A Host Ca\(^{2+}\)/Mn\(^{2+}\) Ion Pump Is a Factor in the Emergence of Viral RNA Recombinants

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SUMMARY

Viruses change rapidly due to genetic mutations, and viral RNA recombination in RNA viruses can lead to the emergence of drug-resistant or highly virulent strains. Here, we report that host Pmr1p, an ion pump that controls Ca\(^{2+}\)/Mn\(^{2+}\) influx into the Golgi from the cytosol, affects the frequency of viral RNA recombination and the efficiency of replication. Inactivation of PMR1 leads to an ~160-fold increase in RNA recombination of Tomato bushy stunt virus (TBSV) in yeast, a model host. Expression of separation-of-function mutants of Pmr1p reveals that the ability of Pmr1p to control the Mn\(^{2+}\) concentration in the cytosol is a key factor in viral RNA recombination. Indeed, a high Mn\(^{2+}\) concentration in a cell-free TBSV replication system increases the recombination frequency, and knockdown of Ca\(^{2+}\)/Mn\(^{2+}\) exporters in plants increases virus replication and RNA recombination. Thus, a conserved host protein could affect the adaptive evolution of RNA viruses.

INTRODUCTION

Host-virus interaction is a continuously changing battlefield, where viruses have major advantages due to their rapid evolution, which can be much faster than the evolution of the host. Therefore, it is not surprising that RNA viruses are widespread in nature and that they cause many diseases in humans, animals, and plants. Viruses evolve rapidly due to genetic mutations and recombination (Chare and Holmes, 2006; Roossinck, 2003). RNA recombination can dramatically alter the features of the recombinant virus, allowing it to escape immune recognition or other natural host resistance (Worobey and Holmes, 1999). Most RNA recombination events are driven by viral replicase as a result of template switching during nascent RNA synthesis (Cheng and Nagy, 2003; Kim and Kao, 2001; Nagy and Simon, 1997). Indeed, mutations within viral replication proteins have affected RNA recombination in several RNA viruses (Nagy et al., 1995; Serviene and Nagy, 2003). The role of the host in the formation and emergence of new viral RNA recombinants is poorly understood, notwithstanding its obvious potential significance.

To identify host genes affecting viral RNA recombination, genome-wide screens representing ~95% of host genes have recently been performed with Tomato bushy stunt virus (TBSV), utilizing a short replicon (rep)RNA in yeast as a model host (Cheng et al., 2006; Li et al., 2008b; Serviene et al., 2005, 2006). These screens have led to the identification of 32 yeast genes that either inhibit or accelerate TBSV RNA recombination. Most notably, single deletion of a set of yeast genes has led to ~50- to ~200-fold higher levels of recombinant viral RNAs than that found in the parental yeast strain (Serviene et al., 2005). The recombinants emerged in the absence of artificial selection to facilitate their appearance, suggesting that their formation is an efficient process.

Among the identified host factors affecting TBSV recombination, the role of the yeast Xm1p 5'-3' exoribonuclease and the Nicotiana benthamiana ortholog Xm4p were characterized in some detail (Cheng et al., 2006). These exoribonucleases were found to inhibit RNA recombination by efficiently degrading the recombination intermediates generated by putative endoribonucleases acting on the TBSV RNA (Cheng et al., 2006, 2007; Jaag and Nagy, 2009). Thus, the exoribonuclease is a suppressor of viral RNA recombination. The function of other host factors in viral RNA recombination is currently unknown (Nagy, 2008).

In this work, we identified the highly conserved PMR1 gene involved in TBSV recombination. We found that inactivation of PMR1, which codes for an ATPase-driven Ca\(^{2+}\)/Mn\(^{2+}\) pump in yeast, led to increased levels of TBSV RNA recombination as well as higher viral RNA accumulation. Pmr1p (plasma membrane ATPase related) controls Ca\(^{2+}\) and Mn\(^{2+}\) influx to the Golgi from the cytosol (Ton and Rao, 2004). Use of separation-of-function Pmr1p mutants in yeast, as well as a cell-free TBSV replication assay, led to the discovery that the ability of Pmr1p to control Mn\(^{2+}\) levels is the critical factor in TBSV recombination. Knocking down the similar Ca\(^{2+}\)/Mn\(^{2+}\) pumps LCA1 and ECA3 in plants also resulted in enhanced TBSV recombination and replication. Thus, this work unravels how a conserved host factor can contribute to the complexity of host-virus interactions.

RESULTS

Inactivation of the Yeast Pmr1p Ca\(^{2+}\)/Mn\(^{2+}\) Pump Increases TBSV Recombination

Among the identified genes affecting viral RNA recombination from the genome-wide screens (Cheng et al., 2006; Serviene et al., 2005, 2006), deletion of hur1 had the most unusual effect on the emergence of TBSV recombinants. We found four types of highly abundant recombinant RNAs in hur1Δ yeast (Serviene et al., 2005)(Figure S1A available online). To define whether deletion of HUR1, which is a dubious gene, or the deletion of the overlapping highly conserved PMR1 gene is involved in TBSV recombination, we performed genome-wide screens with the short replicon RNA in yeast. We found that the deletion of PMR1 led to a ~160-fold increase in TBSV RNA recombination, similar to that observed for the deletion of HUR1. These results suggest that the PMR1 gene is involved in TBSV recombination.
recombination, we made nonoverlapping partial deletions in these genes (Figure 1A), followed by launching TBSV repRNA replication from expression plasmids. Northern blot analysis revealed 160-fold more efficient accumulation of TBSV recombinants in pmr1-5Δ than in the parental (WT) yeast (Figure 1B, lanes 6 and 7 versus lanes 2 and 3), whereas the generation of TBSV recombinants increased only 2.5-fold in hur1-5Δ yeast (Figure 1B, lanes 4 and 5). Sequencing across the recombination junction sites confirmed the formation of recRNAs in pmr1-5Δ yeast (Figures S1B and S1C). These findings indicate that PMR1 is a critical host gene involved in TBSV RNA recombination. A complementation experiment involving expression of WT Pmr1p from a yeast plasmid in pmr1-5Δ yeast demonstrated that Pmr1p suppressed TBSV recombination efficiently (by 90-fold) (Figure 1C, lanes 3 and 4).

**The Mn²⁺ Pump Function of Pmr1p Is Critical for TBSV Recombination in Yeast**

Pmr1p is an ATPase-driven Ca²⁺/Mn²⁺ exporter/pump in yeast (Ton and Rao, 2004). Pmr1p controls Ca²⁺ and Mn²⁺ influx to the Golgi from the cytosol. To test whether the Ca²⁺ and/or Mn²⁺ transport function of Pmr1p affects TBSV recombination, we expressed separation-of-function mutants of Pmr1p in the pmr1-5Δ background. These experiments revealed that expression of the Pmr1p-D53A mutant, deficient in Ca²⁺ but still active in Mn²⁺ transport (Wei et al., 2000), inhibited the emergence of TBSV recombinants by 22-fold (Figure 1C, lanes 7 and 8) when compared to yeast lacking functional Pmr1p (Figure 1C, lanes 1 and 2). In contrast, expression of the Q783A mutant of Pmr1p, which is deficient only in Mn²⁺ transport (Wei et al., 2000), reduced the formation of TBSV recombinants by ~4-fold (based on the ratio between recombinants and the repRNAs) (Figure 1C, lanes 5 and 6). Expression of the Pmr1p-E329Q mutant, which is partially deficient in both Ca²⁺ and Mn²⁺ transport, inhibited the accumulation of TBSV recombinants by ~11-fold (Figure 1C, lanes 9 and 10). Altogether, these results support a key role for Pmr1p-driven Mn²⁺ transport in TBSV recombination, with the role of Pmr1p-driven Ca²⁺ transport evidently less important.

**Mn²⁺ Enhances the Frequency of Recombination in a Cell-Free TBSV Replication Assay**

To define how Pmr1p-driven Mn²⁺ transport could affect TBSV recombination, we took advantage of a recently developed yeast cell-free TBSV replication system (Pogany and Nagy, 2008; Pogany et al., 2008), which supports authentic, full-cycle replication when programmed with externally added TBSV repRNA and recRNAs from WT and mutant yeast strains. The newly formed recRNAs and the repRNA are shown with arrows. The numbers on the right side of the panel show the changes in recRNA and repRNA accumulation for the pmr1-5Δ yeast when compared with the WT parental yeast. The numbers at the bottom show the ratio of recRNAs versus repRNAs. (A) Schematic representation of nonoverlapping deletions made in the HUR1 and PMR1 genes. The two deleted nonoverlapping regions, which are replaced by KanMX4 sequence, are indicated with black boxes. (B) Northern blot analysis for detection of (+) strands of TBSV DI-72 repRNA and recRNAs from WT and mutant yeast strains. The newly formed recRNAs and the repRNA are shown with arrows. The numbers on the right side of the panel show the changes in recRNA and repRNA accumulation for the pmr1-5Δ yeast when compared with the WT parental yeast. The numbers at the bottom show the ratio of recRNAs versus repRNAs. (C) Northern blot analysis testing the effect of separation-of-function mutations in PMR1 on the formation of TBSV recRNAs. Mutations Q783A, D53A, and E329Q inhibit Mn²⁺, Ca²⁺, and Ca²⁺/Mn²⁺ transport function of the Pmr1p pump, respectively. Note that samples were taken earlier in these experiments, which resulted in lower recRNA ratios than in (B) above. See also Figure S1 for the recombination junction sequences of TBSV recRNA.

Figure 1. The Pmr1p ATPase-Driven Ca²⁺/Mn²⁺ Pump Inhibits TBSV RNA Recombination in Yeast

We have also tested the highly recombinogenic Δ70RII(+) repRNA, which contains a recombination hot spot sequence exposed at the 5’ end (Cheng et al., 2006). The cell-free replication assay programmed with Δ70RII(+) repRNA supported a 12-fold increased level of TBSV recRNAs in the presence of
Figure 2. Mn\(^{2+}\) Stimulates TBSV RNA Recombination in a Cell-Free Virus Replication Assay

(A) The cell-free TBSV replication assay was programmed with DI-72(+) repRNA. In addition to 5 mM Mg\(^{2+}\), the assay also included 0, 0.25, and 0.5 mM MnCl\(_2\), respectively. Denaturing PAGE analysis of the \(^{32}\)P-labeled repRNA products obtained is shown. The full-length repRNA and the recRNAs are marked. Each experiment was done three times.

(B) The effect of various salts on replication and recombination of the highly recombinogenic \(\Delta 70RII(+)\) repRNA in vitro. In addition to 5 mM Mg\(^{2+}\), the cell-free TBSV replication assay contained 0, 0.06, 0.125, 0.25, 0.5, and 1 mM amounts of MnCl\(_2\), CaCl\(_2\), and NaCl, respectively. Note that recRNA B is likely used to make the slower-migrating recRNAs (marked as C and D products) during the high-frequency recombination events (lanes 9–12), so the accumulation pattern is different for these recRNAs.

(C–E) The graphs show the relative accumulation of recRNA (the most abundant recRNAs were chosen) and repRNA in the in vitro assay.

(F) (Top) The cell-free TBSV replication assay containing either 0, 0.25, 0.5, 1, 2, and 5 mM MnCl\(_2\) or 0.25, 0.5, 1, 2, and 5 mM MgCl\(_2\) was programmed with DI-72(+) repRNA. See further details in (A). (Bottom) The graphs show the relative accumulation of recRNA and repRNA in the in vitro assay. Note that the accumulation levels of recRNAs (marked with B and C) and the repRNA (indicated with A) in the in vitro assay performed in the presence of 5 mM MgCl\(_2\) were chosen as 100%.

(G) The cell-free TBSV replication assay was programmed with the highly recombinogenic \(\Delta 70RII(+)\) repRNA. See further details in (F). See Figure S2 about the lack of inhibition by MnCl\(_2\) on suppression of RNA recombination by Xrn1p.
high Mn$^{2+}$ (Figures 2B and 2D), whereas high Ca$^{2+}$ decreased both the efficiency of repRNA replication and the formation of recRNA species (Figures 2B and 2E). Increasing the level of NaCl did not have a significant effect on repRNA replication or the formation of recRNA species (Figures 2B and 2C). Sequencing confirmed the recombinant nature of the recRNA molecules (data not shown) and the similar profiles of recombinants generated in vivo (in yeast as well as in single-plant cell cultures) (Cheng et al., 2006; Serviene et al., 2005) and in vitro.

To test whether Mn$^{2+}$ or Mg$^{2+}$ is favored by the tombusvirus replicase complex, we added them separately to the replicase assay. These experiments revealed that repRNA replication and RNA recombination, respectively, were up to 25× and 11× higher in the presence of Mn$^{2+}$ over Mg$^{2+}$ for the WT DI-72 repRNA (Figure 2F, compare product A in lanes 2 and 7 for replication and product B in lanes 4 and 9 for recombination), whereas they were up to 25× and 100× higher with Δ70RII(+)-repRNA (Figure 2G, compare product A in lanes 1 and 6 for replication and product B in lanes 1 and 6 for recombination). These data suggest that Mn$^{2+}$ within the range of 0.5–2 mM is favored over 5 mM Mg$^{2+}$ for both replication and recombination by the tombusvirus replicase in vitro. Overall, the results from the cell-free replication assay convincingly demonstrated the critical role of Mn$^{2+}$ concentration in TBSV recombination.

Because the host Xrn1p 5′-3′ exoribonuclease is an inhibitor of TBSV RNA recombination (Cheng et al., 2006; Jaag and Nagy, 2009), it is possible that Mn$^{2+}$ could affect TBSV RNA recombination indirectly by inhibiting the recombinase suppressor activity of Xrn1p. To test this model, we used purified recombinant Xrn1p in the cell-free assay (Figure S2). These experiments showed that the recombinase inhibition effect of Xrn1p was not affected in the presence of Mn$^{2+}$, excluding that Mn$^{2+}$ affects TBSV recombination via regulating the Xrn1p pathway.

**Knocking Down the Levels of the Plant LCA1 and ECA3 Ca$^{2+/Mn^{2+}}$ Pumps Enhances TBSV Recombination**

To confirm that the above findings on the key role of the Ca$^{2+/Mn^{2+}}$ exporter in TBSV recombination in the yeast model host are also valid in plant hosts, we first expressed a known Arabidopsis Ca$^{2+/Mn^{2+}}$ exporter, called AtEca1p (Liang et al., 1997), in pmr1-5 Δ yeast, which led to inhibition of TBSV RNA recombinants by ~40-fold (Figure 3A, lanes 3 and 4). These data promote the idea that the plant Eca1p Ca$^{2+/Mn^{2+}}$ transport protein might play a role in regulation of emergence of TBSV RNA recombinants.

Because Arabidopsis is not a natural host for TBSV, we decided to test the role of the Ca$^{2+/Mn^{2+}}$ transport proteins in TBSV RNA recombination using Nicotiana benthamiana, an experimental host. To inhibit the activity of Ca$^{2+/Mn^{2+}}$ exporters, such as Lca1p (homolog of Arabidopsis Eca1p) (Johnson et al., 2009) and Eca3p (Liang et al., 1997; Mills et al., 2008), we used a virus-induced gene silencing (VIGS) approach to reduce LCA1/ECA3 levels, resulting in ~90% downregulation of LCA1 mRNA (Figure 3E, lanes 1–4). Replication of tombusvirus genomic RNA increased 3.4±0.6-fold in the LCA1/ECA3 double-knockdown plants when compared to the nonsilenced plants (Figure 3B, lanes 1–9). Moreover, replication of two different repRNAs also increased 3- to 4-fold in the knockdown plants (Figures 3C and 3D). In addition, the accumulation of tombusvirus RNA recombinants increased 18-fold for DI-RIΔ and ~16-fold for DI-AU-FP repRNAs in the LCA1/ECA3 knockdown plants when compared to the nonsilenced plants (Figures 3C and 3D). The recombinants generated in the LCA1/ECA3 knockdown plants were similar to those obtained in pmr1-5 Δ yeast (Figure S1D). Time course experiments showed the largest difference in repRNA and recRNA accumulation on the third day after initiation of virus replication (Figures S3A and S3B). Because both tombusvirus replication and recombination increased in the LCA1/ECA3 knockdown plants, these data support the model that Lca1p/Eca3p Ca$^{2+/Mn^{2+}}$ pumps play important roles in TBSV RNA replication and recombination in a plant host.

**DISCUSSION**

Viral RNA recombination is one of the major forces in increasing variability of RNA viruses, enhancing viral fitness, and accelerating their adaptation to new hosts (Aaziz and Tepfer, 1999; Roossinck, 2003; Worobey and Holmes, 1999). Viral RNA recombination is affected by several factors, such as the viral replication proteins and various features of the viral RNA templates (Nagy and Simon, 1997). The emerging picture from genome-wide studies with TBSV and yeast host is that selected host genes also affect RNA recombination (Li et al., 2008b; Serviene et al., 2005, 2006), suggesting complex interaction between a given virus and its host during viral adaptation and evolution.

This paper provides a new picture of how host factors might affect viral RNA recombination. Inactivation of Pmr1p Ca$^{2+}$/Mn$^{2+}$ pump in yeast or double knockdown of LCA1 and ECA3 Ca$^{2+/Mn^{2+}}$ pumps in plants led to vastly increased rates of TBSV recombinant RNA accumulation. These changes in TBSV replication and recombination are likely due to the tombusvirus replicase, which switched to a super-active mode in pmr1-5 Δ yeast or in LCA1/ECA3 knockdown plants. This not only led to increased levels of TBSV recRNA replication, but also to an even higher level of RNA recombinant accumulation. The presented data conclusively demonstrate that these effects are due to a defect in Mn$^{2+}$ pump function in pmr1-5 Δ yeast or in LCA1/ECA3 knockdown plants and to the elevated Mn$^{2+}$ amount in an authentic cell-free replication assay.

We observed that the presence of increased amounts of Mn$^{2+}$ results in a smaller increase in RNA recombination in vitro than in cells but results in a greater increase in replication in vitro. One possible explanation is that the in vitro data are based on a cell-free replication/recombination assay, in which there is only a single complete cycle of replication taking place: from (+)repRNA to (−)repRNA followed by the production of the final abundant (+)repRNA progeny and recRNAs. On the contrary, in the in vivo experiments, there are many sequential full cycles of replication, allowing the more competitive recRNAs to outcompete the original repRNA in pmr1-5 Δ yeast. Another possibility is that the altered amount of Mn$^{2+}$ in pmr1-5 Δ yeast or in LCA1/ECA3 knockdown plants could affect cellular polymerases, other enzymes, and/or host responses that may contribute as well to the increased accumulation of repRNA and recRNAs.

Unlike the Mn$^{2+}$ pump function of Pmr1p, the Ca$^{2+}$ pump function does not seem to affect TBSV RNA recombination based on
expression of separation-of-function mutants in yeast. Pmr1p mediates ATPase-driven intracellular Ca\(^{2+}\)/Mn\(^{2+}\) transport under normal physiological conditions by controlling Ca\(^{2+}\) and Mn\(^{2+}\) influx to the Golgi from the cytosol (Ton and Rao, 2004). Mutations in the mammalian orthologs of Pmr1p, called SERCA (sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase) and hSPCA1, which also control Ca\(^{2+}\)/Mn\(^{2+}\) homeostasis (Strayle et al., 1999), cause autosomal dominant human diseases and also affect normal cardiac function and life span (Vangheluwe et al., 2006). Altogether, Pmr1-like ATPase-driven intracellular Ca\(^{2+}\)/Mn\(^{2+}\) ion pumps are present in eukaryotes and play important roles in controlling cytosolic Ca\(^{2+}\)/Mn\(^{2+}\) ion levels.

Based on the known cellular function of Pmr1p, as well as the presented in vitro and in vivo results on viral recombination, we propose that the Pmr1p (Lca1p/Eca3p in plants) Ca\(^{2+}\)/Mn\(^{2+}\) pump regulates TBSV RNA recombination by keeping the Mn\(^{2+}\) concentration low in the cytosol. In the presence of active Pmr1p in the cell, the RNA-dependent RNA polymerase (RdRp) within the viral replicase utilizes the far more abundant Mg\(^{2+}\) over Mn\(^{2+}\), leading to low-frequency template switching and infrequent RNA recombination (Figure 4A). On the other hand, deletion/inhibition of the Pmr1p Ca\(^{2+}\)/Mn\(^{2+}\) pump leads to an
promoting the more-efficient use of Mn\textsuperscript{2+} by the viral RdRp, a high recombination frequency. To explain the types of recombinants isolated binding to the TBSV replicase. This results in “sloppy” RNA synthesis and level of TBSV RNA synthesis also increases at a higher Mn\textsuperscript{2+} concentration that formation of duplicated and triplicated viral sequences. In addition, the overall templates repeatedly during complementary RNA synthesis, leading to the

Recombination, Based on Regulation of the Mn \textsuperscript{2+} Level in the

Figure 4. A Model Explaining the Role of the Yeast Pmr1p in TBSV Recombination, Based on Regulation of the Mn \textsuperscript{2+} Level in the Cytosol

(A) In the presence of Pmr1p, the amount of cytosolic Mn\textsuperscript{2+} is low, due to the efficient transfer of the Mn\textsuperscript{2+} from the cytosol to the Golgi. Therefore, the TBSV RdRp has the best chance to use Mg\textsuperscript{2+} during RNA synthesis, which then leads to high-fidelity RNA synthesis and a low frequency of template-switching RNA recombination. Note that TBSV replication takes place on the cytosolic surface of peroxisomes. TBSV replicases are shown as spherules on the surface of the peroxisome.

(B) In the absence of Pmr1p, the Mn\textsuperscript{2+} concentration increases ~5-fold in the cytosol, allowing Mn\textsuperscript{2+} to efficiently compete with the more-abundant Mg\textsuperscript{2+} for binding to the TBSV replicase. This results in “sloppy” RNA synthesis and a high recombination frequency. To explain the types of recombinants isolated (see Figures S1A and S1B), we predict that the TBSV replicase may switch templates repeatedly during complementary RNA synthesis, leading to the formation of duplicated and triplicated viral sequences. In addition, the overall level of TBSV RNA synthesis also increases at a higher Mn\textsuperscript{2+} concentration that makes the replicase a more-efficient (super-active) enzyme.

~5-fold increased level of cytosolic Mn\textsuperscript{2+} (Mandal et al., 2000), promoting the more-efficient use of Mn\textsuperscript{2+} by the viral RdRp (Figure 4). In turn, this leads to high-frequency template switching and the abundant formation of RNA recombinants, as shown in the cell-free replication assay (Figure 2). Our model predicts that, through regulating the cytosolic Mn\textsuperscript{2+}, the Pmr1p Mn\textsuperscript{2+}/Mn\textsuperscript{2+} ion pump affects TBSV RNA recombination via changing the activity of the viral RdRp. Of interest, high Mn\textsuperscript{2+} also affects the activity of the reverse transcriptase/RNaseH (Bolton et al., 2002; Vartanian et al., 1999) and the in vitro template activity of RdRps of several RNA viruses, making the polymerase action less specific for templates and stimulating nucleotide misincorporation (Alaoui-Lsmaili et al., 2000; Arnold et al., 2004; Hardy et al., 2003; Poranen et al., 2008; Yi et al., 2003). These data suggest that the effect of Ca\textsuperscript{2+}/Mn\textsuperscript{2+} ion pumps on RNA recombination described in this paper could be general and widespread among viruses. Thus, Ca\textsuperscript{2+}/Mn\textsuperscript{2+} ion pumps might play an important role in viral RNA recombination and evolution.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Expression Plasmids**

Construction of expression plasmids for yeast and plant-based assays are described in the Supplemental Information.

**TBSV Replication and Recombination Assay Based on Yeast Cell-Free Extract**

Replication and recombination of the TBSV repRNAs were carried out in yeast, *N. benthamiana*, or a yeast cell-free extract as described previously (Cheng et al., 2006; Pogany and Nagy, 2008; Wang and Nagy, 2008). Yeast, strain BY4741, was transformed with the combination of pGSTGADHis92s and pHisGBKHis33 to express p33 and p92 replication proteins. The cell-free yeast extract was prepared in buffer A (30 mM HEPES-KOH [pH 7.4], 100 mM potassium acetate, 2 mM magnesium acetate, and 1 mM DTT) as described (Pogany and Nagy, 2008). Replication and recombination of the TBSV repRNAs were carried out in the cell-free extract as described previously (Pogany and Nagy, 2008). In brief, the assay was performed in the cell-free replication (CFR) buffer containing 50 mM HEPES-KOH (pH 7.4), 150 mM potassium acetate, 5 mM magnesium acetate, 0.2 M sorbitol, and 0, 0.06, 0.125, 0.25, 0.5, and 1 mM amounts of MnCl\textsubscript{2}, CaCl\textsubscript{2}, and NaCl, respectively. The 20 µl assay also contained 2.0 µl of cell-free extract prepared from yeast (Pogany and Nagy, 2008; Pogany et al., 2008); 0.4 µl Actinomycin D (5 mg/ml); 2 µl of 150 mM creatine phosphate; 2 µl of 10 mM ATP, CTP, and GTP; 0.25 mM UTP; 0.3 µl of ATP-UTP; 0.2 µl of 10 mg/ml creatine kinase; 0.2 µl of RNase inhibitor; 0.2 µl of 1 M DTT; and 0.5 µg RNA transcript. The reaction was incubated at 25°C for 3 hr. The reaction was terminated by adding 110 µl stop buffer (1% SDS and 0.05 M EDTA [pH 8.0]), followed by phenol/chloroform extraction, isopropanol/ammonium acetate precipitation, and a washing step with 70% ethanol. Denaturing polyacrylamide gel electrophoresis and gel imaging was done as described (Pogany and Nagy, 2008).

**Virus-Induced Silencing of LCA1 and ECA3 in Nicotiana benthamiana Plants**

The *Arabidopsis* genome contains four members of the ATP-driven P-type Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pumps, termed ECA1–4 (Li et al., 2008a; Liang and Sze, 1998; Mills et al., 2008). Among these, ECA1 and ECA3 genes have been shown to affect Mn\textsuperscript{2+} transport. While Eca1p is localized in the ER, Eca3p is present in the Golgi or endosome membranes (Li et al., 2008a; Liang and Sze, 1998; Mills et al., 2008). Tomato has one ATP-driven P-type Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pump characterized, termed LCA1, which has been shown to complement Pmr1p and affect Mn\textsuperscript{2+} transport in yeast (Johnson et al., 2009). Sequences in the two nonoverlapping fragments of the *N. benthamiana* ECA1-like gene, termed NbLCA1N (Figure S4A) and NbLCA1C (Figure S4B), showed 94% similarity to the tomato LCA1 gene while only sharing 67%–73% similarity with the tomato ECA3-like gene, but only 55%–57% similarity with the tomato LCA1 gene and AtECA1/2/4 genes. Based on this information, we decided to knock down the expression levels for both LCA1 and ECA3 genes in *N. benthamiana*.

For the knockdown experiments, we used VIGS using agroinfiltration with a mixture of *Agrobacterium* strains carrying pTRV1, pTRV2-NbLCA1N, pTRV2-NbLCA1C, and pTRV2-NbECA3 as described previously (Wang and Nagy, 2008). The soil of the agroinfiltrated plants was supplemented with 3 ml 200 mM MnCl\textsubscript{2} solution to increase the level of Mn\textsuperscript{2+} in the plants. The mRNA level of NbLCA1 was examined at the ninth day after infiltration by
RT-PCR with primers #2810 (AGACCTTGAGAATCGAGGCACACGC) and #2811 (AGGGCGAGAAGGTGTTGGAATGA). Selected newly emerged (LC41/ECA3 silenced) leaves were agroinfiltrated with mixed Agrobacterium strains carrying pGd-CN/pGd-ΔRI or pGd-CN/pGd-DIAU. Then, 3, 4, and 5 days after agroinfiltration, circles of −1 mg leaf samples were punched out using the agroinfiltrated leaves and processed in a 96-well plate in liquid nitrogen for total RNA extraction.

To demonstrate that additional putative Ca²⁺/Mn²⁺ pumps could not complement the silenced LC41/ECA3 pumps in N. benthamiana, we treated the plants by pipetting 3 ml of 200 mM MnCl₂ at the bottom of the plant stem to increase the Mn²⁺ uptake. We observed that the new leaves of the silenced plants died due to Mn⁺⁺ toxicity, whereas the control plants (nonsilenced Ca²⁺/Mn²⁺ pumps cannot complement the silenced TGGCATAGCATAC) using pYC-DI72 as template. To detect the CNV genomic CACTATAGGAAGGGTCCGCTTCCACAAGTGA and #2755 (AGTGTAAATC) and #151 (2008a). A distinct endosomal Ca²⁺/Mn²⁺ pump affects root growth through the secretory process. Plant Physiol. 147, 1675–1689.


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