Border Control—A Membrane-Linked Interactome of Arabidopsis

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Cellular membranes act as signaling platforms and control solute transport. Membrane receptors, transporters, and enzymes communicate with intracellular processes through protein-protein interactions. Using a split-ubiquitin yeast two-hybrid screen that covers a test-space of 6.4 × 10^6 pairs, we identified 12,102 membrane/signaling protein interactions from Arabidopsis. Besides confirmation of expected interactions such as heterotrimeric G protein subunit interactions and aquaporin oligomerization, >99% of the interactions were previously unknown. Interactions were confirmed at a rate of 32% in orthogonal in planta split–green fluorescent protein interaction assays, which was statistically indistinguishable from the confirmation rate for known interactions collected from literature (38%). Regulatory associations in membrane protein trafficking, turnover, and phosphorylation include regulation of potassium channel activity through abscisic acid signaling, transporter activity by a WNK kinase, and a brassinolide receptor kinase by trafficking-related proteins. These examples underscore the utility of the membrane/signaling protein interaction network for gene discovery and hypothesis generation in plants and other organisms.

Genome projects have provided the inventories of genes and predicted proteins, yet we can only begin to understand how organisms function or interact with their environment once we understand the functions of the proteins in the proteome and their wiring diagrams. Plants, as sessile organisms, are particularly efficient in acclimating to the dynamics of their environments. Plant growth, development, homeostasis, and acclimation require mechanisms that monitor changes in the environment, coordinate cells and compartments, and adjust transport of ions and metabolites across cellular membranes. Sensing, signaling, and transporter regulation are mediated through interactions with membrane proteins. Largely because of technical challenges caused by the hydrophobicity of membrane proteins, only a small number of membrane protein interactions are known. Membranes contain thousands of proteins whose biochemical or physiological functions have not been identified experimentally and are thus classified as "unknowns." Identification of genetic and molecular interactions is a promising way to assign functions to the unknowns in the genome (1–3).

To uncover membrane protein interactions, we performed a systematic binary interaction screen using a yeast two-hybrid system specifically developed for membrane protein interactions: the mating-based split-ubiquitin system (mbSUS) (4, 5). The split-ubiquitin system identified interactions between integral membrane proteins that form homo- and hetero-oligomeric complexes such as sucrose and ammonium transporters, potassium channels, and aquaporins (4–6). The mbSUS was also instrumental for identifying regulators of yeast ammonium and auxotrophy at two stringency conditions (fig. S4). We replicated tests for complementation of histidine auxotrophy at 1070 Cub-fusions comprised >3 × 10^6 interaction tests performed in duplicate and at two stringency conditions (a total of four assays per pair), with a quantifiable readout of yeast colony growth assayed for complementation of histidine auxotrophy (Fig. 1B). We created imaging and statistical analysis pipelines based on distribution model-based classification so as to determine interaction confidence scores and thresholds for positive interactions (figs. S2 and S3). A total of 30,426 interactions, less than 1% of the pairs interrogated, were defined as positive in the primary screen based on stringent criteria (supplementary materials 2). We evaluated all positive interactions from the primary screen in a secondary screen with 12 additional interaction tests in yeast: six replicated tests for complementation of histidine auxotrophy at two stringency conditions (fig. S4). A network of 12,102 interactions between 1523 membrane proteins tested positive consistently at both stringency conditions and was termed MIND1 (Membrane-linked Interactome Database version 1) (fig. S5).
To evaluate the reliability of MIND1, a random subset of 7770 positive interactions from the primary screen was reanalyzed in a tertiary screen (fig. S4). Results from the secondary screen were largely confirmed by the tertiary screen, which included two histidine auxotrophy assays and a LacZ activity assay; only 12% of secondary screen positives failed in all three tertiary screen assays, whereas 80% were positive in at least two assays (fig. S5). Because we tested the interactions of Arabidopsis proteins in a single-cell heterologous system, the observed associations are not necessarily relevant in planta. Therefore, we also tested 195 interactions in planta using the orthogonal, low-throughput split–green fluorescent protein (GFP) protein interaction assay (Fig. 1C and supplementary materials 2.6). These pairs tested positive in 31.8% of the split-GFP interaction tests [95% confidence interval (CI) (25.6%, 38.5%), bootstrap analysis]. We estimated the in planta

**Fig. 1. The Arabidopsis Membrane-linked Interactome (MIND1).** (A) A positive result in the mbSUS screen is the result of four molecular steps: The interaction of the proteins of interest brings into proximity the Nub and Cub moieties, reconstituting a ubiquitin protein (UBQ) that is then recognized by an endogenous ubiquitin specific protease that cleaves the Cub fusion protein to release a transcription factor (TF). The TF is then free to enter the nucleus and activate marker genes whose expression indicates physical interaction between the proteins of interest. (B) 12,102 interactions in MIND1 generated from two rounds of mbSUS screen. Chart displays protein-protein interactions between a membrane protein with another membrane protein or a soluble protein. (C) The results of orthogonal validation of the MIND1 protein-protein interactions by retesting in the split-GFP assay. Error bars show 95% CI estimated with bootstrap analysis (supplementary materials 2.6). (D) Protein family interaction network. Nodes represent family types and edges indicate interactions between two families. Only families that have more than two proteins are included, and only those family pairs that have 10 or more interactions are displayed. (E) Cellular localization interaction network of MIND1NH. Nodes represent cellular components; edges indicate interactions between or among proteins localized in the respective compartments.
false-positive rate of MIND1 by comparing its split-GFP validation rate to the rate of split-GFP validation for two reference sets of protein pairs (supplementary materials 2.6). A positive reference set of 49 independently reported interactions—randomly sampled from the subset of primary screen protein pairs that were reported to physically interact in the literature—was used to estimate the true-positive rate of interactions tested in the split-GFP assay, which was 38.8% [95% CI (24.5%, 53.1%)], bootstrap analysis]. A negative reference set of interactions—randomly sampled from the primary screen protein pairs—was used to estimate the false-positive rate of interactions tested in the split-GFP assay, which was 2.5% [95% CI (0.5%, 5.8%), bootstrap analysis]. MIND1 interactions tested positive at a rate that is not significantly different from the independently reported positive interactions (positive reference set, $P = 0.364$, permutation test) and over 12-fold higher than noninteracting protein pairs (negative reference set, $P$ value = 0, permutation test). The MIND1 in planta false-positive rate was estimated to be 19.2% [95% CI (0, 46.6%), bootstrap analysis], although the small fraction of interactions tested in the split-GFP assay precludes a statistically reliable point estimate of the false-positive rate.

The Potential of Low Stringency Interactions for Discovery

MIND1 does not include interaction pairs that tested positive in only one or two out of the four tests performed in the primary screen. However, we provide these interactions online (www.associomics.org) because they could be biologically relevant. For example, four different SWEET transporter hetero-oligomers were detected in only one out of the four primary screen replicate tests. On the basis of this information, we

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**Fig. 2. Enriched connections between GO biological process groups in MIND1.** Nodes represent different GO groups and are color-coded by group type as shown. Edges represent statistically enriched connections between GO groups in the complete MIND1 (solid lines) or in MIND1NH (dashed lines) determined by comparing with 10,000 randomized networks and using a false discovery rate $Q < 0.05$ as threshold.

**Fig. 3. Interactions between abscisic acid signaling-related proteins.** Three types of evidence (genetic evidence, GO annotation, and abscisic acid responsive gene expression) were used to classify MIND1NH nodes as related to abscisic acid signaling. Interactions involving hubs and unconnected abscisic acid–related proteins are not included.
performed a systematic analysis to detect homo- and hetero-oligomerization among all 17 Arabidopsis SWEETs and found 8 homo- and 47 hetero-oligomers using the split-ubiquitin system (12). Several of these pairs were confirmed in planta by using the split-GFP assay, suggesting that oligomerization is a general feature of SWEETs and highlighting that pairs that did not yield highly reproducible results may be valuable for inclusion in specific hypothesis-driven studies.

**MIND1 Network Characteristics**

MIND1, like other biological networks (13, 14), exhibits small-world properties (short distance among proteins coupled with local order indicated by high clustering coefficient) (supplementary materials 3) and follows a heavy-tailed degree distribution with a reliance on high-degree hubs (proteins with many interactions) (fig. S6). Because hubs have many interaction partners, individual interactions of a hub have low specificity. Thus, hub proteins with degree >70 were removed to generate a non-Hub network (MIND1NH) of 3354 interactions (fig. S7). Interacting proteins often share similar expression patterns (15); therefore, we compared expression correlation for MIND1- and MIND1NH-interacting protein pairs. Relative to noninteracting pairs, only MIND1NH pairs were enriched for expression correlation (fig. S8). However, both MIND1- and MIND1NH-interacting protein pairs were enriched for GO functional similarity (fig. S9), indicating that a hub protein was more likely to share a functional annotation with its interaction partners than an expression pattern. MIND1 interactions are complementary to existing interactome networks from Arabidopsis in that over 99% of the interactions had not been previously reported (13). This is not unexpected because different interactome assays typically yield complementary data sets (14, 16–18) and because the clone sets analyzed were different (only 212 MIND1 proteins were also screened by the Arabidopsis Interactome Mapping Consortium) (fig. S10) (13).

**MIND1-Derived Functional Predictions**

Because 327 proteins (21%) in MIND1 lack GO biological process annotation, the network can potentially contribute to functional predictions (fig. S11). For example, the small, single transmembrane-domain–containing protein AT5G61630 [a potential Ras guanosine triphosphatase (GTPase); www.greenphyll.org] had three interactors that were annotated “water channel activity” (GO:0015250), intimating a role in regulating water transport (table S2). We systematically generated functional predictions for the proteins in MIND1NH by identifying functional annotations that were overrepresented among each protein’s interacting partners relative to the proteins present in the network (table S2). For the above example, the annotation “water channel activity” is overrepresented in the interaction partners of AT5G61630 (P = 5.68 × 10⁻⁶, Fisher’s exact test). We also generated functional predictions for the proteins in MIND1 by first isolating highly connected network clusters (putative functional modules) and then identifying annotations enriched within these clusters. Hierarchical clustering of MIND1 resulted in a large super-cluster dominated by interactions involving hub proteins, whereas hierarchical clustering of MIND1NH resulted in numerous smaller clusters (fig. S13). Two additional module detection methods (supplementary materials 3.7) identified MIND1NH clusters with enriched GO annotations, potentially indicating functional modules (table S4 and fig. S14). For example, the unknown gene described above, AT5G61630, belongs to a cluster that contains five aquaporin isoforms and is enriched for “water transport” (GO:0006833, Bonferroni-corrected P value = 1.36 × 10⁻³, Fisher’s exact test), a function that may pertain to other members of the cluster, including AT5G61630.

Individual interactions, irrespective of the network context, also guide functional predictions. For example, we found interactions between the nitrate transceptor CHL1 (AT1G12110) and the potassium transporter KT2 (AT2G40540) as well as the lysine-deficient protein kinase WNK8 (AT5G41990). CHL1’s transport activity or conformation was affected by these interactions, as demonstrated with the fluorescent transport activity sensor NiTracl (19). WNK8 is a key player involved in dose- and duration-dependent sugar signaling, which controls endocytosis of the G protein–coupled receptor RGS1 (20). Interesting parallels exist in the animal kingdom, in which WNK kinases play important roles in controlling ion transport processes (21).

**Characterization of Hubs in MIND1**

Disruption of network hubs typically has pleiotropic effects (22). Purifying selection acts more strongly on genes with functions essential for biological fitness and results in reduced evolutionary rates (23). Consistently, MIND1 hubs showed evidence of purifying selection in that Kₐ/Kₐ [the ratio of the number of nonsynonymous substitutions per potential nonsynonymous site (Kₐ)] based on Arabidopsis lyrata orthologs was low (mean Kₐ/Kₐ = 0.15) and significantly lower than Kₐ/Kₐ for all Arabidopsis proteins with A. lyrata orthologs (mean Kₐ/Kₐ = 0.21, P value = 0.028, Student’s t test) (supplementary materials 3.8 and table S3). Although we cannot exclude the possibility that the high number of protein interactions for an individual MIND1 hub could be an artifact, the functions of the hubs are consistent with general roles that require interaction with a large number of target proteins; out of the 33 MIND1 hubs with known or inferred molecular function, 21 have putative functions in protein modification (chaperones/thioredoxins, signal peptidases, or proteases) or protein sorting [soluble N-ethylmaleimide–sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) or cornichon] (table S3). Moreover, subcellular localization of 16 MIND1 hub GFP-fusions, including nine unknowns, showed that all but one localized to endomembranes, which is consistent with roles in membrane protein modification or sorting (fig. S15 and table S3).

**Analysis of MIND1 at the Level of Protein Families and Biological Processes**

Analysis of interactions at the protein family level can be used to predict genetic redundancies, interaction motifs, and potential interactions with paralogs. A gene family–based network (Fig. 1D, figs. S16 and S17, and table S5) revealed overrepresentation of interactions involving proteins of unknown function. The unknowns showed numerous interactions with transporters, indicating undiscovered transport or transport-regulatory functions among these proteins (Fig. 1D).

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**Fig. 4. AP2C1 regulates the potassium channel KAT1.** (A) The Blondel clustering algorithm revealed an abscisic acid–related cluster (Blondel cluster 93) in MIND1NH, that includes KAT1 and AP2C1. (B) Current-voltage relationship of the current in Xenopus laevis oocytes injected with KAT1 or AP2C1 or with both cRNAs upon perfusion with 100 mM KCl. In all recordings, the holding potential was set at −40 mV, and voltage steps were applied to potentials ranging between −40 and −200 mV with −20-mV decrements. Data are means ± SE from at least three different individual oocytes.
Aggregating interactions by predicted subcellular localization yielded a location-based network of connections between different cellular compartments predicting higher-level association patterns (Fig. 1E). MIND1 proteins are predominantly plasma membrane-localized, and these plasma membrane proteins show abundant interactions with other plasma membrane proteins and with cytosolic partners (Fig. 1E).

To investigate potential interaction motifs among biological processes, we examined interactions at the level of GO biological processes. Intrabiological process interactions are less abundant in MIND1 as compared with other published networks (fig. S18) (13). We generated a meta-network comprising enriched interactions between biological processes. This network revealed potential interaction motifs such as “Receptor-like Kinase/Pelle (RLK) signaling pathway” linking to “protein catabolism” (Fig. 2).

**Regulation of Transport Activity by Hormones**

To strengthen functional predictions from MIND1, the protein interaction network was overlaid with transcriptome and other annotation data. For example, 311 proteins in MIND1 are related to the hormone abscisic acid according to transcriptomic, genetic, or other experimental evidence (24). MIND1 interactions connected many of these proteins into an abscisic acid–related subnetwork (Fig. 3 and fig. S19). Similar subnetworks obtained for other hormones also represent interaction sets that are strengthened by multiple data sources (figs. S19 to S26).

Transport is highly regulated at the transcriptional and posttranscriptional levels, and hormone signaling often targets membrane-related processes. The activity of potassium channels—which contribute to potassium acquisition from soil, regulation of enzyme activities, adjustment of membrane potential and turgor, and regulation of cellular homeostasis and electrical signaling—is regulated by protein kinases and phosphatases (25). A key mechanism by which abscisic acid promotes stomatal closure is through inhibition of transporters such as the potassium channel KAT1 (26). Abscisic acid responses are mediated by a co-receptor complex that consists of abscisic acid receptors (PYR/PYL/RCAR proteins) and clade-A protein phosphatase 2Cs (PP2C) (27). Although much of abscisic acid signaling proceeds through clade-A PP2C regulation of SnRK2 kinases and SnRK2 regulation of transcription factors, clade-A PP2Cs and SnRK2 kinases can also directly regulate the activity of K+ channels (28, 29).

Abscisic acid signaling proteins in MIND1 share extensive connections with transporters and other membrane proteins (Fig. 3 and fig. S19). For example, the clade-B PP2C, AP2C1, interacts with the K+ channel KAT1 in a cluster enriched for abscisic acid–related GO annotations (Fig. 4A); Blondel cluster 93 (table S4) is enriched for GO:0009738—“abscisic acid signaling pathway” (Bonferroni-corrected \( P = 1.25 \times 10^{-5} \), Fisher’s exact test)—and GO:0071215—“cellular response to abscisic acid stimulus” (Bonferroni-corrected \( P = 1.41 \times 10^{-5} \), Fisher’s exact test). Although clade-B PP2Cs are not canonical abscisic acid coreceptor components and are not known to regulate potassium channels, when coexpressed with KAT1 in oocytes, AP2C1 completely inhibited channel activity (Fig. 4B). KAT1 functions in stomatal opening;

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**Fig. 5. Functional interaction of BRI1 with trafficking proteins.** Split-GFP assay for VAMP727, SYR22, and BRI1, and brassinolide (BL) hypersensitivity of vamp727−/−; syp22 and VAMP727 overexpressed (OX) plants. (A) Yellow fluorescent protein (YFP) fluorescence and bright field images (left, fluorescence channel; right, bright field). (a and b) Reconstitution of YFP fluorescence from nYFP-VAMP727+ BRI1-cCFP; (c and d) nYFP-SYP22+BRI1-cCFP; (e and f) BRI1-cCFP+nYFP (negative control). Scale bars, 20 μm. (B) VAMP727 overexpression plants were grown under indicated concentrations of BL, and hypocotyl lengths were measured. (C) Seedlings were grown under various concentrations of BL, and hypocotyl lengths were measured. (B and C) Error bars indicate SE. Experiments were repeated at least three times. (D) Wild type and vamp727−/+;syp22 were grown for 5 days with 100 nM or without BL. BL-mediated hypocotyl elongation and primary root growth inhibition (coiling) were affected in vamp727−/+;syp22 compared with wild-type plants.
Receptor Kinase Signaling and Regulation

The RLK family of transmembrane receptors is larger in plants as compared with animals and likely serves as a predominant mechanism of communication across membranes. However, characterization of RLKs is complicated by apparent genetic redundancy (31). MIND1 contains 554 interactions for 175 full-length RLK proteins, extending RLK associations within membranes and with soluble signaling proteins (fig. S27). MIND1 contains >20 RLK interactions with small GTPases (fig. S27). At present, no canonical pathway for intracellular RLK signaling analogous to the metazoan protein–coupled receptor (GPCR) transmembrane signaling through intracellular heterotrimeric G proteins has been defined in plants. However, small GTPases and related proteins have been identified as intracellular RLK signaling components (32). RLK-GTPase signaling could act in several ways—for example, via direct signal transduction from RLK activation to ROP activation. We observed numerous RLK interactions with Rab-GTPases, proteins involved in trafficking (33), potentially indicating Rab involvement in trafficking of RLKs shown for RabF2b regulation of FLS2 endocytosis (34). Extending RLK-GTPase associations by using MIND1 and additional interactome data sets (35) reveals additional potential GTPase interaction motifs that could help advance the understanding of the role of small GTPases (fig. S27).

Regulation of Brassinosteroid Signaling by Trafficking Proteins

Membrane proteins are maintained at appropriate steady-state levels by a balance of delivery to the plasma membrane and recycling through endocytosis. Receptor endocytosis plays a key role in controlling activation and signal termination (36). For example, upon activation by ligands, mammalian RLKs are subject to accelerated lysosomal degradation (37). In Arabidopsis, brassinosteroids such as brassinolide are perceived by the RLK BRI1 and its coreceptor BAK1. Ligand-independent trafficking of BRI1 between plasma membrane and early endosomes and degradation in the vacuole has been observed (37, 38), and the Membrane Steroid Binding Protein (MSBP1) was shown to trigger BAK1 endocytosis in a brassinolide-independent manner (39). Although considerable evidence implicates BRI1-trafficking in brassinosteroid signaling, the underlying mechanisms are still ambiguous (40), and the interactions found in MIND1 may help solve some of the open questions regarding RLK trafficking. Here, we found that BRI1 interacts with the R-SNARE VAMP727, a candidate for regulating BRI1 trafficking (table S1).

Membrane Protein and Signaling Protein Interactome for Functional Genomics

Membrane proteins are central components of many cellular processes, often through coordinated action with either membrane or soluble interaction partners. The 12,102 protein-protein interactions in MIND1 (www.assocomics.org) expand the functional genomics knowledge base for the reference plant Arabidopsis and serve as a resource for gene discovery and hypothesis generation. Analysis of MIND1 in conjunction with other data sources has not only confirmed known interactions but also has uncovered connections that shed light on how membrane proteins are regulated at the levels of trafficking, accumulation, and activity.

References and Notes

9. J. D. Lewis et al., BMC Genomics 13, 8 (2012).

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Supplementary Materials

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Supplementary Text

Figs. S1 to S30

References (1–82)

Tables S1 to S6

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