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ScienceDirect

Journal of Genetics and Genomics 42 (2015) 319–330

JGG

METHOD

Fast-Suppressor Screening for New Components in Protein Trafficking, Organelle Biogenesis and Silencing Pathway in *Arabidopsis thaliana* Using DEX-Inducible *FREE1-RNAi* Plants

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Received 9 February 2015; revised 21 March 2015; accepted 27 March 2015
Available online 14 May 2015

ABSTRACT

Membrane trafficking is essential for plant growth and responses to external signals. The plant unique FYVE domain-containing protein FREE1 is a component of the ESCRT complex (endosomal sorting complex required for transport). FREE1 plays multiple roles in regulating protein trafficking and organelle biogenesis including the formation of intraluminal vesicles of multivesicular body (MVB), vacuolar protein transport and vacuole biogenesis, and autophagic degradation. FREE1 knockout plants show defective MVB formation, abnormal vacuolar transport, fragmented vacuoles, accumulated autophagosomes, and seedling lethality. To further uncover the underlying mechanisms of FREE1 function in plants, we performed a forward genetic screen for mutants that suppressed the seedling lethal phenotype of *FREE1-RNAi* transgenic plants. The obtained mutants are termed as suppressors of *free1* (*sof*). To date, 229 putative *sof* mutants have been identified. Barely detecting of FREE1 protein with M_3 plants further identified 84 FREE1-related suppressors. Also 145 mutants showing no reduction of FREE1 protein were termed as RNAi-related mutants. Through next-generation sequencing (NGS) of bulked DNA from F_2 mapping population of two RNAi-related *sof* mutants, *FREE1-RNAi* T-DNA inserted on chromosome 1 was identified and the causal mutation of putative *sof* mutant is being identified similarly. These FREE1- and RNAi-related *sof* mutants will be useful tools and resources for illustrating the underlying mechanisms of FREE1 function in intracellular trafficking and organelle biogenesis, as well as for uncovering the new components involved in the regulation of silencing pathways in plants.

KEYWORDS: Suppressors; FREE1; Endomembrane trafficking; *Arabidopsis*; NGS

Abbreviations: AFE, allele frequency estimation; DEX, dexamethasone; ESCRT, endosomal sorting complex required for transport; FREE1, FYVE domain protein required for endosomal sorting 1; MVB, multivesicular body; NGS, next generation sequencing; *sof*, suppressor of *free1*.

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http://dx.doi.org/10.1016/j.jgg.2015.03.012

INTRODUCTION

The plant endomembrane system contains several functionally distinct membrane-bound organelles including endoplasmic reticulum (ER), golgi apparatus, trans-golgi network (TGN) or early endosome, prevacuolar compartment (PVC) or multivesicular body (MVB) or late endosome, and vacuoles (Lam et al., 2007; Reyes et al., 2011; Gao et al., 2014a). Membrane trafficking within the endomembrane system is essential for protein and lipid material transport and exchange within and in-between cells. In plants, the trafficking system functions for both fundamental cellular activities and also responds to environmental stresses (Samaj et al., 2006; Bar and Avni, 2014; Teh and Hofius, 2014).

Plant cells have highly regulated membrane trafficking pathways that are conserved among eukaryotic cells, but also seem to have evolved additional features with some of the components present in multiple copies (Cvrckova et al., 2012). The plant endomembrane machinery was largely established from homology-based studies (Bassham et al., 2008). For example, the endosomal sorting somplex required for transport (ESCRT) machinery regulates the homeostasis of plasma membrane protein by sorting them into the MVB which eventually fuse with the lytic vacuole to degrade the ubiquitinated plasma membrane (PM) cargo molecules (Henne et al., 2011; Cai et al., 2014). Most of our current knowledge on ESCRT in plants comes mainly by homology with studies performed on yeast and humans. However, orthologs of the subunits of ESCRT-0 have not been identified in plants (Leung et al., 2008).

A recent study of the plant unique FYVE domain containing protein FREE1 (FYVE domain protein required for endosomal sorting 1) showed that FREE1 localizes to the PVC, and interacts with the ESCRT-I component Vps23 to regulate the formation of intraluminal vesicles (ILVs) in PVCs/MVBs thereby facilitating PM protein degradation in the vacuole (Gao et al., 2014b). Mutant plants without functional FREE1 (T-DNA and RNAi mutants) are seedling lethal, defective in MVB ILV biogenesis and vacuolar sorting of membrane proteins. They also show abnormalities in vacuolar trafficking and accumulate small fragmented vacuoles and autophagosomes (Gao et al., 2014b, 2015). FREE1 was found to directly interact with SH3P2, a unique regulator of plant autophagy (Zhuang et al., 2013; Zhuang and Jiang 2014), thus controlling autophagosome-vacuole fusion and finally autophagic degradation in plants (Gao et al., 2015). These studies have unveiled a direct link between the ESCRT machinery and autophagy process, and demonstrate the multiple functional roles of FREE1 in regulating MVB formation, vacuolar protein transport, vacuole biogenesis and autophagy pathway. Because of the multiple functions of FREE1 in *Arabidopsis*, it is likely that plants have evolved unique mechanisms and components responsible for the FREE1-containing ESCRT pathway. It is now clear that FREE1 has dual roles in regulating membrane trafficking: 1) through FREE1 N-terminal direct interaction with ESCRT1 components VPS23, FREE1 regulates MVB biogenesis and PM protein vacuolar degradation; 2) through FREE1 C-terminal direct interaction with the autophagosome regulator SH3P2 (Zhuang et al., 2013; Zhuang and Jiang 2014), FREE1 regulates autophagosome formation and autophagic degradation (Gao et al., 2015). In addition, the abnormalities in *free1* mutant including the dysfunction of vacuolar protein transport and autophagic degradation point to a defect in membrane fusion caused by FREE1 depletion. However, it is still not understood why and how FREE1 depleted cells contain fragmented small vacuoles and how FREE1 loss-of-function leads to seedling death. It is likely that the functions of proteins required for docking and/or fusion with vacuoles might be disrupted in the *free1* mutant. Alternatively, FREE1 might work together with other proteins to regulate membrane fusion and vacuole biogenesis through as yet undefined mechanisms.

To address how FREE1 fulfills its multiple roles in regulating membrane trafficking and organelle biogenesis in plants, we performed a suppressor screen using a FREE1 loss-of-function mutant as the starting material. With the emerging technology of next-generation sequencing (NGS), the mutated gene can be identified through an NGS-mapping approach in a short time (Manavella et al., 2012). Here, we report on the NGS-based high-throughput suppressor identification method to find mutants that can rescue the *free*1 seedling lethal phenotype using inducible *FREE1-RNAi* plants. Upon dexamethasone (DEX) induction, the *FREE1-RNAi* transgenic plants are seedling lethal with the FREE1 protein barely detectable. Seeds from *FREE1-RNAi* plants were treated with EMS for suppressor screening. M_2 seeds were sprayed on plates with DEX to identify surviving plants, which we name *sof* (suppressors of *free1*. Because some of the isolated mutants could be caused by a disruption of the RNAi process, we also performed a second screen through Western blot analysis using FREE1 antibodies. We selected mutants with significantly reduced FREE1 proteins as putative *sof* mutants (termed as FREE1-related *sof* mutants) for further characterization. Using this method, we have successfully isolated 84 FREE1-related *sof* mutants. These SOF probably encode proteins involved in controlling vacuole biogenesis and vacuolar trafficking. All of these FREE1-related *sof* mutants and RNAi-related mutants will be useful resources for members of the international research community working on novel mechanisms of regulating organelle and RNA biogenesis in plants.

RESULTS

Establishment of the mutant pool and screening process

Using the Columbia ecotype (Col) as the wild type, *FREE1-RNAi* M_0 seeds were successfully generated with the wild type transformed with *pTA7002* construct containing the hairpin *FREE1-RNAi* (Fig. 1A). T_3 seeds from individual hygromycin resistant T_2 lines were screened on both DEX and hygromycin conditions. Line 11# was chosen as mutagenesis material because 3/4 of its T_3 showed hygromycin resistance and lethal phenotype on DEX, supporting the single copy insertion of *pTA7002-FREE1-RNAi*. Homozygous Line 11#