MLO-mediated pollen tube reception.

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Title
Mildew Resistance Locus function in pollen-ovary reception is linked to its oligomerization and subcellular

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One Sentence Summary
MLOs can substitute for NORTIA function in pollen tube reception if they localize to a
Golgi compartment prior to pollen tube arrival and retain the ability to homo-oligomerize.

Footnotes
D.S.J. and S.A.K. designed experiments; D.S.J., J.Y., A.C.W, and E.L.K cloned
constructs and conducted genetic analyses; D.S.J. and B.E.S. designed, conducted and
analyzed FRET experiments; D.S.J. and S.A.K. wrote the manuscript.

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Abstract
Sexual reproduction in flowering plants requires communication between synergid cells
and a tip-elongating pollen tube (PT) for the successful delivery of sperm cells to the
embryo sac. The reception of the PT relies on signaling within the synergid cell that
ultimately leads to the degeneration of the receptive synergid and PT rupture, releasing
the sperm cells for double fertilization. In Arabidopsis thaliana, NORTIA (NTA), a
member of the Mildew resistance Locus O (MLO) family of proteins, plays a critical role
in the communication processes regulating PT reception. In this study, we determined
that MLO function in PT reception is dependent on MLO protein localization into a Golgi-
associated compartment before PT arrival, indicating that PT-triggered regulation of the
synergid secretory system is important for synergid function during pollination.

Additionally, a structure-function analysis revealed that MLO homo-oligomerization, mediated by the N-terminal region of the protein, and C-terminal tail identity both contribute to MLO activity during PT reception.

Introduction

Intercellular communication is central to the development and maintenance of all multicellular organisms. In plants, communication between cells controls processes ranging from organ development and tissue patterning (Chaiwanon et al., 2016; Otero et al., 2016) to defense responses (Yi and Valent, 2013; Stahl and Faulkner, 2016) and reproduction (Higashiyama and Takeuchi, 2015; Bircheneder and Dresselhaus, 2016). The importance of cell-cell communication is evident during reproduction in flowering plants as the attraction and reception of the male gametophyte (pollen tube (PT)) by the synergid cells of the female gametophyte (also known as the embryo sac) are essential for reproductive success (Beale and Johnson, 2013; Higashiyama and Takeuchi, 2015).

The synergid cells have two primary functions in reproduction: PT attraction and intercellular communication during PT reception (Russell, 1992; Higashiyama et al., 1998). Synergid cells are responsible for emitting LURE peptides that are perceived by the approaching PTs to guide them to the embryo sac (Okuda et al., 2009). PT reception then initiates as the PT makes contact with the synergid cells and pauses its growth to communicate with the receptive synergid at the filiform apparatus, a membrane-rich region at the micropylar end of synergid cells (Denninger et al., 2014; Hamamura et al., 2014; Ngo et al., 2014). Signal transduction pathways in the synergid
and the PT lead to PT rupture, releasing the sperm cells for double fertilization, and to
the degeneration of the receptive synergid.

PT reception is a complex process with key genes expressed in both the synergid cell
and PT facilitating this communication (Kessler and Grossniklaus, 2011; Beale and
Johnson, 2013). The synergid FERONIA/LORELEI receptor complex plays a role early
in PT reception (Escobar-Restrepo et al., 2007; Li et al., 2015; Liu et al., 2016), while
NORTIA (NTA, also known as AtMLO7), a member of the Mildew resistance locus O
(MLO) family of proteins, acts downstream of the receptor complex to facilitate PT
reception by an unknown mechanism. In nortia homozygous mutants, PT-synergid
communication is disrupted. Instead of bursting to release the sperm cells, PTs continue
to grow within the embryo sac, a phenotype known as PT overgrowth (Kessler et al.,
2010). Recently it was found that calcium oscillations in the synerigid cells begin upon
the arrival of the PT at the filiform apparatus (Denninger et al., 2014; Hamamura et al.,
2014; Ngo et al., 2014). The PT reception components FERONIA and LORELEI are
both necessary for the signaling process that initiates these calcium oscillations. In
contrast, nta-1 mutant ovules retain these oscillations but at lower amplitudes,
suggesting that NTA could play a part in modulating these calcium signals, but not in
initiating them (Ngo et al., 2014). The incomplete expressivity of the nta mutant
phenotype in which some ovules carrying the mutation are fertilized normally, suggests
that a threshold level of calcium (or some other signaling molecule) in synergids is
necessary for PT reception.
MLOs are seven-transmembrane proteins first identified as susceptibility factors for the fungal pathogen powdery mildew (Buschges et al., 1997). The MLO family was first described in barley (Hordeum vulgare L.) where mutations in the mlo locus conferred resistance to powdery mildew (Buschges et al., 1997). MLOs have a conserved calmodulin-binding domain (CaMBD) in their C-terminal cytosolic tail that actively binds calmodulin in a Ca\(^{2+}\)-dependent manner both \textit{in vitro} and \textit{in vivo} (Kim et al., 2002; Bhat et al., 2005). There are 15 MLO proteins in \textit{Arabidopsis thaliana} with representatives from five of the seven MLO clades in the plant kingdom (Devoto et al., 2003; Kusch et al., 2016). MLO proteins have been shown to function as a part of dynamic signaling pathways in response to various stimuli. Both NTA and Barley MLO (HvMLO) actively redistribute within their respective cell types to the site of invasion/penetration (the filiform apparatus in synergids or powdery mildew penetration sites) (Bhat et al., 2005; Kessler et al., 2010). However, the functional significance of such redistribution remains to be elucidated. Similarities between MLO function in pathogen susceptibility and PT reception make determining the role MLO proteins play in either system invaluable to understanding widely conserved intercellular communication pathways within plant cells.

Taking advantage of the diversity within the \textit{Arabidopsis} MLO family (Supplemental Fig. S1), we determined that the localization of AtMLO proteins within a Golgi-associated compartment in the synergid cell before PT arrival correlates with but does not completely explain MLO function in PT reception. This suggests that many coincidental characteristics influence MLO function within this pathway. A structure-function analysis
via domain swap experiments revealed that the N-terminal region of NTA is sufficient to
confer NTA activity on MLO8 and may play a role in homo-oligomerization. Additionally,
we found that the identity of NTA’s C-terminal tail is important for MLO-mediated PT
reception and controls specificity of function even between closely-related MLO
proteins.

Results

Expression of MLO Proteins in the Synergids of nta-1 Reveals Differences in their
Ability to Mediate Pollen Tube Reception

Previous work with AtMLO (MLO) proteins involved in powdery mildew infection showed
that MLO2, MLO6, and MLO12, all members of the same monophyletic clade, were
involved in powdery mildew susceptibility (Consonni et al., 2006). By analogy to the
"powdery mildew clade", we wondered if MLO10 and MLO8, both part of the same
clade as NTA (Fig. 1A, clade III), could have redundant functions within PT reception.
We first asked whether these closely related MLOs are expressed in synergid cells as
NTA is. Native promoter genomic GUS (Fig. 1, C–E) and GFP (Fig. 1, H–K) fusions of
MLO10 and MLO8 were analyzed and compared to NTA to determine their expression
patterns in unfertilized ovules. NTA was limited to synergids in both GUS (Fig. 1C) and
GFP (Fig. 1, H–I) expressing lines, similar to previous reports (Kessler et al., 2010).
MLO10 (Fig. 1D) and MLO8 (Fig. 1E) were expressed in ovules, but only MLO8 was
expressed near the synergids. Additional analysis with MLOpro::MLO-GFP constructs
revealed no GFP accumulation within the synergid cells of either MLO10 (Fig. 1J) or
MLO8 (Fig. 1K) expression lines. However, MLO8-GFP was detected just outside of the
In order to better understand how NTA functions in PT reception, we utilized the sequence diversity found within the MLO family by testing divergent MLOs for their ability to function in MLO-mediated PT reception (Fig. 2A). In addition to NTA, MLO10, and MLO8, we also chose MLO2 (from clade V) and MLO1 (from clade II), as even more distant proteins within the MLO phylogeny in respect to NTA. All MLO proteins tested in our experiment have the same predicted topology, with 7 membrane spanning domains and a calmodulin-binding domain in the C-terminal cytoplasmic tail (Supplemental Fig. S1). MLO2 and MLO1 are both expressed in ovules, but not in synergid cells (Fig. 1, F–G and L–M). All five of these MLOs were then ectopically expressed in the synergid cells of nta-1 using MYB98pro, a synergid-selective promoter (Kasahara et al., 2005), in order to assay their ability to rescue the nta-1 PT reception phenotype. These constructs also incorporated a C-terminal GFP fusion onto each MLO (MYB98pro::MLO-GFP) in order to confirm expression and determine distribution/localization within the synergid cell.

In nta-1 mutants, ovules remain unfertilized due to PT overgrowth during PT reception (Kessler et al., 2010), thus the percentage of unfertilized ovules can be used as a measure for the ability of an introduced construct to complement the nta-1 phenotype. Unfertilized ovule counts revealed that NTA-GFP expressed under MYB98pro could fully rescue nta-1 in comparison to NTA-GFP expressed under its native promoter.
(Supplemental Fig. S3), indicating that the MYB98pro drives expression in the synergid cells at sufficient levels for NTA activity. Both MLO10-GFP and MLO2-GFP significantly rescued nta-1 when expressed in synergids, while MLO8-GFP and MLO1-GFP did not significantly reduce the percentage of unfertilized ovules compared to nta-1 (Fig. 2A). MLO10 has the highest sequence similarity and is the most closely related to NTA according to phylogenetic analysis (Fig. 1A). Interestingly, MLO8 cannot rescue nta-1, although it is in the same sub-clade as NTA, while MLO2, a clade V MLO, does complement nta-1. This suggests that MLO-mediated PT reception requires specific properties of an MLO and that not all MLO proteins can function in the pathway.

MLOs Differ in their Distribution within Synergids and in their Localization in the Endomembrane System

We hypothesized that an MLO’s ability to complement the nta-1 phenotype when expressed in synergids may be dependent on whether it is localized within the appropriate subcellular location. To assay MLO distribution in the synergid cell prior to PT arrival, unfertilized ovules from lines transformed with each MLO-GFP construct were imaged using confocal laser scanning microscopy (CLSM). As was previously reported (Kessler et al., 2010), NTA accumulated within the endomembrane system of the synergid cell and was distributed throughout the entire cell but was excluded from the filiform apparatus prior to PT arrival (Fig. 2B). MLO10 and MLO2 distribution were similar to NTA; however, some GFP signal was also detected in the filiform apparatus (Fig. 2, C, E). MLO8 was predominately distributed toward the filiform apparatus with additional GFP signal detected throughout the rest of the cell (Fig. 2D), while MLO1 was
primarily localized to the filiform apparatus (Fig. 2F). Quantification of MLO-GFP distribution within the synergid cell confirmed that MLO10 and NTA are significantly less distributed toward the filiform apparatus than either MLO8 or MLO1 (Supplementary Fig. S4). Interestingly MLO2 varied in its distribution, with some synergid having GFP distributed towards the filiform apparatus and others being more cytoplasmic (Supplementary Fig. S4, I–K). Overall, the presence of the ectopically expressed MLOs in the filiform apparatus of synergid in unfertilized ovules made it difficult to assess whether active redistribution in response to PT arrival was conserved among the proteins analyzed. However, these results demonstrate that the presence of an MLO protein at the filiform apparatus during PT reception is not sufficient to rescue nta-1 (MLO8 and MLO1), and that either an MLO’s localization within the specific compartments that NTA localizes within or the active process of redistribution to the filiform apparatus may be key to MLO function in PT reception.

While MLO distribution within the synergid cell varies, the ability of MLO10, MLO8, and MLO2 to localize within cytoplasmic compartments similar to those seen in NTA-GFP lines may represent an important characteristic for MLO function in this pathway (Fig. 2, B–E). Therefore, determining the identity of the NTA-localized compartment may be important for understanding MLO function. To this end, the subcellular localization of NTA was defined using a stable co-expression assay in synergid cells with markers for specific organelles. Previously reported markers for Golgi, Endoplasmic Reticulum, and Peroxisomes (Liu et al., 2016) were co-expressed with MYB98pro:NTA-GFP in synergid cells (Fig. 3, A–C). NTA predominantly co-localized with the Golgi marker, with
additional GFP signal detected outside the range of the mCherry channel (Fig. 3A). This suggests that NTA is localized within Golgi bodies and may also be associated with post-Golgi vesicular trafficking. We then performed a co-localization analysis using the same Golgi marker co-expressed with the MLOs used in this study to determine if the others accumulate in this compartment similar to NTA (Fig. 4). MLO10 (Fig. 4A) partially co-localized with the Golgi marker at similar levels as NTA. MLO2 (Fig. 4C) co-localized with the Golgi marker to a lesser extent than either MLO10 or NTA, while MLO8 (Fig. 4B) and MLO1 (Fig. 4D) showed no overlap with the Golgi marker in synergid cells.

Since the differences detected in subcellular localization between closely related MLOs correlated well with their ability to rescue nta-1 (neither MLO8 nor MLO1 co-localized with the Golgi marker), we wanted to further elucidate the identity of the MLO8-localized compartment and compare it to NTA. To this end, MYB98pro::SYP61-mCherry, a trans-Golgi Network (TGN) localized marker (Sanderfoot et al., 2001; Viotti et al., 2010), was co-expressed in synergids with NTA-GFP, MLO10-GFP, and MLO8-GFP in order to determine if these closely related MLOs differentially co-localized within TGN-associated compartments (Fig. 5). NTA had no overlap (Fig. 5A) while MLO10 and MLO8 both partially co-localized with the TGN marker in synergids (Fig. 5, B–C). This further demonstrates a distinct difference between NTA and MLO8, revealing that localization within the proper compartment could be functionally significant. Interestingly, the partial co-localization of MLO10 (which fully rescues nta-1) with both Golgi and TGN markers suggests that the presence of an MLO within endomembrane compartments outside of the range of NTA does not have a negative impact on function.
These distribution/localization experiments revealed that MLO proteins are trafficked differentially within synergid cells. Multi-spanning membrane proteins can be initially directed into the membrane through a variety of different mechanisms, typically encoded as signals within the protein (Goder and Spiess, 2001). In an effort to predict the presence of signal peptides in MLO proteins, sequences of all 15 MLOs were examined using the SignalP 4.0 prediction server (Petersen et al., 2011). Interestingly, we found that out of the 15 Arabidopsis MLOs only NTA, MLO10, and MLO8 had significantly predicted cleavable N-terminal signal peptides (Supplemental Fig. S5). This suggests that this subclade (NTA, MLO10, and MLO8) of the clade III MLOs may be type I multi-spanning membrane proteins (Goder and Spiess, 2001) further differentiating them from the other 12 Arabidopsis MLOs. To determine if NTA’s predicted signal peptide influences its localization and function within PT reception, we cloned NTA without this signal peptide (NORTIA Δsp) and expressed it in the synergid cells of nta-1 under control of MYB98pro (Supplemental Fig. S6). Removing NTA’s signal peptide appeared to have no effect on its function or distribution in the synergid cell prior to PT arrival (Supplemental Fig. S6, A–D). We then generated 35Spro::NTA-GFP and 35Spro::NORTIA Δsp-GFP constructs and performed a transient co-expression assay in Nicotiana benthamiana epidermal cells with a previously published Golgi-mCherry marker (Nelson et al., 2007). Similar to its localization in synergids, NTA partially co-localized with the Golgi marker (Supplemental Fig. S6, H–J), while NORTIA Δsp-GFP predominately accumulated in subdomains of the plasma membrane with no overlap with the Golgi marker (Supplemental Fig. S6, E–G). The presence of a signal
peptide could help explain the MLO localization/distribution patterns observed within synergid cells, however, this is not enough to account for function in PT reception or for localization within Golgi-associated compartments, as MLO8 does not rescue the nta-1 PT reception phenotype and localization within Golgi was not detected (Figs. 2A and 4B). Additionally MLO2, which has no predicted signal peptide, and NORTIA Δsp are able to rescue nta-1’s PT reception phenotype and are distributed similarly to full length NTA in the synergid cell. This suggests that synergids may have cell-type specific mechanisms that determine the localization of this class of membrane proteins.

The N- and C-Terminal Domains of NTA are Important for Function

MLO8 is closely related to NTA, sharing 61% identity and 73% similarity at the amino acid level. The differential subcellular localization patterns and inability to complement the nta-1 PT reception phenotype make MLO8 a good tool for dissecting regions of the NTA protein that are important for its function in PT reception. In order to identify functionally significant regions in the NTA protein, we performed a targeted structure-function analysis of NTA via domain swap experiments with MLO8. Regions swapped were chosen based both on sequence dissimilarity between the two MLOs and on domains previously found to be important for MLO function (Elliott et al., 2005; Chen et al., 2009). Chimeric MLOs were expressed in the nta-1 mutant background under control of MYB98 pro and ovule counts from T2 homozygous transgenic lines were used as a proxy for determining functionality in PT reception. Protein-GFP fusion distribution was analyzed for each line and revealed significant accumulation of all chimeric proteins with varying localization patterns in comparison to the full length MLOs (Supplemental
Chimeric proteins that were able to complement *nta-1* were not primarily confined to the Golgi-associated compartment as seen with NTA; in addition to Golgi localization, proteins accumulated within the synergid vacuole. Comparable variations in localization have been previously reported in a similar structure-function analysis of MLO family proteins (Chen et al., 2009), indicating that chimeric fusions of MLOs may partially disrupt intramolecular interactions within the chimeric proteins and subsequent regulation of their trafficking within the cell.

An N-terminal swap, including the N-terminus through the first extracellular loop (NEL1, Fig. 6B), demonstrated this region to be functionally significant for MLO-mediated PT reception. NTA[NEL1]-MLO8 could partially rescue *nta-1* in comparison to full length NTA while MLO8[NEL1]-NTA was not significantly different than full length MLO8 in its ability to complement the *nta-1* phenotype (Fig. 6B). This domain was further subdivided into swaps of only intracellular loop 1 (IL1) and extracellular loop 1 (EL1). Interestingly, constructs with either MLO’s EL1 (NTA-MLO8[EL1]-NTA and MLO8-NTA[EL1]-MLO8) were capable of rescuing *nta-1* (Fig. 6C) while swaps of IL1 conferred no significant changes from the full-length proteins’ ability to rescue (Supplemental Fig. S9A), indicating that NTA’s EL1 is sufficient to confer NTA activity on MLO8, but the MLO8 EL1 does not compromise NTA’s function unless it is combined with the MLO8 IL1 and N-terminus. Additionally, swaps of intracellular loop 2 (IL2), a prominent domain in both size and variability, had no significant impact on either MLO’s function (Supplemental Fig. S9F). In a previous study it was shown that the NEL1 domain of MLO4 could confer partial function to MLO2 in rescuing the root curling phenotype of *mlo4-1*; however, the
reciprocal swap placing MLO2’s NEL1 domain onto MLO4 remained functional (Chen et al., 2009). Here we have identified the NEL1 domain of NTA as necessary for its role in PT reception and sufficient in conferring function to MLO8.

The C-terminal tails of MLO proteins have been described as intrinsically disordered, a characteristic that may contribute to specificity in function between closely related MLOs (Kusch et al., 2016). In comparison to NTA, MLO8 has a longer C-terminal tail following the conserved Calmodulin Binding Domain (CaMBD) (Supplemental Fig. S1). To test if their C-terminal intracellular domains provide functional specificity, NTA/MLO8 C-terminal domain swaps were analyzed in nta-1. NTA’s C-terminus on MLO8 (MLO8-NTA[CTerm]) rescued nta-1 at similar levels as full length NTA whereas MLO8’s C-terminus on NTA (NTA-MLO8[CTerm]) rescued at an intermediate level and was significantly different than NTA (Fig. 6E). When this domain swap was expanded, incorporating both the third intracellular loop (IL3) and the third extracellular loop (EL3), similar results were observed in which NTA[TM5]-MLO8 did not fully rescue and MLO8[TM5]-NTA rescued nta-1 compared to full length NTA (Fig. 6D), demonstrating that there is no change in the effect on function with these additional domains. This suggests that the identity of NTA’s C-terminal tail domain is crucial for its function and that this domain is necessary and sufficient for MLO-mediated PT reception.

In our analysis of full-length MLO proteins expressed in synergid cells, distribution within the synergid cell and co-localization with a Golgi marker correlated well with their ability to function in PT reception. Based upon these observations, we hypothesized that the
functional NTA/MLO8 domain swaps were primarily distributed throughout the cell and not toward the filiform apparatus and that they would localize within Golgi in a similar manner as NTA, MLO10, or MLO2. To test this, we quantified MLO-GFP distribution for the NEL1 and CTerm domain swaps within the synergid cell and also performed a co-localization analysis with the same Golgi marker expressed in synergid cells (Supplemental Fig. S8 and Fig. 7, respectively). Interestingly, the domain swaps that failed to fully function in PT reception (MLO8[NEL1]-NTA and NTA-MLO8[CTerm]) were more similar to NTA in their distribution than their functional reciprocal swaps (Supplemental Fig. S8). Additionally, partial co-localization with the Golgi marker was detected with all four swaps analyzed (Fig. 7) and we could not identify MLO8-like exclusion of any of the chimeric protein-GFP fusions from the Golgi-associated compartments. Even MLO chimeric proteins with disrupted function (NTA[NEL1]-MLO8 and NTA-MLO8[CTerm]) partially co-localized with the Golgi marker in synergids. This suggests that an MLO’s ability to function in PT reception is not solely dependent on localization within a specific part of the endomembrane system (although at least some accumulation appears to be required as MLO1 does not rescue nta-1) but requires some additional characteristic of the protein influenced by their N- and C-terminal domains.

The N-Terminal Domain of NTA Influences Efficient Homo-Oligomerization

The domain swap constructs that were compromised in their ability to complement nta-1 did not show MLO8-like exclusion from the Golgi compartment, indicating that other molecular properties may have been disrupted in these chimeric proteins. MLO
proteins have previously been shown to homo-oligomerize and conserved cysteine residues residing within EL1 were proposed to play a role in this interaction (Elliott et al., 2005). Since the replacement of the NTA NEL1 domain with MLO8’s NEL1 fully disrupted its ability to function, we hypothesized that homo-oligomerization was important for NTA’s function and that the NEL1 domain swap inhibited this interaction. Using a FRET-based assay in *N. benthamiana* epidermal cells, we tested whether NTA and MLO8 were able to homo- or hetero-oligomerize *in planta* and whether the NEL1 chimeric proteins generated could successfully recapitulate this characteristic. FRET was determined using a correlation analysis comparing the fluorescence emission of the donor (mCerulean3 (Cer3) (Markwardt et al., 2011)) to the emission of the acceptor (YFP (Nagai et al., 2002)) detected after four subsequent rounds of acceptor photobleaching. Fluorescence emission from both channels was compared using a Pearson product-moment correlation analysis in which the more negative the correlation coefficient the stronger the FRET signal, as this would indicate a proportional increase in donor fluorescence and corresponding decreases in acceptor fluorescence (Supplemental Fig. S10; See Materials and Methods). Since NTA localized within Golgi compartments in *N. benthamiana* epidermal cells (Supplemental Fig. S6, H–J), we generated a positive control for the detection of FRET via acceptor photobleaching in NTA-localized compartments, in which Cer3 and YFP were fused in tandem onto NTA’s C-terminal tail (NTA-Cer3-YFP). This ideal standard had a median donor-acceptor correlation coefficient of -0.9864 confirming that FRET could be reproducibly detected within NTA-associated Golgi and post-Golgi vesicles (Fig. 8). NTA-Cer3 was co-expressed with GUS-YFP and analyzed in each experiment as a negative control (with
a median coefficient of 0.5217). FRET was detected at similar levels in test pairs that included: NTA-Cer3 + NTA-YFP and NTA[NEL1]–MLO8-Cer3 + NTA[NEL1]–MLO8-YFP (with median coefficients of -0.93405 and -0.8906, respectively). FRET was detected at significantly lower levels than the first group in the test pairs: MLO8-Cer3 + MLO8-YFP, NTA-Cer3 + MLO8-YFP, and MLO8[NEL1]–NTA-Cer3 + MLO8[NEL1]–NTA-YFP (with median coefficients of -0.6815, -0.5859, and -0.4126) (Fig. 8). While all pairs examined were significantly different than the negative control, this experiment indicates that MLO8[NEL1]–NTA, which does not rescue nta-1, homo-oligomerizes less efficiently than full length NTA under our experimental conditions. Reciprocally, no difference was detected between homo-oligomerization efficiencies of NTA or NTA[NEL1]–MLO8, which both rescue nta-1. Taken together, these data suggest that the homo-oligomerization of NTA could be an intrinsic part of its function in PT reception and that disruption of NTA’s NEL1 domain may reduce the efficiency of this interaction, thus compromising its function.

Discussion

PT reception requires a chain of signaling events in both the arriving pollen tube and within the receptive synergid cell (Kessler and Grossniklaus, 2011). In this study, we have further defined the role of NTA, an MLO protein involved in the female side of this signaling process. The analysis of MLOs closely related to NTA revealed a divergence in expression patterns within reproductive tissues and in their ability to function within PT reception. Previous studies of Arabidopsis MLOs have identified functional redundancy and co-functional relationships within discrete monophyletic clades of the
MLO family (Consonni et al., 2006; Chen et al., 2009). Our data indicates that, of the MLOs presented in this study, NTA is the only MLO that is specifically expressed within synergid cells and likely functions independently from other closely related MLOs. The ectopic expression of full-length MLO proteins in the synergid cells of nta-1 revealed that specific characteristics of an MLO are required for function in PT reception. The closely related MLO8 (clade III) does not complement nta-1 while the more distantly related MLO2 (clade V) could, further demonstrating this divergence in function.

Our data showed that the ability of other MLO proteins to complement the nta-1 PT reception phenotype was closely correlated with the ability of the synergid-expressed MLO to accumulate in a Golgi-associated compartment before PT arrival. However, analysis of chimeric MLO proteins during a structure-function analysis revealed that localization/retention within this Golgi-associated compartment is not the sole determinant of MLO function in PT reception. Accumulation within the synergid endomembrane system prior to PT arrival is conserved between those MLOs (and chimeric constructs) capable of functioning in this pathway. Previous work showed that NTA redistributes to the filiform apparatus during PT reception, indicating that precise control of the synergid secretory system and polarized transport may be necessary for MLO function in this pathway (Kessler et al., 2010). Instances of enhanced trafficking of vesicles and induction of specialized secretory mechanisms in response to pathogens have been widely reported in plants (Kwon et al., 2008; Nielsen et al., 2012; Ding et al., 2014), including responses specific to powdery mildew infection (An et al., 2006; An et al., 2006). Polar redistribution events observed in NTA-mediated PT reception and in
MLO-mediated fungal penetration suggests a conservation of signal-induced MLO transport. Additionally, the SNARE proteins ROR2 (barley) and PEN1 (*Arabidopsis*) are required for *mlo* pathogen resistance, providing evidence that MLO-mediated responses require activation of the secretory pathway with reported focal accumulation of ROR2 occurring at the site of infection (Bhat et al., 2005; Hückelhoven and Panstruga, 2011). NTA seems to follow a similar paradigm, with NTA’s redistribution dependent on signaling via FERONIA (FER), a member of the CrRLK1L-type family of receptor like kinases and a key player in the early stages of PT reception (Escobar-Restrepo et al., 2007; Kessler et al., 2010; Ngo et al., 2014).

Secretory pathway regulation has also been shown to be important for controlling intercellular signaling during compatibility responses in *Brassica* species (Samuel et al., 2009; Safavian and Goring, 2013). In early pollen recognition events, an induction of secretory activity in stigmatic papillae was observed during compatible pollinations while an active inhibition of trafficking was noted in incompatible pollinations (Safavian and Goring, 2013). This increase in secretory activity occurs in a polarized manner toward the pollen grain upon successful signal transduction within the receptive papilla cell, and is directly associated with the hydrating pollen grain. The parallels between secretory regulation of polarization during pollen:stigma and PT:synergid interactions suggest that precise control of the secretory pathway in response to extracellular signals may be an important characteristic of multiple levels of pollen:pistil interactions during pollination. It will be interesting to determine if similar inhibition of trafficking activity occurs during
instances of PT overgrowth as described during incompatible pollinations and if MLO-mediated processes are involved in both.

Our experiments expressing full-length MLO proteins in synergid cells shows that one important determinant of MLO functionality in PT reception is the ability to localize within an endomembrane-associated compartment prior to PT arrival and thus presumably before FER-mediated signaling occurs. While distribution and localization within the synergid cell varied between MLOs, the accumulation of MLO proteins involved in PT reception within endomembrane compartments of the synergid cell could be required for multiple reasons. One hypothesis is that proper timing of polar redistribution to the filiform apparatus is critical for PT reception and that the retention of MLOs within either the Golgi or TGN prevents this redistribution until proper signaling is achieved as a PT communicates with the receptive synergid. This suggests that the presence of an MLO (or an MLO-associated compartment) at the filiform apparatus prior to PT arrival is not ideal and may even be inhibitory to the synergid cell by functioning too early. However the data presented here does not support this hypothesis since MLO2 and several chimeric MLO fusions rescued nta-1 even though instances of MLO-GFP distribution toward the filiform apparatus prior to PT arrival were observed. A second possibility is that NTA retention facilitates its co-localization and/or interaction with some unknown co-factor required for MLO-mediated PT reception. This co-factor could be involved in forming a complex with NTA and/or other components of the PT reception machinery or could be a secreted signaling molecule that needs to be escorted to the filiform apparatus for communication with the arriving PT. Further dissection of the required
components involved in MLO-mediated processes will help elucidate the exact mechanism of MLO function.

The differences observed in MLO distribution within the synergid cell and localization between multiple cell types (NTA vs. NORTIA Δsp localization in the synergid compared to tobacco epidermal cells) may represent cell-type specific processes regulating the incorporation and retention of MLOs within the required endomembrane compartments of the synergid cell; data that supports the second hypothesis proposed above. Mechanisms regulating membrane protein polarization in plant cells have been well described, especially related to the asymmetric distribution of *Arabidopsis* PIN-FORMED (PIN)-type auxin transport proteins (Luschnig and Vert, 2014). However, it is unclear just how conserved such polarization mechanisms are within the synergid cell.

Synergids are highly polarized cells that have two purposes in plant reproduction. Before PT arrival, the synergid cells secrete small LURE peptides in order to attract the PT to the micropyle of the ovule (Higashiyama, 2002; Okuda et al., 2009). The second purpose of synergids is to recognize when a PT has arrived and communicate with it facilitating the release of sperm cells so that double fertilization can occur. These distinct purposes necessitate a change in synergid cell biology from an “attractant state” with high level of protein secretion to a “signaling state” where calcium oscillations and FER-mediated signal transduction cascades prepare the synergid for PT reception (Kessler and Grossniklaus, 2011; Hamamura et al., 2014; Ngo et al., 2014; Higashiyama and Takeuchi, 2015). Interestingly, no PT reception mutants have been reported as having defects in PT attraction, providing evidence for distinct processes
regulating both pathways (Huck et al., 2003; Escobar-Restrepo et al., 2007; Kessler et al., 2010; Tsukamoto et al., 2010; Lindner et al., 2015). This suggests that a change in secretory mechanisms occurs as the PT arrives and FER-mediated PT reception initiates. Overall, more work must be done to determine the conservation of mechanisms regulating membrane polarization and protein secretion that govern these distinct roles of the synergid cell.

Homo and hetero-oligomerization has been demonstrated to be functionally important in various multispanning membrane protein families, where oligomerization is critical for processes such as signal transduction (Maurel et al., 2008), pore or channel formation (Musil and Goodenough, 1993; Véry and Sentenac, 2002), as well as subcellular localization within the endomembrane system (Weisz et al., 1993). While the homo-oligomerization of MLO proteins was previously established (Elliott et al., 2005), the functional significance of this interaction and the domains contributing to dimerization were not described. Here we have shown the homo-oligomerization of NTA in planta with a FRET-based interaction assay and demonstrated a reduction of this dimerization upon disruption of an N-terminal domain (NEL1). Further confirmation of the NEL1 domain’s importance in the effective dimerization of NTA was established in the reciprocal detection of efficient homo-oligomerization of the NTA[NEL1]-MLO8 chimeric protein, which fully complements the nta-1 unfertilized ovule phenotype. It should be noted that since this experiment was based on a transient assay in N. benthamiana epidermal cells and not in the synergid cells of A. thaliana where NTA functions in PT reception, care must be taken in the interpretation of these interaction data. However,
similar localization of NTA-GFP within the Golgi of both cell types demonstrates some conservation of its native characteristics in our assay. Furthermore, the data presented in this study suggests a reduction in the efficiency of interactions between those MLOs incapable of functioning in PT reception (MLO8 and MLO8[NEL1]–NTA) and as such more validation is required to fully elucidate the role of homo-oligomerization in MLO-mediated PT reception.

Calcium is an important signaling molecule in plants with reported roles in systemic responses, cell-cell communication, and intracellular signaling (Dodd et al., 2010; Gilroy et al., 2014). Oscillation of calcium in the synergid cell upon PT arrival is dependent on FER-mediated signaling during PT reception (Hamamura et al., 2014; Ngo et al., 2014). In nta-1 mutants, PT reception is not impaired in every ovule. Incomplete penetrance of the nta-1 phenotype suggests that some ovules have normal fertilization if calcium oscillations within the synergid cell can reach a certain threshold (Ngo et al., 2014). CaM-binding capacity of NTA may in some way modulate calcium signaling during PT reception. In this study, we found that swapping the C-terminal tail (which contains the conserved MLO CaMBD) of NTA with MLO8’s partially disrupted its ability to function in this pathway. However, NTA’s C-terminal tail on MLO8 was able to function in MLO-mediated PT reception, therefore we speculate that this domain may confer the ability to modulate calcium signaling within the synergid cell above this threshold when it is present in a closely related MLO. The MLO C-terminal tail was recently described as intrinsically disordered, suggesting that this domain influences functional specificity throughout the family (Kusch et al., 2016). These data further support the hypothesis
that fundamental differences affecting function exist in the C-terminal domains of MLO proteins.

While this study significantly contributes to our understanding of MLO-mediated PT reception, an exact mechanism of NTA’s function throughout gametophytic interaction remains to be determined. NTA itself could be involved in signaling with the PT to trigger release of the sperm cells, or, alternatively, other factors contained in the NTA-associated compartments could be delivered to the filiform apparatus along with NTA to assist in communication with the PT. Our domain swap experiments have indicated that both homo-oligomerization, influenced by the N-terminal domain of NTA, and C-terminal tail identity are important for NTA function in PT reception. Future work on NTA’s active redistribution during PT reception and its relationship to calcium oscillations within the synergid cell will provide more insight into how MLO proteins function during cell-cell communication.

Materials and Methods

Plant Growth Conditions

Full length MLOs and domain swap constructs were transformed into homozygous nta-1 insertion mutants in the Wassilewskija (Ws-2) background. Wild-type Ws-2 (Ws) and nta-1 were used as the controls for all ovule count experiments. Native promoter GUS and GFP fusion constructs were transformed into the Col-0 ecotype. All Arabidopsis thaliana were grown at 22°C in long day conditions (16 hours of light and 8 hours of dark) in a custom built grow room. Seeds were sterilized and plated on ½ MS plates
with transgenic lines either grown on ½ MS plates supplemented with 20mg/L hygromycin (full length MLO and domain swap ovule count lines and native promoter lines) or planted directly on soil (endomembrane marker co-expression lines) and kept in the dark at 4°C for two days for cold-stratification. 5-7d old seedlings were transplanted from plates to soil (ProMix Flex supplemented with 40g MARATHON pesticide and Peter’s 20:20:20 (NPK) fertilizer at recommended levels) and grown until flowering for further analysis. Seeds sown directly on soil were treated with BASTA herbicide spray for three successive days starting at 5 days after germination and resistant plants were maintained for analysis. *Nicotiana benthamiana* was grown on soil in long day conditions at 22°C in a Conviron ATC13 growth chamber.

**Cloning and Generation of Transgenic Lines**

PCR with PHUSION High-Fidelity Polymerase (NEB, M0535S) was utilized to generate all constructs in this study. Genes were amplified with primers that had attB1 and attB2 sites for recombination via BP reaction into the Gateway-compatible entry vector pDONR207 (Table S1). Native promoter constructs were amplified from Col-0 genomic DNA, while full-length MLO and domain swap constructs were amplified from cDNA derived from rosette leaves or flowers of Col-0. Native promoter entry vectors were recombined via LR reactions into pMDC107 (GFP) and pMDC163 (GUS) (Curtis, 2003) to generate reporter fusion expression vectors. Full-length MLO cDNA entry vectors were recombined via LR reactions into the pMDC83 backbone with the MYB98$_{pro}$ promoter (Muller et al., 2016) to drive synergid-selective expression with a C-terminal GFP fusion. Domain swap and site-directed mutagenesis constructs were made using
primers listed in Table S1 to generate overlapping fragments of specific regions from full-length NTA and MLO8 entry vector cDNA template described above. AttB sites were included on the ends of the chimeric constructs and fragments were pieced together using a PCR-pasting protocol. Amplified chimeras were recombined via BP reaction into pDONR207 and entry vectors were subsequently recombined via LR reaction into the modified pMDC83 backbone described previously. All entry vectors were sequenced to ensure swaps were present within each construct.

MYB98_pro::SYP61-mCherry construct was generated in a single reaction using a 3 fragment Gibson Assembly, with Gibson Assembly Master Mix NEB E2611S (Gibson et al., 2009). SYP61 was amplified from Col-0 flower cDNA and MYB98_pro was amplified using the pMDC83 backbone previously described (Lindner et al., 2015). Fragments were assembled into the pEarlyGate301 backbone following digestion with PacI (NEB R0547S) and BamHI (NEB R3136S) and subsequent gel purification. Primers with overlaps used for amplification are listed in Table S1.

Constructs for transient expression in *N. benthamiana* were generated using entry vectors of full-length MLOs recombined via LR reaction into pMDC83, which has a 2× 35S viral promoter and C-terminal GFP fusion (Curtis, 2003). Gateway-compatible destination vectors with C-terminal mCerulean3 (mCer3), C-terminal YFP and N-terminal YFP were used for all FRET analyses (Myers et al., 2016). Entries described above were recombined into their respective FRET vectors via LR reaction.
nta-1 Complementation Assays

Expression vectors were transformed into the *Agrobacterium tumefaciens* strain GV3101 and subsequently transformed into *nta-1* via the floral-dip method (Bent, 2006). The resulting seeds were plated on ½ MS plates supplemented with 20mg/L hygromycin for selection of transgenic plants. 3-4 different insertion lines for each construct were taken to the T2 generation and homozygous plants were identified using fluorescence microscopy to ensure synergid expression of GFP in every ovule. Fertilization rates of self-pollinated flowers were counted in at least 3 plants from each line. Counts of unfertilized vs. fertilized ovules were performed and compared to controls in order to determine complementation status of each construct in the *nta-1* background. Aniline blue staining of pollen tubes in pollinated pistils was used to confirm that unfertilized ovules in the complementation lines were the result of *nta* mutant-induced pollen tube overgrowth and not other problems with female gametophyte development or pollen tube attraction. Data was graphed and statistically analyzed using Prism (www.graphpad.com). Significance was determined using Analysis of Variance (ANOVA) and Dunnett's multiple comparison test. Full-length MLO complementation lines were compared to both *nta-1* and WS unfertilized ovule counts, resulting in the same significance with both tests (constructs that complement the *nta-1* phenotype were significantly different than the *nta-1* control but not significantly different than the WS control, but only statistical tests with *nta-1* comparisons are shown in the figures for simplicity). Domain swap complementation lines were compared to full-length NTA and MLO8 unfertilized ovule counts.
**GUS Staining of Ovules**

Flowers from 2-3 T1 plants of each native promoter line were emasculated. At 2 days after emasculation, pistils were slit down the septum prior to being fixed for 45 min in 90% acetone at -20°C. Exposed ovules were vacuum infiltrated and incubated overnight in GUS staining solution (10 mM EDTA, 0.1% Triton X-100, 2 mM Fe$^{2+}$CN, 2 mM Fe$^{3+}$CN, 1 mg mL$^{-1}$ X-GLUC (GoldBio) in 100 mM sodium phosphate buffer pH 7.2) at 37°C. Ovules were dissected out of the pistils into chloral hydrate:glycerol:water solution (8:1:2, w:v:v) and imaged using Differential Interference Contrast (DIC) optics on a Nikon Eclipse Ni-U compound microscope.

**Transient Expression in N. benthamiana**

Leaves from 4-6 week old *N. benthamiana* were co-infiltrated with *A. tumefaciens* strain GV3101 harboring either an MLO-GFP construct and –mCherry secretory marker discussed or Cer3 and YFP-fused proteins for analysis of FRET, along with the *A. tumefaciens* strain C58C1 containing the viral silencing suppressor helper complex pCH32 (Hamilton, 1997). For colocalization experiments, *A. tumefaciens* overnight cultures were pre-treated as in (Myers et al., 2016). For FRET analysis, *A. tumefaciens* overnight cultures were centrifuged for 5 min at 4000g and resuspended/washed in 10mM MgCl$_2$ twice. A final suspension of each *Agrobacterium* in 10mM MgCl$_2$ was then co-infiltrated into the leaf. Plants were maintained in the growth chamber and all further analyses took place 2-4 days after infiltration.

**Microscopy**
Young flowers from lines with NTA-GFP driven by NTA’s native promoter and MYB98pro were dissected out in water on a clean microscope slide. Ovules were imaged using a 40× dry objective (NA = 0.75) on a Nikon Eclipse Ni-U compound epifluorescent microscope equipped with an X-Cite 120 LED fluorescent lamp and narrow band eGFP filter cube. Brightfield images were taken using DIC optics and overlaid with fluorescence to determine where NTA-GFP was detected.

Ovules from MLO-GFP lines were dissected out 2 days after emasculating the flower and were removed but retained on the septum for analysis of GFP localization and distribution on a confocal scanning laser microscope (CSLM). Freshly dissected ovules were incubated on a slide in 20μM FM464 (Molecular Probes, T3166) in PIPES Buffer (50mM PIPES, 5mM EGTA and 1mM MgSO4 at pH = 6.8) for 10 min on ice in a humid chamber (pipette tip box with ice in the bottom and moist kimwipes under the slide on the top). Cover slips were placed right before the samples were taken to the microscope and excessive staining solution was removed with a clean kimwipe. Images were taken on a Leica TCS SP8 CSLM using a HC PL APO 40× water immersion objective (NA = 1.10) with GFP excited by a 488nm argon laser and FM464 excited by a 561nm diode laser in sequential mode. Fluorescent signals were detected using two HyD detectors in photon counting mode (single sections) and normal mode (z-series).

Co-localization of secretory markers in mature ovules used similar settings but a more narrow detection window for mCherry fluorescence. Ovules were dissected out but retained on the septum and placed in PIPES Buffer and imaged immediately. For
transient co-localization experiments in *N. benthamiana*, leaf discs were collected near
the site of infiltration, placed in PIPES Buffer and GFP and mCherry signals were
imaged in cells expressing both constructs.

**FRET Detection via Acceptor Photobleaching**

Due to the rapid trafficking of NTA and MLO8-localized compartments within the
epidermal cells of *N. benthamiana*, leaf discs were treated with 20μg/mL Cytochalasin B
(Sigma-Aldrich, C6762) in PIPES Buffer for 10 min at room temperature prior to
acceptor photobleaching. Epidermal cells co-expressing the two constructs to be
assayed were chosen with mCer3 (donor) excited at 458nm and detected using a HyD
detector from 459nm to 512nm, while YFP (acceptor) was excited at 514nm and
detected with a PMT from 512 to 562nm, while using a 458/514 notch filter. Three
images of both mCer3 and YFP were taken before photobleaching and in between each
subsequent period of photobleaching (5 sec of high intensity 514 nm). After four rounds
of photobleaching a final set of three images were taken of both mCer3 and YFP
fluorescence.

Overall drift of punctate fluorescent compartments also made the analysis of FRET via a
calculation of FRET Efficiency (as in previous studies (Karpova et al., 2003; Bhat et al.,
2005)) inaccurate, as increases in fluorescence intensity could be a result of axial shifts
of the sample being imaged. Thus all data gathered was analyzed through the use of a
custom Macro in Fiji (Schindelin et al., 2012). Fluorescence of mCer3 and YFP in the
photobleached region were graphed and compared by determining their Pearson
product-moment correlation coefficient. This enabled the accurate detection of mCer3 fluorescence signal changes over time in response to acceptor photobleaching. The distributions of the correlation coefficients from all assayed pairs within each experiment were compared using the non-parametric Kruskal-Wallis test with FDR-corrected multiple pairwise comparisons determined using the Dunn Test. Statistical analyses for FRET data were all carried out in R, version 3.1.2 (Team, 2014) using the packages dplyr (Wickham and Francois, 2015) and DescTools (Signorell, 2015).

**Figure Construction and Image Processing**

All images were edited using Fiji (Schindelin et al., 2012). MLO protein and ovule diagrams were made in Inkscape v0.91. All graphs were generated in Prism (www.graphpad.com) and figures were constructed in Gimp v2.8.14.

**List of Supplemental Materials**

**Fig. S1.** Protein alignment of the *Arabidopsis thaliana* MLO family

**Fig. S2.** MLO8<sub>pro</sub>::MLO8-GFP expression pattern in mature ovules.

**Fig. S3.** Comparison of NTA<sub>pro</sub>::NTA-GFP and MYB98<sub>pro</sub>::NTA-GFP expression in *nta-1*.

**Fig S4.** Analysis of full length MLO distribution in synergid cells.

**Fig S5.** SignalP 4.0 Prediction results and N-terminal peptide alignment for all 15 MLO proteins in *Arabidopsis thaliana*.

**Fig. S6.** Analysis of NTA without its predicted signal peptide (sp).

**Fig. S7.** MLO-GFP distributions of domain swaps and truncations within synergids.

**Fig S8.** Analysis of NEL1 and CTerm domain swap distribution in synergid cells.

**Fig S9.** Analysis of IL1 and IL2 domain swaps between MLO8 and NTA.
Fig S10. FRET detection via acceptor photobleaching.

Acknowledgments

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Figure Legends

Fig 1. Native MLO expression patterns in Arabidopsis ovules.

Fig. 2 Analysis of MLO proteins expressed in synergids of nta-1.
A) Boxplot of unfertilized ovule counts in T3 plants homozygous for indicated
MYB98<sub>pro</sub>:MLO-GFP constructs in nta-1 mutants. Boxplot represents full range of each dataset with median at the center. B-F) MYB98<sub>pro</sub>:MLO-GFP (green) distribution in synergid cells of unfertilized mature ovules merged with FM464 labeling (magenta). Adj. P-values - “****” = < 0.0001, “*” = 0.05-0.01, “ns” = >0.05. Bars = 10μm (B-F).

**Fig. 3. NTA co-localization with secretory markers in synergids.**
A-C) Co-localization of MYB98<sub>pro</sub>:NTA-GFP (green) with the Golgi (A; Man49-mCherry), ER (B; SP-mCherry-HDEL), and Peroxisome (C; mCherry-PTS1) markers (magenta) expressed under the synergid selective promoter of LRE (Liu et al., 2016) in the synergid cell. Bars = 10μm.

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A-D) MYB98<sub>pro</sub>:MLO-GFP (green) co-expressed with the Golgi marker (LRE<sub>pro</sub>:Man49-mCherry; magenta) in the synergid cell. Bars = 10μm.

**Fig 5. NTA, MLO10, and MLO8 co-localization with a trans-Golgi network (TGN) marker in synergids.**
A-D) MYB98<sub>pro</sub>:MLO-GFP (green) co-expressed with the TGN marker (MYB98<sub>pro</sub>::SYP61-mCherry; magenta) in the synergid cell. Bars = 10μm.

**Fig 6. Analysis of domain swaps between MLO8 and NTA.**
A) Diagram of an MLO protein with described domains and regions annotated. N - N-terminal domain, SP - signal peptide, EL - extracellular loops 1-3, IL - intracellular loops.
1-3, CTerm - C-Terminal intracellular tail, CaMBD - calmodulin-binding domain. B-E) Boxplots of unfertilized ovule counts of T2 homozygous lines of MYB98<sub>pro</sub> expressed domain swap constructs in the nta-1 background. B) N-Terminus through the first extracellular loop [NEL1] domain swap between NTA and MLO8 C) extracellular loop 1 [EL1] domain swap between NTA and MLO8. D) C-Terminal swap including intracellular loop 3, extracellular loop 3, and the C-terminal tail [TM5] between NTA and MLO8. E) C-Terminal [CTerm] domain swaps between NTA and MLO8. Adj. P-values - “****” = < 0.0001, “***” = 0.001-0.0001, “*” = 0.05-0.01, “ns” = >0.05.

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A-D) MYB98<sub>pro</sub>:MLO-GFP (green) co-expressed with the Golgi marker (LRE<sub>pro</sub>:Man49-mCherry; magenta) in the synergid cell. Bars = 10μm.

**Fig 8. Detection of oligomerization of MLO constructs via FRET analysis.**
Boxplot of correlation coefficients from FRET analysis of homo- and hetero-oligomerization of constructs indicated. Groupings of “a”, “b”, “c”, and “d” based on significant differences between each dataset’s distribution as determined using a Kruskal-Wallis test. See also Supplemental Fig. S9. Datasets in all boxplots represent full range of data with median at the center.

**Literature Cited**


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