Golgi-localized MORN1 promotes lipid droplet abundance and enhances tolerance to multiple stresses in Arabidopsis

Zhan Li[1,2†], Yue Gao[2†], Jiapei Yan[2,3], Shuai Wang[2,4], Shu Wang[2,5], Yuanyuan Liu[1], Shaokui Wang[1], Jian Hua[2*]

[1] Guangdong Provincial Key Laboratory of Plant Molecular Breeding, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China

[2] School of Integrative Plant Science, Plant Biology Section, Cornell University, Ithaca, NY 14853, USA.

[*]Corresponding author: jh299@cornell.edu

[†]These authors have contributed equally to this work

[3] Current address: National Key Laboratory of Crop Genetic Improvement, Hubei Hongshan Laboratory, Huazhong Agricultural University, Wuhan 430070, China[4] Current address: Nanjing Agricultural University, Nanjing, China

[5] Current address: Jiangbei New Area Biopharmaceutical Public Service Platform Co., Ltd, Nanjing, China

Key words

Lipid droplet, MORN1, Golgi, stress tolerance, heat, cold, immunity, natural variation

Abstract

Lipid droplet (LD) in vegetative tissues has recently been implicated in environmental responses in plants, but its regulation and its function in stress tolerance are not well understood. Here, we identified a *MORN1* gene as a contributor to natural variations of stress tolerance through genome-wide association study in *Arabidopsis thaliana*. Characterization of its loss of function mutant and natural variants revealed that the *MORN1* gene is a positive regulator of plant growth, disease resistance, cold tolerance and heat tolerance. The MORN1 protein is associated with the Golgi and is also partly associated with LD. Protein truncations that disrupt these associations abolished the biological function of the MORN1 protein. Furthermore, the *MORN1* gene is a positive regulator of LD abundance, and its role in LD number regulation and stress tolerance is highly linked. Therefore, this study identifies MORN1 as a positive regulator of LD abundance and a contributor to natural variations of stress tolerance. It implicates a potential involvement of Golgi in LD biogenesis and strongly suggests a contribution of LD to diverse processes of plant growth and stress responses.

Introduction

Plants have evolved multi-layered mechanisms to respond to environment and cope with stresses. In addition to extensive transcriptome reprogramming in nucleus, multiple organelles, such as chloroplast and endoplasmic reticulum (ER), undergo functional modifications in response to environmental changes and contribute to adaptation and stress tolerance (Liu and Li, 2019) (Song et al., 2021). Lipid droplet (LD), also named lipid body or oil body, is recently also implicated in tress tolerance in plants, besides its conserved function as reservoirs of high-energy metabolites. LDs in leaves, while less abundant than in seeds, have been found to modulate plant growth, drought stress, defense responses, plant senescence, heat tolerance, and cold tolerance (Aubert et al., 2010; Brocard et al., 2017; Fernandez-Santos et al., 2020; Gidda et al., 2016; Kim et al., 2016; Shimada et al., 2014). LDs consist of neutral lipids in the core and phospholipids as a monolayer on the surface as well as LD proteins (or LD coat proteins) inserted into or attached to the phospholipids (Chapman et al., 2019; Chapman et al., 2012). Plant LD coat proteins play important roles in modulating LD number and size as well as plant development and environmental responses (de Vries and Ischebeck, 2020; Huang et al., 2019; Ischebeck et al., 2020; Pyc et al., 2017; Shimada et al., 2018). For instance, oleosins, the primary LD coat proteins in seed, are critical for LD integrity (Shimada et al., 2018). A LD protein CALEOSIN3 (CLO3, or RD20) in guard cells controls stomata aperture in response to light and ABA (Aubert et al., 2010) while CLO3 in mesophyll cell is responsible for the biosynthesis of a critical antifungal compound (Shimada et al., 2014).

LD biogenesis is shown to initiate at the membranes of the (ER in yeasts, animals and plants (Murphy, 2012). Neutral lipid triacylglycerol (TAG) is synthesized in the ER and buds out to form LD (Jackson, 2019; Walther et al., 2017; Weigel and Mott, 2009). Some LD proteins target to the LD surface by routing directly through the ER (Ohsaki et al., 2014; Olzmann and Carvalho, 2019). There is also evidence that Golgi is also be involved with LD formation in mammalian and yeast cells. For instance, the Golgi-localized ARF-related protein 1 mediates LD growth via organizing the membranous structures at LD for TAG transport in specific cell types in mice (Hesse et al., 2013). However, it is not known how prevalent the Golgi and LD association is among various cell types and how much this association contributes to LD biogenesis. In plants, no connection of the Golgi to LD biogenesis is reported.

The Membrane Occupation and Recognition Nexus (MORN) motif was first described as an membrane tethering module of the mammalian Junctophilin Protein 1 (JP1 or JPH1) to facilitate calcium transduction between the plasma membrane (PM) and the ER (Takeshima et al., 2000). This motif is now defined as 23 amino acids (aa) including the originally identified 14 aa sequence YxGxWxxG/DxxxGxG (Li et al., 2019a; Sajko et al., 2020). The MORN motif has been found to exert diverse functions in various tissues and species and to influence protein-lipid and protein–protein interactions (Im et al., 2007; Li et al.,

2019a; Ma et al., 2006; Sajko et al., 2020). In *Arabidopsis thaliana* (referred as Arabidopsis from hereon), a total of 15 proteins contain the MORN motif which is always in repeats in each protein. They include 9 Phosphatidylinositol-phosphate-5-kinases (PIP5Ks) (Ma et al., 2006; Mueller-Roeber and Pical, 2002), Accumulation and Replication of Chloroplasts 3 (ARC3), Translocon at the Inner Envelope Membrane of Chloroplast 100 (TIC100, or EMB1211) (Liang et al., 2010; Shimada et al., 2004) and 4 JPH1-like proteins.

In this study, we used Genome Wide Association Study (GWAS) to identify genetic and molecular basis for environmental responses because variations in genomes have been linked to adaptation to local environment (Exposito-Alonso et al., 2018; Fournier-Level et al., 2011; Hancock et al., 2011; Weigel and Mott, 2009). A MORN motif containing protein MORN1 was identified as a contributor to natural variations of plant immunity at 16 ºC in Arabidopsis from this GWAS. The *MORN1* gene is revealed as a positive regulator of plant growth, disease resistance, heat tolerance and freezing tolerance through characterization of its loss of function mutation alleles, natural variants, and its overexpression lines. *MORN1* is found to be a positive regulator of LD abundance, and its role in LD number regulation and stress tolerance is highly linked. Furthermore, the MORN1 protein is associated largely with the Golgi and partially with LD. The identification of a Golgi-localized MORN1 as a novel regulator of LD abundance as well as multiple environmental stress responses provide new areas to explore on LD biology and suggests a role of LD variation in environmental adaptation.

Results

GWAS identifies the *MORN1* gene as a regulator of disease resistance at 16ºC

A total of 69 natural accessions of Arabidopsis plants were assayed for their resistance to a virulent pathogen *Pseudomonas syringe pathovar tomato* (*Pst*) DC3000 at 16ºC (Fig. 1A). GWAS on differences in the growth of *Pst* DC3000 between at 3 days and 0 day (1 hour) after infection revealed one major QTL centered around position 9600 K on the chromosome 4 (Fig. 1B). This QTL fell below the significance value, but could still to be true due to the small number of accessions used in this association study. Earlier studies have identified true QTLs with a similarly low values using the same sized population (Lu et al., 2021; Wang et al., 2019). Underlying this QTL was a gene coding a MORN motif containing protein. This is one of the 4 JPH1-like proteins in Arabidopsis, and we named it MORN1 and other 3 JPH1-like as MORN2 to MORN4 (Fig. S1A). The Arabidopsis MORN1 to MORN4 proteins are mis-annotated (in Araport v11) as putative histone H3K4-specific methyltransferases due to the presence of MORN-repeat motifs in the animal methyl transferases SET7/9, but these plant proteins do not have a methyltransferase domain. The molecular and biological functions of these proteins are unknown except for the MORN-motif repeat protein regulating flowering (MRF1, or MORN3) that was shown to promote flowering under long

day condition (You et al., 2019). The closest homolog of the MORN1 subfamily (MORN1 to MORN4) is TIC100 in Arabidopsis based on the MORN motif sequences (Fig. S1A).

To determine whether or not the *MORN1* gene has a role in disease resistance regulation, we analyzed a *MORN1* loss-of-function mutant (Salk_073158) from the T-DNA mutant collection (Alonso et al., 2003). This *morn1-1* mutant (referred as *morn1* from hereon) has a T-DNA insertion in the first exon of the gene which is predicted to cause a truncation of protein (Fig. S1B), and it also has greatly reduced *MORN1* RNA expression (Fig. S1C). Another *morn1-2* mutant had a T-DNA insertion in the promoter region but did not have a reduced *MORN1* expression compared to the wild type (Fig. S1B, S1C) and therefore was not used for further study. The growth of pathogen *Pst* DC3000 was 2-fold higher in the *morn1* mutant compared with the wild-type Col-0 at 16ºC (Fig. 1B and 1C). This defect was rescued by the wild-type *MORN1* from Col-0 ($MORN1^{Col\text{-}0}$) as shown by the wild-type level of growth of *Pst* DC3000 in transgenic lines in *morn1* (Fig. 1C). Pathogen growth at 22ºC was also higher in *morn1* (by 1.6-fold each) compared to wild-type Col-0, but the difference was not statistically significant (Fig. 1D). This indicates that *MORN1* positively regulates plant immunity, and this function is strongest at a relatively low temperature and is weak or not significant at normal temperatures. This temperature influenced defect is consistent with the general enhancement of disease resistance at low temperatures (Li et al., 2019b).

***MORN1* positively regulates plant growth**

The *morn1* mutants also exhibited growth defects compared to the wild-type Col-0. At both 16ºC and 22ºC, it had a reduced growth with a lower fresh weight and smaller rosette compared to the wild-type Col-0 (Fig 2A, 2B). The growth defects in rosette size and fresh weight were rescued by the $MORN1^{Col\text{-}0}$ transgene (Fig. 2B, S2A, S2B). In addition, a high expression of *MORN1*was associated with more resistance to *Pst* DC3000 and larger growth among lines carrying the transgene of the genomic fragment of the *MORN1* gene from Col-0. *MORN1* was expressed at a higher level in two lines (line #5 and #8) than the rest of the 9 lines (Fig. S2C), which is likely due to transgene insertion. These two lines had larger rosettes and less pathogen growth compared to other lines (Fig. S2B, S2D). This data indicates that *MORN1* is required for plant growth and disease resistance, and it also suggests that its activity is expression level dependent.

***MORN1* positively regulates freezing tolerance and heat tolerance**

To have a more comprehensive assessment of the biological role of *MORN1*, we examined the *morn1* mutant under abiotic stresses, especially freezing and heat. Plants grown at normal growth temperature 22°C for 2 weeks were subject to -10°C treatment, and lethality was examined after recovery at 22°C for 5 days. The wild-type Col-0 and the *morn1* plants were not significantly different from each other after 4 hr of freezing treatment, and neither survived after 10 h of -10°C freezing treatment (Fig. S2E

and S2F). However, the survival rate was significantly lower in *morn1* compared to the wild type after -10°C freezing treatment of 5 h, 6 h and 8 h (Fig. 2C, S2E and S2F). This function of *MORN1* was supported by an increase of freezing tolerance in *MORN1* overexpressing (*MORN1*-OE) transgenic plants compared to the wild type (Fig. 2C). To examine heat tolerance of *morn1*, plants grown at 22°C for 2 weeks were treated by a moderate heat of 35°C. After four weeks at 35°C, all *morn1* mutant plants died while the wild-type plants were still alive (Fig. 2D). This heat susceptible defect was complemented by the $MORN1^{Col\text{-}0}$ transgene (Fig. 2D). Therefore, *MORN1* also acts as a positive regulator of temperature stress tolerance.

The MORN1 protein is associated with the Golgi and lipid droplets

MORN1 encodes a protein of 513 aa, with two predicted transmembrane segments (153 aa to 198 aa) and 7 MORN motifs (from 260 aa to 419 aa) (Fig. 3A). We analyze the subcellular localization of this protein by its GFP fusion proteins. The MORN1-GFP transgene complemented the growth defects of the *morn1* mutant (Fig. S2G), but no GFP signal was detected in transgenic plants under confocal microscope. We therefore transiently expressed this functional fusion in Arabidopsis protoplasts and observed its localization pattern by confocal imaging. A punctate or droplet-like structure was observed for the MORN1-GFP signal, and this structure was not associated with chloroplasts by viewing with the constructed 3D image (Fig. S3A). A number of organelle markers or stains (Nelson et al., 2007; Rumin et al., 2015) were used to determine the location of MORN1-GFP expressed in *N. benthamiana*. Z-stacked images of MORN1-GFP were merged with each of the marker protein or stain of peroxisome, plastid, ER, LD or Golgi. Significant overlap with MORN1 signal was observed for the cis-Golgi marker mannosidase I-mCherry fusion as well as the LD stained by Nile red (Fig. S3B).

Localization of MORN1 to the Golgi was confirmed by marker proteins of both the cis-Golgi ManI and trans-Golgi SYP61 (Yang et al., 2021) (Fig. 3B). When co-expressed in Arabidopsis protoplasts, 80% (from 40% to 90%) of the MORN1-GFP or GFP-MORN1 signal areas overlapped with the ManI-mCherry signals (Fig. 3C), and 70% (45% to 90%) of the MORN1-GFP or GFP-MORN1 signal areas overlapped with the SYP61-mCherry signals (Fig. 3C). It is worth noting that overexpressing MORN1-GFP, and GFP-MORN1 to a less extent, sometimes induced aggregate like structures of the MORN1 fusion protein itself in protoplasts and caused the Golgi marker protein to exhibit a more ER like structure. Therefore, the overlap measured in this transient expression might be an overestimation or underestimation of the real overlap of MORN1 protein produced at normal level with the Golgi. Nevertheless, this data supports the localization of MORN1 to the Golgi, especially considering that expression of the marker protein in some cells may not be high enough to mark all the Golgi and the ratio of overlap could be an underestimation of the association of MORN1 with the Golgi. Since a large overlap was observed for both cis- and trans- Golgi

markers, MORN1 may be associated with both compartments of the Golgi, or the imaging resolution does not resolve the spatial difference of the two.

In *N. benthamiana*, MORN1-GFP had a large overlap with signals from LD stain Nile red on projected Z-stacked images (Fig. S3B). This association of MORN1 with LD was observed in Arabidopsis protoplasts as well. In optical sections of the confocal images, a large signal overlap was observed between Nile red stain and MORN1-GFP or GFP-MORN1 (Fig. 3D). Another LD stain BODIPY that more specifically detects neutral lipids than Nile red was further used to verify the location of MORN1 relative to LD. Because of the emission signals from BODIPY and GFP are too similar to distinguish, the mCherry fusion of MORN1 was used for co-localization with BODIPY staining. The emission range was set at 590 to 640 nm so that BODIPY signal could not be falsely detected in the mCherry channel. Again, a large overlap was observed between MORN1-mCherry signals and BODIPY staining signals (Fig. 3D). The MORN1 signal was close to the LD or wrapped around the LD (Fig 3D), suggesting that MORN1 was associated with the surface of LD. Quantification of the overlapping areas on the optical section images of protoplasts revealed that an average of 60% (30%-78%) of the MORN1-GFP or GFP-MORN1 signal area overlapped with signals from Nile red staining, an average of 57% (from 15% to 81%) of MORN1-mCherry signal areas overlapped with BODIPY signal areas (Fig. 3B), indicating that about half of MORN1 proteins are associated with LDs. The association of MORN1 with LD was less than the association with the Golgi (Fig. 3B), suggesting that MORN1 is localized to the Golgi but is associated with LD frequently.

The dual association of MORN1 with the Golgi and LD suggests an association of LD with the Golgi. As prior studies showed that the ER has a contact with LD for its initiation, we examined the association of MORN1 with the ER in more detail using an ER marker HDEL-mCherry. Similar to what was observed in *N. benthamiana* (Fig. S3B), very little contact was observed between MORN1-GFP and HDEL-mCherry in protoplast cells (Fig. S3C). In addition, we examined the association of MORN1 with the SEIPIN that was shown to reside at the ER and LD contact site (Cai et al., 2015; Salo et al., 2016; Szymanski et al., 2007). Little overlap of signals was observed between MORN1-mCherry and SEIPIN1-GFP when co-expressed in leaves of *N. benthamiana* (Fig. S3D). These data indicate that MORN1 is not associated with the ER.

The transmembrane region and the MORN domain are important for the Golgi and LD associations, respectively

We further examined the structural basis for the association of MORN1 with Golgi and LD. A series of truncated MORN1 proteins were made with one or more of the domains or motifs deleted: the N-terminal segment (aa1-151), two consecutive transmembrane segments (aa152-198), six MORN1 motifs (aa260-419), and the C-terminal segment (aa420-513) (Fig. 4A). The truncated proteins were fused with

mCherry for co-localization study with BODIPY staining, and they were fused with GFP for co-localization with the Golgi marker ManI-mCherry or for staining with Nile red in protoplasts.

Similar to the full-length MORN1, the Δ420-513 form of MORN1 had co-localization signals with both the Golgi markers and the LD stain (Fig. 4A), suggesting the last 94 aa are not essential for the localization of MORN1. The Δ199-513 form of MORN1 was localized on the Golgi but had no association with LD (Fig. 4A), indicating the N-terminus and the TM segments are sufficient for Golgi localization, and the MORN motifs and/or C-terminal are needed for LD localization. The Δ282-513 form of MORN1 that had the N-terminal part till the first MORN1 motif was also localized on the Golgi but not associated with the LD (Fig. 4A). Similarly, the Δ260-419 form that lacks all 7 MORN motifs was co-localized with Golgi marker but not with LD stain (Fig. 4A). This indicates that MORN motifs are required for LD localization but not Golgi localization. Interestingly, the Δ1-259 form of MORN1 (aa260-513) that lacks the N-terminus and transmembrane segments but contains all the MORN motifs and the C-terminus did not show co-localization with Golgi marker or LD stain (Fig. 4A). Therefore, MORN motifs are required but not sufficient for LD localization and Golgi localization is essential for the LD association of the MORN1 protein.

We further analyzed the biological activities of these truncated proteins by expressing the mutant genes in the *morn1* mutant. Over 27 primary transformants for each of the MORN1 forms were analyzed for their growth phenotype. Compared to transgenic lines containing the full-length wild-type *MORN1* gene, transgenic lines carrying all the mutant forms of *MORN1* exhibited reduced growth (Fig. 4B). The Δ420-513 form that had the same subcellular localization with the wild-type form had a higher activity than other truncated forms, although its activity was still lower compared to the wild-type form in promoting plant growth. These data suggests that Golgi localization and LD association are both important for MORN1 function.

MORN1 is a positive regulator of lipid droplet abundance

MORN1 is associated with LD as shown above by its localization in Arabidopsis protoplasts and *N. benthamiana*, but it was not identified as a LD resident protein in any of the lipid droplet proteome studies (Brocard et al., 2017; Kretzschmar et al., 2020; Zhi et al., 2017). We therefore hypothesize that MORN1 is localized at Golgi and may be associated with LD while LD is undergoing biogenesis or maturation. To test this hypothesis, we analyzed the LDs in the wild type and the *morn1* mutant plants by using the BODIPY stain. In leaf epidermis, the *morn1* mutant had a reduced LD number under both normal growth condition and stress conditions (Fig. 5A). Quantification of the BODIPY signals revealed a significantly reduced LD number per leaf area in the *morn1* mutant compared to the wild type under normal growth condition of 22°C (Fig. 5B). LD number increased drastically at 48 h after infection with virulent

pathogen *Pst* DC3000 in the wild type (Fig. 5A, 5B). A similar extent of increase was observed in the *morn1* mutant after infection, and the LD number in the mutant remained reduced compared to the wild type under pathogen infection (Fig. 5A, 5B). LD number also increased after 48 h of heat (35°C) treatment (Fig. 5A, 5B). An increase of LD number was also observed in the *morn1* mutant but to a much lower extent compared to the wild type (Fig. 5A and 5B). The average LD size was similar between the wild type and the *morn1* mutant (Fig. 5C). The LD number difference between the mutant and the wild type was also observed in protoplasts generated from mesophyll cells (Fig. 5D). Quantification using Z-stack images of BODIPY stain of protoplasts revealed that the *morn1* mutant had a 30% reduction of LD number per cell compared to the wild type (Fig. 5E). The average LD size was also comparable between the wild type and the mutant (Fig. 5F).

We further used the LD coat protein CLO3 to quantify LD amount to complement the LD staining method. A CLO3-GFP protein that is expressed under the native *CLO3* promoter and is present in about 80% of leaf LDs (Shimada et al., 2014) was introduced into the *morn1* background by hybridization to generate isogenic plants of the wild type and the *morn1* mutant carrying the same *CLO3-GFP* transgene. Confocal imaging of epidermis revealed a reduced CLO3-GFP signal in the *morn1* mutant compared to the wild type under non stress condition, after pathogen infection, and after heat treatment (Fig. S4A). Consistent with the microscopy observation, Western blot of total leaf proteins from isogenic lines revealed a similar reduction of CLO3-GFP protein under all conditions tested. The CLO3-GFP was reduced to about half in *morn1* compared to the wild type under normal growth condition (Fig. 5G). After infection with pathogen *Pst* DC3000, the wild type and the mutant had an increase of CLO3-GFP by 12 folds, and the CLO3-GFP remained lower in *morn1* compared to the wild-type Col-0 after infection (Fig. 5G). Heat treatment of 35°C for 24 hours induced CLO3-GFP in the wild type by 5 folds and in the *morn1* mutant by 1.2 folds, leading to a drastically reduced CLO3-GFP in the mutant (Fig. 5G). The CLO3-GFP protein level was slightly higher at 16°C compared to 22°C for both wild type and the *morn1* mutant, and the mutant remained to have a lower CLO3-GFP compared to the wild type (Fig. S4B). The reduced level of the CLO3 protein was not due to a lower transcript level of *CLO3* in the mutant. The RNA level of *CLO3* in the *morn1* was even higher than that in the wild-type Col-0 at 22°C and was even higher in the mutant compared to the wild type at 16°C and 35°C (Fig. S4C). These data indicate that *MORN1* is a positive regulator of LD abundance, and its effect is present under normal growth conditions as well as under biotic and abiotic stresses.

MORN1 does not significantly affect TAG, sterols or phosphatidylcholine content or Golgi-mediated protein secretion

As LD consists of TAG in the core as well as sterols in and phospholipids on the surface , we asked whether or not MORN1 affects their metabolism to regulate LD abundance. Six major TAGs and 22 phosphatidylcholines were analyzed on leaf tissues from plants grown under 16°C, and none of them had significant difference between the wild-type Col-0 and the *morn1* mutant (Fig. 5H and S5B). In addition, all 12 sterols analyzed (stigmasterols, sitosterols and campesterols) had a similar concentration in Col-0 and the *morn1* mutant except for stigmasterol (18:2) which was slightly lower in *morn1* than in Col-0 (Fig. S5A). This indicates that MORN1 does not drastically affect the amount of TAGs, sterols or phosphatidylcholines.

We further asked whether or not MORN1 has a function in vesicle trafficking that is carried out by the Golgi. A glycosylphosphatidylinositol (GPI)-anchored protein was used as a marker for this trafficking process, because defective Golgi function would render it trapped in the Golgi rather than presented on the PM (Bernat-Silvestre et al., 2021; Martiniere et al., 2012). When expressed in Arabidopsis protoplasts, the GFP-GPI fusion protein was localized on the PM in both the wild type and the *morn1* mutant in all protoplasts observed (Fig. 5I).

Together, these data suggest that the MORN1 protein does not have a significant role in TAG metabolism or vesicle trafficking, therefore its regulation of LD abundance is not likely via its effect on TAG metabolism or Golgi function.

Natural MORN1 variants have differential activities in plant stress tolerances and LD number

As *MORN1* was identified as a candidate gene for natural variation, we determined whether or not the *MORN1* variants could confer differential disease resistance. Polymorphisms were observed in regulatory or intergenic regions as well as in the coding regions in the *MORN1* gene among three high resistant accessions (Bsch_0, Kin_0, Sq_8) and three low resistance accessions (Kro_0, Ove_0, Sei_0) (Fig. 6A, S6A, S6B). The three resistant accessions (Bsch_0, Kin_0, Sq_8) and one susceptible accession (Sei_0) had a higher *MORN1* expression than the less resistant Col-0, and two susceptible accessions had a similar expression as Col-0 (Fig. 6B), suggesting that the *MORN1* expression level may contribute to resistance variation. Inspection of coding sequences revealed that *MORN1* variants of the resistant accessions are similar to that of the reference accession Col-0, while *MORN1* variants of the susceptible accessions are similar to each other but distinct from Col-0 (Fig. 6A, S6B), and they differ by 5 amino acids residing outside the transmembrane segments and the MORN motifs (Fig. S6C).

To assess the biological activities of the variants, we isolated the genomic fragments of the *MORN1* gene (including its promoter) from the representative susceptible accession Ove_0 and resistant Kin_0 accessions. The $MORN1^{Ove\_0}$ transgene could not complement the growth defect of the *morn1* mutant (Fig. 6C, and S6D) even though the *MORN1* gene expression was higher in these lines than the wild-type Col-0

(Fig. 6D). In contrast, the *MORN1*$^{Kin\_0}$ transgene complemented the growth defect of the *morn1* mutant (Fig. 6E, and S6D), and it was expressed at a higher level than the *MORN1* gene in Col-0 (Fig. 6F). This indicates the *MORN1*$^{Ove\_0}$ variant has a lower activity than the *MORN* $^{Col-0}$ and *MORN1*$^{Kin\_0}$ variant in promoting plant growth. In addition, lines #2 and #3 of *MORN1*$^{Kin\_0}$ in *morn1* with a higher *MORN1* $^{Kin\_0}$ expression had larger rosettes than line #1 with a lower *MORN1*$^{Kin\_0}$ expression (Fig. 6E and 6F), suggesting an enhancement of growth by a higher expression of *MORN1*.

In addition to the growth promoting activity, the *MORN1*$^{Ove\_0}$ variant also exhibited lower activity than *MORN1*$^{Kin\_0}$ or *MORN1*$^{Col-0}$ in rescuing defects in disease resistance, freezing tolerance and heat tolerance of *morn1*. *MORN1*$^{Ove\_0}$ could not complement the defect of immunity, heat and freezing tolerance in *morn1*, while *MORN1*$^{Kin\_0}$ and *MORN1*$^{Col-0}$ each restored the *morn1* to the wild-type Col-0 phenotype (Fig. 6G, 6H and 6I). This indicates that *MORN1* variations, either in expression (Kin_0 versus Col-0) or protein activity (Col-0 versus Ove_0), can confer differences in growth and environmental responses.

We further examined the effect of *MORN1* variants on LD abundance using *morn1* transgenic lines carrying *MORN1*$^{Ove\_0}$ or *MORN1*$^{Kin\_0}$. BODIPY staining of protoplasts from wild type, *morn1*, and transgenic plants revealed that LD number in *MORN1*$^{Kin\_0}$/*morn1* was higher than that in *morn1* and similar to that of the wild-type Col-0 while LD number in *MORN1*$^{Ove\_0}$/*morn1* was not significantly different from that in *morn1* (Fig. 6J). This indicates that natural *MORN1* gene variants can confer variations of LD numbers in addition to variations of growth and environmental responses.

Discussion

Here, we identified MORN1, a MORN motif containing protein, as a positive regulator of growth and stress tolerances in Arabidopsis. A loss of *MORN1* function resulted in multiple defects, including reduced plant growth, compromised immunity, and hyper-susceptibility to heat and freezing, while a high expression of *MORN1* enhances plant growth and stress tolerances (Fig. 1, 2 and S2). Furthermore, natural polymorphisms in the *MORN1* gene confer variations in growth and stress tolerance and contribute to variations in disease resistance in Arabidopsis natural accessions (Fig. 6 and S6). These data not only indicate an important role of *MORN1* in environmental response but also implicate its contribution to adaptation in natural populations.

The biological function of MORN1 in stress tolerance and growth likely results from its cellular function in promoting LD abundance. MORN1 is a positive regulator of LD abundance. LD number is reduced with the loss of *MORN1* activity and increased when the *MORN1* has a higher activity (Fig. 5 and 6). The abundance of LD has been associated with plant growth and stress tolerance (Pyc et al., 2017; Shimada et al., 2018). The reduced LD number might account for the multiple defects of *morn1* in plants

growth, immunity, heat tolerance, and freezing stress. The *morn1* mutant exhibited a stronger susceptibility at 16°C compared to at 22°C, it is possible that LD proteins such as CLO3 are important for defense responses, reduced LD protein in the *morn1* mutant may lead to reduced disease resistance. Because defense response against *Pst* DC3000 is enhanced at 16°C (Li et al., 2019b), MORN1 might be involved in this lower temperature enhancement of defense response. Although a high correlation of LD number and stress tolerance was found for effects of loss of function, overexpression and natural variants of MORN1 gene, whether or not the regulation of LD number leads to its regulation of plant growth and stress tolerance will still need to be further investigated.

It is not yet known at the molecular level how MORN1 modulates LD number in plants. MORN1 does not significantly affect metabolism of TAG or sterols (Fig. 5H and S5), and it also does not significantly affect protein trafficking through the Golgi as indicated by the marker protein GFP-GPI (Fig. 5I). It is therefore unlikely that the processing of some LD coat proteins in the Golgi is significantly affected in the *morn1* mutant leading to LD biogenesis defect. Our data suggests that MORN1 may act as a bridge between the Golgi and LD and thus promote or maintain the association of LD with Golgi. Structurally, MORN1 could be a Golgi resident protein through its transmembrane region in the Golgi membrane. At the same time, it could associate with LD through the MORN repeats and therefore tether LD to the Golgi. While its Golgi localization only requires the transmembrane region, its LD association requires both transmembrane region and the MORN motifs (Fig. 3). The tethering of LD to the Golgi via the MORN1 protein could affect the biogenesis of LD. LD may initiate from Golgi directly and MORN1 may maintain a longer association of LD with the Golgi to facility material transfer. Alternatively, LD initiated from ER may dock on Golgi through MORN1 for further maturation (Fig. S7). Similar tethering and transfer facilitating activities have been observed in other MORN motif containing proteins. JPH proteins in muscle and neuronal cells tether the ER to the PM for lipid transfer and calcium dynamics between the ER and the PM (Woo et al., 2016), and TIC100 on the chloroplast inner envelope is critical for chloroplast protein import (Loudya et al., 2022). Because of the low expression of MORN1-GFP in transgenic plants, all localization studies were carried out by transient expression in Arabidopsis protoplasts and *N. benthamiana* leaves. Stronger fluorescent proteins might be used as tags for MORN1 localization studies in plants for the future. Whether or not MORN1 has an overlapping function with other MORN proteins to tether LD and Golgi also needs to be investigated.

The study of MORN1 suggests that the Golgi might be a site for LD initiation or maturation. Although the ER is considered as an initiation site for LD biogenesis, the Golgi was recently found to be another initiation site in animal lipid cells. High throughput fluorescence imaging of mammalian cells also revealed that LDs are in contact with multiple organelles, with the ER as the most frequently contacted

organelle followed by the Golgi and the mitochondria (Valm et al., 2017). Proteomic analysis of leaf LDs also suggested a direct contact of Golgi with LD, as a number of Golgi membrane proteins were detected from purified LD proteins in Arabidopsis leaves (Kretzschmar et al., 2020). High throughput and high resolution imaging could be employed to further test this hypothesis in plants.

Variations in the *MORN1* genes contributes to diversity of plant growth, defense and thermotolerance as well as LD biogenesis. This suggests that modulating LD number could be an adaptation strategy in natural population. The expression and protein activity of MORN1 show natural variations which could both contribute to its activity variation. Differences in amino acids are mainly in N terminus before TM domain and the linker between TM and MORN domain (Fig. S5C). The linker region was found to be important for protein-protein interaction to facilitate JHP4 molecular function (Woo et al., 2016). Further detailed targeted mutagenesis should reveal the major variations that are responsible for the phenotypic effects and how these variations might enable adaptation to their local environment.

In summary, this study has identified MORN1 as a regulator of LD abundance and suggests an involvement of the Golgi in LD initiation and/or maturation. This study supports the emerging role of LD in growth and stress tolerance in vegetative tissues. The contribution of *MORN1* to natural variations of disease resistance suggests a role of LDs in environmental adaptation.

Materials and Methods

Plants growth conditions

The Arabidopsis plants were grown in chambers with a light intensity of 70-100 μmol m^{-2} sec^{-1} and a relative humidity of 50-70%. Plants were grown under constant light unless they were used for pathogen growth assay where a 12 hr light/12 hr dark photoperiod was used.

Generation of constructs and transgenic plants

For complementation tests of $MORN1^{Col\text{-}0}$, $MORN1^{Ove\_0}$ and $MORN1^{Kin\_0}$, genomic fragments containing the promoter region of *MORN1* were amplified from the respective accessions and cloned first into PCR8/GW/TOPO vector (Invitrogen) and then the binary vector pMDC99 (Curtis and Grossniklaus, 2003) by Gateway LR clonase (Invitrogen). For MORN1-OE construct, the cDNA of *MORN1* was cloned into vector PMDC32 (Curtis and Grossniklaus, 2003) by the Gateway system. The constructs were transformed into Agrobacterium GV3101 and then Arabidopsis plants via floral dipping method (Clough and Bent, 1998). For localization assays, cDNA of MORN1 and genomic DNA of SEIPIN1 were cloned in the vector pSAT4A-mCherry-N1 (Citovsky et al., 2006) by In-fusion cloning (Vazyme) or in the vector pSAT6-EGFP-N1 (Tzfira et al., 2005) by the Gateway system. All primers used in generating constructs are provided in Supplemental data.

Genome-wide association study

A genome-wide association study was performed on GWAPP (https://gwapp.gmi.oeaw.ac.at/index.html) with a 250K single nucleotide polymorphism SNP dataset (Seren et al., 2012). The 69 Arabidopsis natural accessions used were the same as in the early study (Wang et al., 2019).

Pathogen growth assay

Pathogen growth assays were performed by dipping inoculation as previously described (Gou et al., 2015). Seedlings were grown at 22°C for 14 days before inoculation. The number of bacteria in seedlings was analyzed at 1 h (0 d) and 3 d post inoculation (dpi).

Freezing and heat tolerance assays

Plants were grown at 22°C for 2 weeks before being moved to -10°C for freezing treatment or 35°C for heat treatment. Phenotypic comparison was done on plants grown side by side in the same pot. The heat tolerance test was conducted at least three times and the freezing tolerance test was conducted two times with similar results.

Protoplast isolation and transformation

Protoplast isolation and transfection were performed as previously described (Jung et al., 2015). Transfected protoplasts were inoculated at 22°C for 16 h under dark before being placed on ice for transporting to facility for imagining.

Lipid droplet staining

LD staining in leaves was performed as previously described (Gidda et al., 2016) with slight modifications. The 5^{th} or 6^{th} leaves of Arabidopsis plants were stained with 2 μg/mL BODIPY 493/503 (Invitrogen) in 50 mM PIPES buffer (pH 7.0) under water-pump vacuum for 30 minutes followed by washing with 70% ethanol for 30 seconds and then with PIPES buffer for 5 minutes. LD staining in protoplasts was done by incubating protoplasts in 0.1 μg/mL BODIPY or Nile red for 1 minute.

Confocal microscopy and imaging quantification

Confocal microscopy images were taken using the ZEISS LSM880 confocal microscope equipped with the ZEISS software package. A 20x objective was used for imaging, and images with a zoom factor of 8-bit were acquired with a frame size of 512 x 512 pixels. For BODIPY staining and GFP, signals were excited at 488 nm and emission was obtained at 495-545 nm. For Nile red staining and mCherry fusions, signal was excited at 561 nm and emission was obtained at 590-640 nm. Images were acquired as individual single optical sections or as a Z-stack sections.

All confocal imaging experiments were done at least two times independently with similar results. Images shown are representative from these experiments. The number and area of signals (such as LD)

were quantified on the Z-stacked images or section images using the 'Analyze Particles with the circularity value set at 0.80-1.0 to exclude signals that likely come from clustered LDs, while the area of one signal that overlaps with another signal was quantified on the optical section images using the "ROI manager" function in FIJI software (Schindelin et al., 2012).

Analysis of plant lipids

Leaf tissues were harvested from 21-day-old Arabidopsis seedlings grown at 16°C under 12h/12h light/dark. Lipids were extracted according to the protocol described (Shiva et al., 2018) and submitted to Kansas Lipidomics Research Center for Mass-spec analysis.

Western blot

Total proteins were extracted from 2-week-old seedlings with or without treatments. Anti-GFP antibody (JL-8; Takara Bio) was used at a 1:3000 dilution to detect CLO3-GFP. Anti-mouse IgG antibody linked with HRP (Cell Signaling Technology) was used at a 1: 5000 dilution to detect the primary antibody. Protein signals were quantified using FIJI.

Acknowledgement

We thank Dr. Ruth Welti and Kansas Lipidomics Research Center for TAG measurement and the Arabidopsis Biological Resource Center for the Arabidopsis mutant seeds. We thank Dr. June Nasrallah, Dr. María J.M. Zaragoza and Dr. Ikuko Hara Nishimura for organelle markers, GFP-GPI construct and the CLO3-GFP seeds. We acknowledge funding support from NYSTEM (C029155) and NIH (S10OD018516) to the Imaging facility of Cornell Institute of Biotechnology for the Zeiss LSM880 microscopes. This work is supported by National Science Foundation USA IOS-1946174 to JH, Cornell-ZJU seed grant, and Seed Grant of Cornell Institute of Biotechnology.

Author contributions

JH conceived and supervised the project; ZL, YG, JY, ShuaiW and ShuW performed the experiments, YL and ShaokuiW provided materials, ZL and JH analyzed the data and wrote the manuscript with inputs from all authors.

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Figure legends

Figure 1. Genome wide association study (GWAS) of disease resistance in Arabidopsis.

(A) Distribution of disease resistance index values at 16°C of 69 Arabidopsis natural accessions as used in an early study. Resistance index was defined as $\log_{10}$cfu/mg FW (3dpi)- $\log_{10}$cfu/mg FW (0 dpi) of growth of bacterial pathogen *Pst* DC3000 at 16°C. **(B)** Manhattan plot of GWAS for resistance index at 16°C for chromosome 4. Red arrow indicates the QTL investigated in this study. Polymorphisms in the same linkage group with the top site were colored in orange. **(C)** Growth of virulent pathogen *Pst* DC3000 in wild-type Col-0, *morn1* and two lines of *MORN1*$^{\text{Col-0}}$ /*morn1* plants at 16°C 4 dpi. Plant phenotypes after infection are shown on the left and the quantification of growth of *Pst* DC3000 is shown on the right. **(D)** Growth of *Pst* DC3000 in wild-type Col-0 and *morn1* plants at 22°C at 3 dpi or at 16°C at 4 dpi.

cfu: colony forming unit. FW: fresh weight, dpi: days post inoculation. Shown in all bar graphs are averages and standard deviations (S.D.) of 3 biological repeats. Different letters indicate statistically significant differences by ANOVA test ($p < 0.05$).

Figure 2. The *morn1* mutant is compromised in plant growth and thermotolerance.

(A) Growth phenotype (left) and fresh weight (right) of Col-0 and *morn1* and plants grown for 3 weeks at 16ºC under 12/12 h light/dark. Scale bar, 10 mm. **(B)** Growth phenotypes (left), rosette area (middle) and fresh weight (right) of Col-0, *morn1* and *MORN1*$^{\text{Col-0}}$/*morn1* plants grown at 22ºC for 2 weeks under constant light (Scale bar, 10 mm). **(C)** Morphological phenotypes (left) and survival rates (right) of wild-type Col-0, *morn1* and *MORN1*-OE (overexpression) plants after freezing treatment at -10°C for 6 h. Seedlings were grown at 22°C for 2 weeks and then at -10°C for 6 h, followed by recovery at 22°C for 5 days before being photographed (Scale bar=1 cm). **(D)** Phenotypes (left) and survival rates (right) of wild-type Col-0, *morn1* and *MORN1*$^{\text{Col-0}}$/*morn1* plants under heat. Seedlings were grown at 22°C for 2 weeks and then at 35°C for 4 weeks before being photographed (Scale bar=1 cm).

Shown in bar graphs are averages and standard deviations (S.D.) of more than 15 plants biological repeats (for rosette size and fresh weight). Different letters indicate statistically significant differences by ANOVA test ($p < 0.05$). '**' indicates a significant difference between samples determined by Student's t test at $p < 0.01$.

Figure 3. The MORN1 protein is associated with the Golgi and the lipid droplet (LD).

(A) Diagram of the MORN1 protein: seven MORN motifs (gray) and two transmembrane segments (blue). The numbers below indicate amino acid positions. **(B)** Representative images of fluorescent signals of MORN1 fusion proteins (MORN1-GFP or GFP-MORN1) and the co-expressed Golgi markers (cis-Golgi ManI-mCherry or trans-Golgi SYP61-mCherry) in Arabidopsis protoplasts. **(C)** Ratios of MORN1 signals overlapped with signals from BODIPY, Nile red, ManI-mCherry and SYP61-mcherry. Quantification was done from 7-21 cells as in (B) and (D). **(D)** Representative images of fluorescent signals from MORN1-mCherry, MORN1-GFP, or GFP-MORN1 expressed in protoplasts stained with BODIPY (for the mCherry fusion) or Nile-red (for the GFP fusions). Scale bar in (B) and (D) is 10 μm in main images and 2 μm in magnified images. 'Merged' images in (A) and (C) are from signals from two fluorescent channels.

Figure 4. Requirement of the transmembrane region and the MORN motifs for the Golgi and LD localization of MORN1.

(A) Analysis of co-localization of full-length and truncated MORN1 proteins with the Golgi and LD in protoplasts. For co-localization with the Golgi, GFP fusions of MORN1 proteins were co-expressed with the Golgi marker ManI-mCherry (left panel). For co-localization with LD, cells expressing mCherry fusions of MORN1 proteins were stained with BODIPY (middle panel) except that cells expressing MORN1Δ199-513-GFP fusion were stained with Nile red. Truncations include Δ420-513, Δ199-513, Δ282-513, Δ260-419, and Δ1-259 of MORN1 as diagramed to the right of the images. Shown are representative images of fluorescent signals and merged images from two fluorescent signals and the bright field signal for the MORN1 proteins. Scale bars are 10 μm. Co-localization of MORN protein signals with the Golgi or LD was indicated by "+" and "-" respectively. (B) Morphological phenotype (left), rosette diameter (middle) and fresh weight (right) of T1 plants of morn1 plants transformed with wild-type MORN1 or truncated MORN1 mutant forms: Δ282-513, Δ1-259, Δ260-419, and Δ420-513. Left shows a representative plant for each transgene. Scale bar is 10 mm. Bar graphs of diameter and weight show average and S.D. from at least 27 T1 plants. The number of T1 plants are shown as on top of the bars. Different letters indicate significant differences by ANOVA test ($p < 0.05$).

Figure 5. The *morn1* mutant has reduced LD number compared to the wild type.

(A) LDs shown by BODIPY staining in leave epidermis of wild-type Col-0 and *morn1* mutants before and after treatment with pathogen *Pst* DC3000 or heat stress of 35°C for 48 hr (Scale bar = 20 μm). The 5th or 6th leaves of 3-week-old plants were assayed. Shown are merged images of signals from BODIPY, chloroplasts and the bright field. **(B, C)** Quantifications of LD number (B) and size (C) in 60 μm x 60 μm area of leaf epidermis as in (A). Shown is box plot of data distribution, averages, and quartiles from more

than 20 leaves of each genotype and condition combination. Each data point of LD size is an average of all LDs in each image, and 20-30 images were analyzed for each genotype and condition combination. Different letters indicate statistically significant differences by ANOVA test ($p < 0.05$). **(D)** Z-stacked image of LDs shown as fluorescent signals from BODIPY stain in a representative protoplast cell prepared from the wild-type Col-0 and the *morn1* mutant. **(E, F)** Quantifications of LD number (E) and size (F) in protoplasts from wild-type Col-0 and the *morn1* mutant from Z-stacked images as in (D). Shown are box plots of measurements from more than 25 cells for each genotype. '**' indicates a significant difference between samples determined by Student's *t* test at $p<0.01$. **(g)** Protein amount of CLO3-GFP in wild-type Col-0 and the *morn1* mutant before and after treatments of pathogen *Pst* DC3000 or heat stress of 35°C for 24 hr. Upper panel is the immunoblot of total leaf protein extracts from 2-week-old CLO3-GFP transgenic plants in wild-type Col-0 or *morn1* probed with anti-GFP antibody. Lower panel shows the Coomassie brilliant blue staining of the protein gel around the Rubisco band. Relative amount of CLO3-GFP or Rubisco is shown below the protein band as relative amount (in fold number) to the Col-0 sample of 22°C. **(H)** Contents of major triacyl glycerides (TAGs) in 3-week-old wild-type Col-0 and *morn1* plants grown at 22°C. Shown are average and S.D. from 5 biological repeats (each from5 mg of plants). **(I)** Localization of GFP-GPI (a marker protein for Golgi trafficking) expressed in protoplasts isolated from wild-type Col-0 and *morn1* plants. ManI-mCherry was co-expressed as a Golgi marker. Scale bar is 20 μm. Merged images are from two fluorescent channels and the bright field.

Figure 6. *MORN1* variants have different activities in promoting growth, stress tolerance and LD abundance.

(A) Polymorphisms in the coding region of *MORN1* in 3 accessions (Kro_0, Ove_0 and Sei_0) with low disease resistance index and 3 accessions (Sq_8, Bsch_0 and Kin_0) with high disease resistance index. Shown are browser views from Salk Arabidopsis 1001 Genome Browser (http://signal.salk.edu/atg1001/3.0/gebrowser.php.). Color lines and grey boxes indicate differences from the reference Col-0 sequence at the amino acid level. **(B)** Relative expression of *MORN1* in accessions of Col-0, Kro_0, Ove_0, Sei_0, Bsch_0, Kin_0 and Sq_8. Shown are relative expression to Col-0 with *Actin2* reference gene. **(C-F)** Fresh weight (C, E) and (D, F) relative *MORN1* expression of Col-0, *morn1* and $MORN1^{Ove\_0}$/*morn1* (C, D) or $MORN1^{Kin\_0}$/*morn1* (E, F) plants grown for 2 weeks at 22 ºC under constant light. Shown in (C, E) are average and standard deviation from more than 20 plants. Shown in (D, F) are average and standard deviation from 3 biological repeats. Different letters indicate significant differences among samples by ANOVA ($p < 0.05$). **(G)** Growth of *Pst* DC3000 in 16°C grown plants of the wild-type Col-0, *morn1*, $MORN1^{Ove\_0}$/*morn1* and $MORN1^{Kin\_0}$/*morn1* at 4 days post inoculation (dpi). Error bars

represent standard deviation (S.D.) from three biological replicates in one experiment. Similar results were obtained from three independent experiments. Different letters indicate statistically significant differences by ANOVA test ($p < 0.05$). **(H)** Growth phenotypes (left) and survival rate (right) of wild-type Col-0, *morn1*, *MORN1*$^{Ove\_0}$/*morn1* and *MORN1*$^{Kin\_0}$/*morn1* plants after freezing treatment. Seedlings were grown at 22°C for 2 weeks and treated at -10°C for 5 h, followed by a recovery at 22°C for 5 days before being photographed and assessed for survival (Scale bar=1 cm). (**I**) Growth phenotypes of wild-type Col-0, *morn1*, *MORN1*$^{Ove\_0}$/*morn1* and *MORN1*$^{Kin\_0}$/*morn1* plants under heat. Seedlings were grown at 22°C for 2 weeks and then at 35°C for 4 weeks before being photographed (Scale bar=1 cm). (**J**) LD in protoplasts prepared from Col-0, *morn1*, *MORN1*$^{Ove\_0}$/*morn1* and *MORN1*$^{Kin\_0}$/*morn1*. Left shows representative images of BODIPY staining of protoplasts. Right shows box plots from at least 10 protoplasts per genotype. Different letters indicate significant differences by ANOVA test ($p < 0.05$).

Supplemental Information

Supplemental figures

Figure S1. Phylogenetic tree of MORN motif containing proteins and characterization of the *morn1* mutants.

Figure S2. Characterization of the *morn1* mutant and the *MORN1* complementation lines.

Figure S3. Analysis of MORN1 subcellular localization.

Figure S4. CLO3-GFP levels in wild-type Col-0 and *morn1* after treatment with *Pst* DC3000, heat or cold.

Figure S5. Contents of major sterols and phosphatidylcholinesins in wild-type Col-0 and *morn1* plants.

Figure S6. Characterization of natural variations of the *MORN1* gene in Arabidopsis.

Figure S7. Model of MORN1 function and LD biogenesis.

Supplemental data sheets

Sheet 1 Disease resistance index of 69 Arabidopsis accessions at 16°C.

Sheet 2 Primers used in construct generation and gene expression studies.