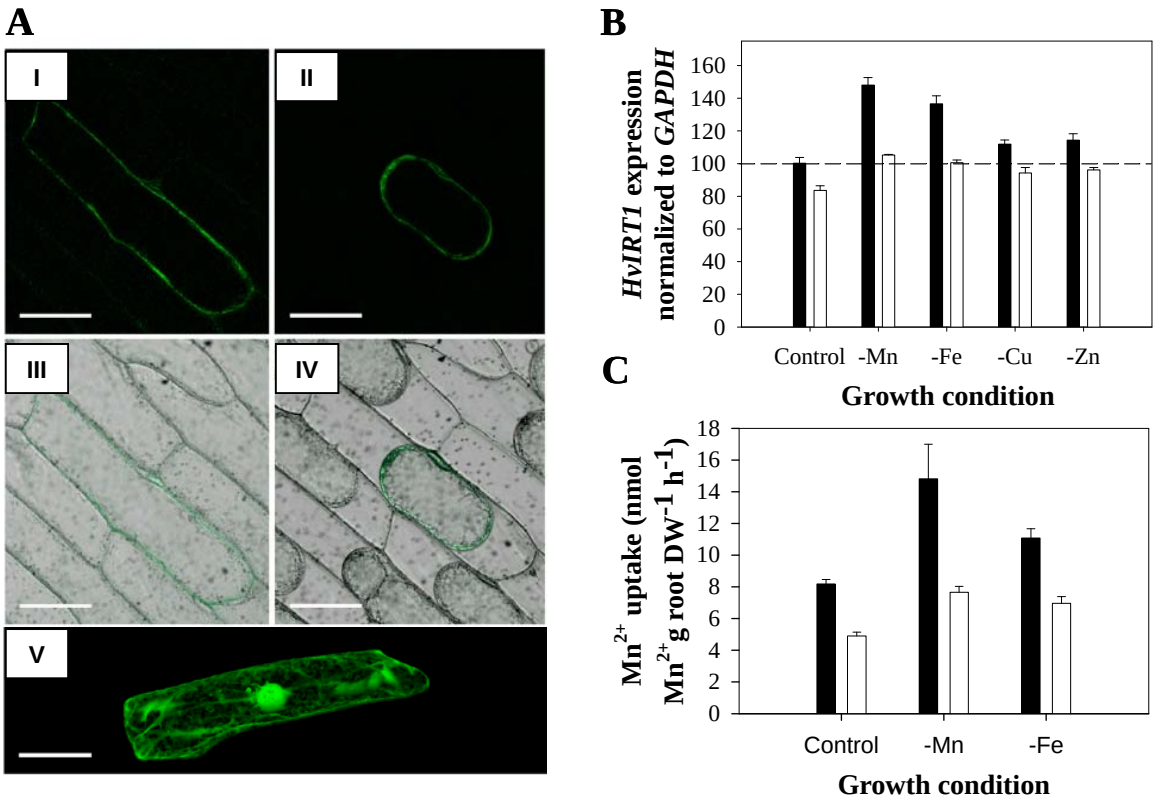

Figure 1. HvIRT1 localizes to the plasma membrane (A, insert I and III). The plasma membrane localization was confirmed by plasmolyzing the cell from insert I in a mannitol solution for 30 min (insert II and IV). Expression of free GFP resulted in an entirely different localization pattern (Insert V). RT-PCR analysis of the *HvIRT1* expression in barley roots was performed for the Mn-efficient genotype Vanessa (black bars) and the Mn-inefficient genotype Antonia (white bars), grown under different nutrient regimes (B). Data are normalized to the expression of the reference gene *GAPDH* and indexed relative to Vanessa plants grown in the control treatment. The effect of Mn and Fe deficiencies on Mn^{2+} uptake rates was determined in the same plants as used for the expression study (C). Data are means ± SE of 3 (Fig. 1B) or 4 replicates (Fig. 1C). Modified after Pedas and co-workers (2008).

Complementation studies in yeast (*Saccharomyces cerevisiae*) showed that HvIRT1 can mediate Mn^{2+}, Zn^{2+} and Fe^{2+}/Fe^{3+} uptake. A novel yeast uptake assay based on ICP-MS analysis of 31 different metal and metalloid ions showed that the HvIRT1 protein, in addition to the three micronutrients, also transported Cd^{2+}. The broad substrate specificity of HvIRT1 is therefore similar to that found for AtIRT1 (Korshunova et al., 1999; Vert et al., 2002). The *HvIRT1* transcript expression is mainly induced by Fe and Mn deficiencies and the transcript level seems to be correlated with the Mn^{2+} uptake capacity (Fig. 1, B and C). Consequently, HvIRT1 is believed to be important in controlling Mn^{2+} uptake in barley roots. Furthermore, the genotypic differences in Mn^{2+} capacity and the expression levels of *HvIRT1* were strongly correlated with the degree of Mn efficiency in barley genotypes, as the Mn-efficient genotype Vanessa showed a higher *HvIRT1*-transcript expression level and a higher Mn^{2+} uptake capacity compared to the Mn-inefficient genotype Antonia (Fig. 1B). These results clearly indicate a role for *HvIRT1* in controlling differential Mn efficiency among barley genotypes. We are in the process of generating RNAi barley mutant lines with reduced activity of HvIRT1 for analyzing the role of HvIRT1 in Mn^{2+} uptake and translocation in barley roots. It has to be noted that the increase in Mn^{2+} uptake capacity upon Mn and Fe deficiency does not need to be directly linked to the level of increased expression of *HvIRT1*, as HvIRT1 may be post-transcriptionally and/or post-translationally regulated, as was shown for AtIRT1 (Connolly et al., 2002).

Mn^{2+} homeostasis

Information about how plants control the intracellular Mn^{2+} level is currently very limited. This is especially the case in monocotyledonous plants, and consequently the need for identification of Mn^{2+} transport proteins is urgent. These proteins may have important roles in Mn^{2+} homeostasis and contributes to the mechanisms controlling the tolerance for Mn deficiency and toxicity, respectively. We have employed a yeast assay using the Mn^{2+} hypersensitive strain Δ*pmr1*. The Δ*pmr1* strain accumulates high Mn concentrations because it is unable to exclude excess Mn^{2+} by exocytosis (Lapinskas et al., 1995). The strain was transformed with a barley cDNA library (Pedas et al., 2008) and the transformed yeast cells were screened for increased Mn^{2+} tolerance. The screen resulted in the identification of two genes encoding the membrane-bound proteins, HvMTP8.1 and HvVIT1, belonging to the cation diffusion facilitator (CDF) family and homologous to the yeast protein Ccc1p, respectively. Expression of the two isolated cDNAs in various yeast mutants indicated that HvMTP8.1 is specific for Mn^{2+} transport, whereas HvVIT1 in addition to Mn^{2+} also is able to transport Fe^{2+} (Fig. 2A, data not shown). A growth assay with increasing Mn^{2+} concentrations was performed showing that the growth of *HvMTP8.1* transformed yeast cells was not inhibited by increasing Mn^{2+} concentrations whereas that of *HvVIT1* transformed yeast cells was, although not to the same extent as for yeast cells transformed with the empty vector pFL6.1 (Fig. 2B). The Mn accumulation in the yeast cells was analyzed by ICP-MS and the Mn content of *HvMTP8.1* transformed yeast cells was reduced compared to the empty vector transformed control cells, whereas the *HvVIT1* transformed cells showed a higher Mn content (Fig. 2C). This indicate that the two proteins have different functions in Mn^{2+} homeostasis, HvVIT1 possibly involved in Mn^{2+} sequestration into an internal organelle, while HvMTP8.1 could be involved in Mn^{2+} efflux.

AtVIT1, which is homologous to HvVIT1, has previously been shown to play a role in Fe^{2+}/Fe^{3+} and Mn^{2+} loading into the vacuole and this function seems to be essential, at least for Fe, in terms of seed germination (Kim et al., 2006). The subcellular localization of HvVIT1 is currently being studied in our laboratory and data will be presented at the conference. MTP11 proteins have also been shown to be involved in controlling Mn tolerance, *e.g.* mutant plant lines are more sensitive towards Mn toxicity. However, these proteins seem to play a role in excluding Mn from the cytosolic space via the secretory pathway (Golgi network),

thereby allowing removal of Mn^{2+} from the cell via exocytocis (Peiter et al., 2007). Yeast assays with HvMTP8.1 indicate a similar role as for the MTP11 proteins (Fig. 2). Ongoing analysis of the membrane localization and transcript expression profile during varying Mn conditions will clarify the role of HvMTP8.1.

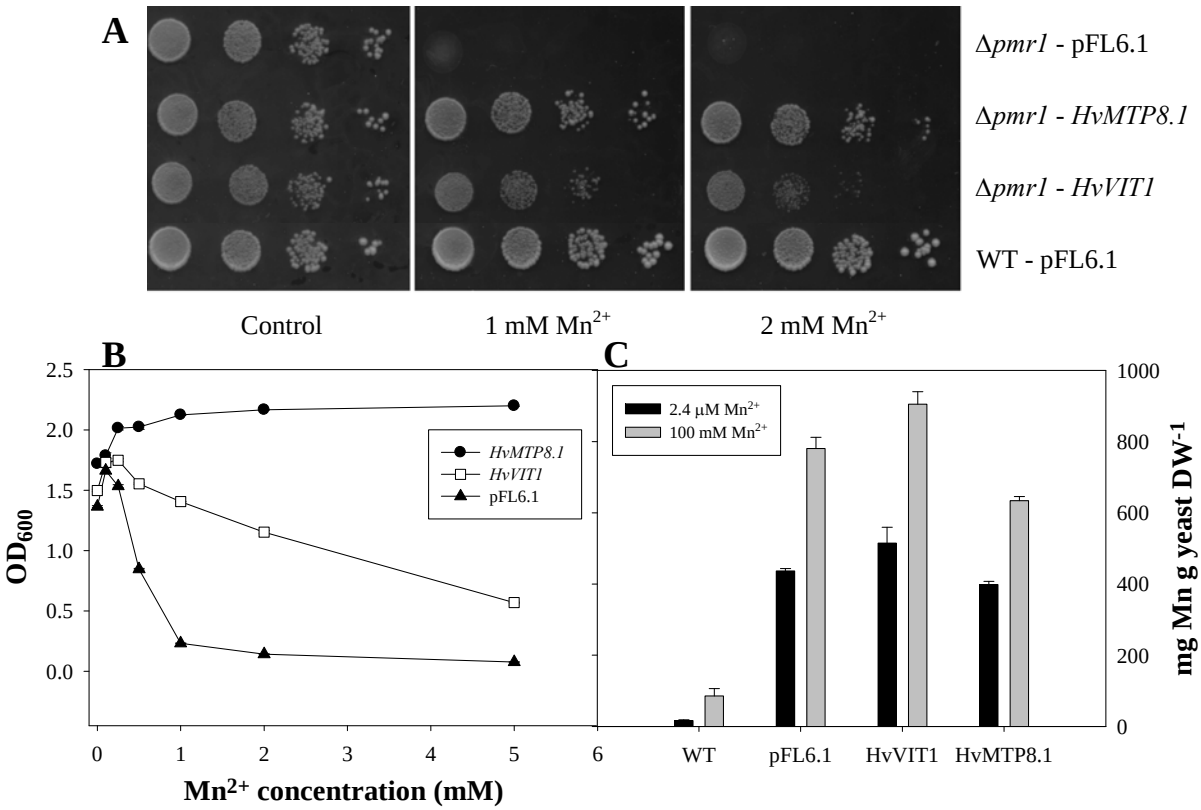

Figure 2. The *HvMTP8.1* and *HvVIT1* cDNAs complement the Mn^{2+}-sensitive phenotype of the Δ*pmr1* yeast strain (A). 48 hours yeast growth assay with increasing Mn^{2+} concentrations resulted in a different response of *HvMTP8.1* and *HvVIT1* transformed yeast cells compared to empty vector (pFL6.1) control cells, respectively (B). Mn accumulation analyzed for wild type yeast and Δ*pmr1* yeast cells transformed with *HvMTP8.1*, *HvVIT1*, or empty vector pFL6.1 after 5.5 hours of growth (C). Data are means ± SE (n = 3).

Conclusions

Plants monitor and respond to the availability of Mn^{2+} in the soil solution. A strict control is therefore needed for plants to be able to absorb sufficient amounts of Mn^{2+} for normal growth and to limit the toxicity when Mn^{2+} is present in excess. The knowledge gained so far has primarily been obtained from studies performed with dicotyledonous plants and it is therefore crucial to elucidate the mechanisms that important crop plants, such as barley and rice, use for perceiving and responding to varying Mn^{2+} conditions, both during Mn deficiency and toxicity. The present work describes the first Mn^{2+} transport proteins identified and characterized from barley. Results from ongoing studies regarding HvIRT1 protein levels, phenotypic characterization of *HvIRT1* RNAi lines, subcellular localization, as well as transcript expression profiles in response to varying Mn levels of *HvVIT1* and *HvMTP8.1,* will be presented and discussed in relation to known pathways within Mn^{2+} homeostasis.

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