Characterization of the small *Arabidopsis thaliana* GTPase and ADP-ribosylation factor-like 2 protein TITAN5

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Highlights

- The small ARF-like GTPase TTN5 has a very rapid intrinsic nucleotide exchange capacity with a conserved nucleotide switching mechanism
- Biochemical data classified TTN5 as a non-classical small GTPase, likely present in GTP-loaded active form in the cell
- YFP-TTN5 is dynamically associated with vesicle transport and different processes of the endomembrane system, requiring the active form of TTN5

Running title

ARF-like GTPase TTN5 functions

Abstract

Small GTPases comprise key proteins in signal transduction that function by conformational switching ability between GDP- and GTP-bound states. The ADP-ribosylation factor (ARF) family is involved in vesicle trafficking and cellular functions. Though evolutionarily well conserved, little is known about ARF and ARF-like GTPases in plants. Here, we characterized functional properties and cellular localization of the essential small ARF-like GTPase TITAN5/HALLIMASCH/ARL2/ARLC1 (hereafter termed TTN5) from *Arabidopsis thaliana*. TTN5 showed rapid guanine nucleotide exchange capacity comparable to that of human counterparts, but a remarkably low GTP hydrolysis reaction. A TTN5^{Q70L} mutant had enhanced nucleotide exchange activity, indicative of intracellular activation, while TTN5^{T30N} with fast nucleotide dissociation can be considered a dominant-negative form. This suggests that TTN5 is present in GTP-loaded active form in the cells. YFP-tagged TTN5 and the two derived mutant variants were located at multiple sites of the endomembrane system in the epidermis of Arabidopsis seedlings and *Nicotiana benthamiana* leaves. While YFP-TTN5 and YFP-TTN5^{Q70L} were highly mobile in the cells, mobility was reduced for TTN5^{T30N}. Colocalization with endomembrane markers in combination with pharmacological treatments resolved localization at membrane sites and showed that YFP-TTN5 and YFP-TTN5^{Q70L} were located in Golgi stacks, multivesicular bodies, while this was less the case for YFP-TTN5^{T30N}. On the

48 other hand, all three TTN5 forms were located at the plasma membrane. Hence, the unusual
49 capacity of rapid nucleotide exchange activity of the small ARF-like GTPase TTN5 is linked
50 with cell membrane dynamics, likely associated with vesicle transport pathways in the
51 endomembrane system.
52

Keywords
54   TTN5; ARF-like; ARL2; endomembrane; GTPase; plasma membrane; vesicle
55

Abbreviations

Arabidopsis	*Arabidopsis thaliana*
ARF-like / ARL	ADP-ribosylation factor-like
BFA	brefeldin A
EE	early endosomes
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
ICQ	intensity correlation quotient
MVB	multivesicular body
TGN	*trans*-Golgi network
TTN5	TITAN 5

Introduction
A large variety of regulatory processes in signal transduction depends on guanine
nucleotide-binding proteins of the GTPase family. Following the identification of common
oncogenes (*HRAS, KRAS,* and *NRAS*) a new class of GTPases has been recognized, that
became known as the RAS superfamily of small GTPases (Bos, 1988; Hall, 1990; Kahn et al.,
1992). RAS proteins have many conserved members in the eukaryotic kingdom. The RAS
superfamily consists of five subfamilies in mammals: the Rat sarcoma (RAS), RAS homologs
(RHO), RAS-like proteins in the brain (RAB), Ras-related nuclear proteins (RAN), and ADP-
ribosylation factor (ARF) subfamilies (Kahn et al., 1992; Ahmadi et al., 2017). In *Arabidopsis*
thaliana (Arabidopsis) only four families are represented, the ROP (Rho of plants), RAB, RAN,
and the ARF (Vernoud et al., 2003). The subfamilies are classified by sequence identity and
characteristic sequence motifs with well-conserved regulatory functions within the cell (Kahn
et al., 1992). Many mammalian small GTPases have been shown to act as molecular switches
in signal transduction. They switch from inactive GDP-loaded to active GTP-loaded GTPase
form. The different activity states enable them to form differential complexes with proteins or
act in tethering complexes to the target membrane. Small GTPases have usually low intrinsic
GDP/GTP exchange and GTP hydrolysis activity and require the regulation by guanine
nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs are
potentially recruited to the inactive, GDP-bound GTPase at their site of action and accelerate
GDP/GTP exchange leading to GTPase activation. GTP binding induces a conformational
change of two regions referred to as switch I and II. The active, GTP-loaded GTPases exert
their function *via* direct interaction with their effectors (Sztul et al., 2019; Nielsen, 2020;
Adarska et al., 2021) until their inactivation by GAPs, which stimulate the hydrolysis of GTP.
Most of the known protein interactions important for their signaling functions occur in the active
conformation of the GTPases. Members of the ARF family contain a conserved glycine at
position 2 (Gly-2) for the characteristic *N*-myristoylation of an amphipathic helix (Kahn et al.,
1992). ARF GTPases are often involved in vesicle-mediated endomembrane trafficking in
mammalian cells and yeast (Just and Peränen, 2016).

In plants, the activities of small GTPases and their functional environments in the plant cells are by far not understood well and only described in a very rudimentary manner. In particular, the ARF family of small GTPases is surprisingly poorly described in plants, although the Arabidopsis ARF family consists of twelve ARF, seven ARF-like, and the associated SAR1 proteins (Singh et al., 2018). The best-studied plant ARF-GTPases, SAR1 and ARF1, act in the anterograde and retrograde vesicle transport between the endoplasmic reticulum (ER) and the Golgi. SAR1 is involved in COPII trafficking from the ER to the Golgi, whereas ARF1 participates in the opposite COPI pathway (Singh et al., 2018; Nielsen, 2020). Another ARF-like protein, ARL1, has perhaps a role in endosome-to-Golgi trafficking (Latijnhouwers et al., 2005; Stefano et al., 2006). This role in vesicle formation within the endomembrane system is well conserved in eukaryotes and raises the question of whether other plant ARF members are also involved in functioning of the endomembrane system. A recent study showed Golgi-related localization for some ARF and ARF-like proteins (Niu et al., 2022) promoting a general involvement of the ARF family in the endomembrane system.

TITAN5 (TTN5)/HALLIMASCH (HAL)/ARF-LIKE2 (ARL2), ARLC1, from here on referred to as TTN5, is essential in plant development. It was identified in two independent screens for abnormal embryo mutants. The *ttn5* loss-of-function mutants are arrested soon after cell division of the fertilized egg cell, without displaying a phenotype in pollen or the male gametophyte, indicating the fundamental, potentially housekeeping role in cellular activities of TTN5 protein (Mayer et al., 1999; McElver et al., 2000; Lloyd and Meinke, 2012). TTN5 is closely related in sequence to human ADP-ribosylation factor-like 2 (HsARL2). HsARL2 has high nucleotide dissociation rates, being up to 4000-fold faster compared to RAS (Hanzal-Bayer et al., 2005; Veltel et al., 2008). HsARL2 is associated with different functions in cells, ranging from microtubule development, also identified for yeast and Caenorhabditis homologs (Bhamidipati et al., 2000; Fleming et al., 2000; Radcliffe et al., 2000; Antoshechkin and Han, 2002; Tzafrir et al., 2002; Mori and Toda, 2013), adenine nucleotide transport in mitochondria (Sharer et al., 2002) and control of phosphodiesterase activity in cilia (Ismail et al., 2011; Fansa and Wittinghofer, 2016). It is thought that HsARL2 requires fast-acting nucleotide exchange and participates in different cellular processes that depend on developmental and tissue-specific factors. With regard to the plant ortholog, it is still completely unknown, which cellular roles TTN5 fulfills in plants. Until today, the function of this small plant GTPase remains elusive at the molecular level. Besides lacking knowledge of the physiological context, the GTPase characteristics and properties of the TTN5 enzyme are not yet demonstrated.

Here, we show that TTN5 is a functional small GTPase with conserved GTP hydrolysis and very fast nucleotide exchange characteristics. TTN5, based on its localization, participates in different processes in the endomembrane system. We characterized TTN5 wild type (WT) and two dysfunctional mutants (T30N and Q70L). Similar mutants have been frequently used as dominant-negative and constitutive-active forms of various GTPases (Dascher and Balch, 1994; Brumm et al., 2020; Fisher et al., 2020; Gimenez et al., 2022). This study lays the foundation for studying the functional relationships of this small GTPase.

Results

TTN5 exhibited atypical characteristics of rapid nucleotide exchange and slow GTP hydrolysis

There is a higher sequence similarity of TTN5 with its animal ARL2 ortholog than to any Arabidopsis ARF/ARL proteins (Figure 1A) (McElver et al., 2000; Vernoud et al., 2003). Several observations indicate that TTN5 plays a fundamental and essential role in cellular activities. Loss of function of TTN5 causes a very early embryo-arrest phenotype (Mayer et al., 1999;

McElver et al., 2000). An essential TTN5 function is also reflected by its regulation and ubiquitous gene expression during plant development and in the root epidermis revealed in public RNA-seq data sets of organ and single cell analysis of roots (Supplementary Figure S1A-B). *TTN5* is strongly expressed during early embryo development where cell division, cell elongation, and cell differentiation take place (Supplementary Figure S1C). Hence, *TTN5* is expressed and presumably functional in very fundamental processes in cells with stronger expression when cells grow and divide.

The molecular switch functions can be presumed for TTN5 (Figure 1B-C). HsARL2 has a fast GDP/GTP exchange characteristic (Hanzal-Bayer et al., 2005; Veltel et al., 2008). However, it had not been known whether the plant TTN5 has similar or different GTPase characteristics as its animal counterparts. We characterized the nucleotide binding and GTP hydrolysis properties of TTN5 and two of its mutants using heterologously expressed and purified proteins and *in vitro* biochemical assays, as previously established for human GTPases ((Eberth and Ahmadian, 2009) workflow in Supplementary Figure S2A-D). The mutants were T30N and Q70L with amino acid exchanges in conserved positions of GTPases. The constitutively active Q70L mutants can presumably not hydrolyze GTP, while the dominant-negative T30N is likely to bind a GEF, preventing the latter from functioning in the proper context. Similar mutants have been frequently used as dominant-negative and constitutive-active forms of various GTPases (Scheffzek et al., 1997; Zhou et al., 2006; Newman et al., 2014). We monitored the real-time kinetics of the interactions of fluorescent guanine nucleotides with TTN5^{WT} and the two potentially dysfunctional variants, TTN5^{T30N} and TTN5^{Q70L} using stopped-flow fluorimetry (Figure 2A-C). Here, 2-deoxy-3-O-N-methylanthraniloyl-deoxy-GDP (mdGDP) and GppNHp (mGppNHp), a non-hydrolyzable GTP analog, were used to mimic GDP and GTP binding to TTN5 proteins. This approach allowed us to measure very fast reaction rates as characteristic of small GTPases, such as HsARL2 and HsARL3 (Hillig et al., 2000; Hanzal-Bayer et al., 2005; Veltel et al., 2008; Zhang et al., 2018). First, we determined the association of mdGDP and mGppNHp in the presence of increasing amounts of nucleotide-free TTN5 proteins (Figure 2B). We found that TTN5 proteins were able to bind both nucleotides, except for mGppNHp binding by TTN5^{T30N} (Supplementary Figures S3A-F, S4A-E). Clearly, TTN5^{Q70L} revealed the highest k_{on} value for mGDP binding (0.401 μM^{-1}s^{-1}), which was nine-fold higher compared to TTN5 (0.044 μM^{-1}s^{-1}) and TTN5^{T30N} (0.048 μM^{-1}s^{-1}), respectively (Figure 2D; Supplementary Figure S3D-F). The k_{on} values for mGppNHp binding were 2-fold lower for TTN5^{WT} (0.029 μM^{-1}s^{-1}) and TTN5^{Q70L} (0.222 μM^{-1}s^{-1}) compared with those for mGDP binding, respectively (Figure 2E; Supplementary Figure S4C, D). The differences of k_{on} for the different nucleotide binding were low. However, it was noted that TTN5^{Q70L} showed a 7.5-fold faster mGppNHp binding than TTN5^{WT}. One remarkable observation was that we did not observe the kinetics of mGppNHp association with TTN5^{T30N} (Figure 2E). Therefore, we monitored the mGppNHp fluorescence in the absence and the presence of nucleotide-free TTN5^{T30N}. As shown in Supplementary Figure S4B, mGppNHp can bind to TTN5^{T30N} so quickly that the association cannot be resolved.

We next measured the dissociation of mdGDP and mGppNHp from the TTN5 proteins in the presence of excess amounts of GDP and GppNHp, respectively (Figure 2C), and found quite interesting differences (Figure 2D, E; Supplementary Figures S3G-I, S4F-H). First, TTN5^{WT} revealed a k_{off} value (0.012 s^{-1} for mGDP) (Figure 2D, Supplementary Figure S3G), which was 100-fold faster as compared to those obtained for classical small GTPases, including RAC1 (Haeusler et al., 2006) and HRAS (Gremer et al., 2011), but quite similar to the k_{off} value of ARF3 (Fasano et al., 2022). Second, the k_{off} values for mGDP and mGppNHp, respectively, were in a similar range between TTN5^{WT} (0.012 s^{-1} and 0.001 s^{-1}) and TTN5^{Q70L} (0.025 s^{-1} and

$0.006\ s^{-1}$), respectively, but differed 10-fold between nucleotides (Figure 2D, E; Supplementary
Figure S3G, I, S4F, H). Hence, mGDP dissociated from proteins 10-fold faster as compared
to mGppNHp. Third, the mGDP dissociation from TTN5^{T30N} (k_{off} = 0.149 s^{-1}) was 12.5-fold faster
than that of TTN5^{WT} and 37-fold faster than the mGppNHp dissociation of TTN5^{T30N} (k_{off} = 0.004
s^{-1}), which are either characteristic of a dominant-negative mutation or a fast-cycling mutation
(Figure 2D-E; Supplementary Figure S3H, S4G).
The dissociation constant (k_d) is calculated from the ratio k_{off}/k_{on}, which inversely indicates the
affinity of the interaction between proteins and nucleotides (the higher k_d, the lower affinity).
Interestingly, TTN5^{WT} binds mGppNHp (k_d = 0.029 µM) 10-fold tighter than mGDP (k_d = 0.267
µM), a difference, which was not observed for TTN5^{Q70L} (k_d for mGppNHp = 0.026 µM, k_d for
mGDP = 0.061 µM) (Figure 2D-E). In contrast, the K_d value for the mGDP interaction with
TTN5^{T30N} was even 11.5- and 50.6-fold lower (3.091 µM) than for TTN5^{WT} and TTN5^{Q70L},
suggesting that this mutant may have a rapid GDP/GTP exchange in cells (Figure 2D).
To obtain the complete GTPase cycle, the ability of TTN5 to hydrolyze GTP had to be
determined. Accordingly, the intrinsic GTP hydrolysis reaction was determined by incubating
100 µM GTP-bound TTN5 proteins at 25°C and injecting the samples at different time points
onto a reversed-phase HPLC column (Figure 2F; Supplementary Figure S5). The determined
GTP hydrolysis rates (k_{cat}) were quite unexpected in two respects (Figure 2G). First, all three
TTN5 protein forms showed quite similar k_{cat} values (0.0015 s^{-1}, 0.0012 s^{-1}, 0.0007 s^{-1};
Supplementary Figure S5). The GTP hydrolysis activity of TTN5^{Q70L} was quite high and
unexpected (0.0007 s^{-1}). We had expected that the glutamine mutations at the corresponding
position as in the case of most other GTPases lead to drastic impairment of the intrinsic
hydrolysis and consequently to a constitutively active GTPase form in cells. Second, the k_{cat}
value of TTN5^{WT} (0.0015 s^{-1}) although quite low as compared to other GTPases (Jian et al.,
2012; Esposito et al., 2019), was 8-fold lower than the previously determined k_{off} value for its
mGDP dissociation (0.012 s^{-1}) (Figure 2E). A fast intrinsic GDP/GTP exchange versus a slow
GTP hydrolysis can have drastic effects on TTN5 activity in cells under resting conditions, as
TTN5 can accumulate in its GTP-bound form, unlike the classical GTPase (Jaiswal et al.,
2013). To investigate this scenario, we pulled down GST-TTN5 protein from bacterial lysates
in the presence of an excess amount of GppNHp in the buffer using glutathione beads and
measured the nucleotide-bound form of GST-TTN5 using HPLC. As shown in Figure 2H,
isolated GST-TTN5 increasingly bonds GppNHp, indicating that the bound nucleotide is rapidly
exchanged for free nucleotide (in this case GppNHp). This is not the case for conventional
GTPases, which remain in their inactive GDP-bound forms under the same experimental
conditions.
In summary, the TTN5 sequence not only contains conserved regions necessary for
nucleotide binding but also TTN5 protein detectably binds nucleotides. Interestingly, the slow
intrinsic GTP hydrolysis rates in combination with the high dissociation rates for GDP indicate
that TTN5 tendency is to be present in a GTP-loaded form. A fast intrinsic GDP/GTP exchange
and a slow GTP hydrolysis can have drastic effects on TTN5 activity in cells under
resting/unstimulated conditions, as TTN5 can accumulate in its GTP-bound form, unlike the
classical GTPases (Jaiswal et al., 2013). On the other hand, the suspected constitutively active
TTN5^{Q70L} still has intrinsic GTPase activity, while the T30N variant had a low affinity for mGDP.
Hence, we proved that TTN5 exhibits the typical functions of a small GTPase based on *in vitro*
biochemical activity studies, including guanine nucleotide association and dissociation, but
emphasized its divergences among the ARF GTPases by its kinetics.
227
YFP-TTN5 is highly dynamic and localizes to different intracellular compartments

Several ARF GTPases function in vesicle transport and are located at various membranous
sites linked with the endomembrane compartments in eukaryotes (Vernoud et al., 2003).
Localization had not been comprehensively studied for TTN5. To obtain hints where in a cell
TTN5 may be localized, we first created transgenic Arabidopsis lines constitutively expressing
YFP-tagged TTN5 (pro35S::YFP-TTN5) and its two mutant forms (negative pro35S::YFP-
TTN5^{T30N}, active pro35S::YFP-TTN5^{Q70L}) and investigated the localization in 6-day-old
seedlings in the epidermis of cotyledons, hypocotyls, root hair zone and in root tips (Figure 3A;
Supplementary Figure S6A). The microscopic observations were made in different planes of
the tissues, e.g. inside the cells across the vacuoles (Supplementary Figure 6) and underneath
the plasma membrane at the cell peripheries (Figure 3). We chose the investigation of YFP-
TTN5 in the epidermis as this is a tissue where *TTN5* was found to be expressed in plants
(Supplementary Figure S1B). YFP-TTN5 signals were detected in the nucleus, in the
cytoplasm, and at or in close proximity to the plasma membrane in the epidermal cotyledon
cells (Supplementary Figure S6B). The same localization patterns were found for mutant YFP-
TTN5 forms (Supplementary Figure S6C-D). The YFP-TTN5, YFP-TTN5^{T30N,} and YFP-
TTN5^{Q70L} signals were also present in a similar pattern in the stomata (Figure 3B-D). In
hypocotyls, intracellular localization of YFP-TTN5 and mutant forms was well observed in
nuclei and in close proximity to or at the plasma membrane (Supplementary Figure S6E-G).
Investigation of the root hair zone showed YFP signals in the cytoplasm and at the plasma
membrane of root hairs (Supplementary Figure S6H-J). In the root tip, YFP-TTN5 localization
was detectable inside the cytoplasm and in nuclei (Supplementary Figure S6K). YFP-TTN5^{T30N}
and YFP-TTN5^{Q70L} revealed the same localization (Supplementary Figure S6L-M). The
localization of YFP-TTN5, YFP-TTN5^{T30N,} and YFP-TTN5^{Q70L} inside the cytoplasm was
punctate indicating that the signals were present in cytosolic vesicle-like structures together
with free signals in the cytosol. This localization pattern was also present in leaf epidermal cells
and stomata of the cotyledons (Figure 3B-D), in the hypocotyls (Figure 3E-G), and in the cells
of the root hair zones and in the root hairs itself (Figure 3H-J). These observed structures point
to an association of TTN5 with vesicle and endomembrane trafficking. A closer inspection of
these structures over time in cotyledons presented mobility of YFP-TTN5 and mutant forms
within the cells (Supplementary Video Material S1A-C). Similar was the case for YFP-TTN5 in
hypocotyl cells (Supplementary Video Material S1D). Interestingly, the mobility of these
punctate structures differed within the cells when the GTPase-negative mutant YFP-TTN5^{T30N}
was observed (Supplementary Video Material S1E). We observed approximately half of the
cells within the hypocotyl with slowed-down or completely arrested movement, which was not
the case for YFP-TTN5 and YFP-TTN5^{Q70L} (Supplementary Video Material S1F). This loss of
mobility of YFP-TTN5^{T30N} may be a consequence of missing effector interaction. We did not
observe the blocked mobility for YFP-TTN5, YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} in cells of the
root elongation zone (Supplementary Video Material S1G-I). No mobility of any YFP-TTN5
form was visible in root tip cells (Supplementary Video Material S1J-L).
To confirm the Arabidopsis data and to better visualize YFP-TTN5, we expressed YFP-
TTN5 constructs transiently in *Nicotiana benthamiana* leaf epidermis cells. We found that YFP-
TTN5, YFP-TTN5^{T30N,} and YFP-TTN5^{Q70L} were also all localized at or in close proximity to the
plasma membrane and in several cytosolic punctate structures, apart from the nucleus, similar
to Arabidopsis cotyledons, hypocotyls and root hair zones (Figure 3K-N). This showed that
YFP-TTN5 localization was similar between Arabidopsis epidermis cells and *N. benthamiana*
leaf epidermis. It should be noted that the 35S promoter-driven YFP-TTN5 constructs did not
complement the embryo-lethal phenotype of *ttn5-1*. On the other side, a triple hemagglutinin-
tagged HA$_3$-TTN5 driven by the 35S promoter did (Supplementary Figure S7A-C). We

expected the lack of complementation to be an effect of the large size of the YFP tag compared to the relatively small HA$_3$-tag. To verify that the localization of the YFP-TTN5 constructs is representative of a functional TTN5, we performed immunofluorescence staining against the HA$_3$-tag in HA$_3$-TTN5 seedlings and compared the localization pattern (Supplementary Figure S7D). The Alexa 488 staining reflecting HA$_3$-TTN5 was clearly visible in root cells and root hairs as expected. HA$_3$-TTN5 was mostly present in punctate structures close to the plasma membrane and in the cytosol, fitting with the YFP-TTN5 localization (Supplementary Figure S7D). Hence, we presume the correct localization of the YFP-TTN5 constructs in the various membrane-associated places in the cytosol including the plasma membrane.

Taken together, YFP-TTN5 and its mutant forms were located in multiple membrane compartments in the epidermis of different Arabidopsis organs and of *N. benthamiana* leaves. YFP-TTN5 and YFP-TTN5^{Q70L} displayed high mobility in the cells, while YFP-TTN5^{T30N} was less mobile. Altogether, these data suggest that TTN5 may have multiple functions as an active GTPase and may be associated with different intracellular structures of the endomembrane system.

YFP-TTN5 associates with components of the cellular endomembrane system

The localization of YFP-TTN5 prompted us to better resolve the membrane structures and to identify the nature of YFP-TTN5-positive cellular compartments. The endomembrane system is highly dynamic in the cell. Well-established fluorescent endomembrane markers and pharmacological treatments help to determine the nature of individual components of the system in parallel to colocalization studies with proteins of interest such as TTN5. To clarify the nature of the compartments, we conducted the colocalization experiments in *N. benthamiana* leaf epidermis where the localization pattern was similar to that in the Arabidopsis cotyledons and root epidermis, as it represents an established system for functional association of the multiple endomembrane components, allowing optimal identification of membrane structures (Brandizzi et al., 2002; Hanton et al., 2009).

At first, we investigated the endoplasmic reticulum (ER)-Golgi connection. This site is characteristic of association with small GTPases like ARF1, involved in COPI vesicle transport from Golgi to the ER (Just and Peränen, 2016). The soybean (*Glycine max*) protein α-1,2 mannosidase 1 (GmMan1) is a glycosidase that acts on glycoproteins at the *cis*-Golgi, facing the ER (Figure 4A). GmMan1-mCherry-positive Golgi stacks are visible as nearly round punctuate structures throughout the whole cell (Nelson et al., 2007; Wang et al., 2016). YFP-TTN5 and mutant variants partially colocalized with GmMan1-mCherry signals at the Golgi stacks (Figure 4B-D). Further, quantitative analysis confirmed the visible colocalization with the marker with Pearson coefficients 0.63 (YFP-TTN5), 0.65 (YFP-TTN5^{T30N}), and 0.68 (YFP-TTN5^{Q70L}) (Supplementary Figure S8A; see also similar results obtained with overlap coefficients), indicating a strong correlation between the two signals. Using object-based analysis, we detected 24 % overlapping YFP-TTN5 fluorescence signals with Golgi stacks, while YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} signals only shared 16 and 15 % overlap with GmMan1-mCherry-positive Golgi stacks (Supplementary Figure S8B). Some YFP-TTN5 signals did not colocalize with the GmMan1 marker. This effect appeared more prominent for YFP-TTN5^{T30N} and less for YFP-TTN5^{Q70L}, compared to YFP-TTN5 (Figure 4B-D). Indeed, we identified 48 % GmMan1-mCherry signal overlapping with YFP-TTN5^{Q70L}-positive structures, whereas 43 and only 31 % were present with YFP-TTN5 and YFP-TTN5^{T30N} respectively (Supplementary Figure S8B), indicating a smaller amount of GmMan1-positive Golgi stacks colocalizing with YFP-TTN5^{T30N}. Hence, the GTPase-active TTN5 forms are likely more present at *cis*-Golgi stacks compared to dominant negative TTN5^{T30N}.

Next, we confirmed the Golgi localization by brefeldin A (BFA) treatment, a commonly used
tool in cell biology for preventing dynamic membrane trafficking events and vesicle transport
involving the Golgi. BFA is a fungal macrocyclic lactone that leads to a loss of *cis*-cisternae
and accumulation of Golgi stacks, known as BFA-induced compartments, up to the fusion of
the Golgi with the ER (Figure 4E). The action of BFA causes a corresponding redistribution of
GmMan1-mCherry (Ritzenthaler et al., 2002; Wang et al., 2016). We found that upon BFA
treatment, GmMan1-mCherry was present in the ER and in BFA-induced compartments. YFP-
TTN5 proteins showed partially matching localization with GmMan1-mCherry upon BFA
treatment proving the connection of TTN5 to Golgi localization (Figure 4F-H). Hence, the
colocalization with GmMan1-mCherry and BFA treatment confirmed the localization of TTN5
with the Golgi stacks and the lower association of both mutant forms with this membrane
compartment.

Second, we investigated localization to the endocytic compartments, endosomes of the
trans-Golgi network (TGN), and multivesicular bodies (MVBs) using the marker RFP-ARA7
(RABF2B), a small RAB-GTPase present there (Kotzer et al., 2004; Lee et al., 2004; Stierhof
and El Kasmi, 2010; Ito et al., 2016) (Figure 4I). These compartments play a role in sorting
proteins between the endocytic and secretory pathways, with MVBs developing from the TGN
and representing the final stage in transport to the vacuole (Valencia et al., 2016; Heucken
and Ivanov, 2018). Colocalization studies revealed that YFP-TTN5 protein was present at
RFP-ARA7-positive MVBs (Figure 4J). Noticeably, overlaps between RFP-ARA7 and YFP-
TTN5^{T30N} fluorescence signals were lower than for the other forms (Figure 3K-L;
Supplementary Figure S8C-D). We obtained a Pearson coefficient for YFP-TTN5 and YFP-
TTN5^{Q70L} together with RFP-ARA7 of 0.78, whereas a coefficient of only 0.59 was obtained
with YFP-TTN5^{T30N} confirming the visual observation (Supplementary Figure S8C; see also
similar results for overlap coefficients). Object-based analysis showed that, RFP-ARA7-
positive structures had an overlap with YFP-TTN5 (29 %) and even more with YFP-TTN5^{Q70L}
(75 %) unlike with YFP-TTN5^{T30N} (21 %) (Supplementary Figure S8D). Based on this, YFP-
TTN5^{Q70L} and YFP-TTN5 tended to colocalize better with ARA7-positive compartments than
YFP-TTN5^{T30N}.

To prove MVB localization, we treated plant cells with wortmannin, a common approach to
studying endocytosis events. Wortmannin is a fungal metabolite that inhibits
phosphatidylinositol-3-kinase (PI3K) function and thereby causes swelling of the MVBs (Cui et
al., 2016) (Figure 4M). RFP-ARA7-expressing cells showed the expected typical wortmannin-
induced formation of doughnut-like shaped MVBs (Jaillais et al., 2008). The coexpressed YFP-
TTN5 constructs partially colocalized with these structures (Figure 4N-P) proving YFP-TTN5
and the two mutants are present in MVBs. YFP-TTN5^{Q70L} was located even to a greater extent
to MVBs than wild-type YFP-TTN5 and much more than the YFP-TTN5^{T30N} mutant, suggesting
an active role of YFP-TTN5^{Q70L} in MVBs like the lytic degradation pathway or the recycling of
proteins, similar to ARA7 (Kotzer et al., 2004).

Finally, to investigate the connection of TTN5 with the plasma membrane, we colocalized
the YFP-TTN5 proteins with the dye FM4-64, which can emit fluorescence in a lipophilic
membrane environment and marks the plasma membrane in the first minutes following
application to the cell (Bolte et al., 2004) (Figure 5A). All three forms of TTN5 colocalized with
FM4-64 at the plasma membrane in a similar manner (Figure 5B-D). To further prove plasma
membrane localization, we performed mannitol-induced plasmolysis. All three YFP-TTN5
protein forms were located similarly to FM4-64-stained Hechtian strands, which are thread-like
structures attached to the apoplast visible upon plasmolysis that is lined by plasma membrane
(Figure 5E-G).

In summary, these colocalization experiments showed that YFP-TTN5 locates in different
membrane sites of the endomembrane system, including Golgi, MVBs, and plasma
membrane. We figured that similar to other ARF proteins, TTN5 might participate in a highly
dynamic vesicle trafficking process. Indeed, when we recorded the dynamic movement of YFP
signals inside *N. benthamiana* leaf epidermis cells, YFP-TTN5 and YFP-TTN5^{Q70L} colocalized
with GmMan1-mCherry and revealed high motion over time, while this was less the case for
YFP-TTN5^{T30N} (Supplementary Video Material S2A-C).
One potential cellular trafficking route is the degradation pathway to the vacuole. We,
therefore, investigated YFP-TTN5 localization in late endosomal compartments that might be
involved in vacuolar targeting. FM4-64 is used as a marker for vacuolar degradation targeting,
since following plasma membrane visualization FM4-64-stained endocytic vesicles become
apparent at later stages as well as vacuolar membrane staining (Ueda et al., 2001; Emans et
al., 2002; Dhonukshe et al., 2007; Ivanov and Vert, 2021). Hence, we colocalized YFP-TTN5
with FM4-64-positive compartments at later time points. Next to YFP-TTN5 colocalization with
FM4-64 at the plasma membrane, we detected colocalization with fluorescent compartments
in the cell, which was similar for the two mutant forms (Figure 5H-J). This indicates that YFP-
TTN5 may be involved in the vacuolar targeting of plasma membrane material, irrespective of
the mutations.
In summary, YFP-TTN5^{T30N} tended to be less mobile and dynamic and colocalized less with
endomembrane structures compared to YFP-TTN5 and YFP-TTN5^{Q70L}.

Discussion
This work showed that the small ARF-like GTPase TTN5 has a very rapid intrinsic nucleotide
exchange capacity with a conserved nucleotide switching mechanism. Enzyme kinetics
indicate that TTN5 might be primarily present in a GTP-loaded active form in a cell. YFP-TTN5
was dynamically associated with vesicle transport and different processes of the
endomembrane system. The active TTN5^{Q70L} mutant was capable of nucleotide switching, and
YFP-TTN5^{Q70L} had localization properties comparable with YFP-TTN5. The dominant negative
YFP-TTN5^{T30N} mutant, on the other hand, had a lower nucleotide exchange capacity than the
other TTN5 forms and it differed significantly in localization properties depending on cell types.
Therefore, this mutation affected the cellular localization and dynamics of TTN5.
403
TTN5 exhibits characteristic GTPase functions
TTN5 was classified as an ARL2 homolog of the ARF GTPases based on its sequence
similarity. The sequence analysis suggested nucleotide binding (McElver et al., 2000) which is
reinforced by structural prediction suggesting the formation of a nucleotide-binding pocket by
the binding motifs. Nucleotide association and dissociation of TTN5, TTN5^{T30N,} and TTN5^{Q70L}
proved that TTN5 along with the two mutant forms can bind guanine nucleotides. The k_{on} value
for TTN5^{Q70L}, which is described as a GTP hydrolysis-defective mutant (Dascher and Balch,
1994), was clearly higher than that of the wild-type form, indicating that this mutant can bind
GDP faster than TTN5 to form the nucleotide-bound form. Compared with other members of
the Ras superfamily, it was in the range of HRAS (Hanzal-Bayer et al., 2005) and around ten
times slower than the fast association of RAC1 (Jaiswal et al., 2013). The k_{on} values of
TTN5^{T30N} and TTN5 were nearly the same, indicating no effect of the mutation on the GDP-
binding characteristics as was expected in the absence of a GEF. Intrinsic nucleotide exchange
measurements of TTN5 and TTN5^{Q70L} have shown remarkably fast nucleotide exchange rates,
when compared to other well-studied RAS proteins. The intrinsic nucleotide exchange reaction
rates for RAC1, RAC2, and RAC3 have been mentioned around 40.000 s (Haeusler et al.,
2006). Our data show that TTN5 is faster in nucleotide exchange rate and very similar to that

of human homolog ARL2 (Hanzal-Bayer et al., 2005; Veltel et al., 2008). This explains that
TTN5 tends to quickly replace GDP for GTP and transforms from an inactive to an active state.
This behavior indicates that TTN5 does not require interaction with GEFs for activation. This
explanation seems also the case for TTN5^{Q70L}. Small GTPases with substitutions of the
glutamine of the switch II region (*e.g.*, Glu-71 for HsARF1 and ARL1, Glu-61 for HRAS) are
constitutively active (Zhang et al., 1994; Van Valkenburgh et al., 2001; Karnoub and Weinberg,
2008). Therefore, TTN5^{Q70L} is likely to exchange GDP rapidly to GTP and switch itself to stay
in an active form as suggested by the fast intrinsic nucleotide exchange rate. Interestingly,
dominant negative TTN5^{T30N} resulted in an even higher dissociation rate constant k_{off}. The
calculated K_d confirmed the higher nucleotide-binding affinity for GDP of TTN5 and TTN5^{Q70L}
compared with TTN5^{T30N}. Reports on human ARL2, ARF6, and ARL4D showed that their
corresponding T30N mutants led to a decreased affinity to GDP similar to TTN5^{T30N} (Macia et
al., 2004; Hanzal-Bayer et al., 2005; Li et al., 2012).
Interestingly, a comparison of mdGDP with mGppNHp revealed a higher GTP affinity for all
three versions, with the highest for TTN5^{Q70L}. These high GTP affinities in combination with the
fast GDP exchange rates and extremely slow hydrolysis pinpointed to a GTP-loaded TTN5
even in the rested state, which is very uncommon for small GTPases. This atypical behavior
is already reported for a few non-classical RHO GTPases like RHOD or RIF (Jaiswal et al.,
2013). This unusual GTP-bound active state along with the lacking N-myristoylation and
phylogenetic distances (Boisson et al., 2003; Vernoud et al., 2003) strengthens that there are
major differences between TTN5 and other ARF family members. The similarity between the
wild type and TTN5^{Q70L} is consistent with the previous report on human ARL2 in which wild-
type and Q70L proteins showed only a little difference in binding affinity (Hanzal-Bayer et al.,
2005). Additionally, an equivalent ratio of nucleotide affinity was found between HRAS and
HRAS^{Q61L}, but with a much higher affinity typical for small GTPases (Der et al., 1986). Since
Gln-70 at the switch II region is important for GAP-stimulated GTP hydrolysis (Cherfils and
Zeghouf, 2013), we assume that nucleotide exchange activity is unaffected by this amino acid
substitution.
To date, no GEF protein for TTN5 is reported. The Arabidopsis genome encodes only two
of the five mammalian GEF subgroups, namely the large ARF-GEF subgroups, the BFA-
inhibited GEF (BIG), and the Golgi Brefeldin A (BFA)-resistance factor 1 (GBF/GNOM) family
(Memon, 2004; Wright et al., 2014; Brandizzi, 2018). Potential interactions with these proteins
are of high interest and can also point to functions of TTN5 as a co-GEF as it is proposed for
HsARL3 and HsARL2 with their effector BART by stabilizing the active GTPase (ElMaghloob
et al., 2021). Especially, interactions at the nucleotide-binding site, which are prevented in the
TTN5^{T30N} mutant, will be of great interest to study further functions and interaction partners of
TTN5.
Taken together, the categorization as a non-classical GTPase has three implications:
First, the very slow hydrolysis rate predicts the existence of a TTN5 GAP. Second, TTN5^{T30N}
is a dominant negative mutant and in the presence of a GEF, it cannot bind GDP. Third, the
TTN5^{Q70L} hydrolysis rate is not decreased.

YFP-TTN5 is part of the endomembrane system

The ARF family of small GTPases is well known for its involvement in vesicle trafficking and
coat assembly with diverse localization patterns within the cell. We observed YFP-TTN5 to be
localized at different cellular compartments which is typical for several members of this family
and emphasizes its involvement in endomembrane trafficking (Memon, 2004; Sztul et al.,
2019). A detailed colocalization analysis showed that both *cis*-Golgi and MVB-positive
structures colocalized to a higher proportion with the YFP-TTN5^{Q70L} compared with YFP-

TTN5^{T30N}. This could be an indicator of the site of TTN5 action, considering our knowledge of
the activation of ARF GTPases and TTN5 homologs in other organisms. They are usually
recruited or move to their place of action upon interacting with their specific GEF, which leads
to GDP to GTP exchange-dependent activation (Sztul et al., 2019; Nielsen, 2020; Adarska et
al., 2021). Though our biochemical data implies no need for a typical GTPase-GEF interaction
for activation, GEF interaction can be still important for the localization. Most of the effector-
GTPase interactions take place in their GTP-bound form (Sharer and Kahn, 1999; Hanzal-
Bayer et al., 2005). One exception is the role of TTN5 homologs in microtubule dynamics.
ARL2/Alp41-GDP interacts with Cofactor D/Alp1^D (Bhamidipati et al., 2000; Mori and Toda,
2013). Another possibility is a hindrance of dimerization by the T30N mutation. ARF1 protein
dimer formation is important for the formation of free vesicles (Beck et al., 2009; Beck et al.,
2011) associated with cell mobility which was disturbed in YFP-TTN5^{T30N}-expressing cells. The
colocalization of YFP-TTN5 with ARA7-positive structures even still in the wortmannin-induced
swollen state, triggered by the homotypic fusion of MVBs (Wang et al., 2009), may indicate
that TTN5 performs similar functions in relation to ARA7. ARA7 is involved in cargo transport
in the endocytic pathway to the vacuole, with a role, for example, in the endocytosis of plasma
membrane material (Ueda et al., 2001; Sohn et al., 2003; Kotzer et al., 2004; Ebine et al.,
2011). Colocalization of YFP-TTN5 with FM4-64-labeled endocytosed vesicles leads to the
assumption of a TTN5 involvement in endocytosis and the degradation pathway to the vacuole.
Our colocalization data with the different markers reinforces the suggestion that TTN5 has
functions in vesicle trafficking.
A potential explanation of the localization to similar compartments of YFP-TTN5 and YFP-
TTN5^{Q70L} compared to inactive YFP-TTN5^{T30N} can be based on a special feature of TTN5 in
the ARF family. ARF GTPases are mostly myristoylated on Gly-2, which is essential for their
membrane binding. TTN5 as well as ARL2 and ARL3 lack this myristoylation though Gly-2 is
present (Boisson et al., 2003; Kahn et al., 2006). ARL2 and ARL3 are still able to bind
membranes, probably only by their N-terminal amphipathic helix as it was established for
SAR1, with an ARL2 membrane-binding efficiency being nucleotide-independent (Lee et al.,
2005; Kapoor et al., 2015). We suggest similar binding efficiency for TTN5 as all YFP-tagged
TTN5 forms localized to membranous compartments. But based on the varying colocalization
efficiency, with the YFP-TTN5^{T30N} signal being less prominent at the Golgi and MVBs,
compared to YFP-TTN5 and YFP-TTN5^{Q70L}, we hypothesize a nucleotide- or nucleotide
exchange-dependent specificity for different membranes with TTN5 in the resting state being
predominantly present close to the plasma membrane and being active in the endomembrane
system. Interestingly, with respect to the intracellular dynamics, we observed that the TTN5^{T30N}
mutant had a different behavior in different organ types. This might be due to differing GEFs
being differentially expressed. Likewise, it is conceivable that the constitutively expressed
TTN5 has different effector binding partners.
This broad diversity of biological functions of TTN5 homologs associated with different
signaling cascades is also reflected by very different protein partners for that. Few homologs
of human ARL2 interaction partners are present in Arabidopsis. It is therefore exceedingly
interesting to identify interacting proteins to determine whether TTN5 performs similar
functions as HsARL2 or what other role it may play. Such interactions might also explain why
TTN5 is essential in plants with regard to a potential GTP-dependence for TTN5 function which
fits to already known functions of other ARF GTPases (Sztul et al., 2019; Nielsen, 2020;
Adarska et al., 2021). In addition, ARF proteins are affected by a similar set of GEFs and
GAPs, indicating an interconnected network in ARF signaling. ARF double knockdowns
revealed specific phenotypes, suggesting redundancy in the ARF family (Volpicelli-Daley et

al., 2005; Kondo et al., 2012; Nakai et al., 2013; Adarska et al., 2021). The investigation of the TTN5 connection in the ARF family might reveal a missing link in ARF signaling and cell traffic.

In our study, the pro35S::YFP-TTN5 and mutant forms did not complement the *ttn5-1* mutant phenotype although there is constitutive expression of *TTN5*. There could be differences in TTN5 levels in some cell types. Overexpression of ARF1 did not affect intracellular localization compared to endogenous tagged-ARF1 but differed in function to form tubulated structures (Bottanelli et al., 2017). Though constitutively driven, the YFP-TTN5 expression may be delayed or insufficient at the early embryonic stages resulting in the lack of embryo-lethal complementation. On the other hand, the very fast nucleotide exchange activity may be hindered by the presence of a large YFP-tag in comparison with the small HA$_3$-tag which is able to rescue the embryo-lethality. This represents a potential limitation for the localization of small GTPases with rapid nucleotide exchange in plants.

Conclusion

In this study, we identified TTN5 as a functional GTPase of the ARF-like family. TTN5 had not only sequence similarity with human ARL2 but also the two proteins share a very rapid nucleotide exchange capacity in contrast to other characterized ARF/ARL proteins. TTN5 has a faster nucleotide dissociation rate to a slower GTP hydrolysis rate and a higher affinity to GTP compared to GDP. Thus, TTN5 is a non-classical GTPase that most likely accumulate in a GTP-bound state in cells. The nucleotide exchange capacity affected the localization and dynamics of YFP-tagged TTN5 protein forms and associated TTN5 with the endomembrane system. In the future, the identification of potential TTN5 GEF and GAP proteins as well as other interaction partners and effector proteins will be of great interest to clarify the role of TTN5 in endomembrane trafficking and cell-physiological responses.

Material & Methods

Arabidopsis plant material and growth conditions

The Arabidopsis *ttn5-1* mutant was previously described (McElver et al., 2000). Heterozygous seedlings were selected by genotyping using the primers TTN5 intron1 fwd and pDAP101 LB1 (Supplementary Table S1). For pro35S::YFP-TTN5 and pro35S::HA$_3$-TTN5 constructs, *TTN5*, *TTN5^{T30N,}* and *TTN5^{Q70L}* coding sequences were amplified with B1 and B2 attachment sites for Gateway cloning (Life Technologies) using the primer TITAN5 n-ter B1 and TITAN5 stop B2 (Supplementary Table S1). The obtained PCR fragments were cloned *via* BP reaction (Life Technologies) into pDONR207 (Invitrogen). pro35S::YFP-TTN5 and pro35S::HA$_3$-TTN5 constructs were created *via* LR reaction (Life Technologies) with the destination vector pH7WGY2 (Karimi et al., 2005) and pALLIGATOR2 (Bensmihen et al., 2004), respectively. Agrobacteria were transformed with obtained constructs and used for stable Arabidopsis transformation (adapted by (Clough and Bent, 1998). Arabidopsis seeds were sterilized with sodium hypochlorite solution (6 % Sodium hypochlorite and 0.1 % Triton X-100) and stored for 24 hours at 4°C for stratification. Seedlings were grown upright on half-strength Hoagland agar medium (1.5 mM Ca(NO$_3$)$_2$, 0.5 mM KH$_2$PO$_4$, 1.25 mM KNO$_3$, 0.75 mM MgSO$_4$, 1.5 µM CuSO$_4$, 50 µM H$_3$BO$_3$, 50 µM KCl, 10 µM MnSO$_4$, 0.075 µM (NH$_4$)$_6$Mo$_7$O$_{24}$, 2 µM ZnSO$_4$, 50 µM FeNaEDTA and 1 % sucrose, pH 5.8, supplemented with 1.4 % Plant agar (Duchefa)] in growth chambers (CLF Plant Climatics) under long-day condition (16 hours light at 21°C, 8 hours darkness at 19°C). Seedlings were grown for six days (six-day system) or 17 days with the last three days on fresh plates (two-week system).

Nicotiana benthamiana plants were grown on soil for 2-4 weeks in a greenhouse facility under long-day conditions (16 hours of light, 8 hours of darkness).

Point mutant generation of *TTN5*

pDONR207:TTN5 was used as a template for site-directed *TTN5* mutagenesis. Primers T5T30Nf and T5T30Nr (Supplementary Table S1) were used to amplify the entire vector generating the TTN5^{T30N} coding sequence and primers TQ70Lf and T5Q70Lr (Supplementary Table S1) were used to amplify the entire vector generating the TTN5^{Q70L} coding sequence. The PCR amplifications were run using the following conditions: 95°C, 30 s; 18 cycles of 95°C, 30 s/ 55°C, 1 min/ 72°C 8 min; 72°C, 7 min. The completed reaction was treated with 10 units of DpnI endonuclease for 1 h at 37°C and then used for *E. coli* transformation. Successful mutagenesis was confirmed by Sanger sequencing.

In vitro GTPase activity assays

An overview of protein expression and purification is shown in Supplementary Figure S2A. Recombinant pGEX-4T-1 bacterial protein expression vectors (Amersham, Germany) containing coding sequences for *TTN5*, *TTN5^{T30N}* and *TTN5^{Q70L}* were transferred into *E. coli* BL21 (DE3) Rosetta strain (Invitrogen, Germany). Following induction of GST-TTN5 fusion protein expression according to standard procedures. Cell lysates were obtained after cell disruption with a probe sonicator (Bandelin sonoplus ultrasonic homogenizer, Germany) using a standard buffer (300 mM NaCl, 3 mM Dithiothreitol (DTT), 10 mM MgCl$_2$, 0.1 mM GDP, 1 % Glycerol, and 50 mM Tris-HCl, pH 7.4). GST-fusion proteins were purified by loading total bacterial lysate on a preequilibrated glutathione Sepharose column (Sigma, Germany) using fast performance liquid chromatography system (Cytiva, Germany) (Step 1, affinity-purified GST-TTN5 protein fraction). GST-tagged protein fractions were incubated with thrombin (Sigma, Germany) at 4°C overnight for cleavage of the GST tag (Step 2, GST cleavage) and applied again to the affinity column (Step 3, yielding TTN5 protein fraction). Purified proteins were concentrated using 10 kDa ultra-centrifugal filter Amicon (Merck Millipore, Germany). The quality and quantity of proteins were analyzed by SDS-protein gel electrophoresis (Bio-Rad), UV/Vis spectrometer (Eppendorf, Germany), and high-performance liquid chromatography (HPLC) using a reversed-phase C18 column (Sigma, Germany) and a pre-column (Nucleosil 100 C18, Bischoff Chromatography) as described (Eberth and Ahmadian, 2009) (Supplementary Figure S2B-D).

Nucleotide-free TTN5 protein was prepared from the TTN5 protein fraction (Eberth and Ahmadian, 2009) as illustrated in Supplementary Figure S2E. 0.5 mg TTN5 protein was combined with 1 U of agarose bead-coupled alkaline phosphatase (Sigma Aldrich, Germany) for degradation of bound GDP to GMP and Pi in the presence of 1.5-fold molar excess of non-hydrolyzable GTP analog GppCp (Jena Bioscience, Germany). After confirmation of GDP degradation by HPLC, 0.002 U snake venom phosphodiesterase (Sigma Aldrich, Germany) per mg TTN5 was added to cleave GppCp to GMP, G, and Pi. The reaction progress of degradation of nucleotides was analyzed by HPLC using 30 µM TTN5 in 30 µl injection volume (Beckman Gold HPLC, Beckman Coulter). After completion of the reaction, in order to remove the agarose bead–coupled alkaline phosphatase, the solution was centrifuged for 10 min at 10000 *g*, 4°C, which was followed by snap freezing and thawing cycles to inactivate the phosphodiesterase. mdGDP (2-deoxy-3-O-N-methylanthraniloyl GDP)- and mGppNHp 2'/3'-O-(N-Methyl-anthraniloyl)-guanosine-5'-[(β,γ)-imido]triphosphate)-bound TTN5, TTN5^{T30N} and TTN5^{Q70L} were prepared by incubation of nucleotide-free forms with fluorescent nucleotides (Jena Bioscience, Germany) in a molar ratio of 1 to 1.2. The solution was purified from excess amount of mdGDP and mGppNHp by using prepacked gel-filtration NAP-5 Columns (Cytiva,

Germany) to remove unbound nucleotides. Protein and nucleotide concentration were determined using the Bradford reagent (Sigma Aldrich, Germany) and HPLC, respectively.

All kinetic fluorescence measurements including nucleotide association and dissociation reactions were monitored on a stopped-flow instrument system SF-61, HiTech Scientific (TgK Scientific Limited, UK) and SX20 MV (Applied Photophysics, UK) at 25°C using nucleotide exchange buffer (10 mM K_2HPO_4/KH_2PO_4, pH 7.4, 5 mM $MgCl_2$, 3 mM DTT, 30 mM Tris/HCl, pH 7.5) (Eberth and Ahmadian, 2009). Fluorescence was detected at 366 nm excitation and 450 nm emission using 408 nm cut-off filter for mant-nucleotides (Hemsath and Ahmadian, 2005).

To determine the intrinsic nucleotide exchange rate, k_{off}, 0.2 µM mdGDP- and mGppNHp-bound proteins were combined with a 200-fold molar excess of 40 µM non-fluorescent GDP in two different set of experiments, respectively. The decay of the fluorescence intensity representing mdGDP and mGppNHp dissociation and replacement by non-fluorescent nucleotide were recorded over time (Supplementary Figure S2G). Moreover, to determine the nucleotide association rate, k_{on}, of mdGDP and mGppNHp to the nucleotide-free GTPase, 0.2 µM fluorescent nucleotides were mixed with different concentrations of nucleotide-free TTN5 variants. The increase in the fluorescent intensity was obtained by the conformational change of fluorescent nucleotides after binding to the proteins (Supplementary Figure S2H).

The data provided by stopped-flow were applied to obtain the observed rate constants. Dissociation rate constants or nucleotide exchange rates (k_{off} in s^{-1}) and pseudo-first-order rate constants or observed rate constants (k_{obs} in s^{-1}) at the different concentrations of the protein were obtained by non-linear curve fitting using Origin software (version 2021b). The slopes obtained from plotting k_{obs} against respective concentrations of proteins were used as the second-order association rate constants (k_{on} in $µM^{-1}s^{-1}$). The equilibrium constant of dissociation (K_d in µM) was calculated from the ratio of k_{off}/k_{on}. In order to investigate the intrinsic GTP-hydrolysis rate of TTN5 variants, the HPLC method is used as described (Eberth and Ahmadian, 2009). As an accurate strategy, HPLC provides the nucleotide contents over time. The GTPase reaction rates were determined by mixing 100 µM nucleotide-free GTPase and 100 µM GTP at 25°C in a standard buffer without GDP. The GTP contents were measured at different times and the data were fitted with Origin software to get the observed rate constant.

Nicotiana benthamiana leaf infiltration

N. benthamiana leaf infiltration was performed with the Agrobacterium (*Agrobacterium radiobacter*) strain C58 (GV3101) carrying the respective constructs for confocal microscopy. Agrobacteria cultures were grown overnight at 28°C, centrifuged for 5 min at 4°C at 5000*g*, resuspended in infiltration solution (5 % sucrose, a pinch of glucose, 0.01 % Silwet Gold, 150 µM Acetosyringone), and incubated for 1 hour at room temperature. Bacterial suspension was set to an OD_{600}=0.4 and infiltrated into the abaxial side of *N. benthamiana* leaves.

Subcellular localization of fluorescent protein fusions

Cloning of YFP-tagged TTN5 constructs is described in the paragraph 'Arabidopsis plant material and growth conditions'. Localization studies were carried out by laser-scanning confocal microscopy (LSM 780, Zeiss) with a 40x C-Apochromat water immersion objective. YFP constructs and Alexa Fluor 488 stainings were excited at 488 nm and detected at 491-560 nm. mCherry or FM4-64 fluorescence was excited at 561 nm and detected at 570-633 nm.

658 Wortmannin (10 µM, Sigma-Aldrich), BFA (36 µM, Sigma-Aldrich) and plasma membrane
659 dye FM4-64 (165 µM, ThermoFisher Scientific) were infiltrated into *N. benthamiana* leaves.
660 FM4-64 was detected after five min incubation. Wortmannin and BFA were incubated for
661 25 min before checking the treatment effect. Plasmolysis was induced by incubating leaf discs
662 in 1 M mannitol solution for 15 min. Signal intensities were increased for better visibility.
663

Whole-mount Immunostaining
Whole-mount immunostaining by immunofluorescence was performed according to the
protocol described by (Pasternak et al., 2015). Briefly, Arabidopsis seedlings were grown in
the standard condition in Hoagland media for 4-6 days. Methanol was used to fix the seedlings.
The seedlings were transferred to a glass slide and resuspended in 1x microtubule-stabilizing
buffer (MTSB). Seedlings were digested with 2 % Driselase dissolved in 1x MTSB at 37°C for
40 mins. Following digestion, permeabilization step was performed by treating the seedlings
with permeabilization buffer (3 % IGEPAL C630, 10 % dimethylsulfoxide (DMSO) in 1× MTSB
buffer) at 37°C for 20 mins. Then blocking was performed with a buffer consisting of 5 % BSA
for 30 min at room temperature. They were incubated overnight with rabbit anti-HA antibody
(1: 200 dilution, Abcam ab9110) at 37°C for 2 hours. After two washes with 1x MTSB, seedlings
were incubated with Alexa Fluor 488-labeled secondary antibody (1:200 goat anti-rabbit IgG,
Invitrogen, A32731) for 2 hours at 37°C. After five steps of washing with 1x PBS, coverslips
were mounted on slides with the antifade reagent (Prolong glass Antifade Mountant with
NucBlue Stain, Invitrogen). Fluorescence microscopy was conducted as described in the
previous section.
680

JACoP based colocalization analysis
Colocalization analysis was carried out with the ImageJ (Schneider et al., 2012) Plugin Just
Another Colocalization Plugin (JACoP) (Bolte and Cordelières, 2006) and a comparison of
Pearson's and Overlap coefficients and Li's intensity correlation quotient (ICQ) was performed.
Object-based analysis was done for punctate structures, adapted by (Ivanov et al., 2014).
Colocalization for both channels was calculated based on the distance between geometrical
centers of signals and presented as percentage. Analysis was done in three replicates each
(n = 3).

Structure prediction
TTN5 structure prediction was performed by AlphaFold (Jumper et al., 2021). The molecular
graphic was edited with UCSF ChimeraX (1.2.5, (Goddard et al., 2018), developed by the
Resource for Biocomputing, Visualization, and Informatics at the University of California, San
Francisco, with support from the National Institutes of Health R01-GM129325 and the Office
of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious
Diseases.
697

698 ***In silico* tool for gene expression analysis**
699 RNA-seq data relies on published data and was visualized with the AtGenExpress eFP at
700 bar.utoronto.ca/eplant (Waese et al., 2017).
701

702 **Statistical analysis**

703 One-way ANOVA was used for statistical analysis and performed in OriginPro 2019. Fisher
704 LSD was chosen as a post-hoc test with $p < 0.05$.
705

706 **ACCESSION NUMBERS**
707 Sequence data from this article can be found in the TAIR and GenBank data libraries under
708 accession numbers: *ARA7* (TAIR: AT4G19640), *GmMan1* (Uniprot: Q0PKY2) and *TTN5*
709 (TAIR: AT2G18390).
710

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Figure legends

Figure 1: TTN5, a homolog of small ARF-like GTPases. (A), Sequence alignment of TTN5
with human HsARL2, Arabidopsis AtARF1, HsARF1, and HsHRAS created with Jalview
(Waterhouse et al., 2009). The conserved G-motifs (G1-G5; indicated by red lines) are defined
for the TTN5 and HRAS sequence. The secondary structure of TTN5 is depicted by red lines
and corresponding cartoon (α-helix in green; β-sheet in orange). Conserved residues of TTN5
relevant in this work are highlighted by boxes: Gly-2, Thr-30 and Gln-70. (B), Model of the
predicted GTPase nucleotide exchange and hydrolysis cycle of TTN5. TTN5 switches from an
inactive GDP-loaded form to an active GTP-loaded one. GDP to GTP nucleotide exchange
and GTP hydrolysis may be aided by a guanidine exchange factor (GEF) and a GTPase-
activating protein (GAP). (C), Predicted protein structural model of TTN5; magenta, marks the
GTP-binding pocket; N-terminal amphipathic helix is highlighted in orange; conserved Gly-2 in
green; T30 and Q70, mutagenized in this study, shown in sticks. The model was generated
with AlphaFold (Jumper et al., 2021), and adaptation was done with UCSF ChimeraX 1.2.5
(Goddard et al., 2018).

Figure 2: Biochemical properties of TTN5 proteins suggest that TTN5 is present in a
GTP-loaded active form in cells. (A), Schematic illustration of the stopped-flow fluorescence
device for monitoring the nucleotide-binding kinetics of the purified TTN5 protein
heterologously expressed in bacteria (Supplementary Figure S2A-D). It consists of two
motorized, thermostated syringes, a mixing chamber, and a fluorescence detector. Two
different reagents 1 and 2 are rapidly mixed and transferred to a fluorescence detection cell
within 4 ms. One of the reagents must contain a fluorescent reporter group. Here, mdGDP and
mGppNHp were used to mimic GDP and GTP. (B), Schematic illustration of the nucleotide
association. Nucleotide-free TTN5 (reagent 1; preparation see Supplementary Figure S2E)
was rapidly mixed with mdGDP (reagent 2). A fluorescence increase is expected upon
association of mdGDP with TTN5. Similar measurements are performed with mGppNHp
instead of mdGDP. (C), Schematic illustration of the intrinsic nucleotide exchange. mdGDP-
bound TTN5 (reagent 1) is mixed with a molar excess of GDP (reagent 2). A fluorescence

decrease is expected upon mdGDP dissociation from TTN5 and binding of free GDP. Similar measurements are performed with mGppNHp. (D-E), Kinetics of association and dissociation of fluorescent nucleotides with TTN5 proteins (WT, TTN5^{T30N}, TTN5^{Q70L}) are illustrated as bar charts. The association of mdGDP (0.1 µM) or mGppNHp (0.1 µM) with increasing concentration of TTN5^{WT}, TTN5^{T30N}, and TTN5^{Q70L} was measured using a stopped-flow device (see A, B; data see Supplementary Figure S3A-F, S4A-E). Association rate constants (k_{on} in µM^{-1}s^{-1}) were determined from the plot of increasing observed rate constants (k_{obs} in s^{-1}) against the corresponding concentrations of the TTN5 proteins. Intrinsic dissociation rates (k_{off} in s^{-1}) were determined by rapidly mixing 0.1 µM mdGDP-bound or mGppNHp-bound TTN5 proteins with the excess amount of unlabeled GDP (see A, C, data see Supplementary Figure S3G-I, S4F-H). The nucleotide affinity (dissociation constant or K_d in µM) of the corresponding TTN5 proteins was calculated by dividing k_{off} by k_{on}. When mixing mGppNHp with nucleotide-free TTN5^{T30N}, no binding was observed (n.b.o.) under these experimental conditions. (F-G), GTP hydrolysis of TTN5 proteins determined by HPLC. (F), Schematic illustration of the GTP hydrolysis measurement. (G), GTP-bound TTN5 proteins (100 µM) were incubated at room temperature at different time points before injecting them on a reversed-phase HPLC system. Evaluated data (data see Supplementary Figure S5) resulted in the determination of the GTP hydrolysis rates (k_{cat}) illustrated as bar charts. (H), TTN5 accumulated in a GTP-loaded active form. GST-TTN5^{WT} (46.5 kDa) was purified from bacterial cell lysates at three different volumes in the presence of 0.1 µM unbound free GppNHp using glutathione beads. The nucleotide contents and the protein purities were determined by HPLC and Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. The presence of much higher amounts of GppNHp-bound versus GDP-bound GST-TTN5 protein indicates that TTN5 rapidly exchanged bound nucleotide und accumulated in this state.

Figure 3: TTN5 was present in punctate structures. Microscopic observations were made in a plane underneath the plasma membrane at the cell peripheries. (A), Schematic representation of an Arabidopsis seedling. Images were taken at three different positions of the seedlings and imaged areas are indicated by a red rectangle. (B-J), YFP-TTN5, YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} protein localization in Arabidopsis seedlings *via* fluorescent confocal microscopy. (B-D), YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were observed in stomata (indicated by empty white arrowhead) and in the epidermis of cotyledons in punctate structures (indicated by filled white arrowhead). (E-G), Localization in the hypocotyls showed the same pattern of punctate structures. (H-J), YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were present in punctate structures in the root hair zone and in root hairs (indicated by filled magenta arrowhead). (K), Schematic representation of a *N. benthamiana* plant, used for leaf infiltration for transient expression. Imaged areas are indicated by a red rectangle. (L-N), YFP-TTN5 signals in *N. benthamiana* leaf epidermal cells. YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} are present in punctate structures (indicated by white arrowheads) and in the nucleus (indicated by empty magenta arrowheads). Scale bar 50 µm.

Figure 4: YFP-TTN5 associated with the endomembrane system in *N. benthamiana* leaf epidermal cells. YFP-TTN5 and its variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were localized in *N. benthamiana* leaf epidermal cells with specific markers *via* fluorescent confocal microscopy. (A), Schematic representation of GmMan1 localization at the *cis*-Golgi site. (B-D), Partial colocalization of YFP-TTN5 proteins with the Golgi marker GmMan1-mCherry at *cis*-Golgi stacks (filled white arrowheads). Additionally, YFP-TTN5 constructs were expressed individually in vesicular-like structures (empty white arrowheads). (E), Schematic representation of GmMan1 localization at the ER after redistribution of the Golgi to the ER

upon brefeldin A (BFA) treatment. (F-H), Redistribution of Golgi stacks was induced by BFA
treatment. GmMan1-mCherry and YFP-TTN5 constructs were present in the ER and in
coexpressing punctate structures. (I), Schematic representation of ARA7 localization at the
trans-Golgi network (TGN) and multi-vesicular bodies (MVBs). (J-L), Colocalization of YFP-
TTN5 proteins with the MVB marker RFP-ARA7. (M), Schematic representation of ARA7
localization in swollen MVBs upon wortmannin treatment. (N-P), MVB swelling was obtained
by wortmannin treatment. ARA7-RFP was colocalizing with YFP-TTN5 constructs in these
swollen MVBs. Chemical treatment-induced changes were imaged after 25 min incubation.
Colocalization is indicated with filled arrowheads, YFP-tagged construct expression alone with
empty ones. Corresponding colocalization analysis data is presented in Supplementary Figure
S8. Scale bar 10 µm.

Figure 5. YFP-TTN5 colocalized with endocytosed plasma membrane material. (A),
Schematic representation of FM4-64 plasma membrane localization and endocytosis. (B-J),
YFP-tagged TTN5 and mutant forms YFP-TTN5^{T30N} andYFP-TTN5^{Q70L} were localized in
N. benthamiana leaf epidermal cells together with the plasma membrane dye FM4-64 *via*
fluorescent confocal microscopy. (B-D), YFP-TTN5, YFP-TTN5^{T30N} and YFP-TTN5^{Q70L}
colocalized with FM4-64 at the plasma membrane. (E-G), YFP-TTN5 protein plasma
membrane localization was tested by mannitol-induced (1 M) plasmolysis. The formation of
Hechtian strands is indicated with filled arrowheads. (H-J), Internalized FM4-64 was present
in YFP-TTN5 and the respective GTPase-variant coexpressing vesicle-like structures.
Colocalization is indicated with filled arrowheads. Scale bar 10 µm.

Supplementary Figure S1. *TTN5* **gene expression based on transcriptome data.**
Ubiquitous expression of *TTN5* in (A), aerial organs of different developmental stages, (B), of
seedling root tissues based on single cell analysis, and (C), in successive stages of embryo
development. Data was visualized with AtGenExpress eFB tool (bar.utoronto.ca/eplant,
(Waese et al., 2017).

Supplementary Figure S2. Heterologous expression and purification of TTN5 protein as
well as preparation of its nucleotide-free form. (A), Overview of protein purification and
preparation of nucleotide-free form of TTN5 in the presence of excess GDP. (B-C), The
chromatograms represent the GST-affinity chromatography to obtain GST-TTN5 and TTN5
variants, before and after GST-cleavage by thrombin. (D), Coomassie Blue SDS-PAGE of
GST-TTN5 (left panel; 46.5 kDa) and TTN5 after GST-cleavage by thrombin and a second
GST-affinity chromatography (right panel; 21 kDa). (E), Schematic illustration of nucleotide-
free TTN5 preparation. In the first step, the purified GDP-bound TTN5 is incubated with alkaline
phosphatase in the presence of 1.5-fold molar excess of Gpp(CH$_2$)p, which is a non-
hydrolyzable GTP analog and, unlike GDP, resistant to alkaline phosphatase. After GDP is
completely degraded, phosphodiesterase is added to the reaction to degrade Gpp(CH$_2$)p to
GMP, G and Pi. The nucleotide content is monitored in each step by HPLC. The solution with
nucleotide-free TTN5 is deep-frozen and thawed twice, aliquoted and stored at -80°C.

Supplementary Figure S3. Kinetic measurements of mdGDP interaction with TTN5
proteins. (A-C), Association of mdGDP (0.1 µM) with increasing concentrations of TTN5^{WT}
(A), TTN5^{T30N} (B) and TTN5^{Q70L} (C). (D-F), Association rate constant (k$_{on}$) is determined by
plotting the k$_{obs}$ values, obtained from the exponential fits of the mdGDP association data (A-
C) against the TTN5 (D), TTN5^{T30N} (E) and TTN5^{Q70L} (F) protein concentrations. (G-I),
Dissociation of mdGDP from TTN5 (G), TTN5^{T30N} (H) and TTN5^{Q70L} (I) proteins (0.1 µM) is
determined in the presence of excess amounts of unlabeled GDP (20 µM). The dissociation
rates (k$_{off}$) are obtained from the exponential fitting of the data by Origin software. All results

are shown as bar charts in Figure 2D. The principles of the assays are illustrated in Figure 2A-
C.

Supplementary Figure S4. Kinetic measurements of mGppNHp interaction with TTN5
proteins. (A-E), Association kinetics. (A, C), Association of mGppNHp (0.1 µM) with increasing
concentrations of TTN5^{WT} (A) and TTN5^{Q70L} (C). (B) When mixing mGppNHp with nucleotide-
free TTN5^{T30N}, no binding was observed under these experimental conditions. Instead,
association of mGppNHp (0.1 µM) with two different titrations of 0.5 µM TTN5^{T30N} was
measured, indicated by arrows, to confirm the fast binding of TTN5^{T30N} with mGppNHp by
increasing fluorescence intensity, determined by fluorimeter. (D-E), Association rate constant
(k_{on}) is determined by plotting the k_{obs} values, obtained from the exponential fits of the
mGppNHp association data (left panels) against the TTN5^{WT} (D), and TTN5^{Q70L} (E) protein
concentrations. Note that TTN5^{T30N} did not show association with mGppNHp, therefore middle
space is left empty. (F-H), Dissociation of mGppNHp from TTN5^{WT} (F), TTN5^{T30N} (G) and
TTN5^{Q70L} (H) proteins (0.1 µM) is determined in the presence of excess amounts of unlabeled
GppNHp (20 µM). The dissociation rates (k_{off}) are obtained from the exponential fitting of the
data by Origin software. All results are shown as bar charts in Figure 2E. The principles of the
assays are illustrated in Figure 2A-C.

Supplementary Figure S5. GTP hydrolysis reaction rates of the TTN5 proteins. (A-C),
The GTP hydrolysis reaction rates (k_{cat}) of TTN5^{WT} (A), TTN5^{T30N} (B) and TTN5^{Q70L} (C) proteins
(100 µM) were measured at 25°C. Aliquots of the reaction mixture at the indicated time points
were analyzed by HPLC as described in Materials and Methods. The obtained results are
shown as bar charts in Figure 2G. The principle of the assay is illustrated in Figure 2F.

Supplementary Figure S6. YFP-TTN5 localization in Arabidopsis seedlings. Microscopic
observations were made in a plane across the centers of most cells. (A), Schematic
representation of an Arabidopsis seedling. Images were taken at four different positions of the
seedlings and imaged areas are indicated by a red rectangle. (B-M), YFP-TTN5 signals in
Arabidopsis seedlings *via* fluorescent confocal microscopy. (B–D), YFP protein localization
was observed in the epidermis of cotyledons. YFP-TTN5 and its two mutant variants YFP-
TTN5^{T30N} and YFP-TTN5^{Q70L} were present in nucleus (indicated by empty white arrowhead)
and cytoplasm and at or in close proximity to the plasma membrane (indicated by filled white
arrowhead) in the epidermis of cotyledons. (E-G), localization in the hypocotyls showed clear
presence in the cytoplasm (indicated by empty magenta arrowhead), next to nuclear and
plasma membrane-related localization. (H-J), YFP-TTN5 and its two mutant variants YFP-
TTN5^{T30N} and YFP-TTN5^{Q70L} were present in nuclei, cytoplasm, at or close to the plasma
membrane in the root hair zone and in root hairs (indicated by filled magenta arrowhead) with
clear cytoplasmic localization. (K-M), localization at the root tip showed clear expression in
nuclei and cytoplasm, visible due to the smaller size of the vacuoles. (N), schematic
representation of a *N. benthamiana* plant, used for leaf infiltration for transient expression.
Imaged areas are indicated by a red rectangle. (O-Q), YFP-TTN5 signals in *N. benthamiana*
leaf epidermal cells *via* fluorescent confocal microscopy. YFP-TTN5 and its two mutant
variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were present in nucleus and cytoplasm and at or in
close proximity to the plasma membrane. Scale bar 50 µm.

Supplementary Figure S7. Phenotypes of *ttn5-1* T-DNA insertion line and whole-mount
immunolocalization. (A), Schematic representation of the *TTN5* exon-intron structure and T-
DNA insertion in the *ttn5-1* allele. The numbers below the exon numbers indicate base pairs
(bp). (B), Early embryo arrest phenotype of a homozygous *ttn5-1$^{-/-}$* seed on the left in
comparison with a wild type (WT) seed of the same silique on the right; arrowheads, indicate

enlarged nuclei in the arrested embryo (filled) and endosperm (empty). Early embryo arrest phenotypes were previously shown by (Mayer et al., 1999; McElver et al., 2000). Scale bar 1 cm. (C), Siliques of heterozygous *ttn5-1*^{+/-} plants, containing ca. 25 % white seeds with arrested embryos (filled arrowheads) and ca. 75 % regular green seeds (empty arrowheads). HA$_3$-TTN5 line crossed to *ttn5-1*^{+/-} was able to rescue embryo lethal seed phenotype. (D), Whole-mount immunostaining of HA$_3$-TTN5 seedlings. HA$_3$-tagged TTN5 localization was detected by α-HA/Alexa 488 immunostaining. HA$_3$-TTN5 was present in vesicle-like structures in the root cells (indicated by filled white arrowhead) and in root hairs (indicated by filled magenta arrowhead) comparable to YFP-TTN5 (Figure 3). Images are presented in a maximum intensity projection of several z-layers for a better visualization. Scale bar 50 µm.

Supplementary Figure S8. YFP-TTN5 is colocalized with GmMan1-mCherry and mRFP-ARA7. Colocalization analyses of the three YFP-TTN5 variants with specific markers using ImageJ (Schneider et al., 2012). (A), JACoP-based colocalization analysis (Bolte and Cordelières, 2006). Comparison of Pearson's and Overlap coefficients for *cis*-Golgi located GmMan1-mCherry with the three YFP-TTN5 variants in vesicle-like structures was done. All YFP-TTN5 variants colocalized similarly with the *cis*-Golgi marker. (B), Object-based analysis was performed for vesicle-like structures based on distance between geometrical centers of signals. YFP-TTN5-positive structures overlapped most with GmMan1-mCherry-positive Golgi stacks, compared to YFP-TTN5[T30N] and YFP-TTN5[Q70L]. GmMan1-mCherry-positive Golgi stacks overlap most with YFP-TTN5[Q70L]-positive structures followed by YFP-TTN5 and YFP-TTN5[T30N]. (C), JACoP-based colocalization analysis (Bolte and Cordelières, 2006). Comparison of Pearson's and Overlap coefficients for TGN/MVB located mRFP-ARA7 with the three YFP-TTN5 variants in vesicle-like structures was done. YFP-TTN5 and YFP-TTN5[Q70L] colocalized similarly with the mRFP-ARA7, whereas YFP-TTN5[T30N] tended to colocalize less. (D), Object-based analysis was performed for vesicle-like structures based on distance between geometrical centers of signals. More than half of all YFP-TTN5 variants-positive structures overlapped with mRFP-ARA7-positive structures, in the order YFP-TTN5[T30N], YFP-TTN5 and best YFP-TTN5[Q70L]. mRFP-ARA7-positive structures overlapped most with YFP-TTN5[Q70L] and for YFP-TTN5 and YFP-TTN5[T30N] being reduced by ca. 3.5-fold and 2.5-fold, respectively. Analyses were conducted in three replicates each (n=3). One-way ANOVA with Fisher-LSD post-hoc test was performed. Different letters indicate statistical significance (p < 0.05).

Supplementary Video Material S1. YFP-TTN5 is present in dynamic vesicle-like structures. (A-O), Time series of YFP-TTN5, YFP-TTN5[T30N] and YFP-TTN5[Q70L] dynamic expression in vesicle-like structures in Arabidopsis seedlings (A-L), or *N. benthamiana* leaf epidermal cells (M-O) *via* fluorescence confocal microscopy. (A-C), YFP-TTN5 and its two mutant variants YFP-TTN5[T30N] and YFP-TTN5[Q70L] were present in dynamic vesicle-like structures in epidermal cotyledon cells and stomata. (D-F), Mobility in the hypocotyls of YFP-TTN5[T30N] (E) differed by a slower or aborted motion in half of the cells compared to YFP-TTN5 (D) and YFP-TTN5[Q70L] (F). (G-I), YFP-TTN5 and its two mutant variants YFP-TTN5[T30N] and YFP-TTN5[Q70L] were present in the same dynamic vesicle-like structures in the root hair zone and in root hairs as well. (J-L), No clear mobility or vesicle-like structures were observed in the root tips of YFP-TTN5, YFP-TTN5[T30N] or YFP-TTN5[Q70L] seedlings. (M-O), YFP-TTN5, YFP-TTN5[T30N] and YFP-TTN5[Q70L] were present in dynamic vesicle-like structures in *N. benthamiana* epidermal cells in a comparable manner to Arabidopsis seedlings. Scale bar 50 µm.

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Figures

Characterization of the small *Arabidopsis thaliana* GTPase and ADP-ribosylation factor-like 2 protein TITAN 5

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Figure 1

A

B

inactive TTN5 — GDP
GEF
GDP
GTP
active TTN5 — GTP
GAP
Pi

C

T30
Q70

Figure 1: TTN5, a functional small ARF-like GTPase with nucleotide exchange capacity. (A), Sequence alignment of TTN5 with its human homolog ARL2, Arabidopsis, human ARF1, and human HRAS created with Jalview (Waterhouse et al., 2009). The conserved G-motifs (G1-G5; indicated by red lines) are defined for the TTN5 and HRAS sequence. The secondary structure of TTN5 is depicted by red lines and corresponding cartoon (α-helix in green; β-sheet in orange). Here mentioned conserved residues in ARF/ARL proteins are highlighted by boxes; Gly-2, and mutated Thr-30 and Gln-70. TTN5^{T30N} is expected to have a low nucleotide exchange capacity, while TTN5^{Q70L} is expected to have a low GTPase hydrolysis activity. (B), Model of the predicted GTPase nucleotide exchange and hydrolysis cycle of TTN5. TTN5 switches from an inactive GDP-loaded form to an active GTP-loaded one. GDP to GTP nucleotide exchange and GTP hydrolysis may be aided by a guanidine exchange factor (GEF) and a GTPase-activating protein (GAP). (C), Predicted protein structural model of TTN5; magenta, marks the GTP-binding pocket; N-terminal amphipathic helix is highlighted in orange; conserved Gly-2 in green; T30 and Q70, mutagenized in this study, shown in sticks. The model was generated with AlphaFold (Jumper et al., 2021), and adaptation was done with UCSF ChimeraX 1.2.5 (Goddard et al., 2018).

Figure 2

Figure 2: Biochemical properties of TTN5 proteins suggest that TTN5 is present in a GTP-loaded active form in cells. (A), Schematic illustration of the stopped-flow fluorescence device for monitoring the nucleotide-binding kinetics of the purified TTN5 protein heterologously expressed in bacteria (Supplementary Figure S2A-D). It consists of two motorized, thermostated syringes, a mixing chamber, and a fluorescence detector. Two different reagents 1 and 2 are rapidly mixed and transferred to a fluorescence detection cell within 4 ms. One of the reagents must contain a fluorescent reporter group. Here, mdGDP and mGppNHp were used to mimic GDP and GTP. (B), Schematic illustration of the nucleotide association. Nucleotide-free TTN5 (reagent 1; preparation see Supplementary Figure S2E) was rapidly mixed with mdGDP (reagent 2). A fluorescence increase is expected upon association of mdGDP with TTN5. Similar measurements are performed with mGppNHp instead of mdGDP. (C), Schematic illustration of the intrinsic nucleotide exchange. mdGDP-bound TTN5 (reagent 1) is mixed with a molar excess of GDP (reagent 2). A fluorescence decrease is expected upon mdGDP dissociation from TTN5 and binding of free GDP. Similar measurements are performed with mGppNHp. (D-E), Kinetics of association and dissociation of fluorescent nucleotides with TTN5 proteins (WT, TTN5^{T30N}, TTN5^{Q70L}) are illustrated as bar charts. The association of mdGDP (0.1 µM) or mGppNHp (0.1 µM) with increasing concentration of TTN5^{WT}, TTN5^{T30N}, and TTN5^{Q70L} was measured using a stopped-flow device (see A, B; data see Supplementary Figure S3A-F, S4A-E). Association rate constants (k_{on} in µM^{-1}s^{-1}) were determined from the plot of increasing observed rate constants (k_{obs} in s^{-1}) against the corresponding concentrations of the TTN5 proteins. Intrinsic dissociation rates (k_{off} in s^{-1}) were determined by rapidly mixing 0.1 µM mdGDP-bound or mGppNHp-bound TTN5 proteins with the excess amount of unlabeled GDP (see A, C, data see Supplementary Figure S3G-I, S4F-H). The nucleotide affinity (dissociation constant or K_d in µM) of the corresponding TTN5 proteins was calculated by dividing k_{off} by k_{on}. When mixing mGppNHp with nucleotide-free TTN5^{T30N}, no binding was observed (n.b.o.) under these experimental conditions. (F-G), GTP hydrolysis of TTN5 proteins determined by HPLC. (F), Schematic illustration of the GTP hydrolysis measurement. (G), GTP-bound TTN5 proteins (100 µM) were incubated at room temperature at different time points before injecting them on a reversed-phase HPLC system. Evaluated data (data see Supplementary Figure S5) resulted in the determination of the GTP hydrolysis rates (k_{cat}) illustrated as bar charts. (H), TTN5 accumulated in a GTP-loaded active form. GST-TTN5^{WT} (46.5 kDa) was purified from bacterial cell lysates at three different volumes in the presence of 0.1 µM unbound free GppNHp using glutathione beads. The nucleotide contents and the protein purities were determined by HPLC and Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. The presence of much higher amounts of GppNHp-bound versus GDP-bound GST-TTN5 protein indicates that TTN5 rapidly exchanged bound nucleotide und accumulated in this state.

Figure 3

Figure 3: TTN5 was present in punctate structures. Microscopic observations were made in a plane underneath the plasma membrane at the cell peripheries. (A), Schematic representation of an Arabidopsis seedling. Images were taken at three different positions of the seedlings and imaged areas are indicated by a red rectangle. (B-J), YFP-TTN5, YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} protein localization in Arabidopsis seedlings *via* fluorescent confocal microscopy. (B-D), YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were observed in stomata (indicated by empty white arrowhead) and in the epidermis of cotyledons in punctate structures (indicated by filled white arrowhead). (E-G), Localization in the hypocotyls showed the same pattern of punctate structures. (H-J), YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} were present in punctate structures in the root hair zone and in root hairs (indicated by filled magenta arrowhead). (K), Schematic representation of a *N. benthamiana* plant, used for leaf infiltration for transient expression. Imaged areas are indicated by a red rectangle. (L-N), YFP-TTN5 signals in *N. benthamiana* leaf epidermal cells. YFP-TTN5 and its two mutant variants YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} are present in punctate structures (indicated by white arrowheads) and in the nucleus (indicated by empty magenta arrowheads). Scale bar 50 µm.

Figure 4

Figure 4: YFP-TTN5 associated with the endomembrane system in *N. benthamiana* leaf epidermal cells. YFP-TTN5 and its variants YFP-TTN5[T30N] and YFP-TTN5[Q70L] were localized in *N. benthamiana* leaf epidermal cells with specific markers *via* fluorescent confocal microscopy. (A), Schematic representation of GmMan1 localization at the *cis*-Golgi site. (B-D), Partial colocalization of YFP-TTN5 proteins with the Golgi marker GmMan1-mCherry at *cis*-Golgi stacks (filled white arrowheads). Additionally, YFP-TTN5 constructs were expressed individually in vesicular-like structures (empty white arrowheads). (E), Schematic representation of GmMan1 localization at the ER after redistribution of the Golgi to the ER upon brefeldin A (BFA) treatment. (F-H), Redistribution of Golgi stacks was induced by BFA treatment. GmMan1-mCherry and YFP-TTN5 constructs were present in the ER and in coexpressing punctate structures. (I), Schematic representation of ARA7 localization at the *trans*-Golgi network (TGN) and multi-vesicular bodies (MVBs). (J-L), Colocalization of YFP-TTN5 proteins with the MVB marker RFP-ARA7. (M), Schematic representation of ARA7 localization in swollen MVBs upon wortmannin treatment. (N-P), MVB swelling was obtained by wortmannin treatment. ARA7-RFP was colocalizing with YFP-TTN5 constructs in these swollen MVBs. Chemical treatment-induced changes were imaged after 25 min incubation. Colocalization is indicated with filled arrowheads, YFP-tagged construct expression alone with empty ones. Corresponding colocalization analysis data is presented in Supplementary Figure S8. Scale bar 10 μm.

Figure 5

Figure 5. YFP-TTN5 colocalized with endocytosed plasma membrane material. (A), Schematic representation of FM4-64 plasma membrane localization and endocytosis. (B-J), YFP-tagged TTN5 and mutant forms YFP-TTN5^{T30N} andYFP-TTN5^{Q70L} were localized in *N. benthamiana* leaf epidermal cells together with the plasma membrane dye FM4-64 *via* fluorescent confocal microscopy. (B-D), YFP-TTN5, YFP-TTN5^{T30N} and YFP-TTN5^{Q70L} colocalized with FM4-64 at the plasma membrane. (E-G), YFP-TTN5 protein plasma membrane localization was tested by mannitol-induced (1 M) plasmolysis. The formation of Hechtian strands is indicated with filled arrowheads. (H-J), Internalized FM4-64 was present in YFP-TTN5 and the respective GTPase-variant coexpressing vesicle-like structures. Colocalization is indicated with filled arrowheads. Scale bar 10 μm.